Evaluation of the plastidic antioxidant system in *Jatropha curcas* and *Swietenia macrophylla* seedlings submitted to drought stress

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II

Summary

Tropical plants species face a very particular environment with highly variable light intensity, heat, low mineral availability and other abiotic stresses which might have led to the evolution of specific protective mechanisms. The understanding of mechanisms for tolerance over abiotic stress in these plants could help to protect tropical forests, allow a more intelligent grow of economically interesting species and improve crops.

The study of non-model plants, the category for most tropical species, is hindered by the amount of available information and the quality of this data. *Swietenia macrophylla* has not been sequenced, since the end of this work. To perform an evaluation of gene expression it was necessary to clone previously partial sequences for all required genes and to design specific primers for PCR amplification, including the reference genes. This work was supported by phylogenetic analysis, nourished by available sequences of related species. On the contrary, *Jatropha curcas* is on its way to becoming a novel model-species for genetic and eco-physiological studies. The species was sequenced in 2010 and had already some studies of gene expression under abiotic stress. Even when it is a helpful aspect to be able to compare results with other studies, it was demonstrated here that the provided information still needs to be carefully evaluated before it is used. The validity of the information for 2CP, APX, GPX and CAT obtained in two different databases, was corroborated by cloning full cDNA sequences. The characterization of genes belonging to the enzymatic antioxidant system in chloroplasts was performed by studies of exon and for 2CP and APX, the chloroplast targeting was confirmed by transient transformation of *N. benthamiana* with a construct carrying the signal peptide and a reporter gene.

Stress experiments were performed in outdoor conditions in Panama for *Swietenia*, and also in greenhouse conditions in Berlin, for *Jatropha*; they included mainly variations in temperature and irradiance. Physiological parameters such as stem elongation, leaf area and biomass production seemed different between the two species, praising *Jatropha* for its capacity of growth regulation during mild and severe drought stress. Nevertheless, PSII quantum yield and CO_2 assimilation rates in *Swietenia* unveiled an unexpected photosynthetic capacity during drought.

The gene regulation of the antioxidant enzymes in *Swietenia* proved, in most cases, to be similar to the description for other C3 drought sensitive plants such as *Arabidopsis*. Though few times reached statistical significance, there was considered that plants under treatment

experienced a trend for up-regulation in 2CP, APX and GPX during mild stress, for down-regulation during severe stress, and after re-watering, all three genes came back to regular expression. In the case of *Jatropha*, there was not one single trend for each gene. During mild stress, 2CP was not regulated and APX was down-regulated. During severe stress and re-watering, there was reported a pattern of expression for each gene, depending on the growth conditions of each experimental setup. Moreover, CAT was inhibited by low light intensities in *Jatropha* at all phases of the experiment and was not upregulated during mild or severe stress in adequate light intensities.

Based on the final holistic evaluation, it can be confirmed that the regulation mechanism of the antioxidant system in the chloroplasts is more sensitive in *Jatropha*. Counting only the genes evaluated in both species, there was a fine-tuning regulation in the antioxidant system of *Jatropha* that was not observed in *Swietenia*. *Jatropha curcas* can be considered as more tolerant to drought because it can adjust gene transcription levels more specifically, presenting various regulation patterns for each gene and in all evaluated drought conditions, in a balanced way.

Zusammenfassung

Tropische Pflanzenarten sind einer sehr spezifischen Umgebung mit stark schwankender Lichtintensität, Wärme, geringer Mineralverfügbarkeit und anderen abiotischen Stressfaktoren ausgesetzt, die zur Entwicklung spezifischer Schutzmechanismen geführt haben könnten. Das Verständnis von Mechanismen der abiotischen Stresstoleranz könnte helfen, tropische Wälder zu schützen, ein intelligenteres Wachstum von wirtschaftlich interessanten Arten zu ermöglichen und Nutzpflanzen zu verbessern.

Die Untersuchung von Nichtmodellpflanzen, der Kategorie für die meisten tropischen Arten, wird durch die geringe Anzahl an verfügbaren Informationen und die Qualität dieser Daten behindert. *Swietenia macrophylla* ist bis zum Ende dieser Arbeit nicht sequenziert. Um eine Evaluierung der Genexpression durchzuführen, war es notwendig, zuvor Teilsequenzen für alle erforderlichen Gene zu klonieren und spezifische Primer für die PCR-Amplifikation, einschließlich der Referenzgene, zu entwerfen. Diese Arbeit wurde durch eine phylogenetische Analyse gestützt, genährt von verfügbaren Sequenzen verwandter Arten. Im Gegenteil ist *Jatropha curcas* auf dem Weg eine neue Modellspezies für genetische und ökophysiologische Studien zu werden. Die Art wurde 2010 sequenziert und es gibt bereits einige Studien zur Genexpression unter abiotischem Stress. Auch wenn es ein hilfreicher Aspekt ist, Ergebnisse mit anderen Studien vergleichen zu können, wurde hier gezeigt, dass die bereitgestellten Informationen noch sorgfältig ausgewertet werden müssen, bevor sie verwendet werden. Die Gültigkeit der Information für 2CP, APX, GPX und Catalase in zwei Datenbanken wurde durch Klonen vollständiger Sequenzen bestätigt. Die Gencharakterisierung für das enzymatische Antioxidationssystem in Chloroplasten wurde durch Exonstudien durchgeführt, und für 2CP und APX wurde das Targeting der Chloroplasten durch transiente Transformation von *N. benthamiana* mit einem Konstrukt, das das Signalpeptid und ein Reportergen trägt, bestätigt.

Stressexperimente wurden unter Freilandbedingungen in Panama für *Swietenia* und auch in Gewächshausbedingungen in Berlin für *Jatropha* durchgeführt; Sie beinhalteten hauptsächlich Schwankungen in Temperatur und Bestrahlungsstärke. Physiologische Parameter wie Stammlänge, Blattfläche und Biomasseproduktion sind zwischen den beiden Arten unterschiedlich und zeigen, dass Jatropha die Fähigkeit besitzt das Wachstums während unter leichten und schweren Trockenstress zu regulieren. Dennoch zeigten die PSII-Quantenausbeute und die CO_2-Assimilationsraten in *Swietenia* eine unerwartete photosynthetische Kapazität während der Dürre.

Die Genregulation der antioxidativen Enzyme in *Swietenia* erwies sich in den meisten Fällen als ähnlich zu der Beschreibung für andere C3 dürreempfindliche Pflanzen wie *Arabidopsis*. Obwohl kaum signifikant, wurde ein Trend für 2CP, APX und GPX für die Hochregulation bei leichtem Stress, für die Runterregulation während des schweren Stresses und nach dem erneuten Gießen erkannt. Im Falle von *Jatropha* gab es kein Trend in der Genexpression. Bei leichter Belastung war 2CP nicht reguliert und APX wurde herunterreguliert. Während starker Belastung und erneuter Bewässerung wurde ein Expressionsmuster für jedes Gen beobachtet, abhängig von den Wachstumsbedingungen jeder Versuchsanordnung. Darüber hinaus wurde CAT in allen Phasen des Experiments durch niedrige Lichtintensitäten in Jatropha gehemmt beobachtet und wurde während leichter oder starker Belastung nicht hochreguliert.

Basierend auf der abschließenden ganzheitlichen Bewertung kann bestätigt werden, dass der Regulationsmechanismus des antioxidativen Systems in den Chloroplasten in *Jatropha* empfindlicher ist. Betrachtet man nur die bei beiden Spezies untersuchten Gene, so gab es eine Feinregulierung im antioxidativen System von Jatropha, die in Swietenia nicht beobachtet wurde. Jatropha kann als toleranter gegenüber Trockenheit angesehen werden, da sie die Genexpression spezifischer anpassen kann, indem sie verschiedene Regulationsmuster in allen Genen und unter allen Dürrebedingungen in ausgewogener Weise präsentiert.

Resumen

Las plantas tropicales se enfrentan a un entorno muy particular con altas variaciones en intensidad de luz y temperatura, poca disponibilidad de minerales y otros tipos de stress abiótico que podrían haber llevado a la evolución de mecanismos específicos de protección. La comprensión de los mecanismos de tolerancia en estas plantas podría ayudar a proteger los bosques tropicales, permitir un crecimiento más inteligente de especies económicamente interesantes y mejorar los cultivos.

El estudio de plantas no-modelo, la categoría en la que se incluyen la mayoría de las especies tropicales, se ve obstaculizada por la cantidad de información disponible y la calidad de estos datos. *Swietenia macrophylla* no ha sido secuenciada hasta la culminación de este trabajo. Para realizar una evaluación de la expresión génica fue necesario clonar previamente secuencias parciales para todos los genes requeridos y diseñar cebadores (primers) específicos para la amplificación por PCR, incluidos los genes de referencia. Este trabajo fue respaldado por análisis filogenéticos y nutridos con las secuencias disponibles de especies relacionadas. Por el contrario, *Jatropha curcas* está en camino de convertirse en una nueva especie modelo para estudios genéticos y eco-fisiológicos. La especie fue secuenciada en 2010 y ya cuenta con algunos estudios de regulación génica bajo estrés abiótico. Incluso cuando es beneficioso poder comparar los resultados con otros estudios, aquí se demostró que la información provista debe ser cuidadosamente evaluada antes de ser utilizada. La validez de la información para 2CP, APX, GPX y CAT obtenida de dos bases de datos diferentes se corroboró clonando secuencias completas de cDNA. La caracterización de los genes pertenecientes al sistema antioxidante enzimático en los cloroplastos se realizó mediante estudios de exones; además, se confirmó la localización en el cloroplasto de 2CP y APX por transformación transitoria de *N. benthamiana* con un plásmido que portaba el pre-péptido marcador de localización y un marcador fluorescente.

Los experimentos de sequía se realizaron en condiciones al aire libre en Panamá para *Swietenia*, y también en condiciones de invernadero en Berlín, para *Jatropha*; incluyendo principalmente variaciones de temperatura e irradiación. Parámetros fisiológicos como el alargamiento del tallo, el área foliar y la producción de biomasa son diferentes entre las dos especies, distinguiendo a *Jatropha* por su capacidad de regulación del crecimiento durante periodo de sequía leve y grave. Sin embargo, el rendimiento cuántico del PSII y las tasas de

asimilación de CO_2 en *Swietenia* revelaron una capacidad fotosintética inesperadamente alta durante la sequía también.

La regulación génica de las enzimas antioxidantes en *Swietenia* demostró, en la mayoría de los casos, ser similar a la descripción de otras plantas sensibles a la sequía C3, como *Arabidopsis*. Aunque pocas veces alcanzó niveles de significancia estadística, se consideró que las plantas bajo tratamiento experimentaron una tendencia de regulación al incremento para 2CP, APX y GPX durante periodos de estrés leves; de regulación en reducción en los tres genes bajo estrés severo, y después de volver a regar, estos tres genes regresaron a la expresión regular. En el caso de *Jatropha*, no hubo una sola tendencia para cada gen. Durante estrés leve, 2CP no fue regulado y APX fue regulado negativamente. Durante estrés severo y recuperación, se observaron varios patrones de expresión para cada gen, dependiendo de las condiciones de crecimiento de cada experimento. Además, se observó que CAT se inhibía por la baja intensidad de la luz en *Jatropha* en todas las fases del experimento y no se regulaba positivamente durante el estrés leve o severo en condiciones lumínicas adecuadas.

Basado en la evaluación holística final, se puede confirmar que el mecanismo de regulación del sistema antioxidante en los cloroplastos es más sensible en *Jatropha*. Considerando solo los genes evaluados en ambas especies, hubo una delicada regulación del sistema antioxidante de *Jatropha* que no se observó en *Swietenia*. *Jatropha curcas* se puede considerar como más tolerante a la sequía porque puede ajustar los niveles de transcripción de los genes más específicamente, presentando varios patrones de regulación en cada uno los genes y en todas las condiciones de sequía evaluadas, de una manera equilibrada.

List of Abbreviations

A	ampere/absorption
A260	absorbance at λ = 260 nm
ABA	abscisic acid
Act3	Actin 3
APXs	ascorbate peroxidases
Asc	ascorbate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	control plants
°C	Celsius degree
CAT	catalase
CEF	cyclic electron flow
2CP	2-Cys peroxiredoxin
cDNA	complementary DNA
CO2	carbon dioxide
Cu/Zn SOD	copper-zinc superoxide dismutase
dNTPs	deoxyribonucleotides
DTT	dithiothreitol
DW	dry weight
EDTA	ethylenediamintetra-acetic acid
ER	endoplasmic reticulum
ETR	electron transport rate
Fig.	figure
FW	fresh weight
Fv/Fm	maximum quantum yield of PSII
$\Delta F/Fm'$	effective quantum yield of PSII
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GPXs	glutathione peroxidases
HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
IRGA	Infra-red gas analyser
KCl	potassium chloride
kDa	kilo dalton
KH_2PO_4	monopotassium phosphate
K_2HPO_4	dipotassium phosphate
L.	Carl von Linné
LiCl	lithium chloride
λ	wavelength
$MgCl_2$	magnesium chloride
M	molar concentration
mRNA	messenger RNA
n	sample size
n.a.	sample not available
n.m.	not measured
n.s.	not significant
nt	nucleotide
NBT	nitro blue tetrazolium
O_2	molecular oxygen
$^1O_2$	singlet oxygen

$O_2^{\bullet}$	superoxide radical anion
$OH^{\bullet}$	hydroxyl radical
PCR	polymerase chain reaction
PM	plasma membrane
PMSF	phenylmethylsulfonyl fluoride
PSI	photosystem I
PSII	photosystem II
PRXs	peroxiredoxins
PrxQ	peroxiredoxin Q
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
RWC	relative water content
sAPX	stromal ascorbate peroxidase
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
tAPx	thylakoid-bound ascorbate peroxidase
TBS	Tris-buffered saline
TEMED	tetramethylethylene-diamine
U	protein unit
V	volt
v/v	volume per volume
w/v	weight per volume
WC	absolute water content

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Chapter 1
Introduction

1.1. Tropical plants

The tropical zone is located between the Tropics of Cancer and Capricorn, 23 ° latitude north and south. Olson *et al.* (2001) distinguish six types of tropical biomes but there are several classification lists to describe all possible plant communities existing inside. The tropical forest biome is mainly classified by the annual rainfall they receive, from 1000 mm up to 3000 mm per year, including a wide range from dry tropical forest to wet rainforest; temperatures are not dropping below 18°C in the whole year in general. These forests comprise 40-75% endemic species and could contain evergreen or semi-deciduous trees (drop some or all their leaves during the dry season). Tropical rainforest have stratified vegetation, including soil floor, understory, canopy and emergent layer.

As a result of human activities such as agriculture expansion, heavy logging, mining and climate change, they are currently considered threatened ecosystems. Therefore, international politics and programs are created to protect them, starting with reforestation programs. Inside rainfall and altitude, there is also difference between primary and secondary forest, that is visible by the species combination and the adaptations of main plants to the site (Craven *et al.*, 2015).

1.1.1. Tropical forest and climate change

Since the evidence supports climate change, the challenges that these modifications pose for plant growth have become more visible. Effects on plant growth and survival will affect tropical zones, both agricultural lands and forests. Rosenzweig *et al.* (2014) predicts that agricultural activities are going to be affected mostly in the areas of low latitudes, with impoverished countries, corresponding to tropics.

Tropical ecosystems have different climatic, edaphic, floristic and ecological attributes compared to temperate ecosystems. These factors are important when evaluating how plants will respond to increased concentrations of CO_2 (Cernusak *et al.*, 2013). It is not known, for example, how much impact increased temperatures will have on plant performance. Enzymatic activity depends on temperature, so under the elevated temperature in tropical areas, photosynthesis is expected to increase when there is higher CO_2 concentration

(Farquhar *et al.*, 1980). Dougthy and Goulden (2008) propose that tropical forest already experience close to the maximum of tolerance, so with further increasing heat, photosynthesis will decline. Körner *et al.* (2009) even suggest that tropical trees are having no limitation on carbon supply at the current concentrations, so that photosynthesis and further biomass accumulation will have very little modification. The use of stress-susceptible species like *Arabidopsis thaliana* to study drought and salinity has been criticized based on its limited defence mechanisms, affirming that their response might reflect death or senescence instead of useful adaptations (Munns and Tester, 2008; Beritognolo *et al.*, 2011) that cannot be extrapolated to other plants with major prediction for affectation.

Plant species in tropical forests are spread in a wide range of tolerance to shadow. In a region with high irradiation, light or shadow are also indicators of temperature (Wright *et al.*, 2005). Most shade-tolerant species will recruit and survive everywhere, while the most demanding ones will only do so in large forest openings (Wright *et al.*, 2003). Small disturbances often result in replacement by slow-growing shade-tolerant plantlets, regularly belonging to high wood densities and large adult stature. While small disturbances are often happening, multiple tree fall is more frequent due to climate change storms, opening niches for low wood density, light-demanding pioneer species (Cernusak *et al.*, 2013), characteristically from secondary forests.

1.1.2. Republic of Panama

The Republic of Panama is located in central America between latitudes 7° and 10°N, and longitudes 77° and 83°W and has a total area of 74,177.3 km^2. It has coasts at the Pacific Ocean and the Caribbean Sea and neighbours Costa Rica to the west and Colombia (in South America) to the southeast. It has a tropical climate with high humidity and uniform temperatures through the year. Diurnal ranges are low, approximately 6-8°C difference between the hottest and the coolest point; temperature seldomly exceeds 32 °C for more than a short time. Precipitation in the country varies regionally from less than 1,300 millimetres to over 3,000 millimetres per year, condensed during rainy season, which varies from seven to nine months, usually from May to December (Hartshorne, 1981). Although rainy-season thunderstorms are common, the country is outside the hurricane belt. According to the U.N. FAO (2011), nearly 40% of Panama was wooded though tree cover has been reduced by more than 50% since the 1940s. The country hosts at least 10,000 species of vascular plants, of which 12.3% are endemic and 6.5% are protected under IUCN categories I-V.

Of the twelve Holdridge Life Zones in Panama, more than 75% of the country's forest are categorized as: Tropical rainforest, Tropical wet forest, Tropical moist forest and Tropical dry forest (Hartshorne, 1981). In the country, growth and ecological behaviour of different plant species have been reported in natural forests, disturbed areas, reforestation and potted experiments (Leigh *et al.*, 2014; van Breugel *et al.*, 2013; Craven *et al.*, 2018). Big trees giving structure to forest and timber species have been examined for long time, including *Swietenia macrophylla*.
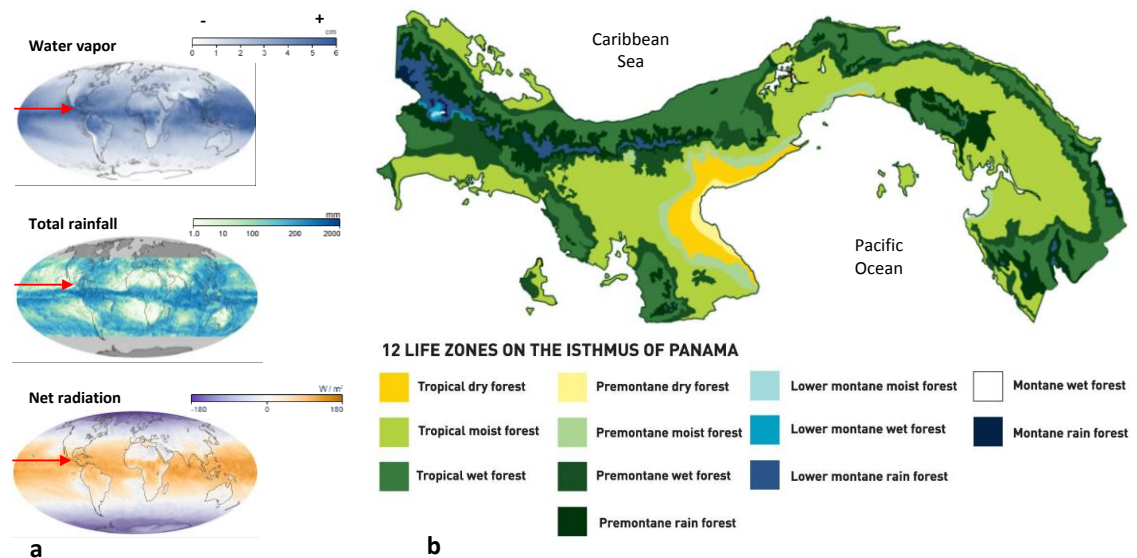
Figure 1.1. Climatic and vegetation evaluations of the Republic of Panama. Global information about climatic variations in the world (a), marking with a red arrow Panama's world situation (info: Global maps from NASA Earth Observatory). Panama forest composition (b), using Holdridge classification (info: Smithsonian Tropical Research Institute platform).

1.1.3. *Swietenia macrophylla*

Swietenia macrophylla King is a species belonging to the *Meliaceae* family, known world-wide as mahogany. In total, the group is formed by 23 species, but in Panama and Costa Rica three are described: *S. macrophylla*, *S. mahagoni* and *S. humilis* (Correa, Galdames & Stapf, 2004; Alvarenga & Florez, 1987). The big-leaf mahogany is a large tree (20-40 m) that is found in wet and dry forests, from Mexico to Brazil. The species is found in low density and can grow on low-ground soils composed mainly on silty loams, acidic and low in nutrients (Norghauer *et al.*, 2008) but in Panama prefers well drained soils with good levels of nutrients (Hall & Ashton, 2016).

Distribution of *S. macrophylla* in Panama is reported between 0-1000 m over sea level (Correa *et al.*, 2004). Carrasquilla (2006) describes *Swietenia macrophylla* ecotypes of Panama as large canopy trees with compound, paripinnate leaves with 6-10 ovate to elliptic leaflets, disposed in alternate positions, that shed all its leaves once per year during the dry season (from November to May). It has capsular, woody fruits of 11-18 cm, bearing five carpels with average of 60 seeds (20 mm long and 9 mm wide) per fruit that are dispersed by air during fruit dehiscence (Carrasquilla, 2006; Correa *et al.*, 2004; Roman *et al.*, 2012) (Fig. 1.2).

For nurseries, seeds need to be collected directly from the tree or from the floor but appear to lose their viability quite soon (Roman *et al.*, 2012). It often does not regenerate successfully after harvesting and studies have shown that it depends on shade, seed position on the soil and watering (Morris *et al.*, 2000; Negreros-Castillo *et al.*, 2003). Studies in artificial plantations in Panama showed high survival of plantlets, and after two years of growth, crowns were relatively small when compared to other species growing together. Trunk diameter after 2 years vary between 3,5 - 6,7 cm depending on the soil type, while crown diameter was about 1 m (Hall & Ashton, 2016). In Costa Rica, young seedlings present higher mortality rates during dry months of the first year (Gerhardt, 1996). Seedlings establish easier in understory than in full canopy gaps. Tree crowns regularly emerge from the forest canopy, but this is possible when they are healthy in their juvenile status; plantlets are often attacked by one specific plague, *Hypsipilla grandella*, common for all Meliaceae (Roman *et al.*, 2012; Hall & Ashton, 2016).

Swietenia has an asynchronous flowering pattern for each inflorescence, branch, even between trees. Only if enough trees are in flowering phase at the same time, opportunities for mating will not constrain genetic diversity within the progeny. Field experiments in Brazilian Amazonas (Grogan & Loveless, 2013) found that the smallest tree observed to flower at any site had 13.8 cm stem diameter, but in general, only trees over 30 cm diameter were blooming. After germination, seedlings have to establish and grow for 2-5 years to flower. Small trees don't produce flowers every year and some have a 5-year rhythm. As tree diameter increased, annual flowering became more frequent, but even some trees that were 30–60 cm diameter did not flower every year. This is important for the conservation of genetic diversity in forest and to establish nurseries for reforestation and greenhouse studies.

Big-leaf mahogany is the most valuable widely traded neotropical timber species. It was harvested and traded since the 16th century to craft luxury items and furniture (Langbour *et*

al., 2011) This species has been heavily logged in primary forests throughout its natural range from Bolivia to the Yucatan Peninsula of Mexico (Alvarenga & Florez, 1987, Negreros- Castillo & Mize, 2014). Since 2002, mahogany has been listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora -CITES (Hall & Ashton, 2016). Each country has regulations for logging in natural populations, but studies have detected that the regression equation used for the model are inaccurate and do not support flowering cycles and gene flow to support natural regeneration (Grogan *et al.*, 2014).

Source: naturoloveyou.sg

Source: skyfruitseed.com

Source: Flickr /
Mauricio Mercadante

Source: ndrc.org
Alamy Stock Photo

Source: NurseryLive.com

Figure 1.2. *Swietenia macrophylla* King. Habitus, leaf disposition, position as emerging tree, seeds and fruit.

Stocks for timber industry are reducing constantly, thus reforestation projects could relieve forests from logging pressures. Evaluation on germoplasms from 67 trees of a various populations of big-leaf mahogany show difference only on relative height growth rate; higher tolerance to *Hypsipylla* has not been detected in any particular group but a wide range of tolerances was reported (Wightman *et al.*, 2008). Wood quality and yield have been evaluated in natural and commercial communities (Moya & Munoz, 2010), comparing it also with common introduced timber species, to find that profitability of *Swietenia* is comparable or higher than *Tectona* or *Pinus* (Griess & Knoke, 2011), even when the attack of *Hypsipylla* cannot be avoided. *Swietenia* has been generally evaluated as a sensitive species towards the environmental conditions due to its low-density distribution in forest. Nevertheless, punctual studies have demonstrated tolerance toward high irradiation (Azevedo & Marenco, 2012; Abdala-Roberts *et al.*, 2014), drought stress (Cordeiro *et al.*, 2009) and heavy petroleum contamination (Perez-Hernandez *et al.*, 2013).

In terms of intra-specific genetic variation in mahogany species, Styles and Khosla (1976) reported the presence of diploid (2n=54) and tetraploid (2n= 108) varieties of *S. macrophylla*; in addition, these authors mention the identification of a polyploid series for *S. mahagoni* in

plantations in Fiji, with chromosome numbers varying from 2n = 12 to 2n = 60. There are suggestions pointing out that *S. macrophylla* and *S. humilis* could be ecotypes of the same species, since natural hybrids from these two species have been found for 100 years, with intermediate characteristics of both progeny lines. Hybridization is spontaneously happening between the tree concurrent species, following mendelian relation of segregation 1:2:1. In total, there is consensus that *Swietenia macrophylla* is a polyploid species with variable chromosome number (24, 46, 54, 60 or 108). As explained above, this species is completely natural, far away for domestication attempts. The high distance from the ground to where flowers are situated (and afterwards seeds) make it difficult to keep track of parenting cross information, and the enormous amount of seeds produced at the same time could present problems to determine parent tree if two or more trees are close to each other.

Besides its value as a timber species, *Swietenia macrophylla* has been identified as a source for high levels of antioxidant and other compounds with potential benefits for human health. Ethyl acetate fraction from seeds show neuronal protection against oxidative stress (Sayyad *et al.*, 2017) while another polar fraction proved promising results as inhibitor for snake venoms (Henao-Duque, 2017). Moreover, 27 phenolic compounds were identified from aqueous leaf extractions with potential in neurodegenerative diseases like Alzheimer and Parkinson (Pamplona *et al.*, 2015). Bark contains tannins used for leather tanning (Herbarium Universidad de Panama).

1.1.4. *Jatropha curcas*

Jatropha curcas was first described by Carl von Linné in 1753 (Missoury Botanical Garden) and is a species belonging to the Euphorbiaceae, a family in which C4 and CAM photosynthesis have evolved. Historically classified as a C3 plant, Winter & Holtum (2015) proved that it has incipient CAM metabolism at specific moments. It is accepted to be native to Central America with important diversification between Mexico and Brazil. It was then brought to the old world by Portuguese sea farers and has since then conquered many ecosystems in Africa, South-East Asia and India (Loureiro *et al.*, 2013; Maghuly & Laimer, 2013; Dias *et al.*, 2012; Koh & Ghazi, 2012). Islam *et al.* (2011) highlight that in general the term "Jatropha" is used to refer to the species *J. curcas*, although there are approximately 170 known species of the plant. The Mesoamerican region presents the greatest genetic diversity population (Ovando-Medina *et al.*, 2011).

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The habitus for this plant is a described as a tall bush or a small tree (up to 6 m height). Has a straight trunk with thick branchlets containing a milky latex, and green alternate leaves with five lobes and a length and width of 6 to 15 cm (Islam *et al.*, 2011; Foidl *et al.*, 1996; Openshaw, 2000), (Fig. 1.3). The plant is mostly monoecious; the inflorescence has 20-60 male flowers per each female flower (Juhász *et al.*, 2009; Rincon-Rabanales *et al.*, 2016), with variations depending on the genotype (Ahmad and Sultan, 2015). Flowers can be cross pollinated with the help of honeybees (Wang & Ding, 2012) and further 36 different insect species, but self-pollination is most common (Rincon-Rabanales *et al.*, 2016). The fruits have an oval shape with three lobes, about 40 mm length and contain 3 seeds (on average), which look like black beans with similar dimensions, of about 18 mm long and 10 mm wide.

Source: B & T World seeds   Source: Quora.com   Source: REUK.co.uk

Source:http://sciencebeingjournal.com

Figure 1.3. *Jatropha curcas* **L.** Habitus, seeds, inflorescence and leaf disposition.

Jatropha seedlings can grow in a wide range of regions, including tropical, subtropical and temperate zones (King *et al.*, 2009). It appears to be more adapted to drier regions in the tropics but is found growing in a broad range of rainfall regimes (250 - 1200 mm/ year), mostly in lower altitudes (0 - 500 m over sea level) with temperatures above 20 °C (Katwal and Soni, 2003; Islam *et al.*, 2011). The lifespan of this perennial plant is about 50 years, it can grow on degraded soils with low fertility and moisture, including stony, sandy, shallow, saline and calcareous soils (Islam *et al.*, 2011; Foidl *et al.*, 1996; Openshaw, 2000; Koh & Ghazi *et al.*, 2012). It can start producing seeds 9 months after germination, with significative yields after 3 years (Islam *et al.*, 2011). Blooming is non-synchronous, induced by rains, but plants can produce fruits 2-3 times per year (Rincon-Rabanales *et al.*, 2016), with fruits in different maturation stages at the same time.

The best moment for collecting fruits, as well as seed storage conditions are shown to be related to the final use of the seed. For germination, the best moment is when fruits are yellow (Ahmad & Sultan, 2015; Zavala-Hernandez *et al.*, 2015), approximately 78 days after anthesis.

But for oil extraction, fruits should be collected from the tree when they are brownish-black, reaching up to 48% oil content (Ahmad & Sultan, 2015) including mostly oleic and linoleic acids. Low seed viability is a problem for establishing plantation by seed propagation. Studies on seed morphology were still scarce five years ago, when Lureiro *et al.* (2013) made a physio-anatomical characterization of all developing stages of *J. curcas* seeds. *J. curcas* seeds are genetically heterozygous which results in high degree of variation. Studies on genotype variability based on physical characteristics was done juveniles (Nietsche *et al.*, 2015), using fifteen accessions and finding modifications between them in traits as inflorescence type, fruit and seed production, oil content and leaf morphology.

Besides traditional uses in medicine and as a living fence, *Jatropha curcas* has become a potential option for biodiesel production. It was catalogued as one of the most important species for biofuel production in Brazil, India, Cuba and many other countries. That is due to high yield of oil production from its seeds, which consist mainly from oleic and linoleic acids (unsaturated fatty acids).

There is an actual debate about the use of plants for producing biodiesel. Currently, the most common used plant species are soybean, rapeseed, canola, sunflower, palm, coconut and corn. There are organizations claiming that those crops should be used primary/only for the food industry (Koh & Ghazi *et al.*, 2012). *Jatropha* does not fall into this category, because almost all of the reported genotypes are toxic for human and cattle consumption (Maghuly & Laimer, 2013; Sudhakar, 2011). The oil extracted from its seeds has low acidity, good stability (compared to soybean), low viscosity (compared to castor) and better cold properties than palm oil. Besides, *Jatropha* oil could be a good alternative fuel with no modifications required in the engine (Koh & Ghazi *et al.*, 2012).

Jatropha curcas has a relatively small diploid genome. Carvalho *et al.* (2008) described 22 chromosomes (2C DNA content of 0.850 ± 0.006 pg or 1C DNA content of 0.416 × 109 bp). *Jatropha* has not yet been completely domesticated, which means that seeds do not produce stable genotypes. So far, most of studies are made with self-collected seeds, directly from the tree. Achten *et al.* (2010) estimate that it will require a minimum of 15 years of conventional breeding before *Jatropha* reaches a level of domestication, but this period could be shortened with the use of transgenesis (Maghuly & Laimer, 2013).

1.2. Environmental stress

Since the beginning of times, living beings have faced challenges in the environment they have lived in. While animals and other motile organisms are able to walk away from their houses when it's not comfortable and secure anymore, plants have to adapt to this situation in another way. Challenges in the environment can include positive and negative affectation of plants. These effects can be products of punctual interaction in one moment of their lives (like during seed development), or during their entire life (like seasonal changes). For all cases, plants have developed chemical modifications to achieve physiological changes, to be fit for those challenges. Many times, these modifications are encoded in the genome and need to be activated, but sometimes it is only possible a partial acclimation during the plants life period which can conduce to long-term adaptation and speciation.

Challenges posed by the environment over the vegetation can be classified on the source of disturbance, as biotic or abiotic stress. Defense against biotic stresses like herbivory, display visible mechanisms such as thorns, trichomes and latex (Wright and Bonser, 1999). Defence against abiotic stresses are mostly hidden to the naked eye, having a biochemical background.

The increases of CO_2 concentration and temperatures have deep impacts on the physiological functioning of plants that modify interactions between plants and other organism, affecting ecosystems. Climate change poses a selective pressure over plants, having the potential to influence evolutionary processes (Beckling *et al.*, 2016).

1.2.1. Abiotic Stress

Abiotic stress includes all things that could affect plants, coming from the atmosphere and the soil where they are situated. The most commonly studied abiotic stresses are: extreme levels of light (high and low), radiation (UV-B and UV-A), temperature (high, low and freezing), water (drought and flooding), heavy metal concentrations, soil pH, salinity (excessive Na+), essential nutrient availability, gaseous pollutants and winds (Pereira *et al.*, 2016). Some adaptations considered as defence mechanisms against herbivores have proved also to be related to environmental stress (da Silva, 2017).

Tolerance and sensitivity towards abiotic stress is unequally distributed among plant species (Beritognolo *et al.*, 2011). There are several studies suggesting that plants spatial distribution

is caused by the tolerance to fresh water availability, shadow, salinity and temperature, all of them directly related to drought tolerance.

1.2.2. Drought tolerance

The osmotic condition in plants is related to the amount of water inside the plant and outside. Regulation of stomata opening has a direct consequence over water balance, because as they open to let in CO_2, they also let the H_2O inside of the plants escape to the atmosphere. Stomata are controlled by salt concentrations in the guard cells; a big loss of water can occur while opening stomata if the vapor pressure in the air is low. Under low water availability in the soil, plants might need to close their stomata; this has to be regulated to reduce photosynthesis as little as possible. Direct sun irradiance can heat up leaves in the tropic, so plants normally open stomata to refresh their surfaces; depending on the frequency, this cooling effect can sum up a problem of drought.

Many tropical forest trees display a pronounced reduction in net photosynthesis and stomatal conductance during midday, that is described directly as an adaptation to high levels or irradiation during midday hours, independent of soil moisture (Cernusak *et al.*, 2013). Net photosynthesis (NPP) can be calculated as the gross primary production minus respiration during the given time, or as the addition of the changes in plant biomass to the non-respiratory carbon allocations, such as reproduction, herbivory, exudation of compounds in the root, defence molecules, etc. (Cernusak *et al.*, 2013). Leaf dark respiration represents more than 30% of plants autotrophic respiration (Chamber & Silver, 2004; Cernusak *et al.*, 2013). Carbohydrates produced in excess during photosynthesis will be consumed by anabolic processes, including accumulation as non-structural carbohydrates (Cernusak *et al.*, 2013).

1.3. Photosynthesis

Photosynthesis is strongly affected by abiotic stress because it requires water and CO_2 to be available. Consequently, variation in their availability will affect metabolism. Most analyses about plant responses to elevated CO_2 has been carried out in template zones, making tropical forest responses less understood so far (Körner *et al.,* 2009). Climate change research predicts enormous modifications in water availability and CO_2 content, keeping the future for tropical forests and its species composition unknown. Considering that tropical plants kept their metabolism adapted to 300 ppm for 8,000 centuries and needed to endure 33% increment of this concentration in 3 centuries due to the industrialization era (Lüthi *et al.,* 2008), it can be

agreed on, that plants are currently under elevated adaptation pressure. Besides, the Intergovernmental Panel of Climate Change (2013) predicts CO_2 will rise over 800 ppm in 2100 (+160% in 4 centuries), including a total increase of 4°C. This is an interesting time to evaluate all modifications that might be happening to cope with all this.

1.3.1. Leaf adaptations

As a defence against drought stress, there are at least two forms of adaptations in leaves: physical and biochemical changes. Physical changes include blade movement and orientation, thicker cuticles, wax layers, trichomes, etc. Some of these modifications act primarily as defences against pathogens, but all of them have also an important role to avoid undesirable water loss.

Biochemical changes include morphological aspects like thicker palisade or mesophyll cells, stomatal distribution and reorganization of vascular tissue but also metabolic modifications such as in photosynthesis. Plants developed different strategies of CO_2 capture with maximum water efficiency in short time, and in the same way as different morphological adaptations are found in related species, the same occurs with the metabolic adaptations.

1.3.2. Photosynthesis pathway

Most tropical plants use the C3 photosynthesis pathway. Abiotic factors, such as unfavourable conditions of light intensity, temperature, CO_2, humidity, water and mineral supply (Schulze *et al.*, 2002), are environmental factors that can restrict C3 plant development, but that are somehow overcome by other pathways. Responses to environmental changes vary among natural and cultivated species.

Species with C4 photosynthetic metabolism often have very low photorespiratory activity and are usually more efficient in the use of water. This confers these plants a greater tolerance to water deficiency, salinity and higher temperatures (Sage and Kubien, 2007; Stepien and Klobus, 2005; Yoshimura *et al.*, 2004; Ward *et al.*, 1999). However, Sage and Mcnkown (2006) have noted that C3 species tend to display greater phenotypic plasticity of photosynthesis than C4 species. (Vitolo *et al.*, 2012). CAM species avoid water loss by keeping stomata closed during the day, and while it is a trait that increases water use efficiency, it involves a lot of energy in the process.

C4 and CAM species have a higher reproductive efficiency than C3 species because of their higher water- and nitrogen-use efficiency (Cernusak *et al.*, 2013, Ramirez *et al.*, 2017). Reproductive efficiency was negatively related to the cost of reproductive structures in C3 species. Besides the traditionally acknowledged relationship with resistance to stressful environments, reproductive strategies of C4 and CAM plants could be also related to an increment in plant reproduction (Ramirez *et al.*, 2017).

Nevertheless, the C3 pathway has in principle, the greatest potential to benefit from increases in CO_2 concentrations due to climate change in the tropics (Cernusak *et al.*, 2013). Benefits include increase in net photosynthesis from approx. 20 µmol CO_2 m^{-2} s^{-2} under current concentrations to 40 µmol CO_2 m^{-2} s^{-2}, when CO_2 concentrations reach 900 ppm, and a reduction in stomatal conductance at 700 µmol CO_2 m^{-2} s^{-2} in 10 forest tree species (Cernusak *et al.* 2013, Winter unpublished). CO_2 concentrations determine stomata opening and lower stomatal conductance results in less water loss by transpiration (Cernusak *et al.*, 2013).

To protect photosynthetic activity, several antioxidant molecules were developed and differentially controlled. Their regulation is reported to be variable between many species, sometimes providing extra tolerance to drought. It is not yet clear if there is a strict correlation in antioxidant system regulation and photosynthesis pathways or if it is a random diversification. There is also the possibility that the regulation of the antioxidant system can be a gradual acclimatization to stress that will finish in changing into the next pathway. Analysis of stress tolerant genotypes from multiple plants species will probably reveal novel tolerance and selective mechanisms in natural populations (Pereira *et al.,* 2016).

1.4.   Reactive Oxygen species production

Over 2.2 billion years ago, the first communities of cyanobacteria started to photosynthesize. Big amounts of oxygen were "discarded" into the air and after some chemical processes, it started to accumulate (Halliwell, 2006). During chemical water split, the liberated energy can generate reactive oxygen species in a cascade of reactions. Previously identified only as toxic products, today they are also recognized as regulatory factors for many biological processes. Nowadays it is clear that there is a major system controlling the redox state of cells, that plays an important role in regulating enzyme activity and many other cellular processes (Buchanan and Balmer 2005, Hisabori *et al.*, 2007). The generation of reactive oxygen species starts with

ground state oxygen, O_2, and organisms over the surface were forced to developed adaptations to defend against O_2 and use its oxidative power.

1.4.1. Types of Reactive oxygen species (ROS)

- **Singlet oxygen**

O_2 electron configuration changes if they take energy from chlorophyll triplets that are produced due to insufficient energy dissipation in the chloroplast (Krieger-Liszkay, 2005), resulting in **singlet oxygen.** $^1O_2$ is highly reactive and can interact with most normal molecules, damaging proteins, lipids, DNA, among others (Halliwell, 2006; Mittler, 2002, Wagner *et al.*, 2004). In chloroplasts, $^1O_2$ can damage D1 proteins and decrease photosynthesis (Trebst *et al.*, 2002). To prevent these destructive effects, plants accumulate low molecular weight antioxidants (e.g. β-carotene and α-tocopherol) that quench $^1O_2$ (Telfer, 2002; Trebst *et al.*, 2002).

- **Superoxide radical**

When one electron is added to O_2 (Mahler reaction), a **superoxide radical** (O_2^*) is produced (Mehler, 1951). The superoxide radical has a moderate activity with a short life-time (2-4 μs) and restricted movement (Halliwell, 1976; Fridovich, 1986). Superoxide can be produced at PSI (Asada *et al.*, 1974) but photosystem II (PSII) seems to be more sensitive (Krieger-Liszkay *et al.*, 2011). Having both, oxidizing and reducing properties, it generates the formation of more reactive ROS via Haber-Weiss (Fenton) reactions and singlet oxygen (Haber and Weiss, 1932; Fridovich, 1986a; Halliwell, 2006), which is considered its most toxic effect (Benov, 2001).

- **Hydroperoxyl**

When superoxide gets protonated, the new molecule is called **hydroperoxyl** (HO_2^*). These radicals can cross membranes and initialize lipid autoxidation (Halliwell and Gutterigde, 2000).

- **Hydrogen Peroxide**

One electron extra to the superoxide radical produces O_2^{2-}, called peroxide ion. In biological terms, it corresponds to H_2O_2, the **hydrogen peroxide**. This molecule by itself is not so reactive since it's not a free radical, but its danger relies on its instability due to a weak oxygen-oxygen bond (Halliwell 2006). It is produced spontaneously in aqueous solution or by detoxification of superoxide (Asada, 1999, Halliwell, 2006). In plant cells the main sources of H_2O_2 are electron transport chains of chloroplasts and mitochondria, β-oxidation of fatty acids and

photorespiration (Asada, 1999; Foyer and Noctor, 2000; Wingler *et al.* 2000). H_2O_2 is a long-living molecule (approx. 1ms) with moderate activity and the ability to diffuse and be actively transported across membranes via aquaporins (Bienert *et al.*, 2006), generating damages and inactivating enzymes away from the generation site (Halliwell, 2006).

- **Hydroxyl radical**

Hydrogen peroxide exposed to UV light can react with soluble iron (Fe^{2+}) and copper (Cu^{2+}) and form **hydroxyl** ($HO^{\bullet}$) through the Fenton reaction, discovered in 1876. $HO^{\bullet}$ has indiscriminate reactivity towards biological molecules (D'Autreaux and Toledano, 2007) and its excess results in programmed cell death (Halliwell, 2006; Moller *et al.* 2007).

- **Ozone**

In case no electron donor is available, UV light can affect O_2 by breaking the oxygen-oxygen bond, first producing O^{*-}, that by oxidizing power will add to a normal molecule of O_2, producing **ozone** (O_3). This by principle is a non- free radical but can easily be converted into one.

1.4.2. Cellular damages

Reactive oxygen species are permanently produced in chloroplasts, mitochondria, peroxisomes, the endoplasmic reticulum, apoplast, cell wall and plasma membrane (Mittler *et al*. 2002). ROS production and scavenging must stay balanced to maintain cell homeostasis (Foyer *et al.* 1994; Hurry *et al.,* 1994; Mittler, 2002; Ensminger *et al.,* 2006). High amounts of ROS are produced as by-products during photosynthesis; at excess of energy, the electrons are transferred from photosystem I to oxygen generating **superoxide anions** (Mehler 1951, Asada *et al.,* 1974). High stress levels lead to increased superoxide production, which can accumulate and convert to **hydrogen peroxide** spontaneously or by oxidizing sulphur-containing amino acids (cysteine and methionine) in enzymes of the Calvin cycle and superoxide dismutases (Cu/Zn-SODs, Fe-SOD) (McCord and Fridovich, 1969; Marklund, 1976; Kurepa *et al.,* 1997). In addition, hydrogen peroxide can oxidize proteins and lipids (Kristensen *et al.*, 2004; Groen *et al.*, 2005).

Singlet oxygen from **chloroplasts** affects membrane proteins, lipids and DNA near the site of production (Asada, 2000). It can interact with some molecules to release free metal that can be used to generate **hydroxyl radicals.** The latter interact with all biological molecules and cause big cellular damages such as lipid peroxidation (Schopfer, 2001; Bailly, 2004) and can

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ultimately lead to cell death (Pinto *et al.* 2003). H_2O_2 and superoxide anions destructive characteristics are primarily due to their contribution to the hydroxyl radical production rather than the direct damage (Karuppanapandian *et al.,* 2011).

1.4.3. Signalling role

Beside this damaging capacity, ROS are important signalling molecules (Maruta *et al.,* 2012). Mittler *et al*. (2004) defined more than 152 genes in *Arabidopsis* that are tightly regulated in the ROS gene network and that affect plant growth and cell metabolism. This has been tested for the inside and outside of chloroplast (Asada, 1999; Foyer and Noctor, 2000; Maruta *et al.,* 2012), mitochondria, peroxisomes and less endangered organelles as cell membrane, vacuole and cytosol. Plant stress hormones such as salicylic acid (SA), jasmonate (JA) and ethylene (ET) are located downstream of the ROS signalling pathway and can be activated to act as a secondary messenger (Ahmad *et al.*, 2016; Kawano, 2003).

Hydrogen peroxide has a retrograde signalling function when it is at low concentrations (Maruta *et al*., 2012). It can diffuse due to modulated changes in membrane permeability or by transport through aquaporins (Bienert *et al.,* 2006). **Hydroxyl radical**, HO$^{\bullet}$, despite very short half-life and high toxicity, seems to operate in H_2O_2 sensing (D'Autreaux and Toledano, 2007).

Even if there is accumulation of the same molecule, the plant is not sending and receiving the same message. While H_2O_2 produced in peroxisomes induces transcripts involved in protein repair responses, when it is produced in chloroplasts induces early signalling responses, including transcription factors and biosynthetic genes involved in production of secondary signalling messengers (Sewelam *et al.,* 2014).

1.5.   Antioxidant system

To avoid cellular damage, plants have evolved a highly efficient antioxidant system (Asada, 1999). Antioxidants are substances that, in small amounts, react with ROS and detoxify them. Especially in high fluctuating environments, strong detoxification systems are needed to work in cellular compartments where ROS are produced (Asada 1999, Noctor and Foyer, 1998). Besides the low molecular weight scavengers, there is a highly effective antioxidant system that regulates ROS balance inside the cell, consisting of enzymatic components.

1.5.1. Non- Enzymatic antioxidant system

The first prevention step to reduce ROS accumulation depends on low molecular weight molecules, such as tocopherol, carotenoids and flavonoids. **Tocopherols** are lipid soluble antioxidants localized in cell membranes, including the thylakoids, to avoid the formation of chlorophyll triplets by quenching singlet oxygen (Halliwell, 2006). **Carotenoids** are lipophilic light harvesting pigments situated in chloroplast, that absorb energy in the range 450-570 nm and quench singlet oxygen (Xiao *et al.*, 2011); they are precursors of ABA biosynthesis. **Flavonoids** are scavenging hydrogen peroxide and hydroxyl radicals in the vacuole (Arora *et al.*, 2000); other common phenolic compounds (e.g. tannins, lignin) similarly possess antioxidant properties.

Ascorbate and glutathione are other important low molecular weight antioxidants (Asada, 1999). **Ascorbate** can be used as substrate by diverse peroxidases, like ascorbate peroxidases (APX) (Asada, 1992) and is involved in regeneration of tocopherol (Mukai *et al.*, 1991). It is the most abundant antioxidant molecule, reaching concentrations of 10-100 mM (Halliwell 1976; Noctor and Foyer, 1998). **Glutathione** is synthetized in two steps catalysed by GSH1 from the chloroplast and GSH2 from the cytosol (Rennenberg, 1982; Halliwell, 1986). It is capable of detoxifying singlet oxygen, superoxide anions, hydroxyl radicals, and hydrogen peroxide (Foyer and Halliwell, 1976) while being regenerated by the ascorbate-glutathione cycle (Noctor and Foyer, 1998).

1.5.2. Enzymatic antioxidant system

The enzymatic antioxidant system includes peroxiredoxins (PRXs), ascorbate peroxidases (APXs), glutathione peroxidases (GPXs), catalase (CATs) and superoxide dismutases (SODs). There are also enzymes required to regenerate substrates to active forms such as monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHR) and glutathione reductase (GR).

- **Peroxiredoxins (PRXs)**

Peroxiredoxins are thiol (non-heme) peroxidases that are localized in the nucleus, cytosol, mitochondria and chloroplasts, present in all kingdoms, with similar sequences and catalytic mechanisms (Baier & Dietz, 1997; Horling *et al.*, 2003). Peroxiredoxins are comprised of three classes: (1) Typical peroxiredoxins, 2-Cystein-Peroxiredoxins (2CP), (2) Non-typical peroxiredoxins, (type II PRX and peroxiredoxin Q) and (3) 1- Cystein-Peroxiredoxin (1CP).

Among the peroxiredoxins, 2CPs (two isoforms in Arabidopsis, 2CP*a* and 2CP*b*) PrxQ and Prx IIE are situated in the chloroplasts (highly abundant) while the others are located in other cellular compartments (Horling *et al.*, 2003). They can reduce H_2O_2 to water but have a broad substrate specificity (Baier and Dietz, 1997). 2CP are functional homodimers that form disulphide bonds, also involved in H_2O_2 signalling (Baier *et al.*, 2004).

- **Ascorbate peroxidases (APXs)**

Ascorbate peroxidases are heme-binding enzymes that catalyse the reduction of hydrogen peroxide (H_2O_2) to water and molecular oxygen at the expense of ascorbate, which they oxidize to monodehydroascorbate radical (MDHA). The latter subsequently disproportionate into dehydroascorbate (DHA) in a spontaneous reaction (Asada, 1999). APXs are localized in the cytosol, peroxisomes, chloroplasts and cytosol. They are nuclear encoded and post-translationally targeted to chloroplasts (Asada, 2000). In the chloroplast, there is one soluble isoform in the stroma (sAPX) and one isoform bound to thylakoid membranes (tAPX) that were differentiated early in plant evolution (Pitsch *et al.*, 2010). In *Arabidopsis thaliana* those two plastidic isoforms are encoded in two different genes, but in other plants they are encoded in one single gene that is spliced alternatively (Ishikawa *et al.*, 1999). Its function depends on availability of ascorbate as a substrate and under low concentrations, they are reportedly inactivated by H_2O_2, suggesting that its sensitivity towards H_2O_2 can allow it to act as signalling molecule (Shigeoka *et al.*, 2002). sAPX and tAPX enzymes for *Arabidopsis* are 33 kDa and 38 kDa proteins, respectively, with increased activities during environmental stress such as drought (Sharma and Dubey, 2005).

- **Glutathione peroxidases (GPXs)**

Higher plant GPXs are non-selenocysteine enzymes (Rouhier and Jacquot, 2005) which catalyze, like PRX, the reduction of hydrogen peroxide (H_2O_2) to water and lipid hydroperoxide to alcohol using small thiol-proteins like thioredoxins as the electron donor (Navrot *et al.*, 2006, Margis *et al.*, 2008). In *Arabidopsis thaliana*, GPXs are encoded by a gene family consisting of eight genes, from which two are localized in the chloroplast (GPX1 and GPX7) (Margis *et al.*, 2008; Chang *et al.*, 2009). Those are small proteins with a molecular weight of approximately 25 kDa and sharing 82% sequence similarity (Milla *et al.*, 2003). GPX1 is located in the thylakoid membrane or in the stroma, while precise location of GPX7 is unclear (Meyer *et al.*, 2005). Chloroplast GPX have an important regulatory and protective role during acclimation to photooxidation and other abiotic stresses. (Milla *et al.*, 2003; Chang *et al.*, 2009).

- **Catalases (CATs)**

Catalases are heme-containing enzymes localized in peroxisomes (Mittler, 2002; McLung, 1997). They have the highest efficiency rates amongst all H_2O_2 reducing enzymes (one molecule CAT can reduce approx. six million H_2O_2 molecules per minute to water) with a relatively high specificity for this ROS species, but acting weak on organic peroxides (Mhamdi *et al.*, 2012). There are three gene isoforms of CAT in *Arabidopsis* expressed and regulated independently (Frugoli *et al.*, 1996).

Environmental stressors can modulate CATs activity. While plants under cadmium stress showed reduced activity (Balestrasse *et al.*, 2001), its activity was increased in plants under drought stress (Luna *et al.*, 2005).

- **Superoxide dismutases (SODs)**

SODs are metalo-enzymes located in the cytosol, peroxisomes, mitochondria and chloroplast (Bowler *et al.*, 1992), where they act as the first oxidative defence against ROS (Scandalios, 1993). Classification of superoxide dismutases is based on their metal cofactor (Bowler *et al.,* 1992; del Rio *et al.*, 1983). *Arabidopsis thaliana* has three iron SOD isoforms (Fe SOD), one manganese SOD (Mn^{2+} SOD) and three copper-zinc SODs (Cu/Zn SOD). They are all encoded in the nucleus and are subject to post-translational allocation to specific compartments. One Cu/Zn SOD and three Fe SOD isoforms are located in the chloroplast (Bowler, 1992). Chloroplast Cu/Zn SOD, CDS2, is composed of two identical 16 kDa units, each containing one atom of Cu and Zn (Kitakawa *et al.*, 1991). Superoxide radical ($O_2^{\bullet}$) is able to accept one electron and two protons resulting in the formation of H_2O_2. This reaction that limits its high radical activity can take place either spontaneously or catalysed by superoxide dismutase (SOD) (Asada, 1999; Halliwell, 2006). SODs activity has been reported to increase in plants exposed to various environmental stresses such as drought (Sharma and Dubey, 2005; Zlatev *et al.*, 2006; Regier *et al.*, 2009), salinity, metal toxicity and UV-B stress.

1.6.   Molecular evaluation of species / State of arts

Evaluation of gene expression in response to abiotic stress reveals that a large portion of the genome can be affected, reflecting plasticity in response and protection. For the plant species under investigation, the molecular and genetic work already performed varies.

1.6.1. *Swietenia macrophylla*

Besides early studies on karyotype description, little research has been done in *Swietenia*. In GenBank from NCBI there were a total of 109 sequences belonging to *Sweitenia* and 43 "related sequences" from other organisms listed under this species, after last check (June 2018); including mostly partial sequences used for phylogenetic studies, barcoding and timber identification. For the chloroplast there are reported some partial sequences including psbB, psbA, D1, NADH plastoquinone oxidoreductase and ATP synthase. Nevertheless, there was reported no information on gene loci, functional genomics, gene expression, molecular abundance profiles or clusters of expressed transcripts.

Genetic structure of population has been assessed via microsatellite analysis (Lemes *et al.*, 2002; 2003, and 2011; Roth *et al.*, 2003; Andree *et al.*, 2008; Alcalá *et al.*, 2015) and pairwise genetic differentiation (Alcalá *et al.*, 2014). Two populations of the pacific area of Costa Rica were distant from each the other groups and from each other, while there was a third group in the Atlantic coast of Costa Rica -Nicaragua, and a fourth group for populations in Mexico, Belize and Guatemala (Roth *et al.*, 2003).

Some proteins extracted from *S. macrophylla* have been tested with different molecular methods for proving efficiency in glucose uptake in muscle cells (Lau *et al.*, 2015), effects on colorectal carcinoma cell (Goh *et al.*, 2014) and chronic hepatitis C virus.

Genome

The genome has not been sequenced yet. High-throughput sequencing analysis of the transcriptome of some neotropical was achieved recently (Brosseau *et al.*, 2014), including some species related to *Swietenia macrophylla*. "*De novo*" genome assembly in non-model species from the tropics starts to become a reality and useful tool for molecular analysis in tropical forests.

1.6.2. *Jatropha curcas*

Germplasm was harvested all over the world with basic information on its genome. Genetic diversity between ecotypes inside the species has been characterized using different tools like amplified fragment length polymorphism (AFLP) (Tatikonda *et al.*, 2009), RAPD and ISSR markers (Khurana *et al.*, 2012) and microsatellites (Maurya *et al.*, 2015), indicating high genetic variation within populations and identifying so far, only one non-toxic accession from Mexico (Vasquez-Mayorga *et al.*, 2017). Genotype studies have been done with own native germplasm (Lureiro *et al.*, 2013; Zonta *et al.*, 2014; Tatikonda *et al.*, 2009; Ahmad & Sultan, 2015; Vasquez-Mayorga *et al.*, 2017) and with germplasm from different countries (Sunil *et al.*, 2009; Padonou *et al.*, 2014).

For gene expression analysis and transcript level evaluation, it's necessary to have reference genes that prove stability during tested conditions. For some time, authors have used one gene as a reference out of the common list to normalize other gene expression (Liu *et al.*, 2014; Li *et al.*, 2008) without confirming the stability. So far, three groups have studied stability of reference genes in leaves. Zhang *et al.* (2013) evaluated stability under different developmental stages and different abiotic treatments and showed that more than one reference gene is needed to normalized samples from two different growth conditions. Using two software packages, they considered actin, GAPDH, TUB5, TUB8 and UBQ-like as the most suitable reference genes during desiccation, cold and various combinations. Rocha *et al.* (2016) included other genes in the evaluation, considering GAPDH, EF1a, PP2A2 and UCP as the most stable under salicylic acid (SA), polyethylene glycol (PEG), sodium chloride (NaCl) stress and all factors combined. *BestKeeper* analysis suggests that two to five genes might be needed to normalize gene expression in *Jatropha curcas*. Experiments on young seedlings to evaluate drought stress via qPCR had been normalized so far using UBS (Cartagena *et al.*, 2014), Actin (Zhang *et al.*, 2015) but there has not been performed analysis of reference genes, so an approximation of the proposed genes by Zhang *et al.* (2013) and Rocha *et al.* (2016) could be a good compromise.

Transcriptome analyses are reported for different plant organs (Xu *et al.*, 2015). Gene expression evaluation on seeds during drought stress (Wu *et al.*, 2015) include even Illumina sequencing technology (Jiang *et al.*, 2012). Cartagena *et al.* (2014) developed one oligo-microarray with more than 45,000 sequences in Japan, to observe the responses on the transcriptome level in *Jatropha curcas* seedlings under drought stress finding that 2,214 genes

were affected. Roots and leaves from *J. curcas* seedlings submitted to drought had transcriptional profile analysis (Zhang *et al.*, 2015) resulting in 4103 differentially expressed genes. RNA sequencing was used also to identify the aquaporins (Zou *et al.*, 2016).

Genes from the enzymatic antioxidant system in the chloroplast have been explored vaguely in *Jatropha curcas*. In 2014, one ascorbate peroxidase (tAPX) was fully sequenced (GenBank accession KF560416), fused to a 35S promoter and used for functional complementation (Liu *et al.*, 2014). They used wobble primers to amplify a conserved region while the 5' and 3' region was explored with a RACE PCR. TargetP predicted chloroplast localization for the full peptide that was confirmed by with GFP fluorescence, using protoplasts. Relative gene expression was measured for various tissues using actin as reference gene; highest values were found in leaves. *Nicotiana tabacum* showed increased salt tolerance when overexpressing this sequence. Another ascorbate peroxidase (GenBank accession KF792062) was sequenced "de novo" using wobble primers and RACE PCR (Chen *et al.*, 2015), but without indication of its homologue to *A. thaliana* or possible subcellular localization; transcript levels in *J. curcas* seedlings subjected to salt stress showed elevated levels in leaves. The sequence was cloned into *Agrobacterium* and used for stable transformation of wild-type *A. thaliana*. Seeds of JcAPX overexpressing lines showed enhanced tolerance when growing on MS medium with different NaCl concentrations.

One copper-zinc dismutase (Cu/Zn SOD) was also amplified using wobble primers and RACE PCR (Liu *et al.*, 2015), further sequenced and used for transformation of *A. thaliana* (GenBank accession KF268341). Transgenic lines were tested on MS media with 150 mM NaCl for root length, rosette area, number of leaves and morphology, conferring tolerance.

<u>Genome</u>

The *Jatropha* genome information is available.

The chloroplast genome was completely sequenced (Asif *et al.*, 2010), determined to have 163,856 base pairs, encoding for 130 genes from which 91 are single copies and 17 are duplicated in thee IR region. This allowed the first molecular studies based on specific sequences.

The whole nuclear genome was sequenced using a combination of the conventional Sanger method and new-generation multiplex sequencing methods (Sato *et al.*, 2011) for a total

length of 285,858,490 base pairs of non-redundant sequences. Information on the genomic sequences and DNA markers was available shortly after, at http://www.kazusa.or.jp/jatropha/. The update on this work (Hirakawa *et al.*, 2012) was done using Illumina sequencing platform, and includes modifications and structural features of transposable elements. Based on a comparison between these tentative sequences of transcripts and the predictions of computer programs, a total of 30,203 complete and partial structures of protein-encoding genes were deduced, increasing about threefold from the previous genome annotation. This resources are continuously enriched by newer sequencing results and gene ontology annotation (Tian *et al.*, 2017).

1.7.   Aim of Study

Maintaining high photosynthetic capacity by regulation of ROS concentration during mild stress or by protecting chloroplasts under highly stressful situations is fundamental for plants. The antioxidant system in the chloroplast is one of the first protection mechanisms acting directly at the site of ROS production. Tropical plants represent a large portion of the existing plant species of the planet but remain largely unknown. Moreover, tropical plants face different environmental conditions than their temperate counterparts, thus their adaptations to abiotic stressors could be different.

In this study, gene expression of the enzymatic antioxidant system in the chloroplast was evaluated for two tropical species with different drought tolerances. *Swietenia macrophylla* spends most of its early years in the understory, protected from major climate fluctuations. *Jatropha curcas* is adapted to grow in open zones with high irradiance and big fluctuations. This work was sustained under the hypothesis that there might be a difference in regulation of their antioxidant system, expecting *Swietenia* to be similar to classic descriptions (*Arabidopsis*) based on its strong C3 pathway, while *Jatropha* could have a different regulation, based on the described incipient CAM characteristics.

To do so, the genes coding for the enzymes in the antioxidant system were studied and partially sequenced (for *Swietenia*) or characterized (for *Jatropha*), before evaluating their expression in seedlings submitted to variable environmental parameters, such as drought and irradiance. The aim of this work was to describe the regulation of genes in the enzymatic antioxidant system during drought stress, under field and lab conditions, relating this regulation to the osmotic balance in the soil. At the end, a comparison between the two mechanisms was made, highlighting their similarities and differences.

Chapter 2
Material and methods

2.1. Plant Material

It was performed a total of four experiments for *Swietenia* (E1-E4) and five experiments for *Jatropha* (E1- E5), under different conditions. As there is not a source or institution that can provide seeds with a stable genotype for any of the studied species, it was necessary to collect seeds from both trees during their regular reproduction time, directly under fruiting trees. It was needed to include more than one mother tree in collects, but most of the experiments were performed aiming to have one maternal lineage, if possible.

2.1.1. Material background

Swietenia macrophylla seed were collected from 3 mother trees at natural sites; three of the experiments had one unique maternal background while one experiment (E4) had two maternal lineages in the used samples. Seed collections was done in Gamboa (1 tree close to the Santa Cruz Laboratory) and Panama City, Panama (1 tree inside the University campus and 1 tree around the offices of the Canal Administration Building, in Ancón) all in the Province of Panama, Republic of Panama.

The mother trees of *Jatropha curcas* were situated in Capira (3 trees in the sidewalks of street to the beach), Alto Campana (street from Interamericana highway to the natural reserve, 3 trees) and Panama City (sidewalk in Ave. de La Paz, 1 tree), all in the Province of Panama, Republic of Panama. There was a total of seven maternal lineages used during all phases of the project. Both outdoor experiments, and experiment 5 (indoors in Berlin) have one maternal lineage in their samples, unique for each experiment. E2 and E3 shared the same pool of maternal lineages, constituted by two mother trees. This pool of samples was not possible to divide by lineage because mother trees were too close to each other and it was impossible to determine where each seed came from.

Collect and exportation permits were officially requested and granted by the Department of protected areas and wildlife in the Ministry of Environment of Panama (ANAM), by the Unit of Access to Genetic Resources (UNARGEN). Collect permits numbers are the following:

- Collect permit: SE/P-15-14 (28.05.2004)
- Exportation permit: SEX/P-23-14
- CITES permit: 01421
- Phytosanitary permit: 0085787 (Ministry of Agroindustry Development -MIDA).

2.1.2.   Growth conditions

All experiments in Panama were performed in Santa Cruz Laboratory, part of the Smithsonian Tropical Research Institute, in Gamboa, Ancon department, Republic of Panama. In figure 2.1, the exact position of the experimental area in Gamboa is marked with an arrow, while the growth chamber positions are marked with stars. For *Swietenia macrophylla*, all experiments were performed in Panama, with conditions similar to outdoors, but protected from potential rain by a glass roof. For *Jatropha curcas*, experiments took place in various environments. Two experiments were performed in Panama and three more in Berlin, in the greenhouse. The period of experiments in Panama was for two months in 2015 and 2016 during summer season in the country, with hot temperatures (25-32 °C during daytime) and lower humidity. Experiments were performed in similar conditions to the environment, variating mainly on light intensity and air humidity. Seedlings were germinated outdoors under partial sunlight, with steady irrigation until experimental start.

Figure 2.1. Images of the experimental area: Santa Cruz Laboratory in Gamboa, Panama. (a) general view of the area of Gamboa; red arrow signals the area were the physiology lab is situated. (b) aerial view of the lab; red stars mark the area where experiments where situated.

For *Swietenia*, experiments 1, 2 and 3 (**E1, E2 and E3**) were performed in an open shelter, with transparent glass roof and no walls (fig 2.2a). There was a reduction of 25-30% in sunlight incidence when compared to the outdoor. Air temperature, light intensity, air humidity and wind speed/direction completely depended on environmental conditions.

Figure 2.2. Images of "cabins" used for experiments in Panama. Experimental sites: (A) open shelter used for "full light experiments", (B) is the mesh darkened cabin used for *Jatropha* growing on shadow. Right picture is a frontal picture of the cabin (C) used for *Swietenia* growing under shadow.

Seeds for E4 were germinated under shadow of 70% and kept there with steady irrigation until starting experiments. At the moment of starting experiments, they were visually more developed than the ones in E3. **Experiment 4** was conducted inside an enclosed shelter, with walls of synthetic dark fabric that reduced light intensity to 70%. This was protected with a transparent plastic roof, that avoided any external precipitation, covered with the same dark fabric to reduce 70-80% of light incidence (Fig. 2.2c). Light supply depended directly from sun irradiance, external wind temperature affected the experimental area but having semi-closed walls helped to retain humidity. The shelter was approx. 200 m^2 and was shared with other plants. This last two element together with the reduction of sunlight incidence regulated temperature and made average difference of about 3°C below outside conditions, allowing milder condition than outside.

For *Jatropha*, **E1** took place in the same glass roofed shelter described previously (Fig 2.2a), and **E2** was done in one enclosed shelter similar to the used before, with dark synthetic fabric covering 40% of sun incidence, but of smaller proportions (Fig. 2.2b). This shelter was aprox. 25 m^2 shared with other plants. Seedlings for these experiments were germinated and maintained in the same conditions as the experiment.

Jatropha **experiment 3** was performed in a greenhouse cabin, belonging to the Institute of Biology, at the FU Berlin, Germany (Fig.2.3). Seedlings were positioned on a table, 1.5 m away from lamps (at the beginning of the experiment). Incandescent/ Fluorescent lights were calibrated to produce 350-500 µmol quanta m^{-2} s^{-1} at the highest position of plant leaves at the beginning of the experiment. There were 12/12h light-dark period. Temperature was computer controlled to keep values of 28-33°C during daytime and 18-20°C during night time. Humidity was not remotely controlled but was kept artificially in ranges of 30% during daytime and 80% during night time. There was no wind effect inside.
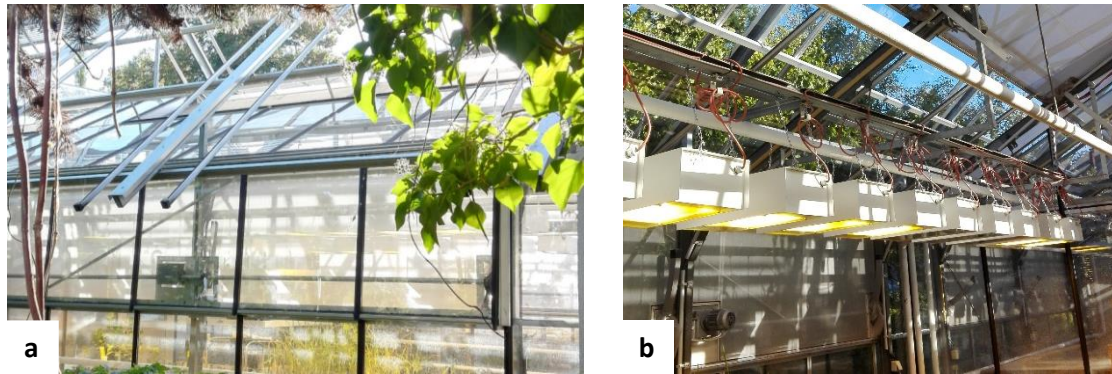
Figure 2.3. Images of cabins in the greenhouse of Freie Universität Berlin, used for experiments. (a) full cabin from outside view, completely covered in glass; (b) illumination system inside chambers and heating pipes.

Experiment 4 was performed in a self-made chamber to inside the greenhouse, with controlled maximum temperature of 28-33°C (daytime) and minimum of 18-20°C (night time). One area over a table was surrounded with dark plastic foil (agrofoil) to reduce light incidence from neighboring lamps. Lamps were regulated to a measured irradiance of 90-200 µmol quanta m^{-2} s^{-1} on the top of plants, at the beginning of experiment. Humidity was kept artificially around 35% during daytime and 80% during night time. **Experiment 5** was performed also in the greenhouse of the Institute of Biology, at the FU Berlin. Temperature was regulated electronically between 28-33°C. Humidity was artificially maintained around 15-45% during daytime to 95% in night time. Lamps were providing 500-700 µmol quanta m^{-2} s^{-1} during 12 h of daytime period. Seeds were germinated under direct light and fertilized several times before starting the experiment.

Seeds for experiments 3, 4 and 5 were germinated under shadow conditions with increased humidity. Three weeks before the start, plants were acclimated to the conditions of the zones were the experiment would take place, with irrigation 3 days a week.

2.1.3. Soil description

Experiments in Panama for both species were done with landfill soil, with clay texture that is common in the surroundings of the Institute Smithsonian for Tropical Research, were experiments were performed. It was not sterilized or extra fertilized. Organic material mostly remained in the soil, just avoiding big trunks or roots. Pots used for both species were 30 cm long and 10 x 10 cm broad, made of black plastic.

For the experiments in the greenhouse of the FU Berlin, a mix of commercial soil was used. This mix consisted of 70 volumes "Topferde" (Einheitserde, Germany), 70 volumes

"Pikiererde" (Einheitserde, Germany), 25 volumes Perligran Classic (Knauf, Germany) supplemented with 0.5 g l-1 dolomite lime (Deutsche Raiffeisen-Warenzentrale, Germany). For experiments 3 and 4 of *Jatropha*, there was no fertilization in the soil after transferring seedlings to bigger pots, only water irrigation. Soil of pots for E5 was fertilized during germination and several times during adjustment to bigger pots, before starting the experiment. During experimental phase there was no extra fertilization.

The cultivation of *Swietenia* and *Jatropha* for the experiments performed in Berlin was started in germination racks (Fig. 2.4; and 2.5). After development of cotyledons and production of first real leaf, seedlings were changed to bigger pots; plants at this moment were aprox. 15cm long.

2.1.4. Plant age

Experiments for *Swietenia macrophylla* were performed with 8-week-old seedlings. These seedlings had developed their cotyledons and produced 4-5 simple leaves at the moment of starting experiments. Most leaves were at least four-week old, with a leathery consistency (Fig. 2.4 b). This age was considered as appropriate because plants have mature leaves at similar developmental status, and stable photosynthesis. Some weeks after, compound leaves will start to appear (fig. 4d) that could mask basic response to drought stress.
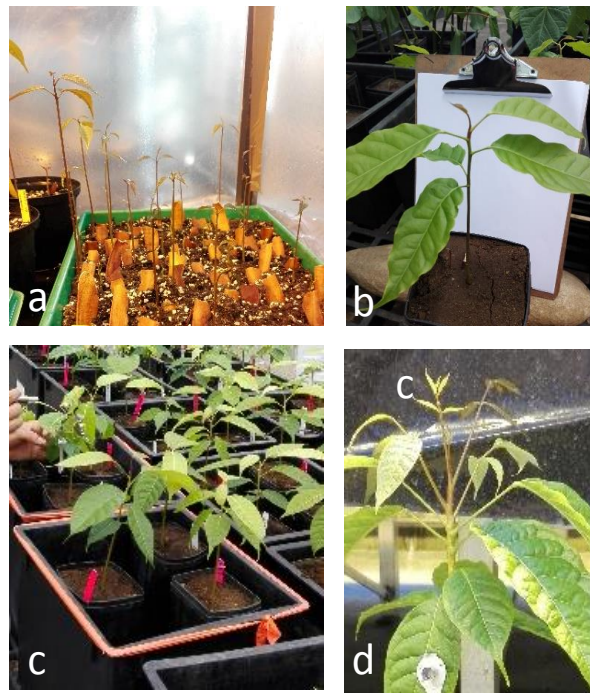
Figure 2.4. Different post-germination status of *Swietenia macrophylla*: (a) 3-7 days after germination; (b), actual experimental size has 4- 5 "real" leaves (8-week old); (c) seedling disposition; (d) 12 weeks old plants start to produce compound leaves on the apex.

If not explicitly otherwise explained, all experiments presented for *Jatropha curcas* were made with 6-weeks-old seedlings. These seedlings had developed their cotyledons and produced 4-5 leaves (including at least three fully developed leaves) at the time point of starting experiments (Fig.2.5). Some leaves had already a coriaceous consistency, distinctive for fully developed leaves.
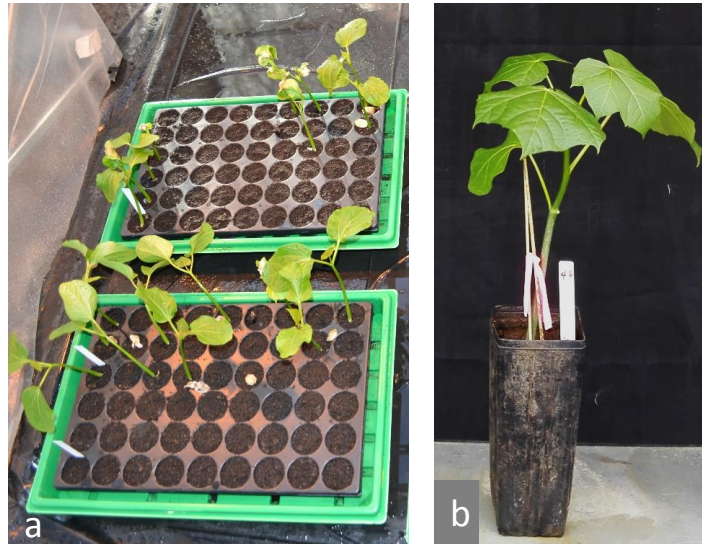
Figure 2.5. Different post-germination status of *Jatropha curcas*: (a) 3-7 days after germination; (b), actual experimental size has 3-5 real leaves, some of them already coriaceous (turgid and hard cell walls).

2.2.   Experimental Design

For both species, the experimental design was similar. A group of well-watered seedlings in the same age were randomly distributed between control and treatment groups. After the evaluation at starting position (day 0), plants were measured along the experimental stress duration, at least one time during moderate stress and once at maximum stress. Stressed plants were then re-hydrated to soil maximum capacity and evaluated after 1 and 3 days (Fig. 2.6).
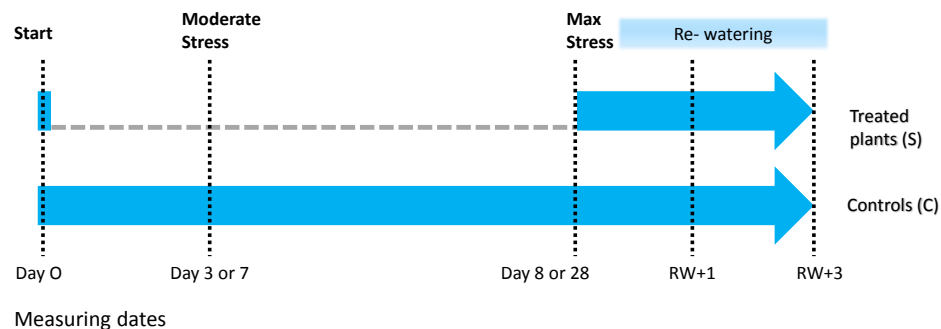
Figure 2.6. Experimental set up: timeline of stress and re-acclimation phase. Plants under drought treatment had irrigation stopped at day 0, getting into a moderate stress 3-7 days later; maximal stress was detected after 28 days (for greenhouse and shadow experiments) or 8 days (full sun experiment). After this moment, all pots were re-watered. Plants were evaluated after 1 and 3 days. Control plants were irrigated every 1-2 days, depending on soil drying. "S" indicates stressed or treated plants, "C" indicates control plants, "RW" stands for Re-Watering.

2.2.1.   Maximum stress determination

Maximum stress was determined mostly visually by wilting leaves and confirmed by photosynthetic activity. Wilting was considered as the modification in leaf blade (horizontal)

position towards the light source, to a position with leaf pointing to the ground; this was result in petiole movement. A plant was described as wilted when most of its leaves were pointing downwards. More details in Chapter 3.

2.3.   Soil status evaluation

After collecting plant material, soil was weighed to determine its fresh weight (FW). A balance with accuracy of 0.002 Kg was used to determine soil weight in Panama, while in Berlin it was a balance with accuracy of 0.001 g. This soil was dried until all water evaporated and its dry weight (DW) was measured and notated. For desiccation, soil was spread over a tray and stored in a drying oven (50-70°C) for two weeks, for experiments in Panama; for the experiments in Berlin, soil was let air dry for 4 weeks before 3h oven drying at 65°, for. Soil water content (WC) is defined for this study as absolute water amount in the soil and is calculated as WC= FW-DW, with unities of Kg H_2O per pot.

The water in pots is lost from the soil mainly because the plant absorbs it. A small fraction of the water is lost in form of soil transpiration (ST). Solar incidence or hot temperatures can increase the evaporation of water in soil surface. Soil transpiration was evaluated with control pots filled with the same soil but without any plant. Three pots were irrigated to max. holding capacity at the same time the control plants were watered, considered as **N**o **P**lant **C**ontrol **W**atered (NPCW). Three pots were not irrigated at all, called **N**o **P**lant **C**ontrol **S**tress (NPCS). At the end, water capacity (WC) for both groups was calculated. To determine the soil transpiration (ST),

$$ST = WC_{NPCW} - WC_{NPCS}$$

Soil transpiration values are expressed as ml H_2O / Kg soil.

2.4.   Atmospheric conditions

Temperature and humidity were recorded for each experiment with Voltcraft Data Logger DL-121 TH. Data collection was done every 10 minutes, to make averages based on hours. For calculation of averages, each day was divided into periodic sections: Early morning (6:00 – 11:00), Midday (11:00-16:00), Late afternoon (16:00 - 20:00) and Night (20:00 - 6:00). Temperature and humidity of each time section was averaged to understand the daily conditions to which plants were adapting.

2.5.   Chlorophyll - *a* fluorescence analysis

MINI-PAM fluorimeter (Walz, Germany) was used for the determination of chlorophyll-*a* fluorescence as indicated by the manufacturer. The maximal (Fm/Fv) and effective quantum yield (ΔF / Fm') were recorded for all plants, after dark acclimation and under regular sun adapted leaves, respectively.

Measurements were done first in light acclimated leaves with the dark leaf clip DLC-8. Closing the clip in the same position for 30 minutes allowed to measure maximum quantum yield. Each plant was measured in three random positions in 2 leaves, and the average was noted. The duration and intensity of the light pulse was 0.8 s and >3000 µmol quanta $m^{-2} s^{-1}$ for single point measurements. At each point, the average for each plant was summed up to obtain group's average value.

Light induction curves were performed with a Junior PAM (Walz, Germany), following manufacturer's indication for standard settings and using a magnetic leaf clip (angle be-tween incident radiation from JUNIOR-PAM and leaf surface, 90°) on 30 mins dark acclimated leaves. The standard saturation pulse is described in the product's manual as 10,000 µmol quanta $m^{-2} s^{-1}$ (intensity level 12) for 0.6 seconds (pulse width).

2.6.   Phenotypical evaluation

Phenotypical growth parameters of seedlings were determined at the beginning and at the end of the experiment. Plant shoot size was defined as the distance from the soil until the tip of the last bud. In control plants, soil was washed out due to irrigation, exposing part of the root; for this case, only the portion of stem was measured.

As a rough description of photosynthetic area of the plants, the leaf number was counted at the beginning and at the end of the experiments. For *Jatropha curcas*, cotyledons were counted as photosynthetic organs as long they remained green.

For *Swietenia*, shoot size is normally not expanding vertically in a substantial way within 2-3 weeks. Therefore, shoot size measurements were not performed for all experiments, only on the ones with longer evaluation period. As a rough indication on biomass production and photosynthetic capacity, leaf amount and total production were recorded.

2.7.   Biomass production

Biomass production was determined for leaves and stems separately. Leaves were weighed directly after cutting them from stems, to determine their fresh weight (FW), with a 0.001 g accuracy digital balance. It included all leaves starting from 1 cm long. Afterwards, they were dried at 80°C for 8-10 days (Panama) or air dried for 4 weeks prior oven drying 3 days for *Swietenia* and 12 days for *Jatropha* (Berlin). The leaf areas of seedlings in Panama were measured with a Leaf Area Scanner (Li-Cor, Nebraska, USA); the ones growing in Berlin were scanned with a desk scanner and measured with ImageJ, using a ruler as a reference to all leaves.

Stem measurements included stems and petioles until the base of the leaf. *Jatropha* has a very fluid latex in the stems; sample fresh weight determination was done as fast as possible to avoid the loss of contained water by latex running. For drying, stems were cut in small pieces (10-15 cm) and disposed on closed envelopes made of paper napkins. After desiccation period, dry weight (DW) was recorded to calculate the water content. For this study, leaf and stem water content corresponds to the absolute amount of water contained in the initial fresh weight. This value was further used in relation to the corresponding dry matter or leaf area.

In previous evaluation of *Jatropha* (data not shown) was observed an apparent difference in petiole size when plants were submitted to stress. For all experiments, the two longest petioles were measured from the position of its axillary bud until the base of the leaf; their average represented the value for the plant.

2.8.   Infra-Red gas analysis (IRGA)

Measuring gas exchange is a common technique used to measure photosynthesis of individual leaves, whole plants or plant canopy (Cernusak *et al*, 2013). The main advantages cited for gas exchange measurements include its instantaneous results, the non-destructive technique and direct evaluation.

The principle of IRGA measurements lies on a physical process: all heteroatomic gas molecules absorb radiation at specific infrared wavebands with specific characteristic in its absorption spectrum (Li-Cor, Nebraska, USA). Infrared gas analyzers (IRGAs) measure the reduction in transmission of infra- red wavebands caused by the presence of CO_2 between the radiation source and a detector. Water vapour is the only gas normally present in the air with an absorption spectrum overlapping the one of CO_2. Since water vapour is usually present in the

air at much higher concentrations than CO_2, this interference is significant, but can be overcome by drying the air.

For this study, an IRGA Li-6400 (Li-Cor, Nebraska, USA) was used for the experiments performed in Panama (Fig. 2.7). Internal CO_2 concentration and temperature settings were 400 ppm and 28°C, respectively. This device was available as a kind support from Dr. Omar Lopez, (INDICASAT, Panama).
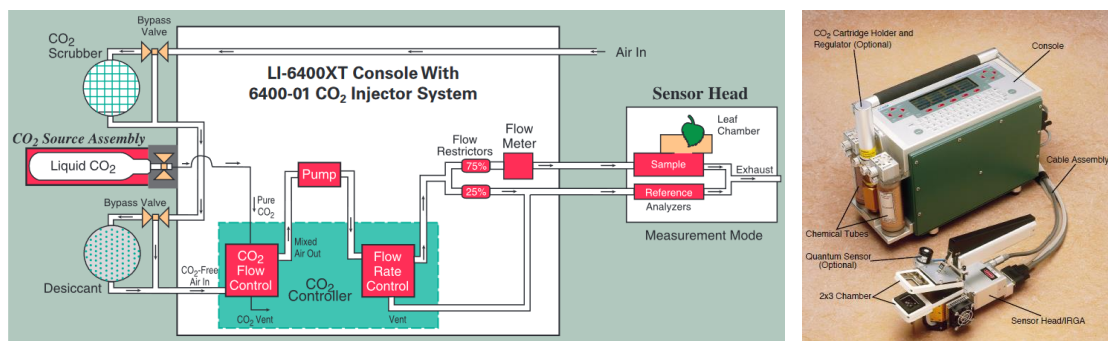
Figure 2.7. Infra Red Gas Analyser (IRGA) system from Li-Cor. The device was used as a closed system (as shown in image) to record net photosynthesis (A) and water stomatal conductancy (g). The chamber included in the sensor head was placed in the middle section of the leaves. Schema and picture of device from Li-Cor 6400 manual.

Variables collected were:

"**Photo**" (A)	Net Photosynthetic rate	(μmol CO_2 m^{-2} s^{-1})
"**Cond**" (g)	Total Conductance to H_2O	(mol H_2O m^{-2} s^{-1})

Variable measurements descriptions and used equations are described in the manual of use for the used equipment.

2.8.1. CO_2 assimilation equations

Gas exchange measurements provide direct measure of the net rate of photosynthetic carbon assimilation. CO_2 exchange systems use enclosure methods, where the leaf is closed in a transparent chamber. The rate of CO_2 fixation is determined by measuring its concentration change in the air flowing across the chamber.

A is net assimilation rate of CO_2 by the leaf (μmol CO_2 m^{-2} s^{-1}).

$$A = \frac{F(C_r - C_s)}{100S} - C_s E$$

where F is the incoming flow rates from the chamber (mol H_2O s^{-1}), Cr and Cs are reference and sample CO_2 concentrations (μmol CO_2 mol air^{-1}), s is the leaf area and E is transpiration rate (mol H_2O m^{-2} s^{-1}), in terms of the LI-6400 measures.

2.8.2. Water conductance

Total water vapor conductance of the leaf g_{tw} (mol H_2O m^{-2} s^{-1}), including stomatal and boundary layer apports, is given by

$$g_{tw} = \frac{E\left(1000 - \dfrac{W_l + W_s}{2}\right)}{W_l - W_s}$$

where W_l is the molar concentration of water vapor within the leaf (mmol H_2O mol air^{-1}), which is computed from the leaf temperature T_l and the total atmospheric pressure, W_s is the sample water mole fractions (mmol H_2O mol air^{-1}).

2.9. Bioinformatics

2.9.1. Databases

Available sequences for *2CP*, *APX* and *GPX* belonging to species related to *Swietenia macrophylla* were browsed from **PeroxiBase** (Fawal *et al.*, 2013; version 2014-2015). Data corresponding to *Arabidopsis thaliana* was used as query to BLAST against all plant sequence in **GenBank**, from the NCBI (Wheeler *et al.*, 2001; release 226.0), to browse more sequences.

Jatropha curcas genome has been sequenced. The information is collected in the **Jatropha Genome Database** (https://www.kazusa.or.jp/jatropha/), produced by the Kazusa DNA Research Institute in Japan (Sato *et al.,* 2011; Sato *et al.,* 2012) using the release version 4.0. Gene sequences corresponding to *Arabidopsis thaliana* found in **Tair**, from the Phoenix Bioinformatics Corporation, were used to blast against *Jatropha* sequences reported in GenBank.

2.9.2 Phylogeny trees

Phylogenetical trees were constructed with the aid of **Angiosperm Phylogeny Website**, version 14 (Stevens, 2001).

2.9.3 Sequence alignments

Multiple sequence alignment was done with **Clustal Omega**, from The European Bioinformatics Institute (EMBL-EBI; Sievers *et al.*, 2011), after the update in 2017. This online program has the possibility to align protein, DNA and RNA sequences.

Pairwise Sequence Alignment was used to identify regions of similarity that may indicate functional, structural and/or evolutionary relationships between two biological sequences (protein or nucleic acid). For alignment of two sequences presumably related, **EMBOS- Needle**

from EMBL-EBI was used (Rice *et al.,* 2000). This online tool can differentiate peptide and nucleotide sequences. It creates an optimal global alignment of two sequences using the Needleman-Wunsch algorithm.

2.9.4   Wobble primer design

Results from Clustal Omega were observed under **Jalview** (Waterhouse *et al.,* 2009), which is a free program for multiple sequence alignment editing, visualisation and analysis developed by the University of Dundee, project of ELIXIR-UK Resource. Specific primer design for qPCR amplification, fragment (or full sequence) was designed following recommendations of Kalendar *et al.* (2011). Primer position was analyzed with **Primer3**, version 4.1.0 (Untergasser *et al.,* 2012 & Koressaar *et al.,* 2007) using recommendations of Thornton & Basu (2010).

Primer sequences were characterized with the **Oligo Calc** (Kibbe, 2007) version 3.27 and the **OligoEvaluator** from Sigma Aldrich.

2.9.5   Discrimination of putative chloroplast isoforms

Sub-cellular localization of *Jatropha curcas* full length sequences were predicted using **TargetP** version 1.1 (Emanuelsson, *et al.*, 2000) and **ChloroP** version 1.1 (Emanuelsson, *et al.*, 1999), both from the Technical University of Denmark, DTU. The prediction for transmembrane helix was performed with **TMHMM** version 2.0 (also from the DTU) and **Phobius** (Käll *et al.,* 2007, Käll *et al.,* 2004), from the Stockholm Bioinformatic Center, at the Universitet Stockholms.

2.9.6   Hydrophobicity test in peptides

The **ProtScale** online tool from the Expasy- Server (Gasteiger *et al.* in Walker, 2005) was used **f**or detecting hydrophobic component in peptides. The sequence was first evaluated based on the algorithm by Kyte & Doolittle (1982). This scale assigns hydrophobicity scores for each amino acid in the sequence based on different chemical and physical properties, offering a visual representation of regions in the sequence that are more or less hydrophobic, based on the amount and type of aminoacids contained. Peptide sequences were also analyzed based on the algorithm by Chou & Fasman (1978) that predicts beta sheet formation, also based on chemical and physical properties of the amino acids.

2.9.7   Gateway cloning

Digital cloning, sequence analysis and plasmid visualization was done with Serial Cloner version 2.6, a free software available online (http://serialbasics.free.fr/Serial_Cloner-Download.html).

2.10.   Transcript analysis

2.10.1. RNA Isolation and quantification

RNA was isolated from 50-100 mg frozen plant material after grinding in liquid nitrogen. Ground material was further pulverized with a Retsch Mixer Mill MM10 (Retsch, Germany). Nucleic acids were extracted using the GeneMATRIX Universal RNA Purification Kit (Roboklon, Berlin, Germany), mostly according to the manufacturer's instruction. The kit uses silica columns and relays on the high binding efficiency of nucleic acids to silica matrixes. Optimization for each species and condition was needed (see Results). Modifications included test of phenolic extraction and buffer usage during mill shaking. To avoid sample contamination with genomic DNA, 1 U RNase-free DNase I (Fermentas GmbH, Germany) was applied on-column digestion.

RNA concentration and quality was assessed with a NanoPhotometer Pearl P300 (Implen, München, Germany), by determining the absorption ratios of the RNA A 260nm /A 230nm and A 260nm / A 280nm, which indicate salt, protein and phenol contaminations, respectively. Samples are regularly considered with good quality if they have ratios close to 2, but for many of the samples coming from experiments in Panama these values were not achieved. Samples were included in analysis if quality ratio was minimum 1.65 and gel electrophoresis showed no degradation.

Electrophoretic separation was performed in a 1.5% (w/v) agarose gel in 1x MOPS buffer. After cooling down to approximately 60 °C, it was supplemented with formaldehyde to a final concentration of 2.5% (v/v). Gels were cast into horizontal gel trays and left for solidification at room temperature. 3µl RNA sample was mixed with 3 µl loading dye and 4.2 µl RNA loading buffer, and incubated for 10 minutes at 65 °C. To avoid reformation of the RNA secondary structure the samples were immediately transferred on ice and cooled until they were loaded onto a gel. Before sample loading, gels were placed in a horizontal electrophoresis chamber containing 1X MOPS buffer; samples were run at 100 V using a BIORAD Power Pac Basic as power supply. After separation, samples were observed under UV light I a chamber, with an integrated camera (INTAS gel documentation camera).

1x MOPS	200 mM 3-(N-morpholino) propane sulfonic acid (MOPS)
	10 mM Na-acetate
	10 mM EDTA
	The pH was adjusted to 7.0

6x DNA loading dye	10 mM Tris-HCl (pH 7.6)
	60 % (v/v) Glycerol
	60 mM EDTA
	0.03 % (w/v) Bromophenol blue

6x RNA loading buffer	60 % (v/v) Formamide
	18 % (v/v) Formaldehyde
	15 % (v/v) 10x MOPS
	1.2 % (w/v) Ethidium bromide

2.10.2.   cDNA synthesis

RNA samples with A 260/ A 230 = 1.65 and concentrations over 75 ng/µl were directly used for first strand cDNA synthesis with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), as described in manual. The kit uses the MultiScribe™ MuLV reverse transcriptase. Primers used for cDNA amplification were a mix of 80% OligodT$_{16}$ primers (Sigma-Aldrich Chemie GmbH, Germany) + 20% random hexamer primers (supplied), instead of just the random hexamer primers. This was done in order to target mRNA and minimize cDNA synthesis from rRNA and tRNA. One microgram RNA was reverse transcribed in a 20 µl total volume of mixture. cDNAs were diluted in a 1:2 ratio with RNase-free water prior to qRT-PCR.

The RNA in samples with low concentration was precipitated by mixing the sample with 0.1 vol 3M NaAC, pH 5.2, and 2.5 volumes 100 % ethanol. If concentration was too low, it was included 1µl glycogen. The mix was vortexed vigorously and stored at -20°C overnight. The next day, the samples were centrifuged at 4°C for 20 minutes at 14,000 rpm, washed with 100 % ethanol and dried with a speed vac. The dry pellet was resuspended in the appropriate volume of RNAase-free water to achieve expected concentrations.

2.10.3.   Quantitative real-time qPCR analysis

Transcript abundance rates were analyzed by quantitative Real Time PCR (qRT-PCR) in the CFX96 thermocycler (BioRAD, Germany), according to the MIQE standards (Bustin *et al*. 2009), using the SYBRGreen method. Primers used for qRT-PCR analysis are presented in Table 2.1 and 2.2. Each biological replicate (each plant) was measured in technical triplicate.

If cDNA was not in the standard concentration, the volume corresponding to the transcription of 50 ng was added, and water in the mix was escalated to 20µl total reaction.

qPCR Master mix included:

              13.44 µl destilled water
              2 µl   10X buffer
              0.8 µl   50 mM $MgCl_2$,
              0.4 µl   5 mM dNTP,
              0.2 µl   10X SYBR Green (Sigma-Aldrich, Germany),
              0.04 µl OptiTAQ (Roboklon, Berlin, Germany)
              0.12 µl Primer mix 50mM (forward + reverse)
              3 µl   cDNA

10X Buffer   160 mM ammonium sulfate
                      1 M Tris-HCl pH 8.3
                      0.1 % (v/v) Tween-20

Amplification program for *Swietenia macrophylla* was the following

95°C			Plate heating
95°C	5 minutes		Initial denaturation
95°C	5 minutes		Denaturation
60°C	1 minute	30X	Annealing
72°C	1 minute		Extension
72°C	1 minute		Final extension

For *Jatropha curcas*, 61 °C was chosen as annealing temperature.

Plate reads were evaluated with machine program and calculated in Excel (Microsoft Word). If not otherwise stated, all final results are expressed as $2^{-\Delta\Delta Ct}$.

2.10.4. Standardization of results

For *Swietenia macrophylla*, the genes used as reference are *Actin* and *Tubulin*. Primers (Table 2.3) were designed based on partial sequences.

For *Jatropha curcas*, stability of some genes during abiotic stresses was evaluated. Six sequences published by Zhang *et al.* (2013) and Rocha *et al.* (2016) were tested (Table 2.4).

For the normalization in transcript abundance of the genes of interest in *Jatropha curcas*, *GAPDH* and *TUB5* were used as reference genes in all experiments.

Table 2.1. List of used primers for qPCR analysis of the genes coding for the antioxidant system in the chloroplast of *Swietenia macrophylla.*

Gene	Orient	Name	Sequence	Tm °C	Amplif.
2CP	Forward	Swm_2CP_f	AGCTGGGCATACTTCATCTG	61,8°C	
	Reverse	Swm_2CP_r	GGAGTCATCCAGCACTCAAC	62,4°C	120 bp
APX	Forward	Swm_APX_f	TTGGACTCATGCCAAGTGTG	64,9°C	
	Reverse	Swm_APX_r	TGTCCGACAGAAATCACTGC	64,0°C	114 bp
GPX	Forward	Swm_GPx_f	TTGCTTCAAGATGTGGTTTG	61,3°C	
	Reverse	Swm_GPx_r	GATTTGATCCAGGTTCTTGC	61,1°C	165 bp

Table 2.2. List of used primers for qPCR analysis of the genes coding for the antioxidant system in the chloroplast of *Jatropha curcas.*

Gene	Orient	Name	Sequence	Tm °C	Amplif.
2CP	Forward	Jcr_q2CP_f	ACACTCCAGGCACTGCAATA	63,4 °C	
	Reverse:	Jcr_q2CP_r	GGTCTGGCTTCATCGACTTC	63,8 °C	91 bp
APX	Forward:	Jcr_qAPX_f	TGCTAGATGGTGTTGCAGGA	64,5 °C	
	Reverse:	Jcr_qAPX_r	CTGCCCGTATCTTCTGCTTC	63,8 °C	96 bp
GPX	Forward:	Jcr_qGPX_f	GAGGACAAGAACCTGGGTCA	64,2 °C	
	Reverse:	Jcr_qGPX_r	GGTCCATTCACATCCACCTT	63,6 °C	100 bp
PrxQ	Forward:	Jcr_qPrxQ_f	TACATCGTCGCACAATGCTT	64,2 °C	
	Reverse:	Jcr_qPrxQ_r	GGAATGCCCCACTCTTTTCT	64,2 °C	96 bp
CAT2	Forward:	Jcr_qCAT2_f	ATGATCCTGTTCGTCATGC	61.2 °C	
	Reverse:	Jcr_qCAT2_r	AGCGGTATCTTTCTCCAGG	60.8 °C	120 bp
SOD	Forward:	Jcr_qSOD_f	CAACAGTGAATGTTCGTGTC	59.3 °C	
	Reverse:	Jcr_qSOD_r	GTTGTTCGGATTGAAATGAG	59.6 °C	119 bp

Table 2.3. List of used as reference genes during qPCR analysis of the genes coding for the antioxidant system in the chloroplast of *Swietenia macrophylla.*

Gene	Orient	Name	Sequence	Tm °C	Amplif.
ACT2	Forward	Swm_Act3.F	GAGCGTGAAATTGTGAGG	59,4°C	
	Reverse	Swm_Act3.R	TCAGGCAACTCGTAGCTC	59,3°C	122 bp
TUB4	Forward:	Swm_Tub4.F	CAGTGGCAAGGAAGATGC	62,1°C	
	Reverse:	Swm_Tub4.R	CCAGTGCAGTTGTCAGCA	62,5°C	114 bp

Table 2.4. List of possible reference genes evaluated for the normalization of sample during qPCR analysis of the genes coding for the antioxidant system in the chloroplast of *Jatropha curcas.*

Gene	Orient	Name	Sequence	Tm °C	Amplif.
EF1-α	Forward	Jcr_EF1.f	AAGATGATTCCCACCAAGCCCA	70,5°C	
	Reverse	Jcr_EF1.r	CACAGCAAAACGACCCAGAGGA	70,5 °C	72 bp
TUB5	Forward	Jcr_TUB5.f	TATGTTCCCAGGGCGGTTCTAATG	70,2 °C	
	Reverse	Jcr_TUB5.r	GGACTGCCCAAAGACAAAGTTATCG	69,2 °C	111 bp
TUB8	Forward	Jcr_TUB8.f	GCAGGGAATAACTGGGCTAAAGGT	67,8 °C	
	Reverse	Jcr_TUB8.r	CTCCACCCAACGAATGACAAACTT	68,8 °C	136 bp
ACTIN	Forward	Jcr_ACT1.f	CTCCTCTCAACCCCAAAGCCAA	70,3 °C	
	Reverse	Jcr_ACT1.r	CACCAGAATCCAGCACGATACCA	70,0 °C	147 bp
18 S	Forward	Jcr_18s.f	CTCAACCATAAACGATGCCGACC	70,1 °C	
	Reverse	Jcr_18s.r	TTCAGCCTTGCGACCATACTCCC	72,0 °C	117 bp
GAPDH	Forward	Jcr_GAPDH.f	TGAAGGACTGGAGAGGTGGAAGAGC	71,6 °C	
	Reverse	Jcr_GAPDH.r	ATCAACAGTTGGAACACGGAAAGCC	71,5 °C	140bp

2.11. Cloning steps

After Real Time PCR amplification (RT-PCR), bands in electrophoretic gel were cut out and cleaned with the Invisorb Spin DNA extraction Kit (Stratec Molecular, Berlin, Germany). This DNA was further used for cloning.

2.11.1 Partial sequence cloning for *Swietenia macrophylla*

The partial sequence fragment amplified with wobble primers was cloned into pCR 2.1 TOPO vector (Fig. 2.8), from a TOPO-TA Cloning Kit (Invitrogen, Life Technologies, Carlsbad, USA), following manual instructions. Quality control of fragment length (identity) was done by DNA gel electrophoresis and plasmid isolation, with a RT-PCR using the same primers.
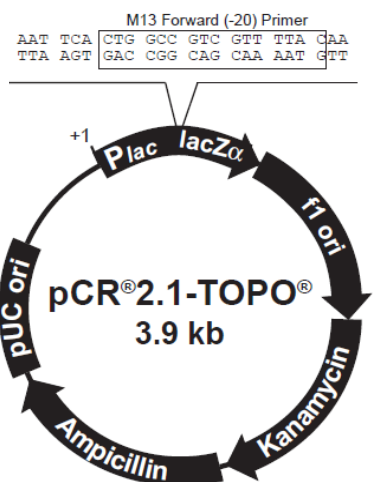
Figure 2.8. pCR2.1-TOPO vector map. Commercial vector used for simple cloning step.

2.11.2 Transformation of *Escherichia coli* (+ colony PCR)

Vectors were cloned into Top 10, *E. coli*. Bacteria were grown on selective plates for 24 hours.

LB media (1 L)	Tryptone	10 g
	NaCl	10 g
	Yeast extract	5 g

Selective plates	LB media	
	Agar	15%
	Kanamycin	50 µg/mL

40 µL of 40 mg/mL X -gal was spread on each LB plate and incubated at 37°C for 24 hours. Five transformed colonies (white) were picked with a pipette tip and inoculated into liquid growing media, using the same composition than in the agar plates, without the solidification agent.

2.11.3 Plasmid isolation

Plasmids were isolated from *E. coli* culture using the following protocol: 3ml bacteria were pelleted by centrifugation in 1.5 ml reaction tubes. The supernatant was discarded. The pellet was resuspended by vortexing it in 100 µl lysis buffer; then, 200 µl SDS solution was mixed by tube inversion. The probes were incubated for 3-5 minutes. After adding 150 µl KAc solution and mixing it by inversion, tubes were incubated in ice for 10 minutes. Centrifugation was carried out at 1500 rpm, 4°C, for 10 minutes. The supernatant containing plasmids was transferred to a new reaction tube and mixed with 0.6 vol isopropanol, vortexing briefly. It was centrifuged for 10 minutes under the same conditions and the resulting supernatant was discarded. The final pellet was washed with 300 µl cold 70 % EtOH before drying in a speed vac; final resuspension was done in 35 µl TE buffer and stored for long term at -20°C.

Lysis buffer	50 mM Tris/HCl, pH 8.0
	50 mM EDTA, pH 8.0
	15% saccharose

| SDS solution | 200 µl NaOH |
| | 1% SDS |

KAc solution	3M KAc
	11.5% EDTA, pH 8.0
	(kept at 4°C)

TE Buffer	10 mM Tris/HCl, pH 8.0
	1 mM EDTA, pH 8.0
	10 µg/ml RNase (fresh added)

Inserts were checked with colony PCR (taking a small amount of bacteria with the tip of a pipette) and plasmid PCR, using M13 forward primer + specific reverse primer, and also by enzyme digestion using the Fast Digest Eco R1 (Thermo Scientific, Carlsbad, USA). For quality extraction and further sequencing, plasmids were isolated with GeneJET Plasmid Mini Prep Kit (Thermo Scientific, Carlsbad, USA).

2.11.4 Glycerol stocks

After confirming by PCR and enzyme digestion a positive clone, bacteria were stored in glycerol stocks. For it, 500 µl of a fresh overnight *E. coli* culture was mixed thoroughly with 500 µl of 80 % glycerol (sterile). Immediately after, bacteria culture suspension was frozen with liquid Nitrogen. It was stored at -80 °C for later re-grow.

2.11.5 *J. curcas* full-length sequence cloning

Jatropha curcas chloroplast genes of the antioxidant system were cloned as cDNA, covering the full open reading frame. Primers used for each gene are described in Table 2.5.

Table 2.5. List of primers used to amplify the full-length sequence of the genes coding for the chloroplast antioxidant system in *Jatropha curcas.* Amplification sizes are based on the sequence provided by the Jatropha Genome Database (Sato *et al*., 2012)

Gene	Orient	Name	Sequence	Tm °C	Amplif.
2CP	Forward	FS_Jc2CP_fw	ATGGCTTGCTCCGCTACTTC	65.5°C	
	Reverse	FS_Jc2CP_r	ATCAGGTGCTGTATTTCCGACCAG	69.0 °C	861 bp
APX	Forward	FS_JcAPX_fw	ATGGCAGACTCCTTGTTACACTT	62.7 °C	
	Reverse	FS_JcAPX_r	TCAATTTCCAAGAAGGGACG	63.8 °C	1191 bp
GPX	Forward	FS_JcGPX_f	ATGGCTTCTGTTCCTTTTCAG	62,1 °C	
	Reverse	FS_JcGPX_r	TCATGCTGCAAGAAGCTTC	61,9 °C	741 bp

After full length sequence amplification by RT-PCR, the product was separated by electrophoresis (see DNA electrophoresis in *Swietenia*). The bands were cleaned with the Invisorb Spin DNA Extraction Kit and cloned into pCR 8 TOPO vector (Fig. 2.8) using the TOPO-TA Cloning Kit (Invitrogen, Life Technologies, Carlsbad, USA), following manual instructions. The plasmid was used to transform TOP 10 *E. coli* and after bacterial growth in selective media (spectinomycin) and plasmid isolation, three positive clones were verified by commercial sequencing, while keeping glycerol stocks of bacteria.

2.11.6 *J. curcas* signal peptide sequence cloning

Sequences encoding for signal peptides of *2CP*, *APX* and *GPX* from *Jatropha curcas* were first cloned into pCR8 (Fig. 2.8). The primer selection is described in the result section. After insert confirmation, the fragments were transferred to the cloning cassette in pMDC3 (Invitrogen, Life Technologies, Carlsbad, USA), via Gateway Cloning (Fig. 2.9), using manufacturer instructions. The correct orientation and expected sequence was first predicted digitally with Serial Cloner, also obtaining the list of digestion sites and their frequencies.
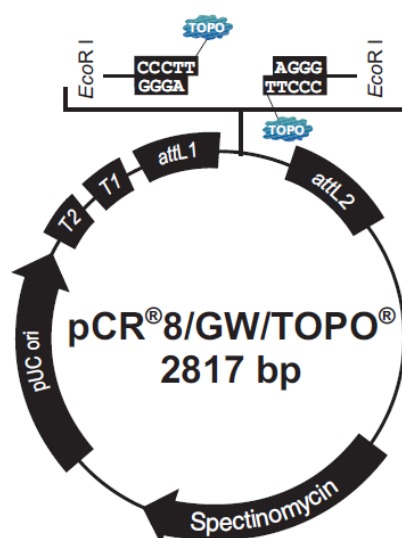
Figure 2.8. pCR8 TOPO vector map. Vector was used for cloning full sequence and signal peptide of *Jatropha curcas* genes. It contains attL1 and attL2 sites, that can be used for Gateway cloning afterwards.
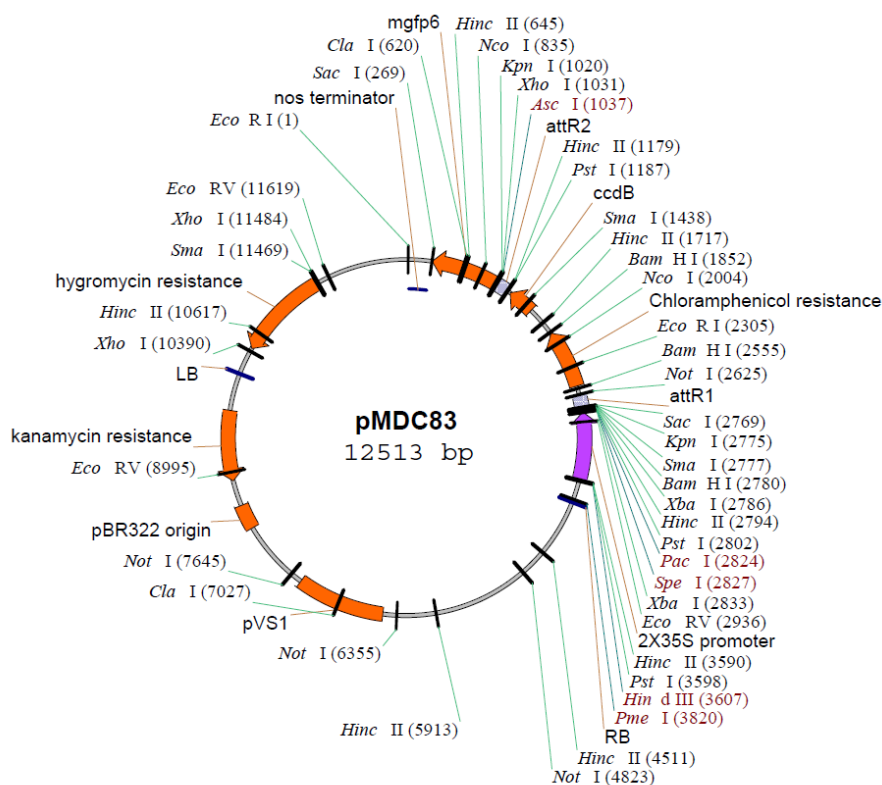
Figure 2.9. pMDC83 vector map. Vector was used as destination vector for cloning signal peptide of *Jatropha curcas* genes. It contains attR1 and attR2 sites, that can be used for Gateway cloning.

Digestion sites used were:

2CP	Spe I + Bss SI
APX	Spe I + Bam HI
GPX	Spe I + Bsr DI

After verification of right orientation of insert with enzyme digestion and PCR, the plasmid was then used for chemical transformation of *Agrobacterium tumefasciens*.

2.11.7 Sequencing

All plasmids were commercially sequenced using the services of Eurofins Genomics (Ebersberg, Germany).

2.12. Transformation of *Agrobacterium*

Confirmed plasmids were used for chemical transformation of *A. tumefasciens* strain GV 3101. One aliquot of competent cells (20-50 µl) was thawed on ice for 30 minutes for each construct. 1 µg of plasmid was added to the reaction tube and was incubated for 5 minutes on ice and 5 minutes on liquid nitrogen. Bacteria were heat shocked by incubating at 37°C for 30 seconds, putting them back to ice afterwards. 500 µl of pre-warmed YEB medium was added to the

tube and incubated for 3 hours at 28°C, under 200 rpm shake. These bacteria was then brought on selective media and incubated for 2 days at 28°C.

YEB media (1 L):   10 g Peptone
                         10 g NaCl
                         5 g Yeast extract
                         Destilled water (calibrated to 1 L)

for plates   + 1.5 % bacterial agar
Antibiotics used: 25 mg/ml Gentamycin + 25 mg/ml Rifampicin + 50 mg/ml Kanamycin

2.12.1. Transformation test

A. tumefasciens colonies growing on selective media were inoculated in 10 ml liquid media and plasmids were isolated with a mini prep. Plasmids were used for transformation of TOP 10 *E. coli*, which grew in LB media containing all three acquired resistances.

Plasmids isolated from *E. coli* were digested with different enzymes after Serial cloner prediction to evaluate them for correct fragment orientation. Positive clones were first re-checked with RT-PCR using M13 forward primer + gene specific signal peptide primer. Positive colonies were then sent for sequencing, for final confirmation.

2.13.   Cellular Localization

2.13.1 Transfection of Nicotiana benthamiana

The bacteria containing the transformation plasmid were grown one day in solid media and inoculated in liquid media for 18-24h at 28°C. *A. tumefaciens* carrying p19K was grown in parallel. Bacteria liquid culture were grown until reaching OD_{600} = 1.8. For harvesting, 100 ml bacterial suspension was centrifuged at 3,000 rpm and resuspended in infiltration media. Concentration was assessed by a spectrophotometer (UltraSpec 2100 Pro Clasic, Amersham BioSciences, Little Chalfont, UK), and re-diluted in infiltration media to reach OD_{600} = 0.8 for the bacteria containing the signal peptide constructs, and to OD_{600} = 8 for the one containing p19K. After resuspension in the infiltration media, bacteria were allowed to acclimate, shaking for 1 hour at room temperature (20°C).

The infiltration solution was then prepared by adding 10 ml of signal peptide construct bacterial carriers, 3ml of the p19K bacterial carrier, and 10μl acetosyringone. p19K presence in the mix should avoid construct silencing in plants, while the acetosyringone allows higher transformation efficiency in plants, in *A. tumefaciens*-mediated transformation procedures.

Infiltration Media   10 mM MES
                            10 mM MgCl2

Nicotiana benthamiana 3-4 weeks old plants were prepared 3 hours in advance by spraying water over the leaves and closing them with a transparent lid, to allow stomata opening. With a 1 ml needleless syringe, bacterial suspension was slowly infiltrated in the abaxial part of three leaves per plants, visually observing that the media was reaching the complete leaf area. Several infiltration points were used per leaf.

Three plants were infiltrated per construct and one additional plant was infiltrated with water as negative control. Plants returned to the greenhouse, stayed covered for the first 10-15 hours, and then were irrigated daily, as regular.

2.13.2 Detection of GFP in leaves

Three days after infiltration, plants were evaluated under fluorescence imaging. Complete pots were placed inside the chamber of the Night Shade (BERTHOLD TECHNOLOGIES GmbH, Bad Wildbad, Germany). The **mGFP6** contained in the vector is excited with its specific wavelength and the image is captured with an integrated camera, including the chlorophyll-*a* fluorescence scale.

2.13.3 CLSM evaluation of GFP target

Transfected leaves were also evaluated under Confocal Laser Scanning Imaging (CLSM). It was used a Leica TCS SP5, from the department of Applied Genetics, at the FU Berlin. LAS X software allowed to set desired conditions for image capture: Argon laser at 30% power; objectives 20X and 60X; pinhole 60.6 µm open. Channels for image analysis used included a bright field, a chlorophyll channel (650-700 nm) and GFP channel (480-500 nm). The pictures were processed only for bright and contrast with Image J.

2.14. Chloroplast Isolation

Protocol for chloroplast extraction was followed as proposed by Klinkenberg *et al.* (2014), with minor adaptations. Dark acclimated plants were collected in the early morning and transported with a dark lid, to avoid starch storage, until the cold room (4°C) where all steps took place. Two infiltrated leaves were grinded with pre-cooled mortar and pestle, together with 3 ml ice cold isolation buffer.

The homogenate was filtered with a 60 µm nylon mesh into a reaction tube. 330 µl of a mix of 40% Percoll (Sigma Aldich) in isolation buffer was placed in the bottom of a reaction tube. On top, 400 µl of the filtered chloroplast containing homogenate was placed carefully and

centrifuged at 4°C, for 6 minutes, gently at 1,700 rpm. Intact chloroplast sedimented as a green pellet while broken ones stayed in the Percoll layer. To increase yields, this procedure was made in triplicate for each construct and after centrifugation phases were put together in one new tube. Quality of the separation was controlled with phase contrast microscopy (data not shown).

Isolation Buffer	0.33 M	Sorbitol
	50 mM	Hepes, pH=7.0
	0.1%	Bovine serum albumine (BSA; (m/v))
	2 mM	EDTA
	1 mM	MgCl2

2.15.   Protein Extraction

After chloroplast extraction, proteins were extracted following the protocol of Klinkenberg *et al*. (2014). Intact chloroplasts were frozen in liquid nitrogen for 3 minutes. Freeze-fractured chloroplasts were resuspended in 50 µl ice-cold Chloroplast Protein Extraction Buffer and centrifuged at 15,000 rpm and 4°C for 10 minutes. The supernatant included the soluble proteins and was deposited in a new tube. The pellet, containing insoluble protein, was washed twice with the extraction buffer before final resuspension in 50 µl of this buffer.

Chloroplast Protein Extraction Buffer

5 mM	EDTA (autoclaved)
10 mM	DTT
100 mM	HEPES, pH 7.2 (steril filtrated)
10%	Glycerol (steril) (m/v)
	Protease inhibitor cocktail (Applied Biosystems, USA)

Self- purified, deionized water (Millipore, Massachusetts, USA).

For total protein extraction, pieces of leaves were deposited in a reaction micro-tube and grinded together with 500 µl Total Protein Extraction Buffer, using an electrical mixer with a pole support (Janke & Kunkel KG type RM 14, now IKA®-Werke GmbH & CO. KG, Staufen, Germany). This homogenate was centrifuged at 4°C, 14,000 rpm for 10 minutes. Supernatant containing the protein was changed to new tube, and further processes equally.

Total Protein Extraction Buffer

150 mM	NaCl
50 mM	HEPES

Adjusted to pH 7.5

2.15.1. Protein Concentration Determination

Protein concentration was calculated via a colorimetric protein assay, based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 (Bradfordt, 1976). 990 µl of the Bradford test solution was mixed by tube inversions with 10 µl of the protein for analysis. After incubation at room temperature (22 °C) for 20 minutes, homogenate was evaluated in a spectrophotometer to absorption of 595 nm.

Obtained values were standardized with bovine serum albumin (BSA) in a dilution series with known concentrations, from 0 to 2 g/ml. Unknown sample absorbance was compared to BSA absorbance, using linear regression to determine their concentration.

Bradford test solution

> 200 µl Protein Assay Dye Reagent Concentrate (BioRad, California, USA)
> 790 sterile water

2.16.   Protein Electrophoresis

5 mg protein was prepared per sample, adjusting the total values of all samples to the same volume with the protein extraction buffer they were suspended in. Protein loading buffer was added accordingly to final volume. Proteins were denaturalized for 5 minutes at 95 °C in a TSC thermoshaker (Biometra Analytic Jena, Jena Germany) and cooled down on ice before loading. Proteins were analyzed on 12 % (v/v) gels according to Laemmli (1970). The separating gel was polymerized between two glass plates and, to provide a straight edge, overlaid with isopropyl alcohol. After the polymerization, the alcohol was removed and the stacking gel was casted. A comb was inserted prior to the polymerization to produce pockets for the samples. Protein electrophoresis was made in a vertical chamber, using SDS running buffer surrounding the plates. Gels were run using a Power Pac Basic (BioRad) at 50 V while protein samples were located in the stacking gel. The separation gel was run for approximately 90 minutes at 150 V, based on observed band separation. The PageRuler Prestained Protein Ladder (Fermentas, Germany) was separated in parallel with the samples.

Protein loading buffer (5x)

> 625 mM   Tris-HCl (pH 6.8)
> 50 %   Glycerol (v/v)
> 20 %   SDS (w/v)
> 0.025 %   Bromophenol blue
> 25 %   distilled water (v/v)
> 1/3 volume ß-Mercaptoethanol (added freshly before usage).

Separating gel

12 %	Rotiphorese Gel (37.5:1) (v/v)
0.375 mM	Tris-HCl (pH 8.8)
0.01 %	SDS (w/v)
0.01 %	APS (w/v)
0.1 %	TEMED (v/v)

Stacking gel

5 %	Rotiphorese Gel (37.5:1) (v/v)
0.1 mM	Tris-HCl (pH 6.8)
0.016 %	SDS (w/v)
0.016 %	APS (w/v)
0.0016 %	TEMED (v/v)

SDS running buffer

200 mM	Glycine
25 mM	Tris
0.1 %	SDS (w/v)

After electrophoresis, protein loading in gels amount was controlled with Coomassie staining.

2.17.   Western Blot

2.17.1 Blotting

Protein in gels were transferred to membranes for immunodetection, following western blot protocol according to Kyhse-Andersen (1984). Gel-Blotting-Paper (Schleicher & Schuell, Düren, Germany) and the nitrocellulose membrane (Roti-NC, Roth, Karlsruhe, Germany) of the correct size were soaked in transfer buffer. Three layers of soaked Gel-Blotting-Paper were placed on the anode. To avoid air bubbles between the layers the papers were flattened with a roller after each layer. The nitrocellulose membrane was placed on top of the papers. Separating gel was carefully placed over the membrane followed by three layers of pre-soaked Gel-Blotting-Paper. Proteins were transferred from the polyacrylamide gel onto the nitrocellulose membrane (Whatman, Germany) using a semi-dry blotting method in FastBlot B44 chamber (Biometra, Germany). The transfer was performed for 45 minutes at constant current of 2 mA per cm^2 of the membrane. For the transfer efficiency control, the membranes were checked with Ponceau Staining, after incubation of 15 minutes. After briefly washing the unbound staining, they were visualized on a desk scanner. The signal of the large subunit of RuBisCO (rbcL) was used as reference. All staining was removed with water before antibody incubation.

Transfer buffer

25 mM	Tris-HCl (pH 8.3)
150 mM	Glycine
10 %	Methanol (v/v)

Ponceau staining solution 0.2 %   Ponceau S (w/v)
                            3 %   acetic acid (v/v)
                           Destilled water

2.17.2 Immunoprecipitation

Blotted membranes were washed for 15 minutes with TBS buffer and blocked with 5% (w/v) fat-free milk for 1 hour in a shaker at room temperature. Subsequently, the membrane was washed three times for 5 minutes with TBST buffer (TBS buffer supplemented with 0.1 % (v/v) Tween-20). The first antibody, a polyclonal GPF (provided kindly by Dr. Lennard Wirthmüller, FU Berlin), was diluted 1:5000 in TBS buffer supplemented with 1 % (w/v) milk powder. The membrane was incubated in the first antibody overnight at 4 °C and washed with TBST 3 times for 5 minutes in order to remove unbound antibodies. Afterwards, it was incubated for 1 hour in the secondary antibody, an anti-rabbit horseradish peroxidase diluted 1:15,000 (Sigma-Aldrich) and washed for unbound antibodies 3 times in TBST for 5 minutes.

TBS buffer   20 mM Tris-HCl, pH 7.6
                  0.8 % (w/v) NaCl

2.17.3 Chemoluminiscence test

1 ml of the two-step developing agent (Western Blotting Substrate Pierce ECL, Thermo Scientific, Waltham, USA) was added to the membranes. This reagent contains luminol, which emits light if it comes in contact with horseradish peroxidase. It was incubated for 2 minutes just before observation. The chemo luminescent reaction was detected by low intensity light at 428 nm in the ImageQuant mini LAS 4000 Biomolecular Imager (GE Heathcare Europe, Little Chalfont, UK).

Chapter 3
RESULTS I – Optimization of experimental parameters

3.1 Seedling establishment and age

To evaluate chloroplast response to drought stress, *Jatropha* and *Swietenia* seedlings were compared at various developmental stages during the juvenile phase.

Growth conditions for *Swietenia* were evaluated to determine the seedlings establishment capacity (from Panamanian seeds). *S. macrophylla* typically germinates within 17 days in the field (Roman *et al.,* 2012), in in Berlin greenhouse it had early development problems, taking more than 6 weeks for germination; the long waiting period provoked fungal infection in seeds (inside the soil). To increase germination and establishment rates, the humidity in the air was increased by covering the germination racks with transparent plastic foil, but newly developed seeds were getting burned tips. This was associated with excessive light intensity, and it was reduced it by creating a region covered with black tissue (Fig. 3.1).

Figure 3.1. Stablishment problems for *Swietenia macrophylla* in Berlin. (a), first leaves were getting burned in the tips; (b), *Swietenia* & *Jatropha* seedlings were sharing the same environment; (c), pots were covered with a black agro-tissue, to reduce light incidence.

As *Swietenia macrophylla* seeds are collected only from trees in reproductive stage and it is on a CITES list of endangered species, the allowed amount for importation (to Germany) is restricted (permits with 50-300 seeds per year). The experiments were planned with 8-weeks-old seedlings, but the amount of plants germinated in a range of 3 days with the same developmental stage, after all germination phases, was too low for molecular analysis in Berlin. It was not possible to maintaining a substantial number of healthy seedlings in the greenhouse, therefore all experiments with *Swietenia* were performed in Panama.

In Panama, seedlings groups were stablished in collaboration in Prof. Dr. Klaus Winter (Smithsonian Tropical Research Institute). 8-week light-adapted plants were submitted to evaluation of the induction curve (Fig. 3.2) with a Infra Red Gas Analyzer (IRGA), using the third leave from the apex, for three plants. Net photosynthesis was affected for the light intensity used in the chamber, increasing until reaching values of 10-12 μmol CO_2 m^{-2} s^{-1}. Potential increase was calculated to be until 1000 μE light, therefore this value was used for all experiments.
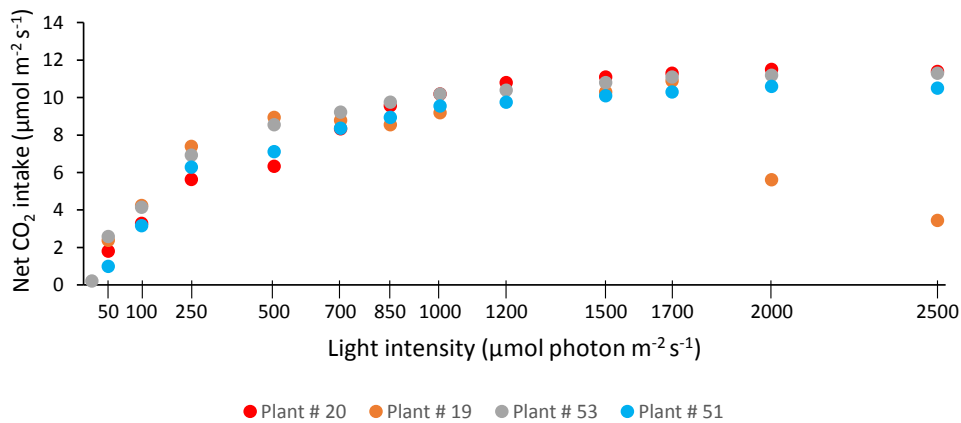
Figure. 3.2. Light induction curve for *Swietenia macrophylla*, using a Li-COR IRGA. Different light intensities were used in the lamp inside the measuring chamber (0, 50, 100, 250, 500, 700, 850, 1000, 1200, 1500, 1700, 2000, 2500 μmol quanta m^{-2} s^{-1}); each color represent one random plant.

Jatropha curcas seeds had low level of germination in Berlin, but almost all developing seedling could establish in the greenhouse. Light intensity and soil composition were the main starting challenge. *J. curcas* is a very resilient species and seeds grew without problem in the soil used normally for *Arabidopsis* (Topf-Erde mix). Establishment of older seedlings (3-4 months-old) that could behave similarly to adults was complicated. The older the plant, the higher risk was found for infection with different pests in the greenhouse (See figure 3.3). Even when regularly watered, some plants developed a disease that made petioles very weak, and the tips of leaves and the main veins started to brown (Fig. 3.3d). Three different types of "sickness" were detected visually, including a red spider, white acars and a fungus.
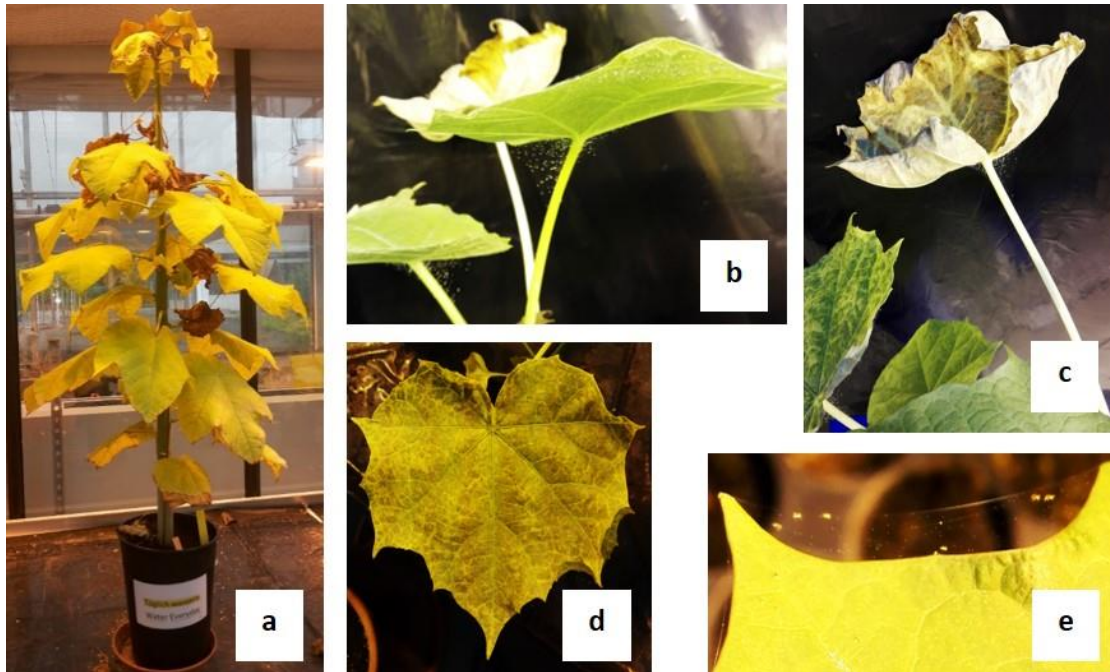
Figure 3.3. Images of *Jatropha curcas* infected during normal growth conditions in the greenhouse. (a) Habitus of a 12-weeks-old seedling, with several dead leaves, (b) and (e) show detail of spider invasion, (c) complete leaf death and high levels of chlorosis in adjacent leaves, (d) brownish leaf veins due to infestation.

To evaluate if it was completely necessary to have 12-old seedlings (or older), a fast evaluation of photosynthetic capacity was performed in 3 leaves from the same plant, situated at the apex, in the middle section, and at the bottom (Fig. 3.4). Three plants, twelve-weeks-old, were submitted to drought stress until the leaves showed symptoms of wilting, together with 3 control plants.

Wilting leaves were used as a visual indicator of water imbalance in the plant. PSII activity was recorded to during this moment to see the effects of this on chloroplast metabolism. Although PSII activity was different in each position evaluated, both for light adapted dark adapted leaves, the difference between control and stress plants is easier observed in leaves situated higher in the apex (Fig. 3.4).

Chlorophyll fluorescence measurement proved a significant difference between stressed and control plants in the higher and middle leaves of all seedlings. Maximum values of quantum yield were observed in dark adapted leaves on the apex, significantly lower in stressed plants (p= 0.004). Under light conditions, effective quantum yield was also higher in the apex, with higher values in control than in stressed plants (p=0.03). Non-photochemical quenching was showed a significant less loss of energy in PSII in apex controls (p=0.04). The most notorious difference was observed in leaves in higher position, which means younger leaves.
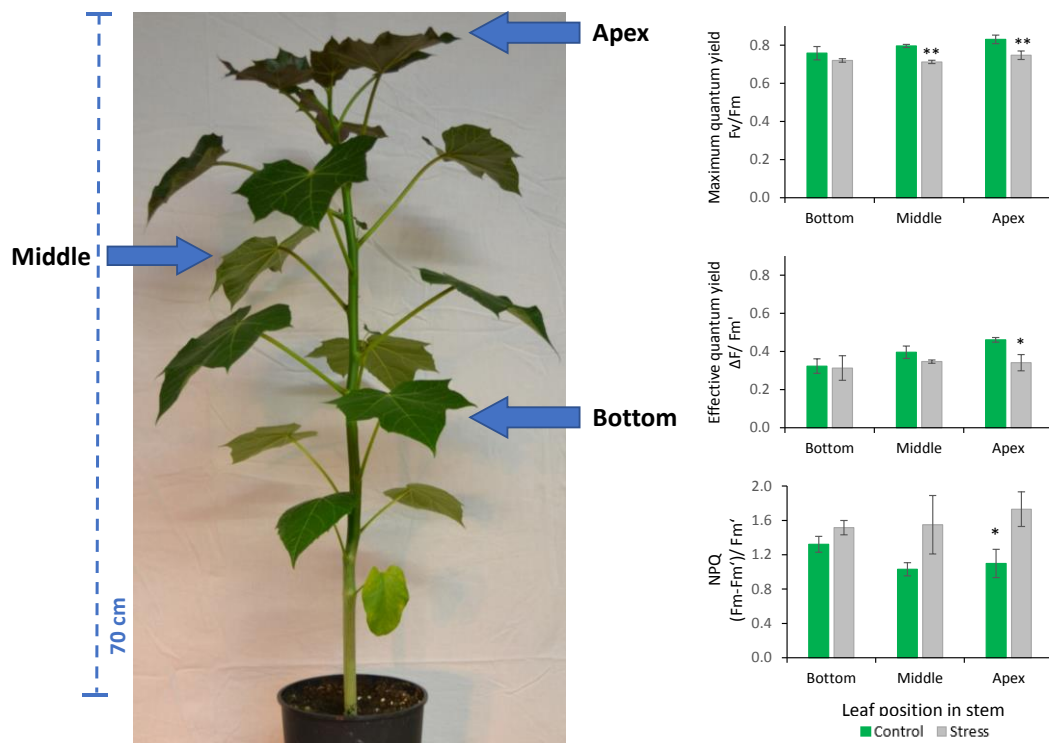
Figure 3.4. Quantum yield of PSII in different leave position on the stem. To the left, description of position for leaves used in analysis. To the right, histograms presenting the chlorophyll flurorescence analysis.

Same plants were submitted to evaluation of the induction curve (Fig. 3.5). In older leaves situated in the lower part of the stem, there is no difference in the induction curve. For the leaves in middle position, results are very similar, and after 3 minutes of the first impulse, values are the same. Only in the leaves close to the apex, the response of stressed plants was distinctive from the controls, since the beginning until the end of the experiment (Fig. 3.5c).

There is a systematic difference in the PSII capacity depending on the leaf's position in the stem (Fig. 3.5d). The closer they are to the apex, the higher access to light they have. Higher levels of irradiation mean that more energy is available that could be used to excite PSII centers, and if plants are not under restrictive conditions could use it for CO_2 assimilation.
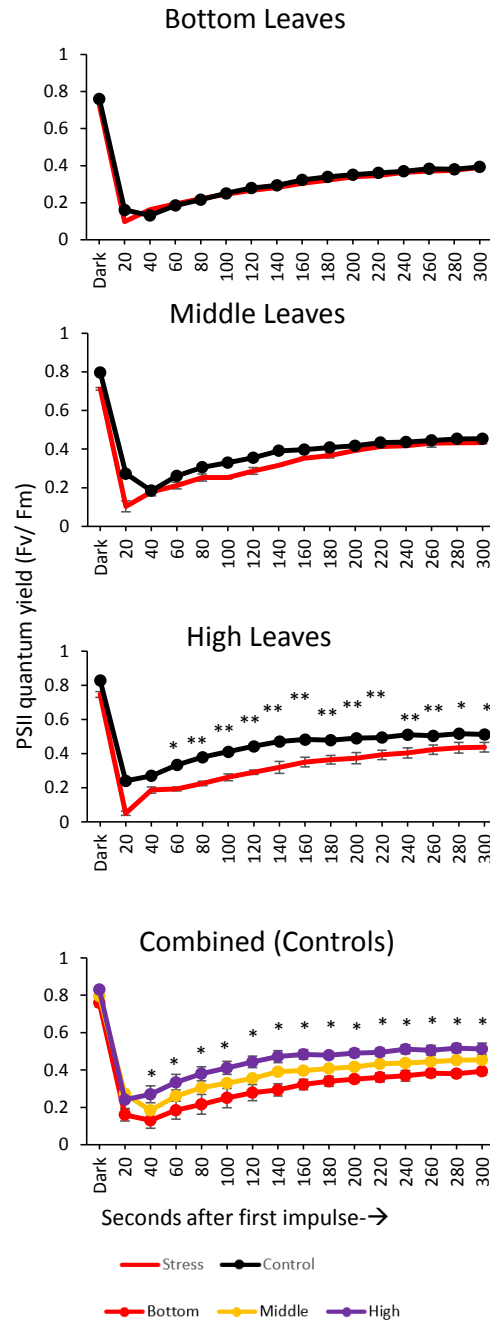
Fig. 3.5. Photosynthetic induction curve of *Jatropha curcas*, divided by leaf position in stem (n=5). Bottom of the plant (2-4th from soil to top, approximately 15-17th from top); middle leaves, approx. 5-7th from top; high leaves, approx. 2-4th fully developed leaf. Starting point: 30 min dark acclimation with clips. 3 measurements per leaf, 3 leaves/ leaf position/plant. Comparison of the induction curve of only control leaves, in different positions in the stem. Stressed plants: subjected to two weeks non-irrigation.

The most appropriate age for studies in this case was 6 weeks-old, because at this point plants had fully developed leaves that could present measurable difference after drought treatment and had a reduced risk of infestation.

3.2 Maximum stress determination

Maximum stress was determined mostly visually by wilting leaves and confirmed by photosynthetic activity. While suspending irrigation for plants, the water balance was affected until a point where the leaves could not keep their turgor; this affected leaf surface appearance and petiole strength. At maximum stress, *Swietenia* plantlets (Fig. 3.6b) had a modification in strength of the base of the leaf, with a slight modification in petiole orientation.
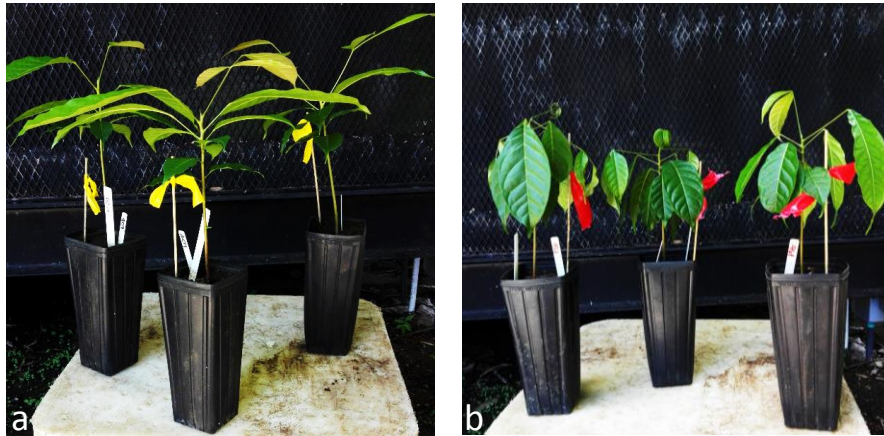
Figure 3.6. Maximum stress in *Swietenia* seedlings. (a) is the regular habitus, (b) is the habitus during drought treatment, with exhibition of wilting leaves.

Jatropha showed two visible droughting responses. Wilting leaves (Fig. 3.7a) were observed in some experiments due to a severe dehydration of petioles, bending itself and the leaf down. In fertilized soils, seedlings just stop growing but leaves were never wilting (Fig. 3.7b). In non-fertilized soils, intermediate responses were also observed (Fig. 3.7c).
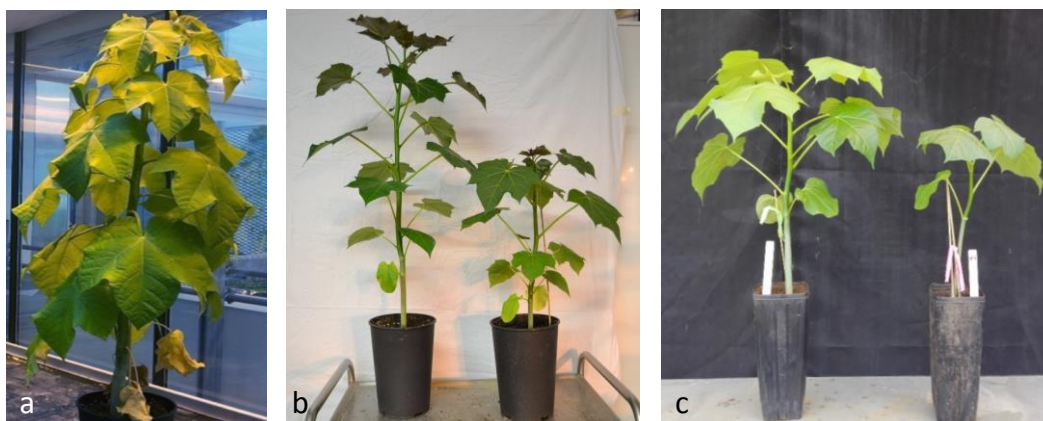
Figure 3.7. Maximum stress in *Jatropha* seedlings. (a) wilting leaves; (b) stress in fertilized soils produce smaller seedlings (E5), right image is the regular habitus and stressed plant is to it left. (c) intermediate stress response observed in E4.

3.3 Atmospheric conditions

During the experimental phase, atmospheric conditions were recorded. For the experiments in Panama during 2016, data was collected by a weather station in Gamboa, and provided by the Smithsonian Tropical Research Institute. For the experiments in Panama during 2017 and all the experiments in Berlin, data was collected with a portable sensor Voltcraft Data Logger DL-121 TH (2017).

3.3.1 *Swietenia macrophylla*

Summary of this information is show in Table 3.1. Registered temperatures indicate that at the experimental site in Panama, there were daily temperatures above 32°C and nights around 23 °C, which represent in average 10°C difference between the hottest moment during daytime and the coolest moment at night. Light intensity was found to be similar in open sun conditions, in experiments performed in 2016 and 2017, while the lowest irradiation range occurred in experiment 4, in shadows.

Table 3.1. Average values for temperature, light intensity and humidity for experiments of *Swietenia macrophylla*. Daily values used correspond to the hottest 8 hours. Night values correspond to the coolest 8 hours. Light intensity (µmol quanta m^{-2} s^{-1}) represents the average of measurements done during sample collection (9:00 – 14:00 h). Extreme values were eliminated.

Swietenia	T° Day	T° Night	Air Hum Day	Air Hum Night	Light intensity
2016 Sun (E1)	**34 °C** (+/-6 °C)	**23 °C** (+/- 2 °C)	**55%** (+/- 8°C)	**95%** (+/- 2%)	**800-1500** (+/- 500µE)
2016 Rain (E2)	**32 °C** (+/-7 °C)	**23,5 °C** (+/-3 °C)	**65%** (+/- 5%)	**97%** (+/- 1%)	**600-1000** (+/- 200µE)
2017 Sun (E1)	**34 °C** (+/- 4 °C)	**22 °C** (+/- 2 °C)	**52,5 %** (+/- 3 %)	**92%** (+/- 3%)	**800-1500** (+/- 500µE)
2017 Shadow (E3)	**31 °C** (+/- 7 °C)	**23 °C** (+/- 3 °C)	**55%** (+/- 5%)	**95%** (+/- 5%)	**150-600** (+/- 400µE)
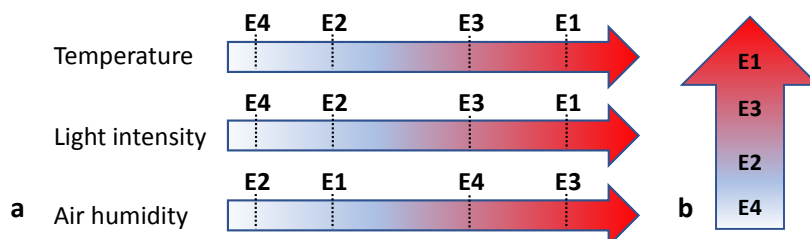
Figure 3.8. Schema of environmental condition stress levels in all experiments for *Swietenia macrophylla*. Each arrow indicates increasing difficulty, from blue (mildest condition) to red (hardest condition). (a) Independent evaluation of factors, (b), combined stress factors.

Adding the effects of all parameters recorded, experimental stress can be categorized from the mildest to the strongest condition (Fig. 3.8). From all four experiments, the strongest stress condition was registered for Experiment 1. The mildest condition in combined stress is considered to happen for Experiment 4.

3.3.2   Jatropha curcas

Registered temperatures indicate that in Panama there were daily temperatures above 33°C and around 23 °C for nights, while the experiments in Berlin had maximum daily temperatures of 29°C and nights of 18 °C (Table 3.2). In general, outdoors experiments were 4-5 °C hotter during daylight than indoor experiments. A very high irradiations was observed in Experiment 1, the double to those in indoor experiments.

In combined stresses (Fig. 3.9), strongest stress condition was experienced by E1 (highest temperature and irradiance, lowest humidity), while the mildest condition is considered to happen for Experiment 5, because of high nutrition available.

An analysis of one classical day in the experiments under full sun in Panama (April 1st, 2017), presents the actual modification in temperature and humidity in hourly values (Fig. 3.10). The environmental values might indicate that maximum or minimum temperatures are not the bigger concern for plants under regular conditions, instead the speed in temperature variation.

One hour after the sunrise at 6 am, starts a steady reduction of the humidity that brings values from 95% to 40% in 8 hours. The most extreme change is in temperature, which stays around 22-24 during night. One hour after sunrise, the surfaces start to heat, and air temperature jumps 1°C each 10mins, reaching 30°C in 1 hour. This corresponds to a dramatic change in one hour, followed by an increasing trend for six hours more, reaching until 36°C while staying at maximum point (37.5 °C) for 2 hours. Cooling off is a slower process that takes the double of time.

Table 3.2. Average values for temperature and humidity for experiments of *Jatropha curcas*. Daily values used correspond to the hottest 8 hours and night values, to the coolest 8 hours. Light intensity (µmol quanta m^{-2} s^{-1}) represents the average of measurements done during sample collection (9:00 – 14:00 h). Extreme values were eliminated.

Jatropha	T° Day	T° Night	Air Hum Day	Air Hum Night	Light intensity
Panama SUN (E1)	36°C (+/-4°C)	22°C (+/- 2°)	50% (+/-3°C)	90% (+/- 3%)	800-1500 (+/- 500µE)
Panama Shadow (E2)	33°C (+/-7°C)	23,5°C (+/-3°C)	55% (+/- 5%)	93% (+/- 5%)	150-600 (+/- 200µE)
Berlin (E3)	29 °C (+/- 2°)	18,5 °C (+/- 1°)	52,5 % (+/- 19%)	78,5 % (+/- 8%)	300-500 (+/- 50µE)
Berlin (E4)	29 °C (+/- 2°)	18,5 °C (+/- 1°)	52,5 % (+/- 19%)	78,5 % (+/- 8%)	100-300 (+/- 50µE)
Berlin (E5)	28 °C (+/- 2°)	17 °C (+/- 1°)	52,5 % (+/- 19%)	80% (+/- 5%)	300-600 (+/- 50µE)
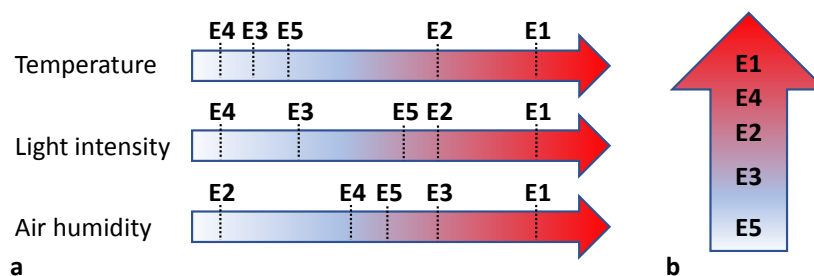
Figure 3.9. Schema of environmental condition stress levels in all experiments for *Jatropha curcas*. Each arrow indicates increasing difficulty, from blue (mildest condition) to red (hardest condition). (a) Independent evaluation of factors, (b), combined stress factors.
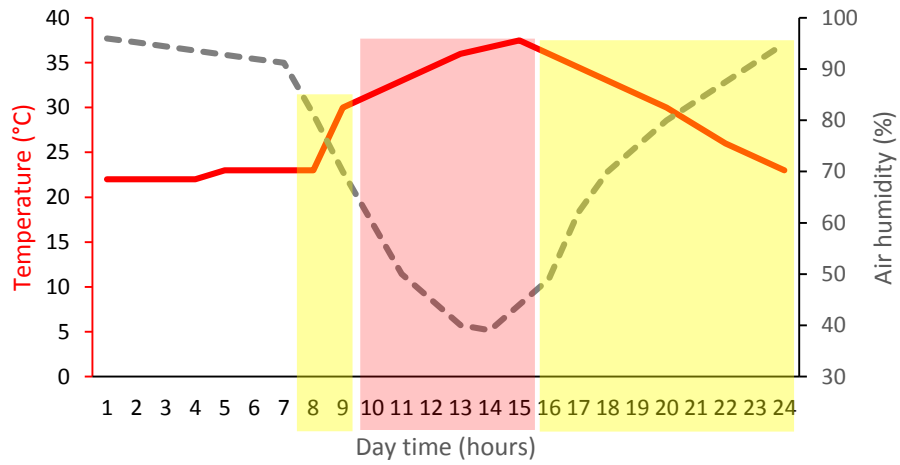
Figure 3.10. Average day for the experiments under full sun in Panama. Measurements were done every 10 minutes. All data is included. Red line represents the temperature, while the gray interrupted line corresponds to air humidity. Red box indicates the maximum temperatures; yellow boxes indicate the increase and decrease period.

CHAPTER 4
RESULTS II– *Swietenia macrophylla*

4.1. Phylogenetic evaluation

Swietenia macrophylla has not been sequenced. Moreover, there is no information about the genes of the enzymatic antioxidant system in the chloroplast in this species. This situation was overcome by cloning a partial sequence based on related plants, browsing sequences from PeroxiBase (Fawal *et al*., 2013) and GenBank (Wheeler *et al*., 2001). The first step in this direction was to determine *Swietenia's* scientific classification (Fig. 4.1), and then search the related genus, families and orders linked to this species (Fig. 4.2).

Kingdom:	Plantae
Phylum:	Magnoliophyta
Class:	Magnoliopsida
Order:	**Sapindales**
Family:	**Meliaceae**
Genus:	*Swietenia*
Species:	*S. macrophylla* (King)

Figure 4.1. Scientific classification of *Swietenia macrophylla*. Information provided by the Missouri Botanical Garden and confirmed in the Angiosperm Phylogeny website.

A phylogenetic tree based on order evolution, constructed with the aid of **Angiosperm Phylogeny Website**, version 14 (Stevens, P.F., 2001), showed the position of *Swietenia* in the evolution, and to estimate the degree of relatedness to the species reporting the available sequences for 2CP, APX and GPX (Fig. 4.2). In the order of Rosids, there are 119 sequenced species reported in the database PlaBi of the University of Aachen, Germany. In the order of Sapindales, they collected 11 genomes, from which there is only one species in the same family (Meliaceae) and tribe than *Swietenia* (*Azadirchta indica*), and nine genomes from *Citrus*, in the "cousin" family Rutaceae.

Taking in mind that genes are prone to be conserved between related individuals, available sequences for each gene of interest (GOI) were collected, including only the ones belonging to the closest related plants to *Swietenia.*
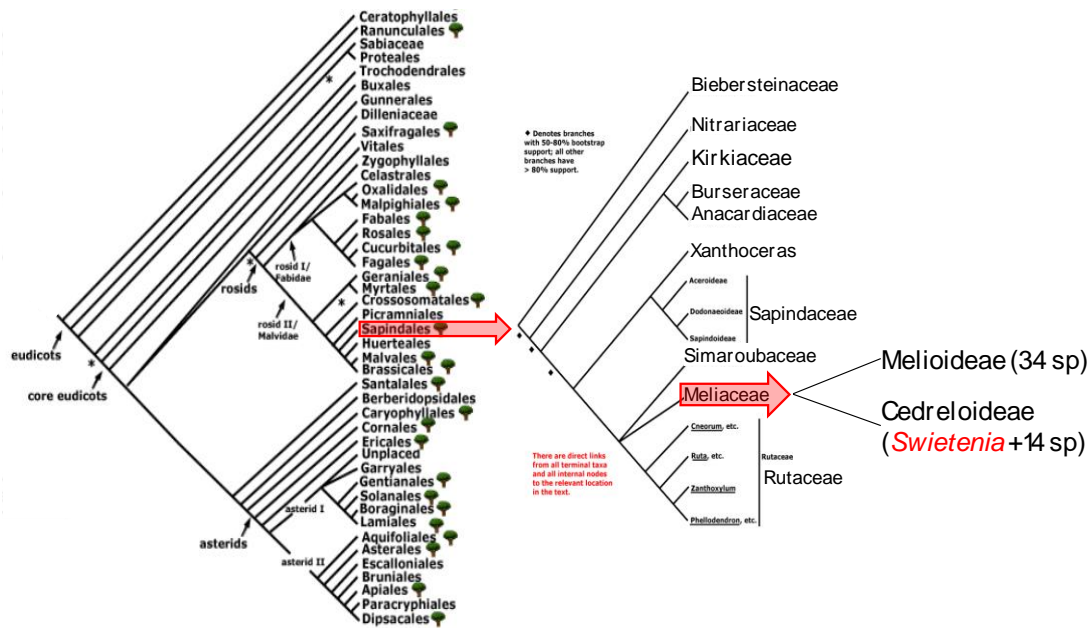
Figure 4.2. Phylogenetic trees describing Meliaceae (Family). Angiosperm tree shows close related orders (left); description of families in the Sapindales (middle); at the end is shown the division of tribes inside Meliaceae, with the containing species

4.2. Generation of Partial Sequences

The process of recognition for partial sequences in the genes of study started with the collection of available sequences in related species. This full-sequences were aligned to discover conserved regions and to design wobbled primers (degenerated primers), that included all variations possible to target a specific region. Each gene was study separately and presented different related sequences mix.

4.2.1 For 2-cys-peroxiredoxin (*2CP*)

2-Cys-Peroxiredoxin sequences were found for related species such as *Arabidopsis thaliana (At), Brachipodium distachyon (Brd), Brasicca napus (Bn), Citrus sinensis (Cs), Curcumis sativus (Cus), Eucalyptus grandis (Eg), Glycine max (Gm), Hevea brasiliensis (Hv), Jatropha curcas (Jc), Malus domestica Md), Medicago trunculata (Mt), Morus notabilis (Mn), Nicotiana tabacum (Nt), Pisum sativum (Ps), Populus trichocarpa (Pt), Spinacia oleraceae (So), Solanum tuberosum (St), Tamaris hispida (Th), Theobroma cacao (Tc)* and *Vitis vinifera (Vv);* abbreviations in parenthesis are used for Fig. 4.3.

Peptide sequences for the species mentioned above were aligned, and to discriminate the conserved regions with less variability, each amino acid (aa) was classified with a color-code using Jalview (Waterhouse *et al*., 2009). All amino acids are coded by a combination of three

of the existing nitrogen bases (A, T, C, G). Few aa are coded by only one combination, like Methionine (ATG), while most aa have multiple coding combinations, like Alanine (GCA, GCT, GCC, GCG). In the alignment, each amino acid (aa) was classified based on the number of codons possible to code for them and visualized in specific colors.

Figure 4.3. Related sequence alignment for *2CP*. Code color was installed, distinguishing between amino acids with 1 codon possible (Yellow), 2 codons (Red) four codons (Purple) and six codons (Blue). Primers position is marked with black arrows.

Areas of high aa conservation including aa with low codon variation (1-2) and separated by minimum 90 aa, were selected for wobble primer design (Fig. 4.3). All amino acids in the same position and all necessary codons for coding them were written down to calculate the variation needed for each position. The proposed primer sequence was then determined as IUPAC compressed nomenclature, with the use of the IUPAC system, that includes **ATCG** + combinations as **R** = G A (purine), **Y** = T C (pyrimidine), **K** = G T (keto), **M** = A C (amino),

S = G C (strong bonds), **W** = A T (weak bonds), **B** = G T C (all but A), **D** = G A T (all but C), **H** = A C T (all but G), **V** = G C A (all but T), **N** = A G C T (any).

IUPAC coded sequence were evaluated with the Oligo Calc (Kibbe 2007) version 3.27, based on the GC content and melting temperature to select the most similar primer pairs. Before primer order, their properties were checked in the OligoEvaluator from Sigma Aldrich.

- **Variation calculations in wobble primers**

Several position for primers were analyzed based on the nucleotide variation inside the peptide sequence. The process for quantification of the variation was the same for all primer positions (in all genes) and can be explained with the evaluation of the final primer pair for 2CP. In this case, the forward primer included the sequence **DFTFVCP**, while the position for the reverse primer was **WKPGEKSM**. Each of these amino acids were translated to the possible nucleotide variation in each position of the codon, as follows:

- **Forward primer**

```
D -   F -   T -   F -   V -   C -   P -   T
GAU   UUU   ACU   UUU   GUU   UGU   CCU   ACU
  C     C     C     C     C     C     C     C      Possible codon
combinations
              A           A           A     A
              G           G           G     G
GAY   UUY   ACN   UUY   GUN   UGY   CCN   ACN    →  Compressed IUPAC
```

- **Reverse primer**

```
W -   K -   P -   G -   E -   K -   S -   M
UGG   AAA   CCU   GGU   GAA   AAA   UCU   AUG
        G     C     C     G     G     C            Possible codon
combinations
              A     A                 A
              G     G                 G
                                    AGU
                                      C
UGG   AAR   CCN   GGN   GAR   AAR   WSN   AUG      →  Compressed IUPAC

CAT   NSW   YTT   YTC   NCC   NGG   YTT   CCA      →  Reverse Complement
```

Variation inside wobble primers represented the amount of sequences mixed to cover all necessary changes and was calculated multiplying the nucleotide variation at each position as follows:

Forward:

G	A	Y	U	U	Y	G	U	N	U	G	Y	C	C	N	A	C	N	IUPAC
1	1	2	1	1	2	1	1	4	1	1	2	1	1	4	1	1	4	Nt var.
	2			2			4			2			4			4		Codon variation

Total variation (TV): 512

Reverse:

C	A	T	N	S	W	Y	T	T	Y	T	C	N	C	C	N	G	G	Y	T	T	C	C	A
1	1	1	4	2	2	2	1	1	2	1	1	4	1	1	4	1	1	2	1	1	1	1	1
	1			16			2			2			4			4			2			1	

(TV): 2048

Forward wobble primer for *2CP* was composed by a mix of 512 variations (possible sequences) and the reverse primer is a mix of 2048 variations. Expected amplification for those primers was 420 bp. After amplification using these wobble primers in a RT-PCR, the partial DNA sequence produced was cloned into a pCR2 vector and verified by commercial sequencing. The result after sequencing was translated into amino acid sequence and aligned with *A. thaliana 2CP* (Fig. 4.4), showing 92% identity and 97% similarity in amino acids, to Ath 2CP-b. Partial sequences covered 50% of *A. thaliana*'s, mostly to the 3' region.

```
At_2CPb    MSMASIASSSSTTLLSSSRVLLPSKSSLLSPTVSFPRIIPSSSASSSSLCSGFSSLGSLT    60
Sm_2CP     ------------------------------------------------------------    0


At_2CPb    TNRSASRRNFAVKAQADDLPLVGNKAPDFEAEAVFDQEFIKVKLSEYIGKKYVILFFYPL   120
Sm_2CP     ------------------------------------------------------------    0


At_2CPb    DFTFVCPTEITAFSDRYEEFEKLNTEVLGVSVDSVFSHLAWVQTDRKSGGLGDLNYPLVS   180
Sm_2CP     DFTFVCPTEITAFSDRYADFEKLNTEILGVSVDSVFSHLA-VQTDRKSGGLGDLKYPLIS    59
           ****************  :*******:************* **************:***:*

At_2CPb    DITKSISKSFGVLIPDQGIALRGLFIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYV   240
Sm_2CP     DVTKSISKSYGVLIPDQGIALRGLFIIDKDGVIQHSTINNLAIGRSVDETMRTLQALQYV   119
           *:*******:*******************:********** .******************

At_2CPb    QENPDEVCPAGWKPGEKSMKPDPKLSKEYFSAI   273
Sm_2CP     QENPDEVCPAGWKPGEKTM-------------   138
           *****************:*
```

Figure 4.4. Alignment for 2CP. *Swietenia macrophylla* (Sm) partial sequence alignment to At 2CPb (AT5G06290).

Primers for qPCR amplification were based on this fragment using **Primer3**, version 4.1.0 (Untergasser *et al.,* 2012 & Koressaar *et al.,* 2007).

4.2.2   For chloroplast ascorbate peroxidase (*cAPX*)

Ascorbate peroxidase sequences from chloroplast isoforms were found for related species such as *Arabidopsis thaliana (At), Brasicca napus (Bn), Citrus sinensis (Cs), Citrus clementine (Cic), Citrus sinensis (Cis), Cucumis melo (Cum), Cucumis sativus (Cs), Eucalyptus grandis (Eug), Glycine max (Glm), Hevea brasiliensis (Hb), Jatropha curcas (Jc), Malus domestica (Mad), Morus notabilis (Mon), Nicotiana tabacum (Nt), Populus trichocarpa (Pt), Solanum lycopersicum (Sol), Solanum tuberosum (Sot), Tamaris hispida (Tah), Theobroma cacao (Tc), Vitis vinifera (Viv);* abbreviations in parenthesis are used for Fig. 4.5.

Peptide sequences for the species mentioned above were aligned, and to discriminate the conserved regions with less variability, each amino acid (aa) was classified with a color code using Jalview (Waterhouse *et al*., 2009) (Fig. 4.5). Primer positions were discriminated in the same way than for *2CP.*

Variation calculations in wobble primers

In the same way as done for 2CP, the peptide region selected for forward and reverse primers was translated to IUPAC coding system and all possible combinations were calculated.

    o   **Forward primer:**

```
H -  D -  A/S -  G -  T -  Y -  N/D - K
CAY GAY DSN GGN ACN TAY RAY AAR
```
→ Compressed IUPAC

Calculated in the same way as previously, this primer represents 12,288 combinations.

    o   **Reverse primer**

```
K -  F -  D -  N -  S -  Y -  F -  K
YTT RAA RTA NSW RTT RTC RAA YTT
```
→ Compressed IUPAC (Rev. Compl.)

Calculated in the same way as previously, this primer represents 2,048 combinations.
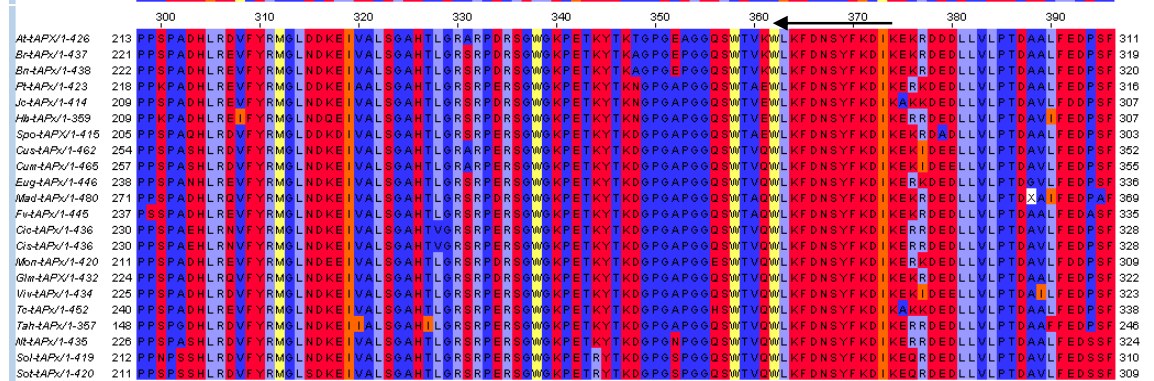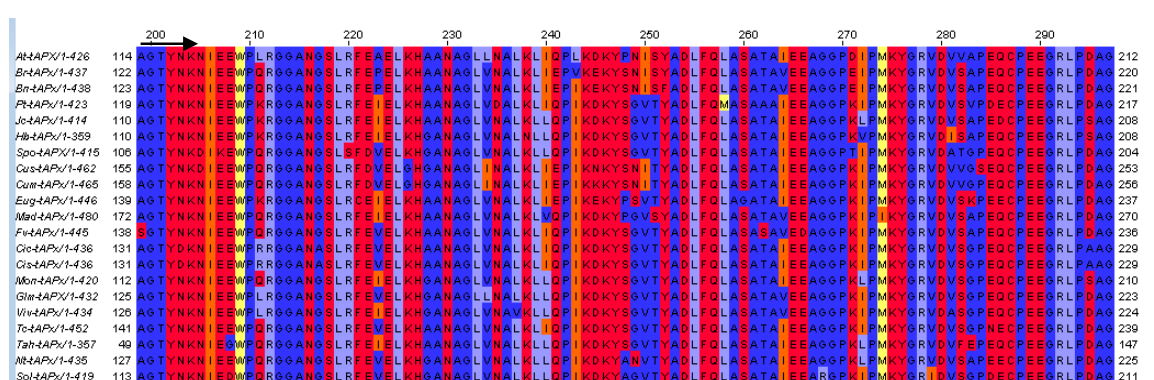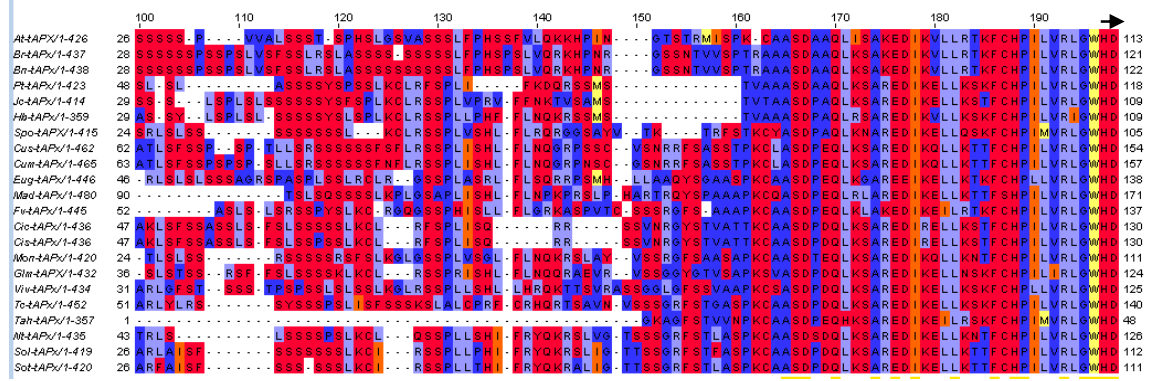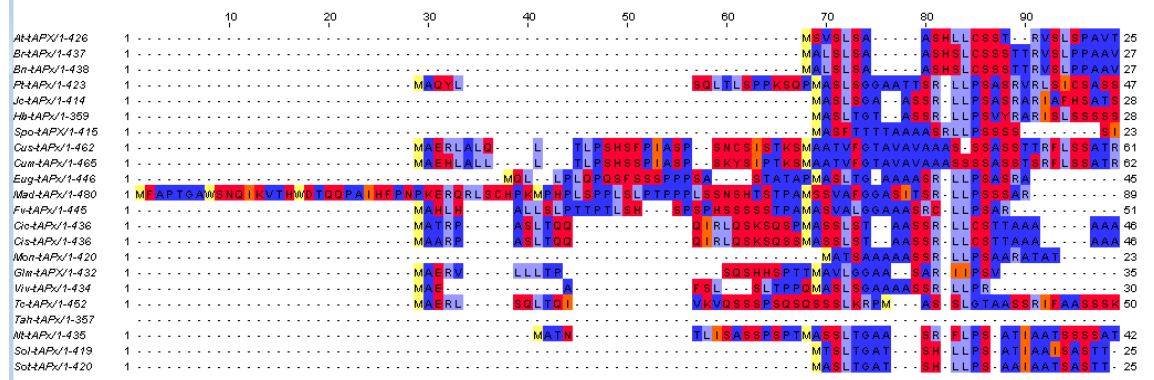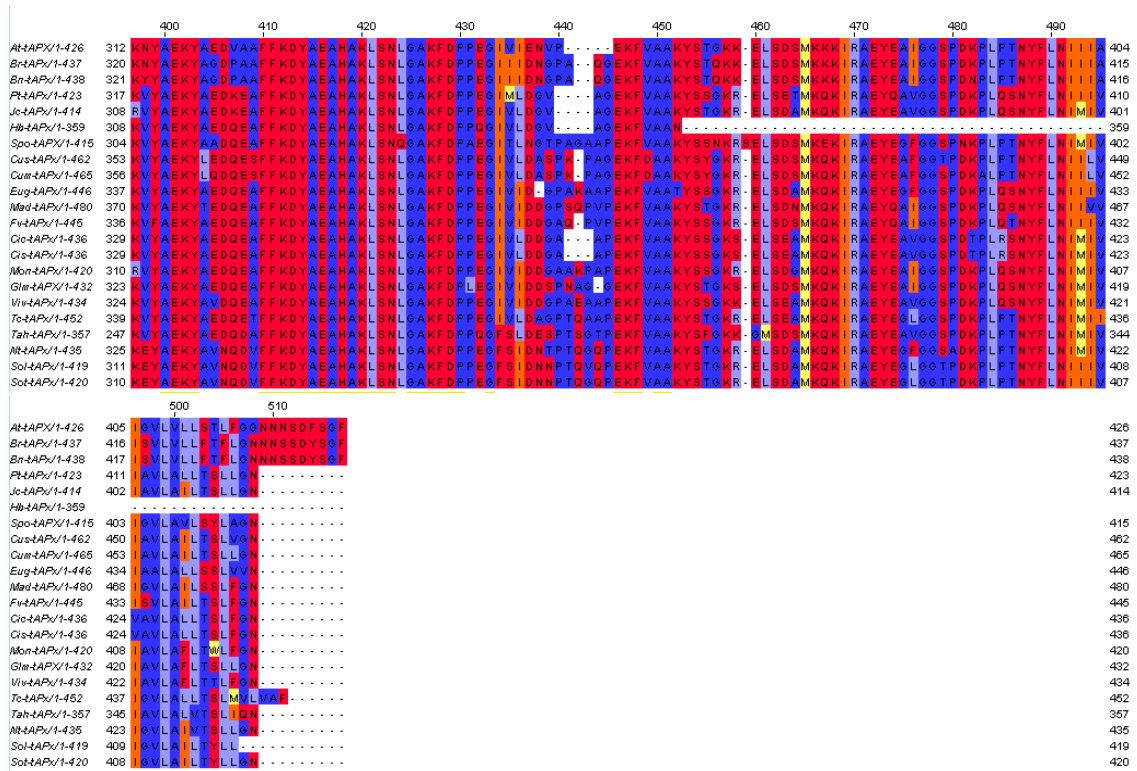
Figure 4.5. Related sequence alignment for APX. Code color was installed, distinguishing between amino acids with 1 codon possible (Yellow), 2 codons (Red) four codons (Purple) and six codons (Blue). Primers position is marked with black arrows.

Expected amplification for those primers is 531bp. After amplification of the cDNA fragment using these wobbled primers in a RT-PCR, this partial DNA sequence was cloned into a pCR2 vector and verified by commercial sequencing.

The result after sequencing was translated into a peptide sequence and aligned with chloroplast APX of *A. thaliana* (Fig. 4.6), showing 87% identity and 93% similarity to *tAPX*, while 88% identity to and 94% similarity to *sAPX*, covering 50%, mostly in the central region.

68

```
At_tAPX        MSVSLSAASHLLCSSTRVSLSPAVTSSSSSPVVALSSSTSPHSLGSVASSSLFPHSSFVL        60
Sm_APX         -----------------------------------------------------------         0


At_tAPX        QKKHPINGTSTRMISPKCAASDAAQLISAKEDIKVLLRTKFCHPILVRLGWHDAGTYNKN        120
Sm_APX         -----------------------------------------------HDGGTYNKN           9
                                                               **.******

At_tAPX        IEEWPLRGGANGSLRFEAELKHAANAGLLNALKLIQPLKDKYPNISYADLFQLASATAIE        180
Sm_APX         IEEWPKRGGANGSLRFEIELKHAANAGLVNALKLLQPIKDKYSGVTYADLFQLASATAIE         69
               ***** *********** *********:*****:**:****  .:.:**************

At_tAPX        EAGGPDIPMKYGRVDVVAPEQCPEEGRLPDAGPPSPADHLRDVFYRMGLDDKEIVALSGA        240
Sm_APX         EAGGPKLPMKYGRVDVSAPEDCPEEGRLPSAGPPSPADHLREVFYRMGLNDKEIVALSGA        129
               *****.:********* ***:********.***********:*******:**********

At_tAPX        HTLGRARPDRSGWGKPETKYTKTGPGEAGGQSWTVKWLKFDNSYFKDIKEKRDDDLLVLP        300
Sm_APX         HTLGRSRPDRSGWGKPETKYTKNGPGAPGGQSWTVEWLKFDNSYFK-------------        175
               *****:****************.***   *******:**********

At_tAPX        TDAALFEDPSFKNYAEKYAEDVAAFFKDYAEAHAKLSNLGAKFDPPEGIVIENVPEKFVA        360
Sm_APX         -----------------------------------------------------------        175


At_tAPX        AKYSTGKKELSDSMKKKIRAEYEAIGGSPDKPLPTNYFLNIIIAIGVLVLLSTLFGGNNN        420
Sm_APX         -----------------------------------------------------------        175


At_tAPX        SDFSGF426
Sm_APX         ------175
```

Figure 4.6. Alignment for *APX*. *Swietenia macrophylla* (Sm) partial sequence alignment to At tAPX (AT5G06290.1).

4.2.3   For glutathione peroxidase (GPX)

Glutathione peroxidase sequences were found for related species including *Arabidopsis thaliana (At), Brasicca napus (Bn), Cicer arietinum (Ca), Cucumis melo (Cm), Gossyphium hirsutum (Gh), Hevea brasiliensis (Hb), Jatropha curcas (Jc), Malus domestica (Md), Medicago trunculata (Mt), Morus notabilis (Mn), Nicotiana tabacum (Nt), Populus trichocarpa (Pt), Lycopersicon esculentum (Le), Theobroma cacao (Tc), Vitis vinifera (Vv);* abbreviations in parenthesis are used for Fig. 4.7.

After the evaluation of conserved regions in related species (Fig. 4.7), wobble primers selected had the following characteristics:

<u>Forward primer seq.</u>   YAYGAYTWYACNGTNAARGAYATH   → 6,144 variations

<u>Reverse primer seq.</u>   YTC NAC YTT RTC RAA DAT NGG RAA   → 1,024 variations
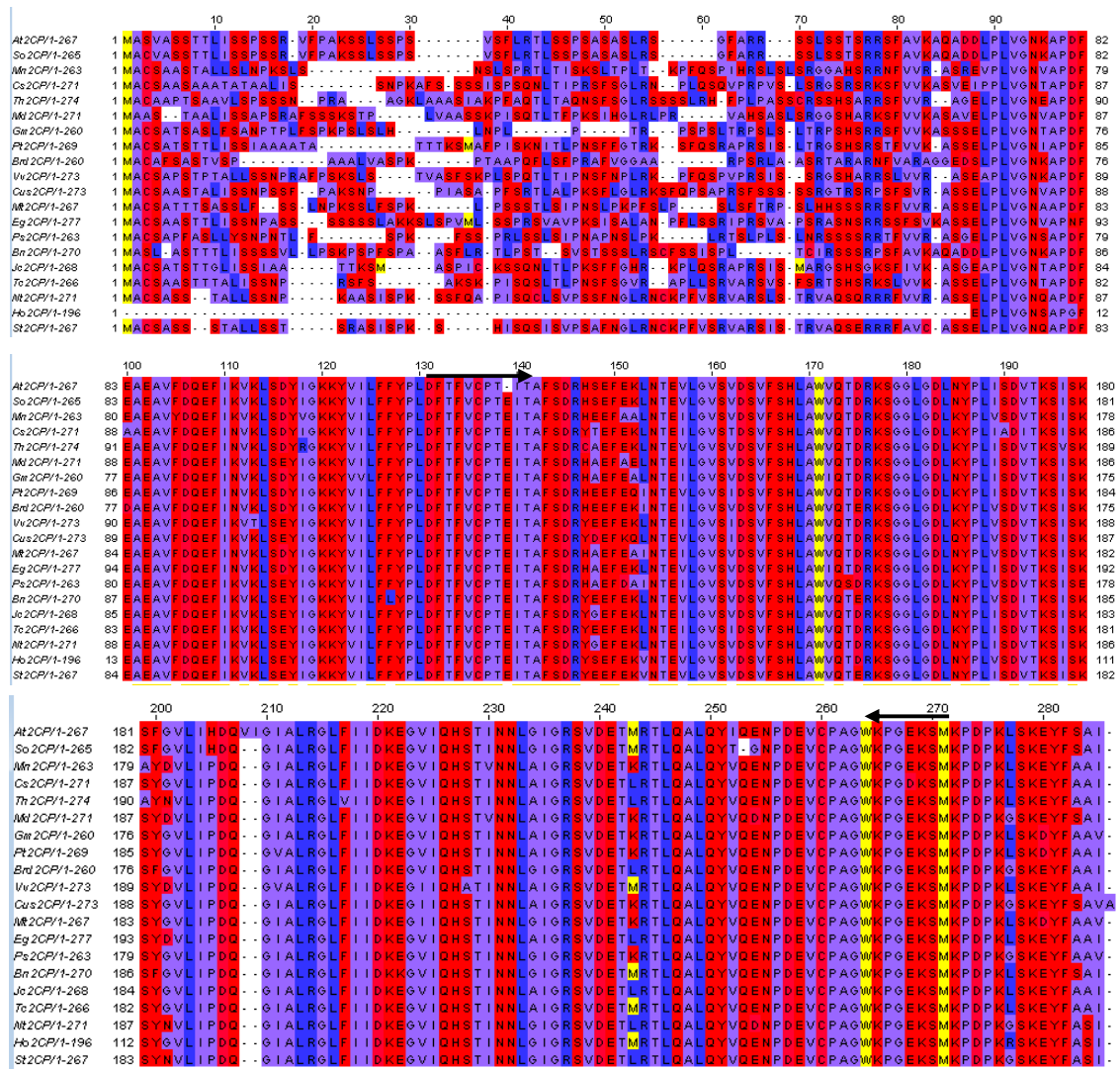
Expected amplification: 360 nt.

Figure 4.7. Related sequence alignment for *GPX*. Code color was installed, distinguishing between amino acids with 1 codon possible (Yellow), 2 codons (Red) four codons (Purple) and six codons (Blue). Primers position is marked with black arrows.

After amplification by RT-PCR, this partial DNA sequence was cloned and verified by commercial sequencing. The result was translated into a peptide and aligned with *A. thaliana* (Fig. 4.8), showing 90% identity and 93% similarity to GPX7, covering 42% condensed in the central region.

```
At_GPX7    MAFSYASFSTPFNGFAANPSPITSAFLGPSLRFSTRTSKTRNPSNGVSVKSSNSHRFLVK    60
Sm_GPX     ------------------------------------------------------------     0


At_GPX7    SKNFSVYARAAAEKSVHDFTVKDIDGNDVSLDKFKGKPLLIVNVASRCGLTSSNYSELSQ    120
Sm_GPX     ---------------HDFTVKDIDGENVSLGKFKGKALLVVNVASRCGLTSSNYSELSH     44
                          **********::***.***** **:*****************:

At_GPX7    LYEKYKNQGFEILAFPCNQFGGQEPGSNPEIKQFACTRFKAEFPIFDKVDVNGPSTAPIY   180
Sm_GPX     LYEKYKTQGFEILAFPCNQFGGQEPGSNPEIKKFACTRFKTEFPIFDKVEVNG------    97
           ******.*****************************:*******:*********:***

At_GPX7    KFLKSNAGGFLGDIIKWNFEKFLVDKKGKVVERYPPTTSPFQIEKDIQKLLAA 233
Sm_GPX     ----------------------------------------------------- 97
```

Figure 4.8. Alignment for *GPX*. *Swietenia macrophylla* (Sm) partial sequence alignment to At GPX7 (AT4G31870).

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To date, there are no previous studies using or comparing stability of reference genes, thus two of the most used ones were cloned in the same way than the genes of interest. For *Swietenia macrophylla*, the genes used as reference were *Actin* and *Tubulin*.

4.2.4   For actin (*ACT*)

Actin sequences were found for related species such as *Ammopiptanthus nanus, Arabidopsis thaliana, Brassica napus, Castanea sativa, Citrus sinensis, Drosera rotundifolia, Eucalyptus grandis, Gossypium arboretum, Gossypium hirsutum, Gossypium raimondii, Hevea brasiliensis, Hibiscus cannabinus, Jatropha curcas, Malus domestica, Mimosa púdica, Moringa oleífera, Morus alba, Oxytropis arctobia, Oxytropis splendens, Pisum sativum, Populus nigra, Populus trichocarpa, Prunus marítima, Pyrus pyrifolia, Quercus robur, Rosa hybrid* and *Rubus idaeus.*

Alignment of related peptide sequences was made based on the conserved regions (as for the other genes), and used to select wobble primers with the following characteristics:

<u>Forward primer seq</u>.   GGCAYCAYACNTTYTAYAA   → 64 variations

<u>Reverse primer seq</u>.   GGNGCNACNACYTTDATYTTCAT   → 768 variations
<u>Expected amplification</u>: 738 nt.

After amplification by RT-PCR, this partial DNA sequence was cloned and verified by commercial sequencing. The result was translated into a peptide sequence and aligned with *A. thaliana* (Fig. 4.9), showing 95% identity and 99% similarity to At ACT-11, covering 66%, condensed in the central and 3' region.

4.2.5   For tubulin (TUB)

Tubulin sequence were found for related species such as *Arabidopsis thaliana, Brassica napus, Camelina sativa, Citrus sinensis, Cucumis melo, Eucalyptus grandis, Glycine max, Gossypium raimondii, Hevea brasiliensis, Jatropha curcas, Malus domestica, Morus notabilis, Phaseolus vulgaris, Populus tremuloides. Tarenaya hassleriana* and *Vigna radiata.*

After alignment of related peptide sequences and based on the conserved regions (as for the other genes), selected wobble primers should amplify 824 nucleotides, and had the following characteristics:

<u>Forward primer seq</u>.   TGGGARYTNTAYTGYYTNGARCAY   → 2,048 variations

<u>Reverse primer seq</u>.   CATNARRCARCANGCCATRTAYTTNCCRTG   →512 variations

After amplification by RT-PCR, this partial DNA sequence was cloned and verified by commercial sequencing. The result was translated into protein and aligned with *A. thaliana* (Fig. 4.10), showing 97% identity and 97% similarity to At TUB α4, covering 60%, condensed in the central and 3' region.

```
At_ACT-11    MADGEDIQPLVCDNGTGMVKAGFAGDDAPRAVFPSIVGRPRHTGVMVGMGQKDAYVGDEA  60
Sm_ACT       ------------------------------------------------------------  0


At_ACT-11    QSKRGILTLKYPIEHGIVSNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREK  120
Sm_ACT       --------------------------------ELRVAPEEHPVLLTEAPLNPKANREK   26
                                             ************************


At_ACT-11    MTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRL  180
Sm_ACT       MTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRL   86
             ************************************************************


At_ACT-11    DLAGRDLTDYLMKILTERGYSFTTSAEREIVRDVKEKLAYIALDYEQEMETANTSSSVEK  240
Sm_ACT       DLAGRDLTDHLMKILTERGYSFTTTAEREIVRDMKEKLAYIALDYEKEMETSKTSSSVEK  146
             *********:***************:********:************:****::*******


At_ACT-11    SYELPDGQVITIGGERFRCPEVLFQPSLVGMEAAGIHETTYNSIMKCDVDIRKDLYGNIV  300
Sm_ACT       SYELPDGQVITIGAERFRCPEVLFQPSMIGMEAAGIHETTYNSIMKCDVDIRKDLYGNIV  206
             *************.*************::********************************


At_ACT-11    LSGGTTMFPGIADRMSKEITALAPSSMKIKVVAPPERKYSVWIGGSILASLSTFQQMWIA  360
Sm_ACT       LSGGSTMFPGIADRMSKEISALAPSSMKIKV-----------------------------  237
             ****:*************:***********


At_ACT-11    KAEYDESGPSIVHRKCF        377
Sm_ACT       ----------------        237
```

Figure 4.9. Alignment for Actin. *Swietenia macrophylla* (Sm) partial sequence alignment to At ACT-11 (AT3G12110).

```
At_TUB-a4    MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQMPSDKTVGGGDDAFNTFFSETGAGK  60
Sm_TUB       ------------------------------------------------CFQHFFSETAAGK  13
                                                             .*: *****.***


At_TUB-a4    HVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD  120
Sm_TUB       HVPRAVFVDLEPTVIDEVRTGAYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD  73
             *********************:**************************************


At_TUB-a4    RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS  180
Sm_TUB       RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS  133
             ************************************************************


At_TUB-a4    VVEPYNSVLSTHSLLEHTDVSILLDNEAIYDICRRSLSIERPTYTNLNRLVSQVISSLTA  240
Sm_TUB       VVEPYNSVLSTHSLLEHTDVSVLLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTA  193
             *********************:***************.***********************


At_TUB-a4    SLRFDGALNVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAFHEQLSVAEITNSAFEPAS  300
Sm_TUB       SLRFDGALNVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVAENTNSAFEPSS  253
             *****************************************:********* *******:*


At_TUB-a4    MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVGTIKTKRTIQFVDWCPTGFKCGINYQPP  360
Sm_TUB       MMAKCDPRHGKYMACCLM------------------------------------------  271
             ******************


At_TUB-a4    TVVPGGDLAKVQRAVCMISNSTSVAEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE  420
Sm_TUB       ------------------------------------------------------------  271


At_TUB-a4    AREDLAALEKDYEEVGAEGGDDEDDEGEEY        450
Sm_TUB       -----------------------------        271
```

Figure 4.10 Alignment for Actin. *Swietenia macrophylla* (Sm) partial sequence alignment to At TUB α4 (AT1G04820).

4.3. Drought Experiments

4.3.1   Experimental description

The aim of the experiments was to evaluate the capacity of the plant to resist drought stress and to recover after re-watering. As the conditions were not equal for all experiments, the total length variated. Each drought period was stopped when stressed plants presented high number of wilting leaves. After maximum stress, plants were re-watered to maximum capacity daily, for up to 3 days. Detailed description of the temperature, humidity and illumination was described in chapter3.

Experiment 1 was performed inside an open shelter with a glass roof (to avoid any direct precipitation), in Gamboa, Panama, during April 2016. Atmospheric conditions were determined by ambient values during summer time in the country, including high irradiation (up to 2500 µmol quanta $m^{-2} s^{-1}$) high temperatures (up to 36°C at midday) and low humidity during daytime (up to 40%). Mild stress was determined after aprox. 5 days, while maximum stress was achieved after 11 days; the plants were re-watered daily for 3 days to maximum capacity, showing visual recovery in this time. 50 seedlings were distributed, having sample number of n=3 per treatment.

Experiment 2 was performed inside an open shelter in Gamboa, Panama, during May 2016. Atmospheric conditions were the regulars for the beginning of rainy season (not expected for the period) in the country, with high irradiation (up to 1800 µmol quanta $m^{-2} s^{-1}$) milder temperatures (up to 32°C at midday) and higher humidy than in E1, including several cloudy days and 4-5 rainy days. Mild stress was determined after aprox. 8 days, while maximum stress was achieved after 16 days; the plants were re-watered daily for 3 days to maximum capacity, showing visual recovery in this time. 50 seedlings were distributed, having sample number of n=3 per treatment.

Experiment 3 was performed inside an open shelter with glass roof in Gamboa, Panama, during April 2017. Atmospheric conditions were the regulars for summer time in the country, with high irradiation (up to 2500 µmol quanta $m^{-2} s^{-1}$) but some cloudy days, high temperatures (up to 35°C at midday) and low humidy during daytime. Mild stress was determined after aprox. 5-8 days, while maximum stress was achieved after 16 days; the plants were re-watered daily for 3 days to maximum capacity, showing visual recovery in this time. 80 seedlings were distributed, having sample number of n=8 per treatment.

Experiment 4 was performed inside shelter in Gamboa, Panama, with a dark plastic mesh covering roof and all lateral sides during May 2017. This mesh reduces the entrance of light in 60%, working also to keep humidity higher. Regular summer conditions were milder in this shelter. The irradiation was 150-850 µmol quanta m^{-2} s^{-1} on daily basis and the temperatures were regularly 2-4° lower than outside. The air humidity was around 30% during daytime. Mild stress was determined after aprox. 14 days, while maximum stress was achieved after 28 days; the plants were re-watered daily for 3 days to maximum capacity, showing visual recovery in this time. 45 seedlings were distributed, having sample number of n=5 per treatment.

4.3.2 Soil Description

4.3.2.1   Soil water content

The amount of water in the soil might be an indication of water status for the plant. The water content (WC) of the soil is reduced due to plant's consumption (for photosynthesis and respiration) or by transpiration. In either case, the reduction of water in the soil affects the osmotic balance in plants when it reaches low values. Soil WC can be expressed in two ways:

- **remaining water available in the pot:** To calculate this, all soil contained in the pots needs to be dried out completely, to estimate the final water content and infer the respective content at each day. It was the method used to estimate WC in soils of pots with seedlings (Fig. 4.11).

- **accumulative water lost during the timeline of the experiment:** This is a direct measurement, product of subtraction of pot weights on previous day and measuring day. The remaining water amount is unknown, but the information presents exactly the amount of water that needs to be withdrew from the seedling's pot weight to figure out plant water consumption. This was the method used to estimate soil transpiration (Fig. 4.12).

In all experiments, a significant reduction in soil water content at the maximum stress was observed (Fig. 4.1). The variation in controls was produced mostly by soil lost during transportation to balance (E1 and E2) and punctual lost during the drying period (E3, RW+3).

After three days of drought, it was observed a significant reduction in WC in E2, while in E1 there was a high variation in the stressed group. From five days on, reduction in soil WC was significant for all measured experiments.

The highest reduction at maximum stress point was observed in E2 after 16 days of drought when soils of stressed plants contained an average of 61 ml H_2O per pot. This value is debatable because it was the only experiment where it was not possible to dry for more than one week and had many lost during measuring.
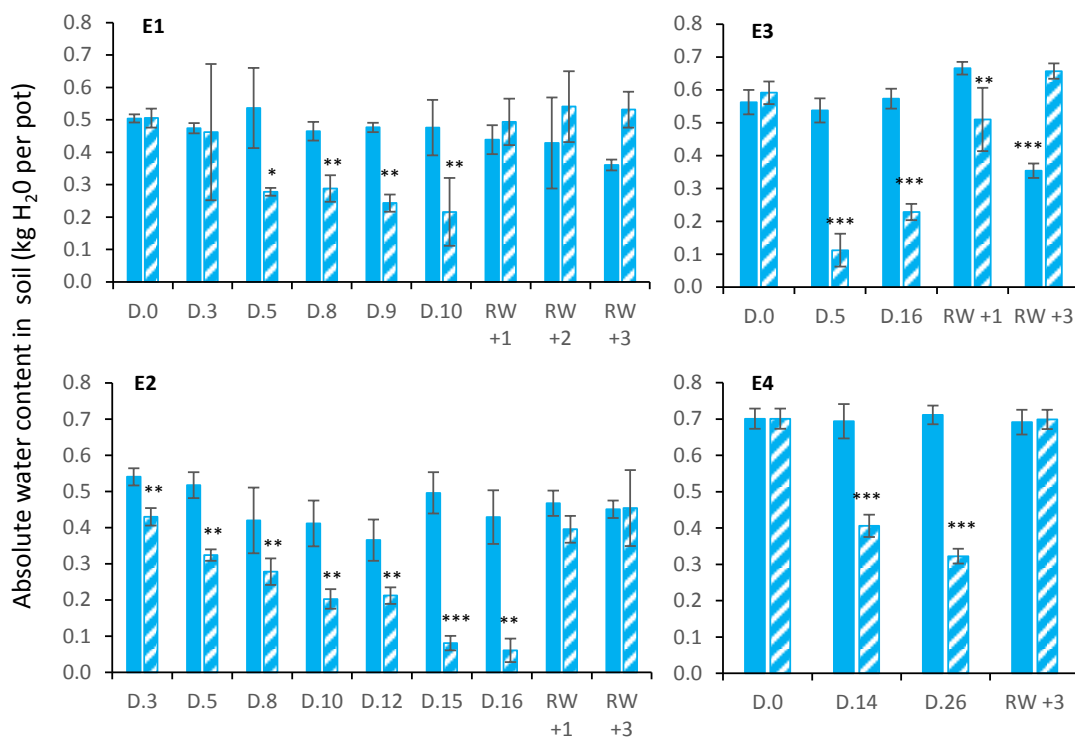
Figure 4.11. Average water content (WC) in seedlings pot for each experiment. Calculated Soil Fresh Weight – Soil Dry Weight. **E1**: n=3; **E2:** n=3; **E3:** n=8; **E4:** n=5. Statistic represent Student's T-test with significance of $p < 0.1$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

All experiments had different control conditions. Absolute water content in soil of control plants of E1, E2, E3 and E4 was around 500 ml, 550 ml, 600 ml and 700 ml, respectively; while minimum absolute water content during the stress period was 216 ml, 61 ml, 228 ml and 320 ml, respectively. One day after re-watering, soil content of previously stressed plants in E1 and E2, as well as after three days in E4, contained similar values of water than their controls. Only experiment 3 did not achieved to re-hydrate to the same value as controls; that might be for a change in the soil textures during drought, that did not allowed water to remain inside pots, letting it run out in excess (visual observation). Another reason could be that plants in this experiment were demanding more water, or that the re-watering regime was not

accurate enough. As experiment 4 was under shadow, soil was keeping more humidity in the inner part of the pots, with a slow reduction of 390 ml H_2O in 26 days. At the driest stress point in this experiment, plants under drought treatment had higher soil WC than stressed plants in the other experiments, corresponding to 32%, 81% and 29% higher than E1, E2 and E3, respectively.

4.3.2.2   Soil Transpiration

The water in the soil is not only used by the plant but also evaporated directly from the soil surface. This is called „soil transpiration ". To evaluate this aspect, three pots that contained only soil were used to measure water lost in pots occurring without plants; they were irrigated at the same time and with the same intensity of seedlings pots. Each measuring date, wet control pots were measured to determine their "dry weight", irrigated to maximum capacity (until water stopped dropping) and measured again to determine the "wet weight" to use as reference for the next day. In this way, the difference in weights between the last irrigation and the "dry weight" corresponded to the lost based on soil transpiration. For dry control plants the procedure was similar, without involving irrigation in between.

 Soil transpiration made a big difference in all experiments. Without plant use, water lost in soils exposed to environmental conditions was 474 ml for E1, 329 ml for E2, 280 ml for E3 and 205 ml for E4 (Fig. 4.12). These values were different from one experiment to the other, based in the environmental conditions experienced, including sun irradiance levels, air humidity and wind. Mild drought conditions in experiment 4 were also confirmed by the low soil transpiration, compared to the other experiments.
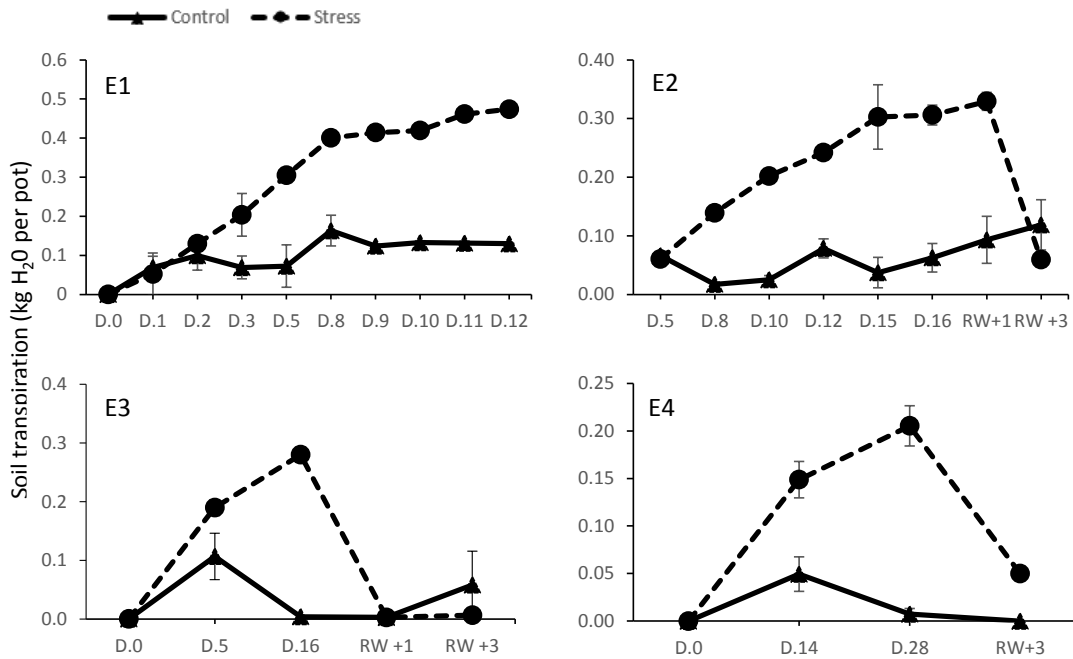
Figure 4.12. Average soil transpiration in control pots with no seedligns, for each experiment; n=3. Calculated as previous weight – actual weight (always as F.W.).

4.3.2.3   Soil composition

Soil in experiments was mostly of clay type. This soil was not extra fertilized and used only once, so it contains the regular amounts of nutrients that plants find in natural conditions (Table 4.1).

Table 4.1. Soil nutrient composition in experiment E4 with *Swietenia*. Starting soil represent the soil used to fill all pots at the beginning of the experiment. At the end of the drought phase, representative samples were taken for both control and stressed seedlings. Nutrient content values are expressed as (mg/ g soil) some elements had **u**nder-**d**etection **v**alues (UDV). Total difference refers to [value in stress- in control], and relative values are compared to control values [Total diff / Value control].

	Al	B	B	Ca	Cr	Cu	Fe	K	Mg	Mn	Na	Ni	P	Zn
							Soil for *Swietenia macrophylla* , in shadow experiment (E4) (mg/g soil)							
Standard	66.72	UDV	0.11	3.43	UDV	0.02	43.40	5.61	5.86	0.94	0.34	UDV	0.48	0.11
Starting Soil	72.54	UDV	0.14	7.34	UDV	0.05	58.55	4.77	13.99	0.67	0.26	UDV	0.85	0.18
D.16 Control	77.73	UDV	0.16	7.95	UDV	0.06	63.63	4.98	15.26	0.65	0.35	UDV	0.92	0.18
D.16 Stress	79.69	UDV	0.17	8.29	UDV	0.07	65.41	5.20	15.58	0.68	0.30	UDV	0.98	0.20
Total Diff.	1.96		0.01	0.34		0.01	1.78	0.22	0.32	0.02	-0.05		0.06	0.02
Rel. Diff.	2.5%		6.1%	4.3%		12.2%	2.8%	4.3%	2.1%	3.3%	-15.3%		6.3%	8.9%

Plants under stress are supposed to arrest growth, therefore the intake of nutrients might be reduced. We can see that the values of some minerals are higher in stress seedling's soil, meaning that it was not taken for plant growth, remaining available. That is the case of aluminum, boron, calcium, copper, iron, potassium, magnesium, manganese, phosphorus and zinc; having respectively 2.5%, 6.1%, 4.3%, 12.2%, 2.8, 4.3%, 2.1, 3.3%, 6.3% and 8.9% more than control. The only element decreasing was sodium (15.3%).

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4.3.3. Physiological Measurements

Several physiological parameters were annotated, to describe plant adjustments during the stress period. Included parameters are: shoot size, biomass (leaves and stem) water content and total production, leaf area, chlorophyll-a fluorescence, CO_2 assimilation (net photosynthesis) and water conductance through stomata.

4.3.3.1.   Shoot size

Swietenia is not known for significant growth in short time, but to confirm this, shoot size was measured at the beginning of the experiment (Fig. 4.13) and along the experiment timeline (Fig. 4.14) for E3 and E4. The starting size of seedlings variated between 15-23 cm, while on E4 variation was 19-24 cm, with some outlayers in each group. Though seedlings with different sizes were distributed homogenously between control and treatment groups, the starting variation is considerable. It is necessary to say that the main batch of seedlings was previously evaluated, for working with plants in apparent same size (visual evaluation) that had at least four leaves with two fully developed ones.
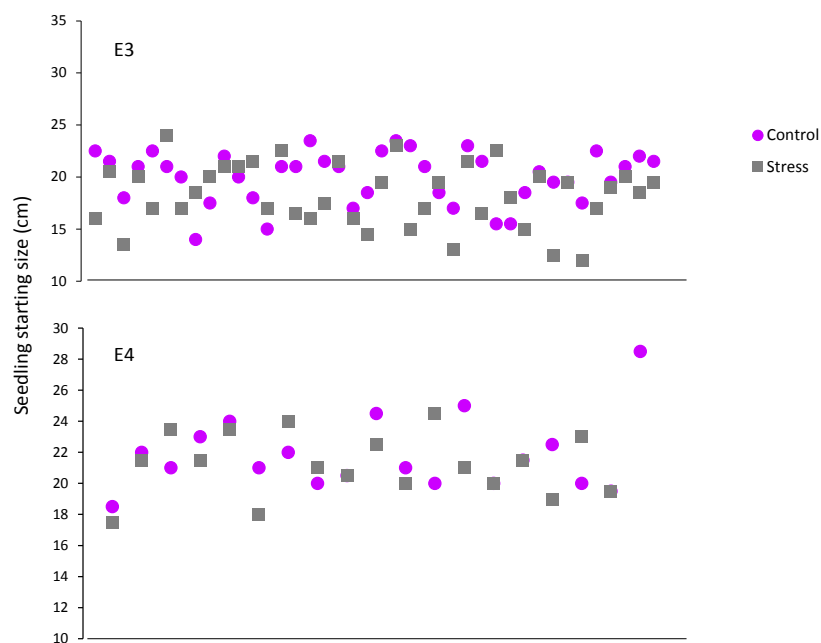
Figure 4.13. Starting shoot size for all seedlings in experiments 3 and 4. Measuring the stem until the last vegetative bud in the apex.

Along the experiment, the size of seedlings at each measuring point presented no difference in E1, E2 (data not shown) or in E3 and E4 (Fig 4.14, marked in bars), when compared to controls. When the actual increase in shoot size for each plant compared to the value on day 0 was analyzed, a difference after 16 days of stress in E3 and after 14 days of stress in E4 ($p < 0.01$) was observed. In both cases the significant difference in growth of control and stressed plants was not changed after the re-watering. These results confirmed the idea that difference in growth is possible to detect only after two weeks of treatment and if using considerable sample size.
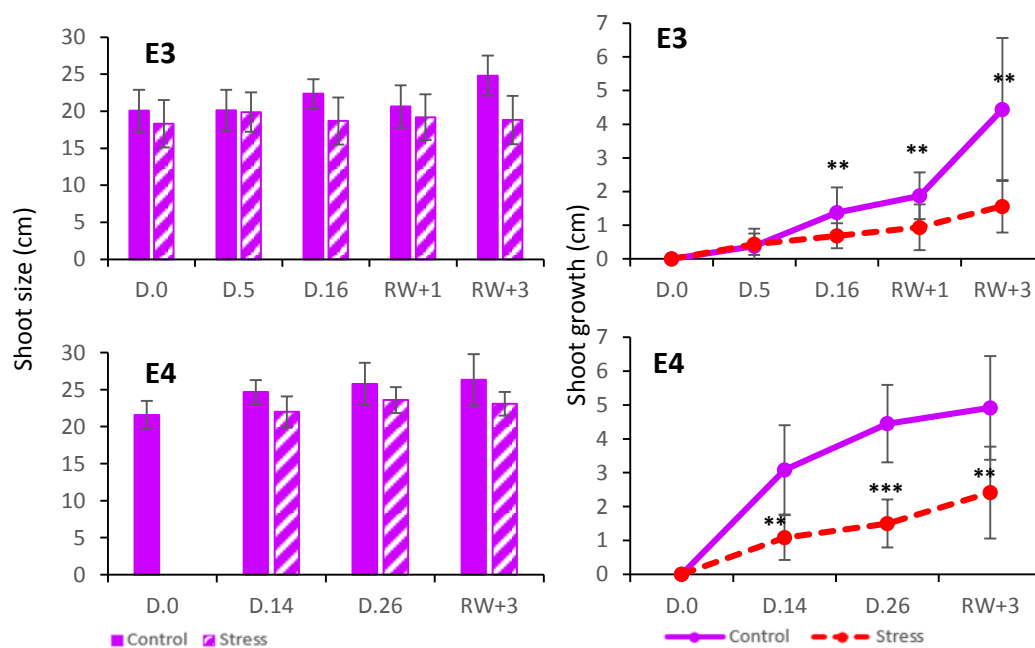
Figure 4.14. Average shoot size for seedlings in each experiment. Measuring the stem until the last vegetative bud in the apex. Sampling size: E3, n=8; E4, n= 5. Statistic represtent Student's T-test with significance of $p < 0.1$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

4.3.3.2.   Biomass production

All biomass above ground was collected and weighed, separating leaves from stems. Regularly, the values of above-ground biomass are measured together, so the first calculations in this experiments were also made as a whole.

Total fresh biomass production (Fig. 4.15), including leaves and stems, did not show variation between control and stressed plants along the experiment in experiments in 2016 (E1 and E2), but in the ones performed in 2017 (E3 and E4) there was a significant difference at maximum stress. In E3, biomass for stressed plants was reduced when compared to controls after 16 days of stress (p< 0.001) and in E4 the difference was only detected after 26 days (p<0.01).
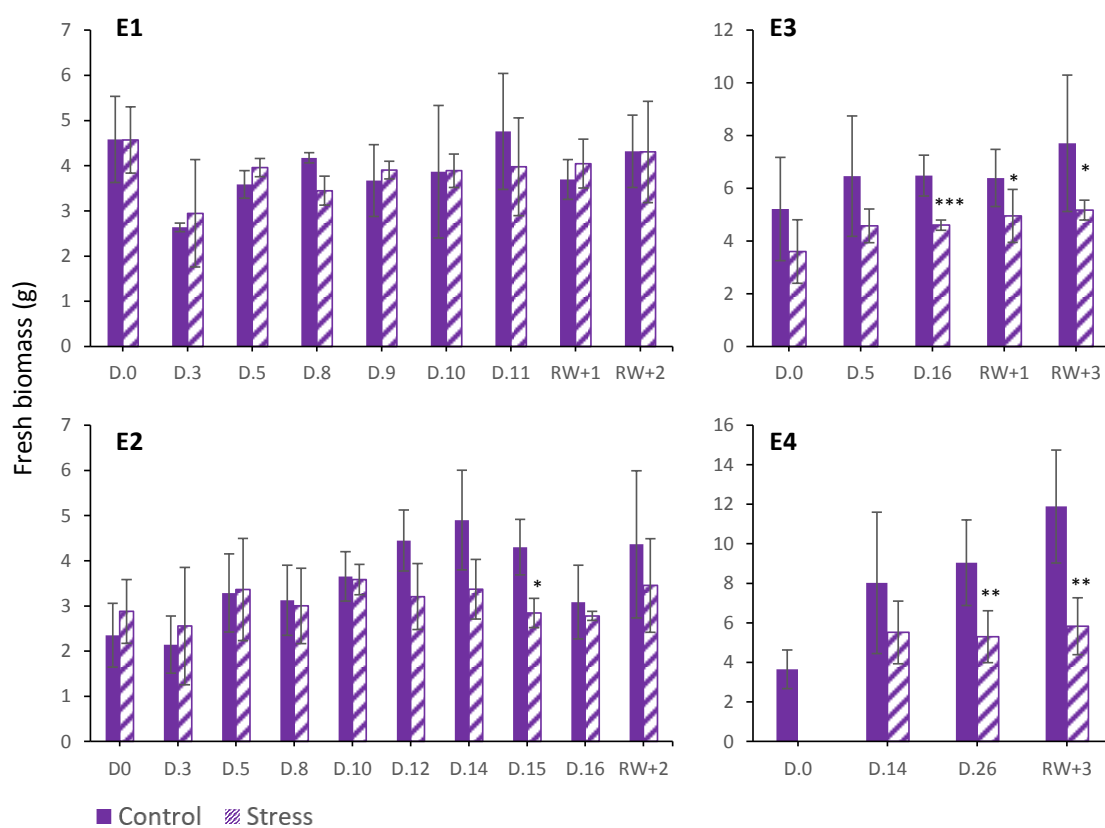
Figure 4.15. Average biomass production (Fresh weight) for seedlings in each experiment. Biomass values included leaf and stems at the moment of collect. Sampling size: **E1**: n=3; **E2**: n=3; **E3**: n=8; **E4**: n=5. Statistic represent Student's T-test with significance of p < 0.1 (*), p < 0.01 (**) and p < 0.001 (***).

The short experimental time in E1 did not allowed to observe variations in biomass, but in E2, that included five days more of stress, a reduction after 15 days (p = 0.03) was observed. Even when there was apparently a difference between control and stressed plants, the high variation in both groups and the low sample number does not allow to discriminate statistically. This problem was overcome in 2017, by increasing the sample number.

4.3.3.3.   Water content in biomass

The biomass collected might variate in the amount of water contained in samples from well irrigated plants and those under drought treatment, but also the allocation of this water could be different in each organ. To test this, leaves and stems were weight and dried separately, and after assessing dry weight for each sample, water content was calculated, producing an average per organ at each measuring point.

In E1 and E2, there was no difference detected in the proportion of water corresponding to the fresh weight of leaves during the drought treatment (Fig. 4.16). The only value with a significance is Day 10 in experiment 2, but the value for stressed plants appear to be higher than in control. As this is not possible during a drought experiment with non-succulent plants and the behavior was only found once between all experiments, this value was considered an outlayer and was therefore dismissed.
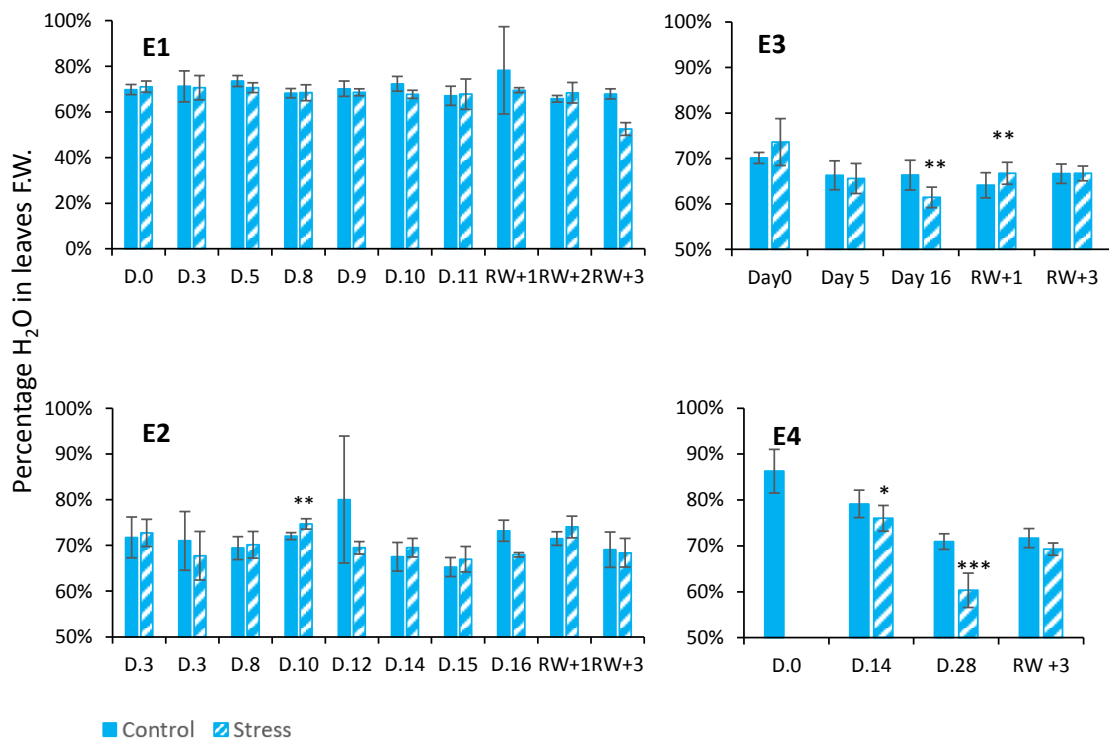
Figure 4.16. Relative water content in leaves for each experiment. Values represent the percentage of the fresh weight that corresponded to water. **E1**: n=3; **E2:** n=3; **E3:** n=8; **E4:** n=5. Statistic represent Student's T-test with significance of $p < 0.1$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

E3 showed lower water proportion in leaves at the end of the dry phase (p= 0.004), same as in E4 after 14 days (p=0.04) and 28 days of drought (p=0.002); and after 3 days re-watering, the proportion of water was the same in controls and pre- drought-treated plants. However, apparently after one day re-watering, stressed plants had a higher proportion of water than controls (p= 0.06) in E3. Since the sample numbering on this experiment does support results,

the only possible explanation is that drought treatment brought stressed plants to an extreme negative osmotic balance, that produced extra absorption at the moment of re-hydration. This trend can be also seen in E2.

For stems (Fig. 4.17), the difference in water content was easier to observe. After 10 days of drought, stressed plants in E1 had significantly less water in their stems than controls (p=0.003), while in E2, this difference was maintained from day 12 (p= 0.04) until day 16 (p= 0.03). In both cases, after re-watering, this difference disappeared.

Maximum stress in experiment 3 produced drier stems in treated plants (p< 0.001); after 1 day of re-watering, treated plants appear to have similar water proportion than in control plants but after three days is there is still less water in seedlings previously stressed. For experiment 4, there is a significant reduction in WC in stems of stressed plants detected after 14 days of drought (p= 0.02) that increases through the drought phase, with p< 0.001 after 26 days of stress. This difference appears to remain still after three days re-watering.
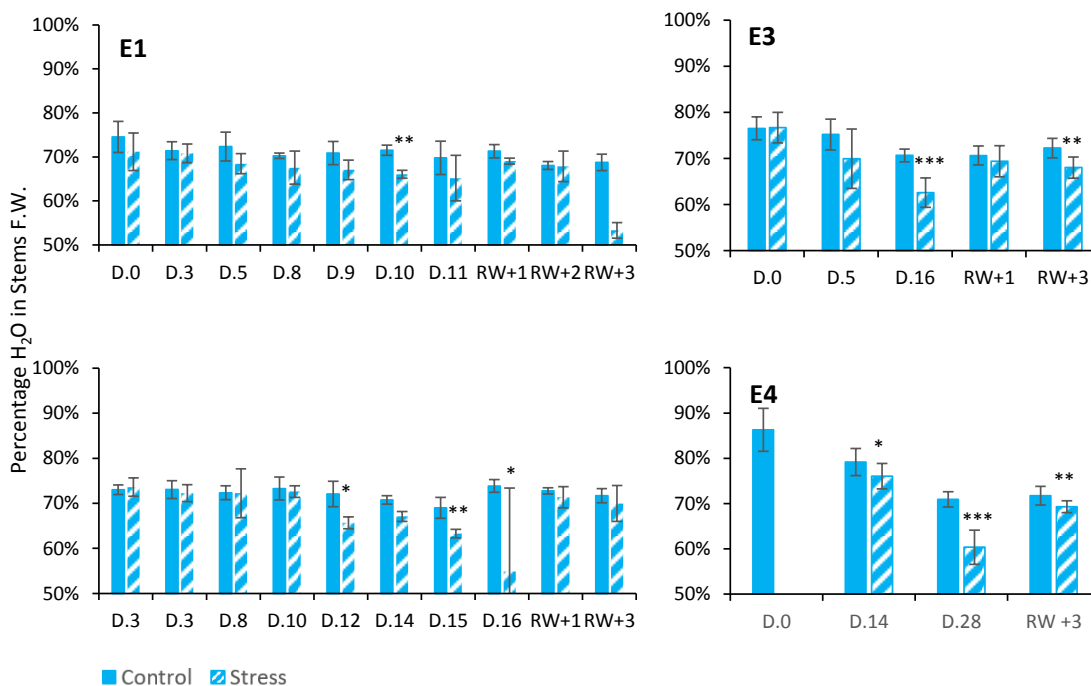
Figure 4.17. Water content in stems for each experiment. Values represent the percentage of the fresh weight that corresponded to water. E1: n=3; E2: n=3; E3: n=8; E4: n=5. Statistic represent Student's T-test with significance of p < 0.1 (*), p < 0.01 (**) and p < 0.001 (***).

Differences in the water content as indicator of osmotic balance in the plant, were easier to observe in stems (Fig. 4.17) than in leaves (Fig. 4.18). Though total re-hydration after 3 days is only confirmed in leaves.

4.3.3.4.   Leaf area

Another way to evaluate plant's growth is based on the leaf area they get until the end of the experiment. Since the process for leaf area evaluation used included detaching all leaves from stems for scanning them, the leaf area values are based on the actual value at the moment of sample collection and its compared to controls on the same day.
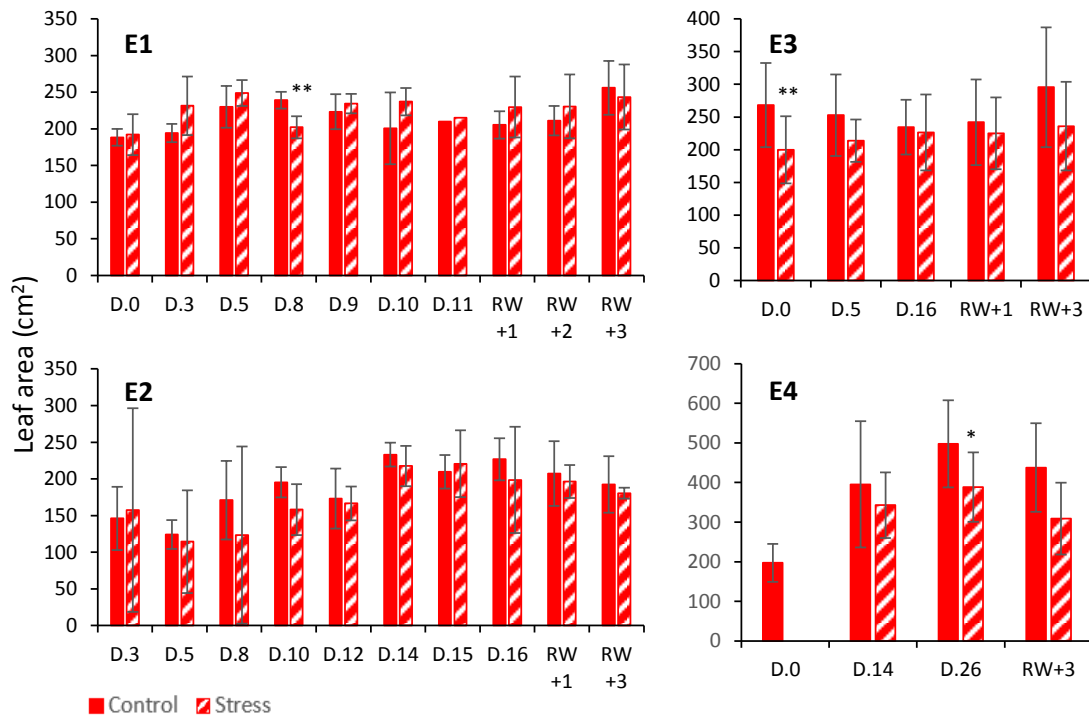
Figure 4.18. Leaf area for seedlings in each experiment. Values represent the area capable of photosynthesis for each plant, in average per measuring date. **E1**: n=3; **E2:** n=3; **E3:** n=8; **E4:** n=5. Statistic represent Student's T-test with significance of p < 0.1 (*), p < 0.01 (**) and p < 0.001 (***).

Drought-stressed seedlings of almost all experiments kept similar leaf areas than controls, during the entire drought process and re-watering. In experiment 1 there is one significant difference (day 8, p < 0.01), but the other values after this day were not significantly different. For E2 and E3, no difference was detected due to drought stress. In the experiment 4, there is a small reduction (p = 0.09) at the end of the dry phase, that is not visible after the re-watering period. In general, it appears that there is not significant leaf production in this period in none of the experiments.

4.3.3.5. Chlorophyll-*a* fluorescence and PSII quantum yield

Chlorophyll fluorescence is a parameter that describes the status of the electron chain and the saturation of reaction center in the photosystems. Energy that enters in the photosynthetic apparatus will be included in the electron transport chain to contribute to CO_2 fixation. When there is an excess of energy, it will be quenched (either by NPQ or heat dissipation). In this way, changes in chlorophyll fluorescence yield reflect changes in photochemical efficiency and heat dissipation.

With a PAM device (**P**ulse **A**mplitude **M**odulated) it is possible to distinguish between quenched and non-quenched fluorescence, hence, the amount of energy that is been "used" for photosynthesis and what is been loss through de-excitation process in the antennas and reaction centers.
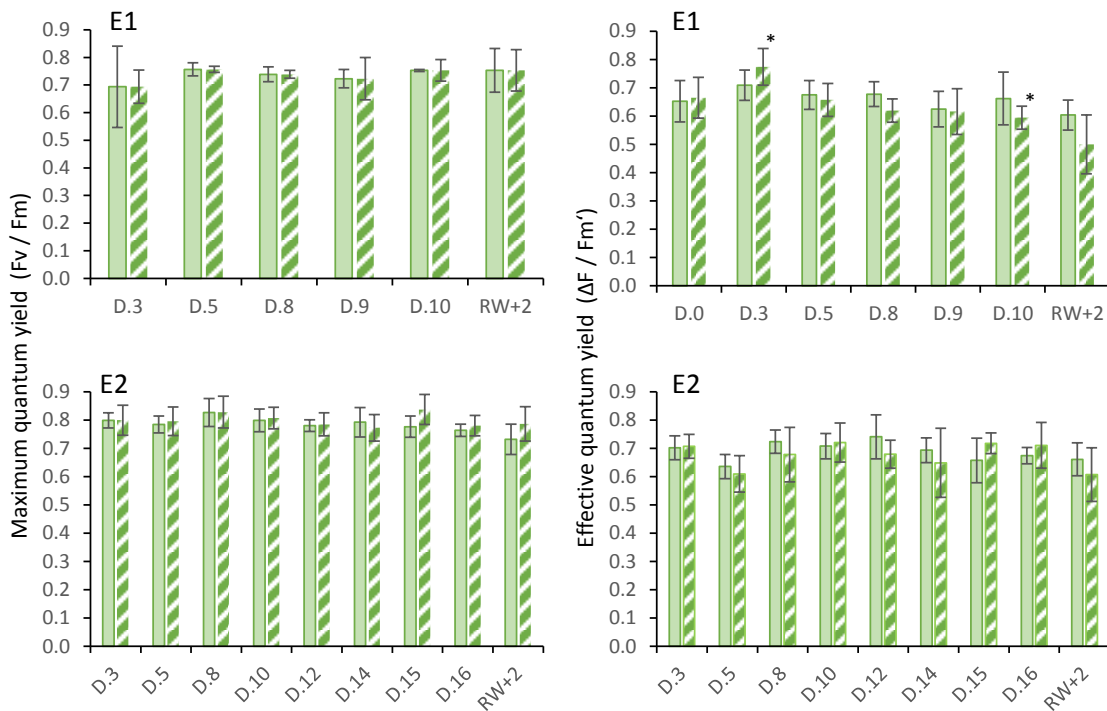
Figure 4.19. Quantum yieald of photosystem II. To the left, all graphs represent "Maximum quantum yield" and was measured with leaves dark adapted with a clip, for 20 minutes. The column on the right represent the "Effective quantum yield", measured with light adapted leaves.

E1 and E2 have no difference in the maximum quantum yield of PS II, all along the experiment, between the two groups (Fig. 4.19). Dark adapted leaves have all reaction centers open, therefore can absorb the maximum of quantum possible. No difference between control and

stress plants during drought treatment, indicates that there has not been degradation of chlorophyll or permanent damage in the photosystems (Maxwell and Johnson, 2000).

In light adapted leaves, reaction centers are partly in use, therefore closed. Less energy from the saturation pulse emitted with the machine will be integrated in the photosynthetic pathway. In the measurements of E1 and E2, a small reduction in the effective quantum yield was increasing along the drought treatment, but never reached significant values. This indicates that both control and stressed plants have similar capabilities to accept the extra light from the saturation pulse, to start photosynthesis. This is paradoxical since the lack of water might stop the energy transport chain, reducing also the photosynthetic capacity; an alternative way to cope with this excess of energy during low water availability might be integrated in this case.

Responses of PSII quantum yield during drought stress in E3 and E4 were not calculated, due to the high amount of samples and the time needed to collect all the other parameters during a considerable short period (4 hours in total).

4.3.3.6.   Photosynthesis activity – CO_2 Assimilation rate (μmol CO_2 m^{-2} s^{-2})

Photosynthesis activity can be estimated with an **I**nfra-**R**ed **G**as **A**nalyzer (IRGA), that can calculate the amount of CO_2 from the air that the plant is fixating. In the experiments performed, the length was determined by wilting leaves and the apparent stop of photosynthesis.

In **E1**, the weather was very sunny and dry. Maximum reduction of photosynthesis was achieved after 10 days, with the reduction trend starting on day 5 (Fig. 4.20). At maximum stress point, control plants had an average net photosynthesis of 9.02 μmol CO_2 m^{-2} s^{-1}, while stressed plants had 4.24 μmol CO_2 m^{-2} s^{-1} (p= 0.08). High biological variance in control plants allowed only a relatively small statistical difference. After re-watering, treated plants increased their activity, to comparable values as in controls.

For **E2,** highest reduction in photosynthesis was observed after 15 days (2.9 CO_2 m^{-2} s^{-1}), but the reduction trend started around day 5. In this case, after the re-watering of soils, stressed plants had similar photosynthesis activity than control plants. The low sample number in this experiment was a determinant factor that hinders the distinction in photosynthesis activity modification in stressed plants.
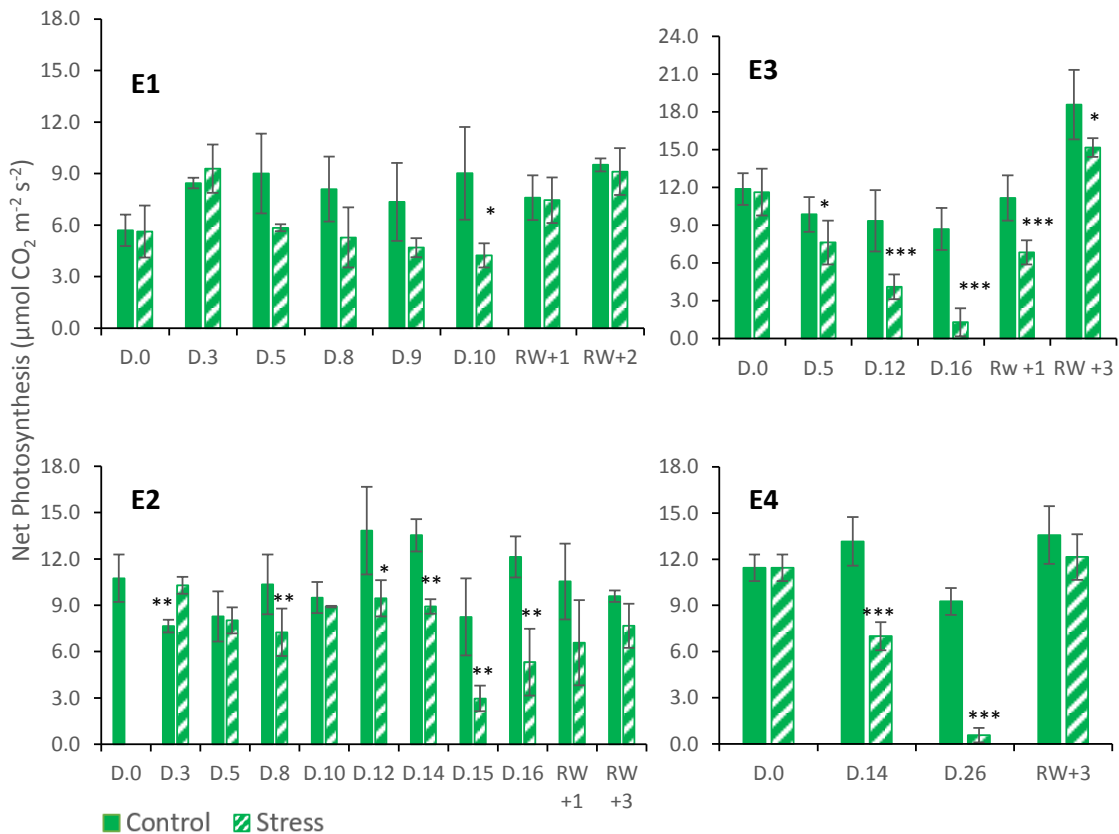
Figure 4.20. Photosynthesis activity in four drought experiments with *Swietenia macrophylla*. E1 and E2 were in Panama, in 2016, while E3& E4 were performed in 2017. Statistic represent Student's T-test with significance of p < 0.1 (*), p < 0.01 (**) and p < 0.001 (***).

With a higher sample number, **E3** presents significant reduction in photosynthesis activity after five days of stress (p= 0.013), that increases until the last day of stress, with very distinct response between control plants (8.7 CO_2 m^{-2} s^{-1}) and stressed plants (1.3 CO_2 m^{-2} s^{-1}, p< 0.0001). In this case, after the re-watering of soils, plants were slowly recovering, reaching values of 6.85 CO_2 m^{-2} s^{-1} after one day, and 15.2 CO_2 m^{-2} s^{-1} after three days re-watering. Even when the levels of photosynthesis achieved after this short time indicate a fast recovery to "regular values", control still differentiate from pre-treated plants, having higher photosynthesis values.

Experiment 4, performed under shadow (40% of sun light), maintained the humidity in the soil longer, which was beneficial for photosynthesis. Due to that, it took 14 days for the first differences to appear. There was a reduction in CO_2 assimilation after 14 days of drought, (p< 0.00001), reaching minimum values (0.05 CO_2 m^{-2} s^{-1}, p< 0.00001) after 26 days. Remarkably, after three days of re-watering, treated plants could recover and pair with control values.

4.3.3.7.   Water conductance through leaf (mol H_2O m^{-2} s^{-1})

Plants close the stomata to avoid losing too much water during drought. This reduce the movement of CO_2 and H_2O molecules in or out. Open stomata indicate that plant have not detected any type of imbalance or that it still represents more benefits than risks.
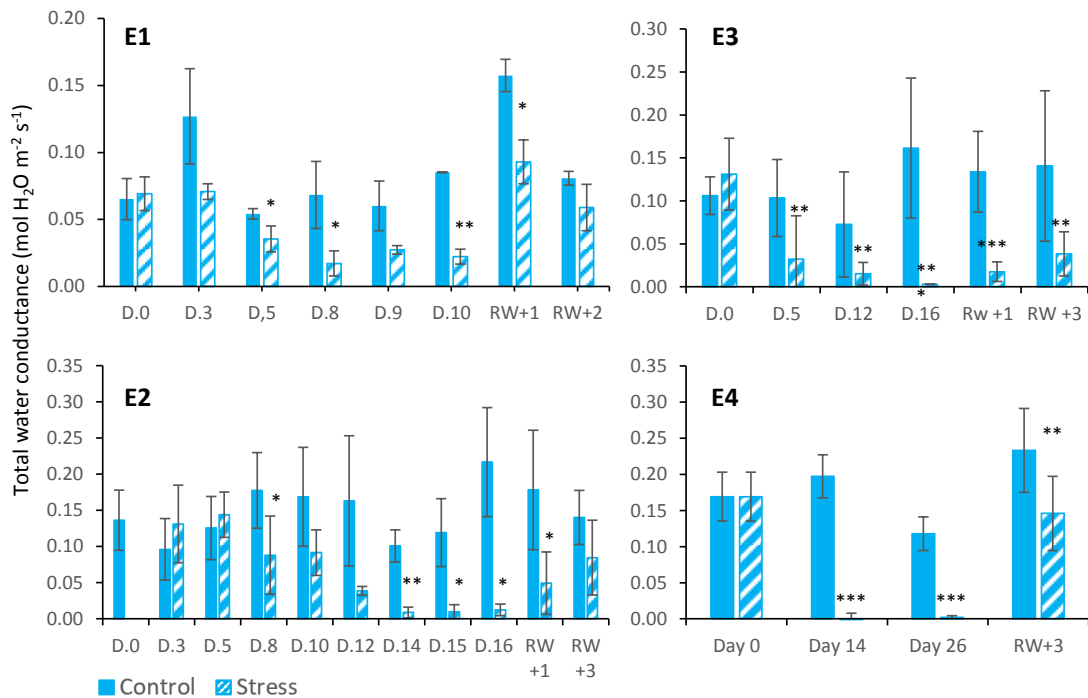
Figure 4.21. Water conductance through leaves in four drought experiments with *Swietenia macrophylla*. E1 and E2 were performed in 2016, while E3 and E4 were performed in 2017. Statistic represent Student's T-test with significance of $p < 0.1$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

In the experiment 1, there is a trend towards reduction of water conductance in stressed plants (Fig. 4.21) starting from day 5 on, reaching p= 0.06 , that continued until 10 days after starting drought treatment with a minimum of water conductance, indicating almost total closure of stomata. Previously stressed plants increase their water conductance after one day of re-hydration, getting values similar to control levels after two days. For Exp. 2, the trend starts on day 8 (p= 0.05), achieving minimum on day 16 (p= 0.04), almost a week after the breakpoint in E1. The difference in water conductance between E1 and E2 are a clear indication of higher humidity in the air during the latter.

Experiment 3 shows a significant reduction after five days drought stress. This reduction gets amplified, reaching p= 0.001, after 16 days of drought. Stomata closure was not possible to correct after three days re-watering, indicating a different adjustment than the one observed in E2. This could be due to the differences in humidity in the air in both experiments, which

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might have caused a damage in the response mechanism of stomata or a reduced water potential inside leave to regenerate from damages. In experiment 4, stomata closure was significant in stressed plants already at mild stress point (day 14), and the re-watering allowed an increase in water conductance, though not completely reaching control values.

4.4. Molecular Evaluation

The samples from the experiments previously evaluated for their physiological behavior towards drought stress, were used to analyze the gene expression of the antioxidant system in the chloroplast, in a search for explanation of their metabolic adjustment during drought and after re-watering. After the non-invasive parameters, two leaves for each plant were frozen in liquid nitrogen, and then grinded.

4.4.1   RNA Extraction

Preparation for gene expression analysis started with extraction of RNA. The kit used for RNA extraction was optimized for *Swietenia* (and *Jatropha*), by increasing in 50µl of buffer R, and adding 2-mercarptoethanol to both buffers during the first step homogenization, keeping tubes always on ice bath and including a final incubation of five minutes before RNA elution from membranes. All extractions were made from 50-100 mg frozen ground material.

The protocol enabled extraction of RNA in good quality out of leaflets of compound leaves from a 15-month old tree, cultivated in the Berlin Botanical Garden (Fig. 4.22a) with concentrations of 250-400 ng/µl and absorption ratios for protein and phenols of 2.00. When using sample material from 8-weeks-old seedlings in experiments in Panama, the quality and concentrations dropped (Fig. 4.22b), reaching concentrations of 5-100 µg/µl while A260/230 and A260/280 had ration of 0.60 -1.50. Evaluation of the difference in the storage quality was tested for samples out of full leaves frozen in liquid nitrogen without grinding, of samples ground under liquid nitrogen with mortar and pestle, and of samples that after grinding were pulverized with an electric beat mill (Fig. 4.22c); apparently very small pulverization was the best option for RNA extraction. If after elution of the membrane, there was a second incubation with 50-100 µl distilled water (Fig. 4.22d), it was possible to increase the total amount of RNA, which was precipitated with sodium acetate, to obtain decent extraction quality with concentrations of 150-350ng/µl RNA and quality ratios around 1.60-1.80.
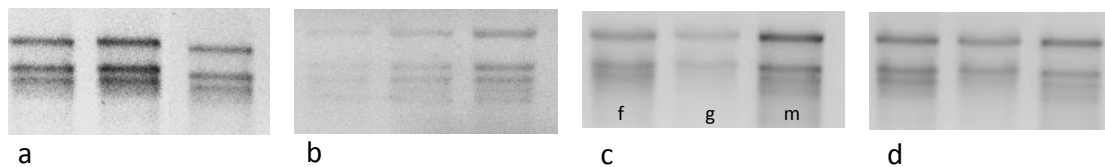
Figure 4.22. RNA extraction for *Swietenia macrophylla* samples from different backgrounds. Three random samples for each RNA extraction test, separated by electrophoresis in gel 1.5% agar. All samples were loaded with 3µl of the extracted RNA; (a) from Berlin Botanical Garden; (b) from Panama; (c) storage conditions: f= frozen full leaves, g= grounded with mortar and pestle, m= use of an electric beat mill; (d) double elution of membrane and precipitation.

For some samples coming from Panama it was impossible to extract RNA. When the problem was quality, the precipitation was not always helpful and new extraction was made out of the same sample. Up to five attempts were made for good quality before giving up on damages samples.

4.4.1.1.   Reference Genes

After describing a partial sequence for actin and tubulin, one set of primers was designed for each gene (ACT1 and TUB1), but none of them worked as expected during test on real time PCR (Fig. 4.23). Actin primers did not amplify anything even when the melting temperature was lowered, while tubulin primer pair amplified several unspecific areas, without distinction, that was also not possible to graduate with temperature or MgCl gradients (data not shown).

Using the same partial sequence, new plasmids were transformed and sent for commercial sequencing. Variations were found in the sequenced cloned in two plasmids for actin and three plasmids for tubulin. New primers positions were tested, two for actin and four for tubulin. New actin primers (both) had specific amplification over the targeted area; for tubulin, primers produced out of the read of plasmid #3 targeted only one sequence, while the primers produced out of the plasmid #2, had unspecific faint bands observed, but at a higher size (Fig. 4.23).

All new reference genes primers were used for qRT-PCR, and their gene expression (Ct values) were evaluated for control and stress samples, considering low standard deviation as a measure of high stability. A combined expression was assessed using one actin primer with one tubulin primer. After all stability analyses, primers selected to amplify reference genes for qPCR evaluation and to normalize the transcript abundance of the genes in the antioxidant system were ACT3 and TUB4.
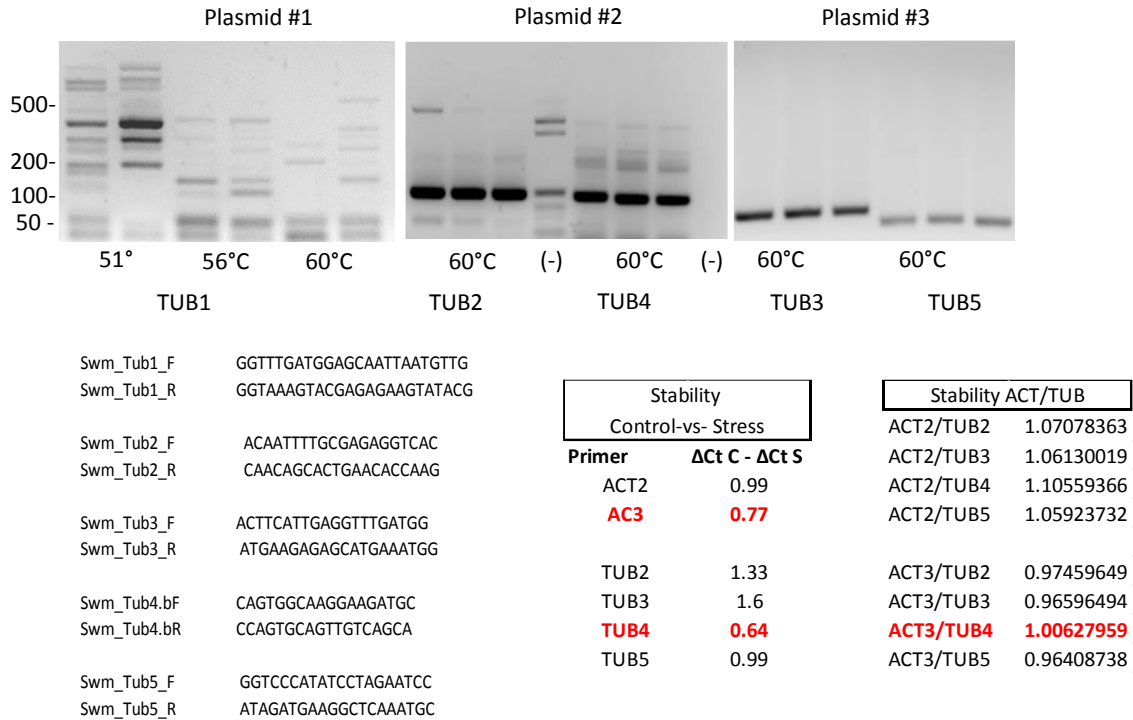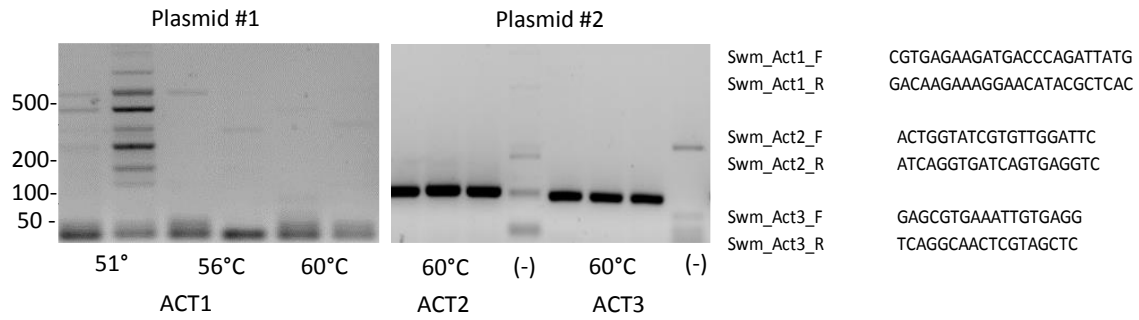
Figure 4.23 Test of Reference Gene qPCR primer amplification. *Swietenia macrophylla* (Sm) partial sequence was used for the design of qPCR specific primer. Primer pairs (per plasmid read) were tested with three samples to evaluate their stability.

Primers designed to amplify *Swm_2CP*, *Swm_APX* and *Swm_GPX* expression were also tested.

For the three primer pairs designed, the electrophoresis showed one single amplification band

(Fig. 4.24), confirming their specific targeting, therefore used further.
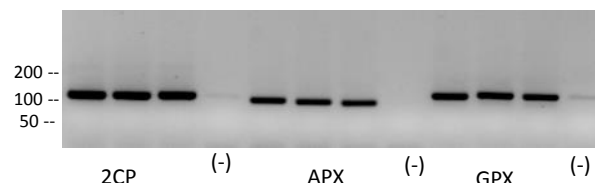
Figure 4.24 Test of amplification for the qPCR primer targeting the genes of interest. *Swietenia macrophylla* (Swm) partial sequence was used for the design of qPCR specific primer. Primer pairs (per plasmid read) were tested with three samples to evaluate their stability.

4.4.1.2. Transcript levels of the antioxidant genes

Samples were normalized to actin and tubulin expression. Expression for the GOI was still highly variable as was the environment. Samples were evaluated as relative expression to the value of controls on the first day measured, but the statistics were applied to the relative expression of the control on the same day. Average values were calculated for 2-3 samples for all experiments. Many samples were degraded or presented no amplification at all during qPCR; that lead to elimination of values for some measuring dates in all experiments and for all genes.

4.4.1.3. 2-Cys-Peroxiredoxin

Using the partial sequence described for *Swm_2CP*, a primer pair was designed to amplify an area of 120 nucleotides (Fig. 4.25). The distribution of primers on the exons covering the coding sequence is not determined, since there were not gDNA sequences available.

…GACTTTACGTTTGTGTGTCCGACAGAAATCACTGCTTTCAGTGACCGCTATGCAGATTTTGAGAAGCT AAACACAGAAATATTGGGTGTCTCTGTTGACAGTGTGTTCTCACACTTGGCATGAGTCCAAACAGATAG AAAATCTGGTGGTCTTGGTGATTTGAAGTATCCACTAATTTCCGATGTTACCAAATCCATTTCAAAATC CTATGGAGTTCTGATCCCTGATCAGGGAATTGCGCTGAGAGGCCTTTTCATTATTGACAAAGAT**GGAGT CATCCAGCACTCAAC**CATTAACAATCTTGCCATTGGCCGTAGTGTTGATGAGACAATGAGAACGCTTCA GGCTTTGCAGTACGTGCAGGAGAATC**CAGATGAAGTATGCCCAGCT**GGGTGGAAACCCGGCGAAAAAAC AATG…

Figure 4.25 Partial sequence for the 2-cys-peroxiredoxin cloned from *Swietenia macrophylla*. Sequence was product of three plasmid reads after amplification with wobble primers. Primers to use for qPCR are marked in bold letters.

This primer pair was then used to amplify 2CP gene expression in samples from the drought experiments. Experiment 1 shows a normalized expression of up to 3-fold increase, compared to values of day 0. Even with the high standard error in all samples, it shows a relative trend (to respective controls) increasing during mild stress (day 5-8), and a decrease during maximum stress under control values (day 16). After re-watering, gene expression went back to normal (Fig. 4.26), though no statistical significance was found at any point. In experiment 2 there was a slight increasing trend in treated plants during mild stress (until day 10), and a reduction at maximum stress (day 16). Re-watering effect was not possible to observe, sample for this point did not produced RNA in good quality, thus cDNA synthesis was not possible. Experiment 3 showed a similar trend as in E1, with upregulation during mild stress (5 days)

and downregulation at maximum stress (day 16). During re-hydration, the gene expression alternated to control levels.

E4 showed no trend at all; the extreme standard error found in control plants at day 14 (values from 0.5 to 35 after normalization and comparison to day 0) might be product of degradation or contamination. At maximum stress as well as after re-watering, 2CP transcript levels in treated and control plants was similar.
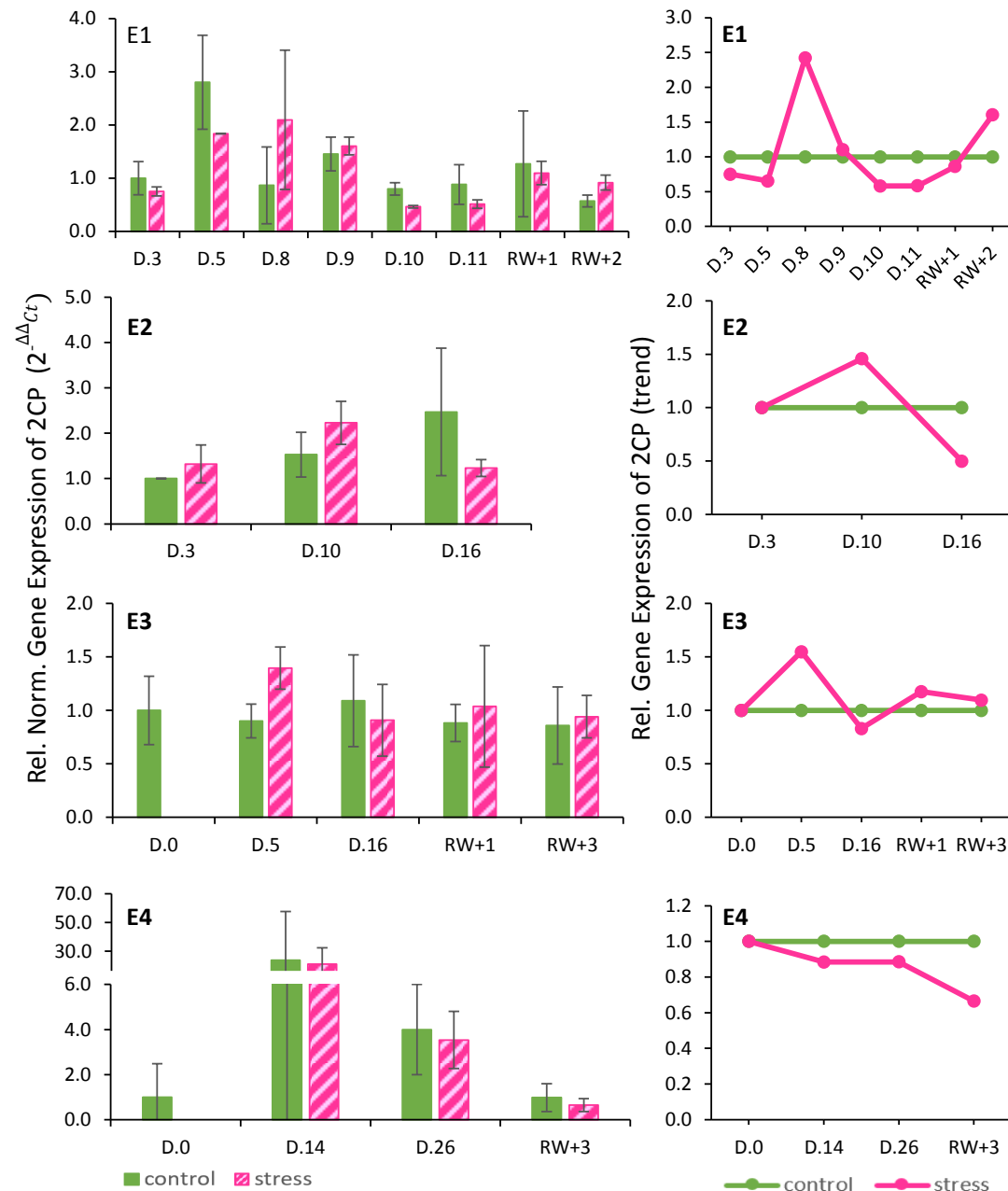
Figure 4.26. 2CP gene regulation during drought experiments. *Swietenia macrophylla* seedlings submitted to drought stress were evaluated with qPCR specific primers. Relative normalized expression to first control is shown in bars, and relative expression to each respective control is shown with lines. Statistic represent Student's T-test with significance of $p < 0.1$ (*).

4.4.1.4.   Ascorbate peroxidase (*APX*)

In the same way, experiment 1 was the only one showing a detailed regulation during the stress gradual increase in the experiment but includes so high standard deviation that does not allow statistical significance (Fig. 4.27). There is upregulation of *APX* during mild stress (day 5-8), but after reaching a maximum point, it was downregulated, from day 9 to day 16. Re-watering brought transcript levels to values similar to that in control plants.

E2 has similar response to E1, with upregulation during mild stress (day 10, p= 0.07), and downregulation during maximum stress (day 16), but all other samples were degraded, not allowing to see intermediate points or the effect of re-watering. In E3, *APX* was also upregulated at day 5, considered mild stress, and was downregulated at day 16, considered maximum stress, though at this point control samples had a large standard deviation; after re-hydration, transcript returned to normal values. Experiment 4 had high standard deviation in their samples and mild stress was not possible to evaluate due to degradation of control samples for this day; at maximum stress is observed downregulation and re-watering brought levels to normal. In the relative expression graph is visible a 50-fold upregulation at mild stress only if compared to values of day 0, but it is important to recognize that due to the variable environmental conditions, gene expression is not supposed to stay stable.
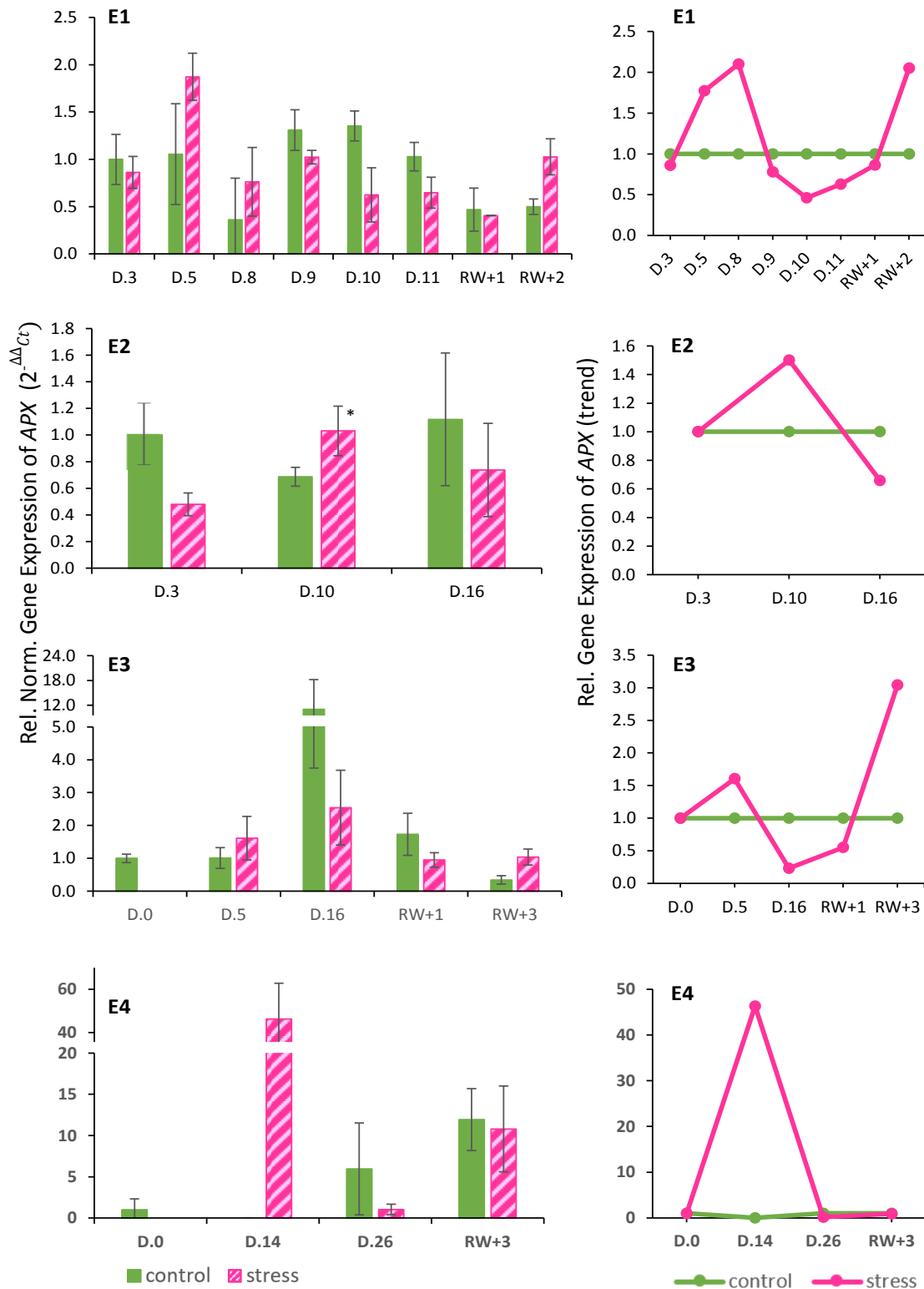
Figure 4.27. APX gene regulation during drought experiments. *Swietenia macrophylla* seedlings submitted to drought stress were evaluated with qPCR specific primers. Relative normalized expression to first control is shown in bars, and relative expression to each respective control is shown with lines. Statistic represent Student's T-test with significance of $p < 0.1$ (*).

4.4.1.5.   Glutathione peroxidase (*GPX*)

In E1 was observed a trend for upregulation of *GPX* during mild stress (day 8) and from this day, there was down regulated until the end of the stress period (Fig.4.28). Re-watering did not bring expression to normal values for this case. Somehow this trend was alike to E4, where max. stress induced downregulation and re-watering did not corrected expression to normal values; in this case the control sample for mild stress (day 14) was degraded, but if it would be compared to control on day 0, there was an upregulation (line from day 0 to day 14 is intermittent, to highlight that this is an assumption, not an observed fact).

Another trend was presented by experiments 2 and 3, where there was a steady upregulation during stress. In E2 there was no control group at day 0 or day 3, but if sample expression is calculated relative to control on day 10, there was an upregulation at day 10 which stayed until measurement corresponding to maximum stress. The transcript levels of GPX in stressed plants during mild stress (d.10) were significantly higher than in controls (p= 0.002).

In E3, transcript levels at maximum stress (d. 16) where 4X higher than in controls, but biological variation did not allow statistical significance; here, the re-hydration brought transcript levels to controls values after one day.
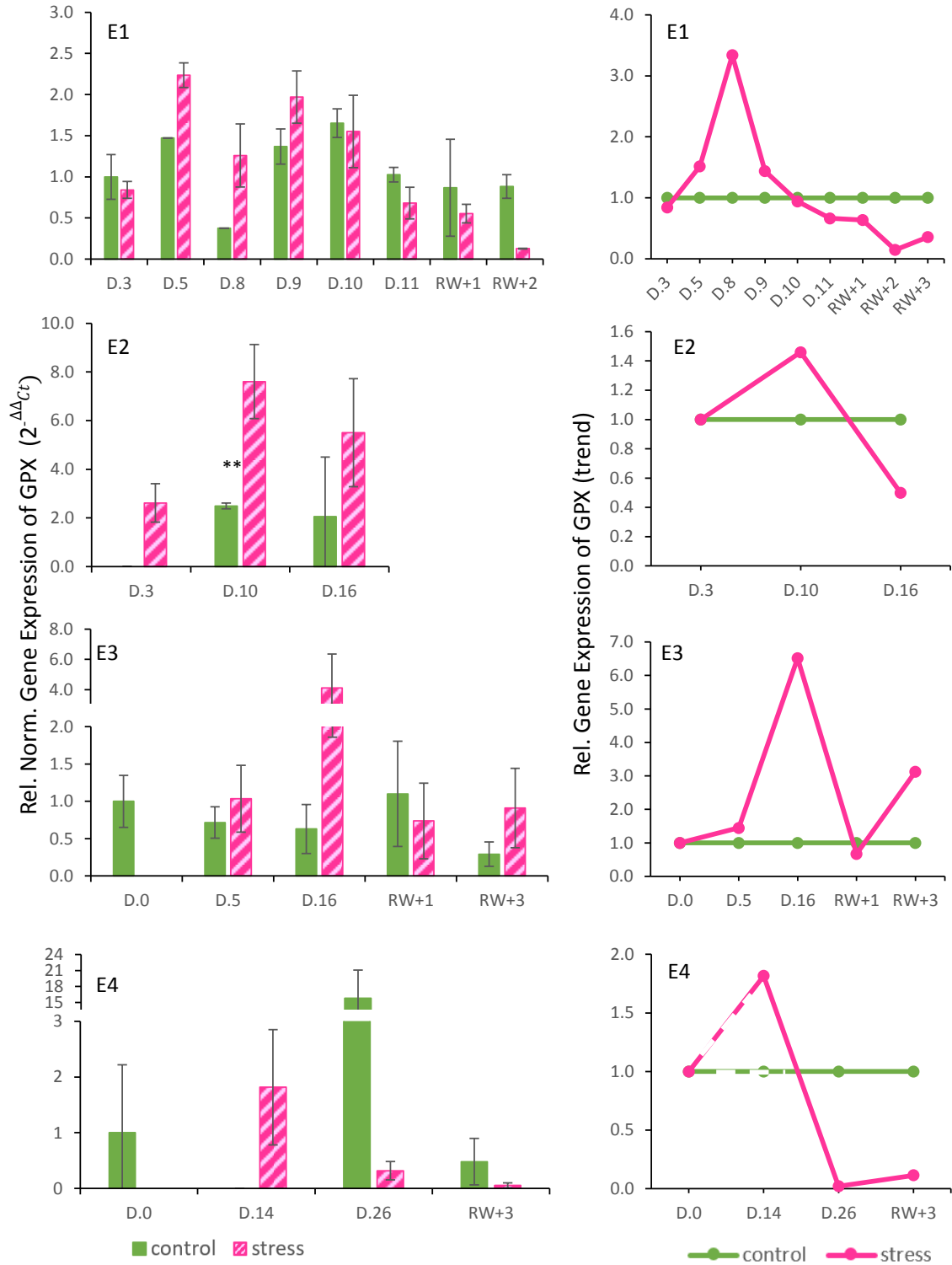
Figure 4.28. GPX gene regulation during drought experiments. *Swietenia macrophylla* seedlings submitted to drought stress were evaluated with qPCR specific primers. Relative normalized expression to first control is shown in bars, and relative expression to each respective control is shown with lines; intermittent line represents a projection, since there was no control this day. Statistic represent Student's T-test with significance of $p < 0.1$ (*), $p < 0.01$ (**).

4.4.1.6.   Combined expressions

Considering that each experiment had unique environmental condition affecting samples, a description based on the response of 2CP, APX and GPX towards each situation might indicate regulation in the antioxidant system (Fig. 4.29). For conditions in **experiment 1**, there was a fast response of APX and GPX after five days of drought, reaching maximum upregulation for the three genes on day 8, considered the mild stress; only at this point was 2CP up regulated. Afterwards all genes show downregulation, and after re-watering, control and stressed plants had similar values for all genes.

Experiment 2 presented a slight upregulation for 2CP and APX during mild stress while GPX was strongly upregulated; at maximum stress, 2CP and APX get downregulated while GPX stays upregulated. In **experiment 3** there was a slight upregulation on all three genes during mild stress (day 5) that got reduced at maximum stress for 2CP and APX, while GPX shows an increment. Re-watering of soils produced similar transcript values in control and treated plants for all genes after one day, but after three days, appeared to induce upregulation in APX and GPX. For **E4**, 2CP was not regulated; for the other genes it is different: there is an upregulation for APX (strong) and GPX (slight) during mild stress, and downregulation for APX and GPX at maximum stress; after re-watering, APX values were similar to controls while GPX remained unchanged (low).

Evaluating each gene regulation based on their behavior on all experiments is also possible. 2CP presented almost always upregulation during mild stress (approx. day 8) and was then downregulated until the end of the stress period, when plants were re-watered, transcript levels in treated plants were similar to controls. APX was responding fast to drought, always showing upregulation during mild stress, up to 5 folds (due to the big standard deviation in E4); after reaching it, the gene was downregulated until reaching lower values than controls, but they returned to regular values with re-hydration. GPX did not present an easy trend. Maximal upregulation was presented at different points and was maintained in some case after mild stress; apparent projection presents increased values that got downregulated at some point and low values at maximal stress that do not return to control levels after watering.
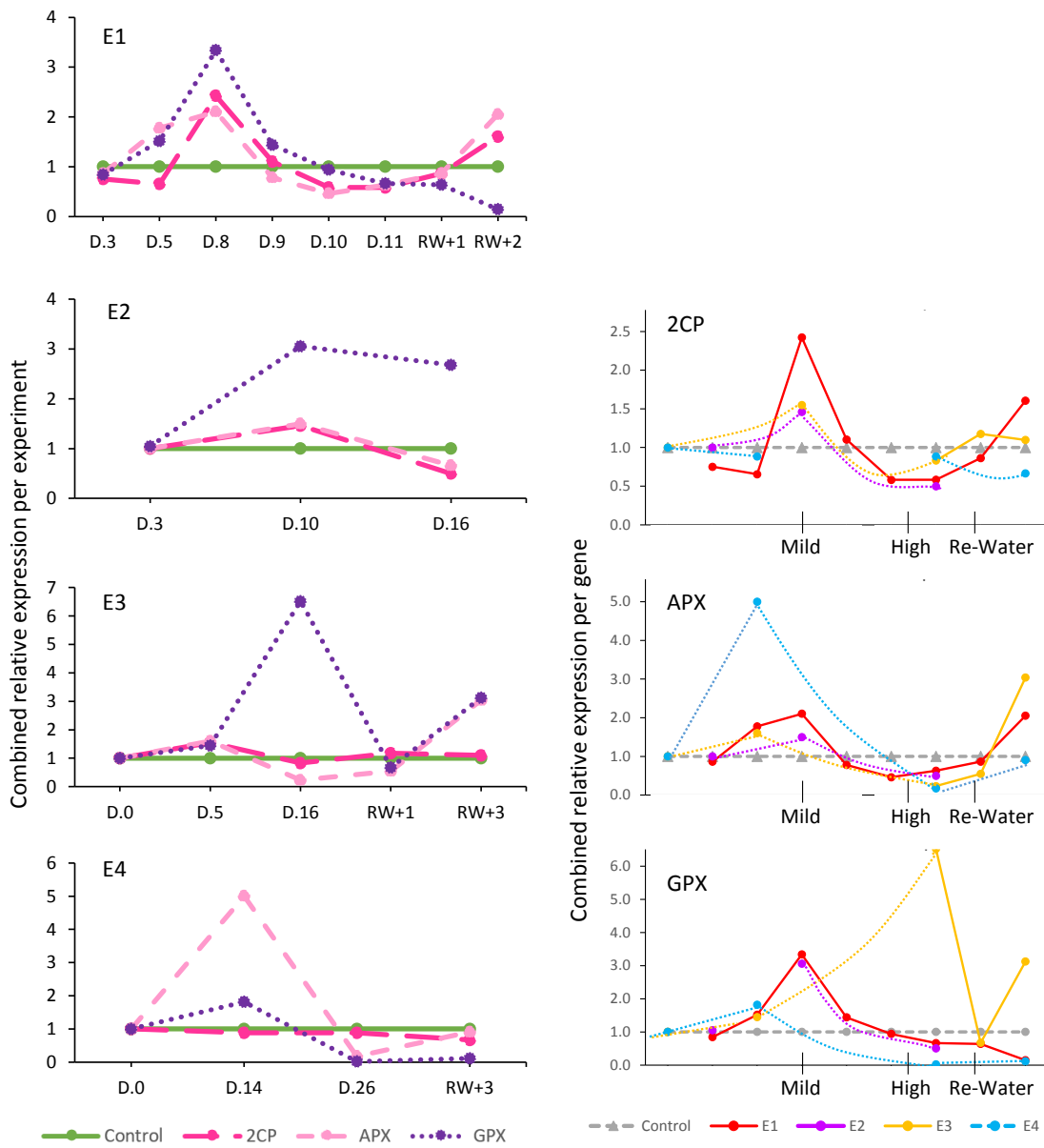
Figure 4.29. Gene regulation during drought experiments. *Swietenia macrophylla* seedlings submitted to drought stress were evaluated with qPCR specific primers. Relative normalized expression is compared for all genes in the same experiment (left), and each gene response in different environmental conditions of drought (right).

Chapter 5
RESULTS III - *Jatropha curcas*

5.1. Sequence information

Sequences for 2CP, APX, GPX, PrxQ, CAT and SOD were taken mainly from two databases: **Jatropha Genome Database** from the Kazuza DNA Research Institute (Sato S. *et al.,* 2011), and the products after **B**asic **L**ocal **A**lignment **S**equencing **T**ool (BLAST) within GenBank of NCBI Database, from the US National Library of Medicine (Wheeler *et al.,* 2001). The options found there were evaluated for similarity between databases, their targeting to chloroplast, and on their similarity to *A. thaliana*'s expected homolog with sequence alignment tools. For A*rabidopsis*, sequence was first check in Peroxibase (Fawal *et al.,* 2013). Sequences were used to design specific primers for q-PCR amplification. Full-length sequence for 2CP, APX and GPX were cloned and confirmed via commercial sequencing. Signal peptides from these three genes were fused to a GFP and cloned in *Agrobacterium* to observe sub-cellular localization. This extra analysis effort was made only on *2CP*, *APX* and *GPX* because they correspond to the major responsive genes of the enzymatic antioxidant system in the chloroplast.

5.1.1. 2-Cys-Peroxiredoxin (*2CP*)

From the Jatropha database, there were two sequences annotated as 2-cys-peroxiredoxins. After sequence alignment, one of them (Jcr4s05382.10) turned out to be a partial sequence of the other, Jcr4s01391.20 (100% similarity in covered area). In NCBI database there was one sequence reported as 2-cys-peroxiredoxine BAS1, chloroplastic-like, with a different name (XP_012074345). The alignment from this sequence and the full sequence of Jatropha database presented 100% amino acid identity in the covered area, but the sequenced suggested an extra loop (19 amino acids) after position 102 for Jcr4s01391.20.

Using primers targeting the full-length sequence, the cDNA for the *Jatropha* ecotype used in this study was amplified. Two independent plasmid clones were sequenced. None showed the loop predicted in the Jatropha Database sequence. *Jatropha* sequences from both databases and the self-sequenced one were aligned with the two isoforms of *Arabidopsis* (Fig. 5.1) demonstrating that the region was only present in Jcr4S01391.20. The said loop is not present in any other 2CP plants sequences, suggesting that the sequence proposed by **Jatropha Genome Database** results from incorrect or alternative splicing.

```
Jcr4S01391.20    --MACS--ATSTTGLISSIAATTKSMASPICKSSQNLTLP-KSFFGHRKPLQSRAPRSIS    55
Ath2CP-b         MSMASIASSSSTTLLSSSRVLLPSKSSLLSPTVSFPRIIPSSSASSSSLCSGFSSLGSLT    60
Ath2CP-a         --MAS--VASSTTLISSPSSRVFPAKSSLSSPSVSFLRTLSSP-SASASLRSGFARRS--    53
XP_012074345     --MAC--SATSTTGLISSIAATTKSMASPICKSSQNLTLPKSF-FGHRKPLQSRAPRSIS    55
FLseq_2CP        --MAC--SATSTTGLISSIAATTKSMASPICKSSQNLTLPKSF-FGHRKPLQSRAPRSIS    55
                   **       ***    *                                        *


Jcr4S01391.20    MARGSHSGKSFIVKASGEAPLVGNTAPDFEAEAVFDQEFIKVKLSEYIGKKYVILFFYPL    115
Ath2CP-b         TNRSASRRNFAVKAQADDLPLVGNKAPDFEAEAVFDQEFIKVKLSEYIGKKYVILFFYPL    120
Ath2CP-a         SLSSTSRRSFAVKAQADDLPLVGNKAPDFEAEAVFDQEFIKVKLSDYIGKKYVILFFYPL    113
XP_012074345     MARGSHSGKSFIVKASGEAPLVGNTAPDFEAEAVFDQEFIKVKLSEYIGKKYVILFFYPL    115
FLseq_2CP        MARGSHSGKSFIVKASGEAPLVGNTAPDFEAEAVFDQEFIKVKLSEYIGKKYVILFFYPL    115
                              ***** ******************** *************


Jcr4S01391.20    DFTFVCPTEITAFSDRYGEFEKLNTEILGVSVDSVFSHLAWVQTDRKSGGLGDLNYPLIS    175
Ath2CP-b         DFTFVCPTEITAFSDRYEEFEKLNTEVLGVSVDSVFSHLAWVQTDRKSGGLGDLNYPLVS    180
Ath2CP-a         DFTFVCPTEITAFSDRHSEFEKLNTEVLGVSVDSVFSHLAWVQTDRKSGGLGDLNYPLIS    173
XP_012074345     DFTFVCPTEITAFSDRYGEFEKLNTEILGVSVDSVFSHLAWVQTDRKSGGLGDLNYPLIS    175
FLseq_2CP        DFTFVCPTEITAFSDRYGEFEKLNTEILGVSVDSVFSHLAWVQTDRKSGGLGDLNYPLIS    175
                 ***************   ********  ********************************* *


Jcr4S01391.20    DVTKSISKSYGVLIPDQPVHVVLSGNIVKSSQNAVLGIALRGLFIIDKEGVIQHSTINNL    235
Ath2CP-b         DITKSISKSFG------------------VLIPDQGIALRGLFIIDKEGVIQHSTINNL    221
Ath2CP-a         DVTKSISKSFG------------------VLIHDQGIALRGLFIIDKEGVIQHSTINNL    214
XP_012074345     DVTKSISKSYG------------------VLIPDQGIALRGLFIIDKEGVIQHSTINNL    216
FLseq_2CP        DVTKSISKSYG------------------VLIPDQGIALRGLFIIDKEGVIQHSTINNL    216
                 * ******* *                   *********************


Jcr4S01391.20    AIGRSVDETLRTLQALQYVQENPDEVCPAGWKPGEKSMKPDPKLSKEYFAAI    287
Ath2CP-b         GIGRSVDETMRTLQALQYVQENPDEVCPAGWKPGEKSMKPDPKLSKEYFSAI    273
Ath2CP-a         GIGRSVDETMRTLQALQYIQENPDEVCPAGWKPGEKSMKPDPKLSKEYFSAI    266
XP_012074345     AIGRSVDETLRTLQALQYVQENPDEVCPAGWKPGEKSMKPDPKLSKEYFAAI    268
FLseq_2CP        AIGRSVDETLRTLQALQYVQENPDEVCPAGWKPGEKSMKPDPKLSKEYFAAI    268
                 ******** ******** ********************************
```

Figure 5.1. Alignment of coding sequences for 2CP. Sequences used belong to *Arabidopsis* (Ath), Jatropha Genome Database (Jcr), GenBank (XP), **F**ull **L**ength sequencing for own plasmid (FLseq). (*) mark exact amino acids. Variations were annotated if were more similar to 2CP-a (in red), to 2CP-b (in purple), or if it was unique to *Jatropha* (in blue).

No significant similarity was observed in the signal peptide fragments, but *Jatropha* sequences and 2CP-a had several consecutive identical amino acids after the starting Methionine. A high level of amino acid conservation was observed in the mature peptide sequence, 96.5% identity and 98.4% similarity. It was possible to observe two positions where *Jatropha* was similar to 2CPa (red in Fig. 5.1), and three other positions were similar to 2CP-b (purple in Fig. 5.1), while six amino acids were specific for *Jatropha curcas*. All modifications were most likely product of a change in one base in the codon.

J. curcas amino acid sequence similarity to 2CP-a was considered after a modification from Isoleucine to Valine, and Valine to Isoleucine. This represent a modification of one base (G-A) at the first position of the codon; the change stayed inside the group of non-polar amino acids. The importing signal was also similar to 2CP-a, with a reduced amount to Serine on it. In the conserved regions, the modifications between amino acids inside the same group like V-I and D-E do not represent big variation because it did not change the charge characteristics of the protein. Similarity to 2CP-b was based on three differentiations: (E) Aspartic acid to (D)

Glutamic acid, both acidic amino acids; (Y) Tryptophan from (H) Histidine, one non-polar and the other, a basic amino acid; and Valine (V) from Isoleucine (I), both non-polar amino acids. The only significative modification was Y-H, but it might not be enough to categorize with conviction the used *Jatropha* sequence as homologue to 2CP-b.

Amino acid changes, unique for *Jatropha*, were spotted in all three used sequences and correspond to changes of K –T (basic to polar), I-V (both non-polar), F –Y (non-polar to polar), G –A (both non-polar), M – L (both non-polar) and S –A (polar to non-polar). Changes to T (tyrosine), Y (lysin) and A (alanine) represent change in the functionality of the amino acid for the coding protein.

- **Exon analysis**

Sequences were evaluated using the genomic and coding sequence provided by the Jatropha Genome Database, together with the self-sequenced one, to determine the size and location of introns and exons to describe the gene model. This was also compared to *Arabidopsis* 2CP a/b gene models (Fig. 5. and 5.3). *Jatropha* sequences have the first and fifth exon different to both isoforms for *Arabidopsis*; exons 2, 3, 4, 6 and 7 have the same size. Exon 5 is three amino acid shorter in FLseq_2CP. Fifth intron (376 aa in FLseq_2CP) was divided on the sequence provided by Jatropha Genome Database in two shorter introns (205 and 110 aa) separated by a 59 aa exon (Fig. 5.4).

Exon	Ath 2CP-a	Ath 2CP-b	Jc 2CP.1	Jc 2CP.2
1	207	228	213	213
2	75	75	75	75
3	82	82	82	82
4	80	80	80	80
5	126	126	123	125
	-	-	-	59
6	114	114	114	114
7	117	117	117	117
Total	**801**	**822**	**804**	**865**

Figure 5.2. Description of exon sizes for 2CP sequences. Comparison of sizes for *Arabidopsis thaliana* (Ath) and *Jatropha curcas* (Jc). Jc 2CP.1 corresponds to XP_012074345 and the self-sequence plasmid. Jc 2CP.2 corresponds to Jcr4S01391.20.

- **Gene model**
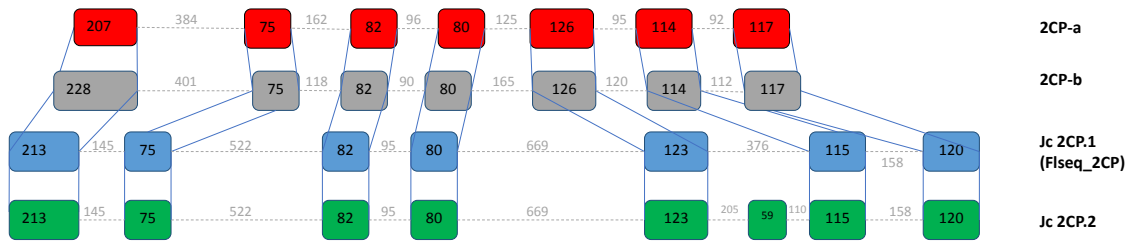
Figure 5.3. Gene model information for 2CP in *J. curcas* and in *A. thaliana*. Comparison based on the information collected for *A.thaliana* (from PeroxiBase), marked in red (2CP-a) and gray boxes (2CP-b) and for *Jatropha*, (from Jatropha Genome Database and full length sequence). Jc 2CP.1, (exons marked in blue boxes) includes XP_012074345 **and** the self-sequence plasmid. Jc 2CP.2 is the prediction for Jcr4S01391.20 sequence.

Alignments of genomic and coding sequence provided by Jatropha Genome Database give some hints about the reason for this extra loop, coding for one extra exon. It can be a result of a mis-splicing. An ATG coding for Methionine, was observed, that could have been read in a shifted frame.

```
genomicSeq    2201 CCAATTATGTAGCCTGTGCATGTTGTCCTCTCCGGCAATATTGTTAAGTC    2250
                             ||||||||||||||||||||||||||||||||||||||||||
cDNA           575 ----------AGCCTGTGCATGTTGTCCTCTCCGGCAATATTGTTAAGTC     614


genomicSeq    2251 GAGCCAGAATGCTGTTCTAGTTAATGTGTTGGATCCATGTTCTCATATTT    2300
                        |||||||||||||||||||
cDNA           615 GAGCCAGAATGCTGTTCTA-------------------------------     633
```

It is not possible to determine with exactitude if the extra loop in *Jcr4S01391.20* is a misread or an extra splicing site that might appear under specific conditions, but we proved that this area was not included in the coding sequence we cloned or the one from GenBank. Further analysis continued using the confirmed sequence, FLseq_2CP.

- **Chloroplast targeting**

The alignment of sequences with the ones of *Arabidopsis* did not give decisive information because of the low conservation in the area (Fig.5.1). Comparison with 20 other plant species showed many differences. The N-terminus in 2CP is not conserved.

TargetP 1.1 (developed by University of Denmark) predicts the subcellular localization of eukaryotic proteins and was used to evaluate this sequence. Description of the site states that "local assignment for proteins is based on the predicted presence of any of the N-terminal pre-sequences: chloroplastic transit peptide (cTP), mitochondria targeting peptide (mTP) or secretory pathway signal peptide (SP)". This method has been proved with *Arabidopsis*

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thaliana and *H. sapiens* (http://www.cbs.dtu.dk/services/TargetP/). RC value (reliability class), is rated from 1 to 5, where 1 indicates the strongest prediction. RC is a measure of the size of the difference between the highest and the second highest output scores.

Jc 2CP	TargetP Prediction Scores					
	cTP	mTP	SP	other	Loc	RC
XP_012074345 Flseq_2CP	0.973	0.016	0.008	0.097	C	1

ChloroP Prediction Scores		
Score	cTP	cTP lenght
0.576	Y	69

Transmembrane prediction	
TMHMM prediction	Probius prediction
No	No

Figure 5.4. Results for TargetP and ChloroP, TMHMM and Phobius, targeting 2CP sequence in the chloroplast. TargetP has four possible targeting compartments but predicts a localization in the chloroplast (higher score). ChloroP program confirms "Yes" =Y, and predicts target peptide size. TMHMM and Phobius are programs to detect transmembrane helix.

For the *Jatropha* 2CP translated sequence studied, *TargetP* gives a score of 0.973 for chloroplast targeting, RC=1, which is the highest probability (Fig. 5.4). *ChloroP* confirms chloroplast targeting with a score of 0.576 and identifies a targeting peptide length of 69 amino acids. *TMHMM* program evaluate the presence of "zero" transmembrane helix in 2CP; *Phobius* program confirms this result and predicts a signal peptide in a specific position between position 2 and 12, that is cleaved between position 16 and 17, followed by a non-cytoplasmic segment.

5.1.2. APX

The Jatropha Genome database includes nine sequences identified as APX. One of the sequences was described as *tAPX* compared to *Arabidopsis* (Jcr4S00512.40). All sequences provided by the database, were compared via BLAST with all plants in general, presenting ascorbate peroxidases homologs. When compared specifically with *Arabidopsis* gene *tAPX* (AT1G77490.1), only one was significative similar (Fig. 5.5).

Sequence from gene Jcr4S00512.40 had the highest score (Fig. 5.5), with double the amount of alignments more than the second and third best alignments. Apparently, there is high conservation in the sequence, 75% similarity was observed with the one of *Arabidopsis*, including the variation from the non-conserved N-terminus (signaling peptide). In Genbank, after BLAST using *Arabidopsis thaliana* as query, it was found one sequence corresponding to APX from *Jatropha* with another name: XM_012222913. Sequence alignment showed high

homology with *J. curcas* gene Jcr4S00512.40 in the covered area, only with a difference of an extra loop of 96 bases present in Jcr4S00512.40 but not in XM_012222913.1. The loop is situated after 374 nt from the start.

Sequence	Identity	Similarity	Score	
Jcr4S00512.40	**66%**	**75%**	**526**	→ *VERY SIMILAR*
Jcr4S06417.20	48%	61%	235	→ *Not similar*
Jcr4S00918.60	47%	58%	217	→ *Not similar*
Jcr4S00177.110	49%	63%	158	→ *Not similar*
Jcr4S00147.10	55%	66%	130	→ *Not similar*
Jcr4S00177.160	35%	50%	121	→ *Not similar*
Jcr4S1955.20	51%	70%	44	→ *Not similar*
Jcr4S00609.60	25%	39%	38	→ *Not similar*

Figure 5.5. Comparison of *J. curcas* APX translated sequence with *A. thaliana* tAPX. "Identity" recalls the percentage of exact the same aa in one position, "Similarity" recalls the alignments of aa with similar substitutes; "Score" refers to the amount of aa alignments made in total.

The sequence reported by the Jatropha Genome database, the start and stop codon are correct (ATG and TGA) but for the sequence reported by *NCBI* the start and stop codon are incorrect (ACA= Threonine and GAT= Aspartic acid). Pairwise alignments show that XM_012222913.1 had a pre-sequence and a post-sequence. It was reported as mRNA from L-ascorbate peroxidase 6, chloroplastic targeted, so this extra area might correspond to some other starting and ending genes.

Using primers targeting the full-length sequence, it was possible to amplify the cDNA for the *Jatropha* ecotype used in this study. *Jatropha* sequences from both databases and the self-sequenced one were aligned with the two isoforms of *Arabidopsis* (Fig. 5.6). Either of the GenBank RNA sequence and the self-cloned cDNA showed the loop after the conserved region **HPILVRLGWHDA,** it was only spotted in Jcr4S00512.40. Two independent plasmid clones were sequenced and the alignment confirmed the same result. The same missing region was also absent in APX sequences from other plants, suggesting that the sequence proposed by Jatropha Genome Database was a result from incorrect splicing, or the presence of extra splicing site.

Signal Peptide

```
Ath_sAPX        MAERVSLTLNGTLLSPPPTTTTT---TMSSSLRSTTAASLLLRSSSS—SSRSTLTLSASS   55
Ath_tAPX        -------------------MSVSLSAASHLLCSSTR—VSLSPAVTSSSSSPVVALSSSS   38
Jcr4S00512.40   MADSL----LHLQLSPSSPMASLSGAASSRLLPSASRARIAFHSATSSSSLSPL-SLSSS   55
FL_JcAPX        MADSL----LHLQLSPSSPMASLSGAASSRLLPSASRARIAFHSATSSSSLSPL-SLSSS   55
XM_012222913.1  MADSL----LHLQLSPSSPMASLSGAASSRLLPSASRARIAFHSATSSSSLSPL-SLSSS   55
                                :      :  *   * *::   :: :   ::  *      :**:*

Ath_sAPX        ---SSLSFVRSLVSSPRLSSSSSLSQKKYRIASVNRSFNSTTAATKSSSSDPDQLKNARE  112
Ath_tAPX        TSPH---SLGSVASSSLFPHSSFVLQKKHPINGT----STRMISPKCAASDAAQLISAKE   91
Jcr4S00512.40   SSSYSFSPLKCLRSSPLVPRVFF---------NK----TVSAMSTVTAASDPAQLKSARE  102
FL_JcAPX        SSSYSFSPLKCLRSSPLVPRVFF---------NK----TVSAMSTVTAASDPAQLKSARE  102
XM_012222913.1  SSSYSFSPLKCLRSSPLVPRVFF---------NK----TVSAMSTVTAASDPAQLKSARE  102
                   : .: **  .          .     .   :   ::** ** .*:*
```

H₂O₂

```
Ath_sAPX        DIKELLSTKFCHPILVRLGWHDAGTYNKNIKEWPQRGGANGSLRFDIELKHAANAGLVNA  172
Ath_tAPX        DIKVLLRTKFCHPILVRLGWHDAGTYNKNIEEWPLRGGANGSLRFEAELKHAANAGLLNA  151
Jcr4S00512.40   DIKELLKSTFCHPILVRLGWHDA------------------------------GLVNA  130
FL_JcAPX        DIKELLKSTFCHPILVRLGWHDAGTYNKNIEEWPKRGGANGSLRFEIELKHAANAGLVNA  162
XM_012222913.1  DIKELLKSTFCHPILVRLGWHDAGTYNKNIEEWPKRGGANGSLRFEIELKHAANAGLVNA  162
                *** ** :.**************                          **:**

Ath_sAPX        LNLIKDIKEKYSGISYADLFQLASATAIEEAGGPKIPMKYGRVDASGPEDCPEEGRLPDA  232
Ath_tAPX        LKLIQPLKDKYPNISYADLFQLASATAIEEAGGPDIPMKYGRVDVVAPEQCPEEGRLPDA  211
Jcr4S00512.40   LKLLQPIKDKYSGVTYADLFQLASATAIEEAGGPKLPMKYGRVDVSAPEDCPEEGRLPSA  190
FL_JcAPX        LKLLQPIKDKYSGVTHADLFQLASATAIEEAGGPKLPMKYGRVDVSAPEDCPEEGRLPSA  222
XM_012222913.1  LKLLQPIKDKYSGVTYADLFQLASATAIEEAGGPKLPMKYGRVDVSAPEDCPEEGRLPSA  222
                *:*:: :*:** .:::*****************.:********. .**:********.*

Ath_sAPX        GPPSPATHLREVFYRMGLDDKDIVALSGAHTLGRSRPERSGWGKPETKYTKEGPGAPGGQ  292
Ath_tAPX        GPPSPADHLRDVFYRMGLDDKEIVALSGAHTLGRARPDRSGWGKPETKYTKTGPGEAGGQ  271
Jcr4S00512.40   GPPSPADHLREVFYRMGLNDKEIVALSGAHTLGRSRPDRSGWGKPETKYTKNGPGAPGGQ  250
FL_JcAPX        GPPSPADHLREVFYRMGLNDKEIVALSGAHTLGRSRPDRSGWGKPETKYTKNGPGAPGGQ  282
XM_012222913.1  GPPSPADHLREVFYRMGLNDKEIVALSGAHTLGRSRPDRSGWGKPETKYTKNGPGAPGGQ  282
                ****** ***:********:**:************:**:************* *** ***
```

C   C

```
Ath_sAPX        SWTPEWLKFDNSYFKEIKEKRDEDLLVLPTDAAIFEDSSFKVYAEKYAADQDAFFKDYAV  352
Ath_tAPX        SWTVKWLKFDNSYFKDIKEKRDDDLLVLPTDAALFEDPSFKNYAEKYAEDVAAFFKDYAE  331
Jcr4S00512.40   SWTVEWLKFDNSYFKDIKAKKDEDLLVLPTDAVLFDDPSFRVYAEKYAEDKEAFFKDYAE  310
FL_JcAPX        SWTVEWLKFDNSYFKDIKAKKDEDLLVLPTDAVLFDDPSFRVYAEKYAEDKEAFFKDYAE  342
XM_012222913.1  SWTVEWLKFDNSYFKDIKAKKDEDLLVLPTDAVLFDDPSFRVYAEKYAEDKEAFFKDYAE  342
                *** :***********:** *:*:*********.:*:* **: ****** *  *******

Ath_sAPX        AHAKLSNLGAEFNPPEGIVI----------------------------------------  372
Ath_tAPX        AHAKLSNLGAKFDPPEGIVIENV-PEKFVAAKYSTGKKELSDSMKKKIRAEYEAIGGSPD  390
Jcr4S00512.40   AHAKLSNLGAKFDPPEGIVLDGVAGEKFVAAKYSTGKRELSDAMKQKIRAEYEAVGGSPD  370
FL_JcAPX        AHAKLSNLGAKFDPPEGIVLDGVAGEKFVAAKYSTGKRELSDAMKQKIRAEYEAVGGSPD  402
XM_012222913.1  AHAKLSNLGAKFDPPEGIVLDGVAGEKFVAAKYSTGKRELSDAMKQKIRAEYEAVGGSPD  402
                **********:*:******:         *************** ** ******** *****

Ath_sAPX        ----------------------------------  372
Ath_tAPX        KPLPTNYFLNIIIAIGVLVLLSTLFGGNNNSDFSGF  426
Jcr4S00512.40   KPLQSNYFLNIMIVIAVLAILTSLL-GN---------  397
FL_JcAPX        KPLQSNYFLNIMIVIAVLAILTSLL-GN---------  429
XM_012222913.1  KPLQSNYFLNIMIVIAVLAILTSLL-GN---------  429
                *** ****** * * ** * * **
```

Figure 5.6. Alignment of coding sequences for APX. Sequences used belong to *Arabidopsis* (Ath), Jatropha Genome Database (Jcr), GenBank (XM), **F**ull **L**ength sequencing for own plasmid (FLseq). (*) mark exact amino acids. Variations were annotated if were more similar to tAPX (in red), to sAPX (bold), or if it was unique to *Jatropha* (in blue). Yellow box indicates the signal peptide and gray box, the transmembrane helix. In red is marked "H₂O₂" for the H₂O₂ binding site, and "C" for the catalytic site.

The importing signal in the N-terminus of the *J. curcas APX* sequences is larger than that of *Arabidopsis* but holds some similarities to it (Fig. 5.6). There is a low level of amino acid conservation in the signal peptide, but a higher conservation observed in the mature peptide sequence, 83.6% identity with 53 amino acid variations in total. Similarity with the thylakoidal isoform of *Arabidopsis* APX (AT1G77490.1) was mostly determined by the

extended sequence in the C-terminus (Fig. 5.7), that might correspond to the trans-helix membrane. Jc *APX* is 9 amino acid shorter than in At *tAPX*, and contained 14 variations specific for *Jatropha*. There were 15 positions were the variation in amino acid, in in the mature peptide sequence, corresponded to At *tAPX*. In total, 88.3% identity was observed between AT1G77490.1 and FLseq_APX. From the 15 aa in variation, 8 aa corresponded to a changed to another amino acid from the same group (small variation), and 7 aa that represent changes in the charge or polarity pattern (K − N), (P − D), (D − T), (E − A), (E − V), (K − E) and (D − N).

From the complete mature peptide sequence, 26 specific amino acid variations were found specific for *Jatropha*. From them, 15 were in between the same group of amino acid, and 11 where changes in the classification, producing probably changes in the functionality. 14 of these differences were found in the C- terminus, corresponding probably to the trans-membrane helix, and 12 amino acid variation were found in the core peptide fragment. Still, when there was high indication of homology to *tAPX*, it was possible to find 12 positions were the direction in changes for amino acids was similar to *sAPX* (AT4G08390), from which 7 were drastic changes of classification.

- **Exon analysis**

Sequences were evaluated using the genomic and coding sequence provided by the Jatropha Genome Database together with our sequence data, to determine the size and location of introns and exons and to describe the gene model. This was also compared to *Arabidopsis* stromal and thylakoidal APX isoforms (Fig. 5.87 and 5.8). All *Jatropha* exons were different in size compared to *Arabidopsis* isoforms.

Jatropha first exon was relatively large like in sAPX. The second exon was smaller, like tAPX. Third exon present in Ath tAPX was absent in the stromal isoform while in *J. curcas* is debatable (Fig. 5.7). gDNA reported by the Jatropha Genome Database does not have precise reads around the area that might correspond to exons 3 and 4 from *A. thaliana* and includes some missing / not identified amino acid region.

Exon	Ath sAPX	Ath tAPX	Jc APX.1	Jc APX.2
1	234	183	238	238
2	268	135	112	112
3	-	121	61 58	25
4	101	101	102	100
5	88	88	90	90
6	68	68	66	66
7	87	87	88	88
8	75	75	74	74
9	78	78	79	79
10	105	105	104	104
11	15	60	63	63
12	-	180	115	115
Total	**1119**	**1281**	**1250**	**1154**

Figure 5.7. Description of exon sizes for APX sequences. Comparison of sizes for *Arabidopsis thaliana* (Ath) and *Jatropha curcas* (Jc). Jc APX.1 corresponds to XM_012222913.1 and the self-sequence plasmid. Jc APX.2 corresponds to Jcr4S00512.40.

In the alignment from the nucleotide sequence of Jcr4S00512.40 and its gDNA, after the second exon there is 650 bases of intron, followed by a 25 base-length exon tailed by a 159 base intron; this before the 4th exon (100 bp). The coding sequence obtained with full length primers in own plant material showed it different. Using the gDNA as template, the alignment presented two exons (61 and 58 base pairs), that, if summed up together would be 119 base exon, alike to tAPX third exon (Fig. 5.7). These results are not be completely reliable: is not possible to confirm if it the three exons are part of one single exon, or if one part corresponds to the 4th exon due to the low quality of the gDNA sequence provided.

From the 4th until the 10th exon, all three sequences have similar sizes. After that, the thylakoidal isoform and *Jatropha* sequences have an 11th exon with about 60 bases, while in sAPX is only 15. The last exon (12th) is absent in the stromal isoform, and in *Jatropha*, it is 65 bases shorter.

- **Gene model**

Figure 5.8 presents a detailed description of the gene models for *sAPX*, *tAPX* (from *Arabidopsis*), and *Jatropha curcas* sequences. It is possible to observe that from third exon on, the two subsequently produced exons could be one single exon evaluated under bad gDNA. Alignments of genomic and coding sequence provided by Jatropha Genome Database (Jcr4S00512.40) give hints about the inconsistent region. There is an area of 207 bases with low quality reads in the genomic DNA (Fig. 5.9). The third exon start with a read of 25 bp (present in the reported nucleotide sequence inside the Jatropha Genome Database, named

CDS_JcAPX in this analysis), and is extended to 61 bases in the self-construct and in XM_012222913.1 (FL_JcAPX).
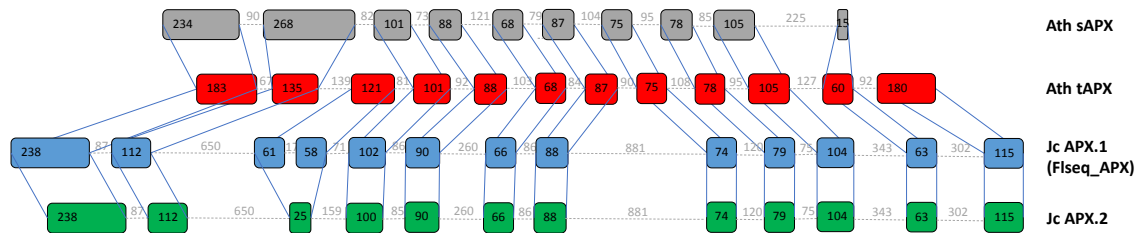
Figure 5.8. Gene model information for APX in *J. curcas* and in *A. thaliana*. Comparison of exon and intron size, based in the information collected. For *A. thaliana,* gene description was obtained from PeroxiBase, and marked in gray (sAPX) and red boxes (tAPX). For *Jatropha*, cDNA amplified and self sequence was aligned with genomic DNA reported in the *Jatropha Genome Database* (Jc APX.1), exons marked in blue boxes; it also corresponds to the same sequence reported for XM_012222913.1 . Nucleotide sequence reported by database was compared with gDNA and exons are marked in green boxes.

The gDNA sequence read in the Jatropha Genome Database was incomplete (Fig. 5.9) and has 17 non-identified bases, nevertheless it is possible to align other part to the full-length cloned sequence (58 bases). Predicted alignment shows that 25 bases are missing in the gDNA read ahead. After this region, an intron of 71 bases is placed, until the start of fourth exon, 102 bases length in FL_JcAPX, 2 bases shorter in the reported CDS.

Data might indicate that the third codon in Jc APX.2 is not only 25 bases long, but most probably it is the sum of this and next coding fragments, that in FLseq_APX has a size of 119 base pairs. A third exon with this size is similar to the one in *Arabidopsis* and other species. Full sequencing of gDNA including this gene must be done to clarify this hypothesis but is not of special interest for this project.

```
gDNA_JcAPX    1051 ATTGATGCTCAAGCCCACTAAAATGCTTATAATTACAGGTTCGACTGGGA    1100
FL_JcAPX       351 ----------------------------------GGTTCGACTGGGA     363
CDS_JcAPX      351 ----------------------------------GGTTCGACTGGGA     363


gDNA_JcAPX    1101 TGGCATGATGCTGGTACATACAACAAGAACATAGAGGAGTGGCCAAAAnn    1150
FL_JcAPX       364 TGGCATGATGCTGGTACATACAACAAGAACATAGAGGAGTGGCCAAAA--    411
CDS_JcAPX      364 TGGCATGATGCT--------------------------------------    376


gDNA_JcAPX    1151 nnnnnnnnnnnnnnnn------------------------GATTTGAAAT    1175
FL_JcAPX       412 --------------AGAGGTGGAGCCAATGGAAGTCTCAGATTTGAAAT    446
CDS_JcAPX      376 --------------------------------------------------    376


gDNA_JcAPX    1176 TGAGCTAAAACATGCAGCGAATGCAGGTAGTTATTTCATATCATGTGTAA    1225
FL_JcAPX       447 TGAGCTAAAACATGCAGCGAATG---------------------------    469
CDS_JcAPX      376 --------------------------------------------------    376


gDNA_JcAPX    1226 CTTTAGCTTGGTTTATAATATTTCTTCCTGACGAGCTGGCGTTTCAGGGC    1275
FL_JcAPX       470 ---------------------------------------------CAGGGC    475
CDS_JcAPX      376 -----------------------------------------------GGGC    404


gDNA_JcAPX    1276 TGGTAAATGCTTTGAAACTCCTTCAGCCAATCAAGGACAAGTATTCTGGT    1325
FL_JcAPX       476 TGGTAAATGCTTTGAAACTCCTTCAGCCAATCAAGGACAAGTATTCTGGT    525
CDS_JcAPX      405 TGGTAAATGCTTTGAAACTCCTTCAGCCAATCAAGGACAAGTATTCTGGT    455


gDNA_JcAPX    1326 GTGACATATGCAGATTTGTTTCAGTTGGCTAGTGCCACTGCTATAGAGGT    1375
FL_JcAPX       526 GTGACACATGCAGATTTGTTTCAGTTGGCTAGTGCCACTGCTATAG----    571
CDS_JcAPX      456 GTGACATATGCAGATTTGTTTCAGTTGGCTAGTGCCACTGCTATAG----    475
```

Figure 5.9. Alignment to determine conflictive area from exons of APX in *J. curcas*. Comparison of exon and intron size, based in the information collected, starting in 3rd exon. Main template is the genomic sequence (gDNA_JcAPX). cDNA from plant material was cloned and self sequenced (FL_JcAPX), and compared with the reported coding sequence for Jcr4S00512.40 in the Jatropha Genome Database (CDS_JcAPX). Controversial exons are marked in yellow.

- **Chloroplast targeting**

In the peptide alignment with *A. thaliana* isoforms (Fig. 5.6), an extended region in the C-terminus was observed. This area was submitted for further analysis with bioinformatics to evaluate targeting predictions.

TargetP predicts for FL_JcAPX a localization in the chloroplast with a score of 0.903 and RW=1 (the strongest prediction possible). *TMHMM server* predicts a transmembrane helix at the end and *Phobious* details the exact position for the transmembrane in the amino acids 377-396 (Fig. 5.10). Data confirms that FL_JcAPX is a homologue to At tAPX, because it exhibits the C-terminal transmembrane helix that differentiates tAPX from sAPX in *Arabidopsis*.
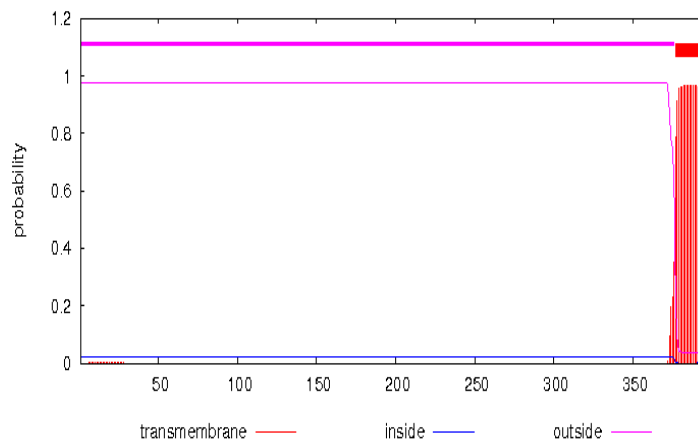
Figure 5.10. Graphical visualization of predictions for FL_JcAPX in TMHMM. Program predicts a transmembrane helix localized at the end of the sequence, marked in red lines in the graph.

Besides, all nine sequences reported in the Jatropha Genome Database as APX were analyzed with *TargetP* and *ChloroP*. Jcr4S00177.160, Jcr4S00512.40 and Jcr4S00609.60 had a chloroplast targeting score in *ChloroP* of 0.506, 0.581 and 0.529 respectively. While the sequences marked as APX6 and APX4 in Jatropha database had a reported peptide length of 29 and 24 amino acids respectively, localization score in *TargetP* was 0.467 and 0.408, with a low reliability class (RC=4). Only Jcr4S00512.40 (marked as tAPX in Jatropha database) had a signal peptide length of 83 amino acids, comparable with the one of *Arabidopsis*. The sequence obtained after full length amplification was more complete than the one provided by the database, so evaluations were continued with FL_JcAPX.

5.1.3. GPX

From the Jatropha Genome Database, eight sequences identified as GPX were found and confirmed when compared to other plants. Two sequences were described as chloroplast GPX. After alignments and blasting using A.th. *GPX* as query, Jcr4S05575.10 and Jcr4S00052.210 were confirmed as homologs to *Arabidopsis* GPX1 & GPX7. Genbank contains a sequence identified as a "chloroplastic phospholipid hydroperoxide glutathione peroxidase 1, mRNA", with another name (NM_001306024.1). Alignment of this peptide sequence with Jcr4S05575.10 and Jcr4S00052.210 showed alignment of 100% on the covered areas, but up to two lost loops at the beginning of the sequence. To investigate in detail the sequence, full-length sequence was amplified using cDNA of plant material from Panama. Three sequences from *J. curcas* were aligned with the *Arabidopsis* GPX sequences, to evaluate conserved regions and differences (Fig. 5.11)

110

Signal Peptide

```
Jcr4S05575.10    MAS---PNYPTTT--------TGDTVKPRVVICV----------------------GDI   26
Jcr4S00052.210   MAS-----VPFQSLKQFQINPKSCSFSPAMAFCIPSMKTSFGPSKSAFLQHGFSLQLPNF   55
FL_JcGPX         MAS-----VPFQSLKQFQINPKSCSFSPAMAFCIPSMKTSFGPSKSAFLQHGFSLQLPNF   55
NM_001306024.1   MAS-----VPFQSLKQFQINPKSCSFSPAMAFCIPSMKTSFGPSKSAFLQHGFSLQLPNF   55
AtGPx01          MVSMTTSSSSYGTFSTVVNSS---RPNSSATFLVPSLKFSTGISNFANLSNGFSLKSPIN   57
AtGPx07          ------MAFSYASFSTPFNGFAANPSPITSAFLGPSLRFSTRTSKTRNPSNGVSVKSSNS   54
                            :                            .:

Jcr4S05575.10    HGYFVKLQNLWSNLQSVISPQEFSSATIIFLGDYCDRGPDTKKVIDFLIHLPSAYPNQKH   86
Jcr4S00052.210   PGFLSKTHSFA--------------------VYARAATEKTIHDYTVKLSNGLIAGIR     93
FL_JcGPX         PGFLSKTHSFA--------------------VYARAATEKTIHDYTVK----------   83
NM_001306024.1   PGFLSKTHSFA--------------------VYARAATEKTIHDYTVK----------   83
AtGPx01          PGFLFKSRPFT--------------------VQARAAAEKTVHDFTVK----------   85
AtGPx07          HRFLVKSKNFS--------------------VYARAAAEKSVHDFTVK----------   82
                  ::  *  : :                       *.    *.: *: ::
```

```
                                          C                        C
Jcr4S05575.10    VF-LSGNHDLAFAAFVGVLPEPANGVSFKEGW--KEYESNEEREGWYKGDGYENMYLQGR   143
Jcr4S00052.210   LQLDIDGNGVPLSKFKGKVLLIVN-VASKCGLTASNYTELSHIYEKYKTQGFEILAFPCN   152
FL_JcGPX         ---DIDGNGVPLSKFKGKVLLIVN-VASKCGLTASNYTELSHIYEKYKTQGFEILAFPCN   139
NM_001306024.1   ---DIDGNGVPLSKFKGKVLLIVN-VASKCGLTASNYTELSHIYEKYKTQGFEILAFPCN   139
AtGPx01          ---DIDGKDVALNKFKGKVMLIVN-VASRCGLTSSNYSELSHLYEKYKTQGFEILAFPCN   141
AtGPx07          ---DIDGNDVSLDKFKGKPLLIVN-VASRCGLTSSNYSELSQLYEKYKNQGFEILAFPCN   138
                   ..:.: :  * *      .* *: : *    .:* . ..    ** :*:* : :  .
```

```
                                                                 C
Jcr4S05575.10    RWAGS-------------------------------------------------VKVR   152
Jcr4S00052.210   QFGGQEPGSNPEIKQFACTRYKAEFPIFDKVDVNGPNTAPVYQFLKSSAGGFLGDLIKWN   212
FL_JcGPX         QFGGQEPGSNPEIKQFACTRYKAEFPIFDKVDVNGPNTAPVYQFLKSSAGGFLGDLIKWN   199
NM_001306024.1   QFGGQEPGSNPEIKQFACTRYKAEFPIFDKVDVNGPNTAPVYQFLKSSAGGFLGDLIKWN   199
AtGPx01          QFGFQEPGSNSEIKQFACTRFKAEFPIFDKVDVNGPSTAPIYEFLKSNAGGFLGGLIKWN   201
AtGPx07          QFGGQEPGSNPEIKQFACTRFKAEFPIFDKVDVNGPSTAPIYKFLKSNAGGFLGDIIKWN   198
                 ::. .                                                :* .
```

```
Jcr4S05575.10    FDAKGIEYKGSIYNAA-PTFESYGV----------   176
Jcr4S00052.210   FEKFLVDKNGKVVERYPPTTSPFQIEKDIQKLLAA   247
FL_JcGPX         FEKFLVDKNGKVVERYPPTTSPFQIEKDIQKLLAA   234
NM_001306024.1   FEKFLVDKNGKVVERYPPTTSPFQIEKDIQKLLAA   234
AtGPx01          FEKFLIDKKGKVVERYPPTTSPFQIEKDIQKLLAA   236
AtGPx07          FEKFLVDKKGKVVERYPPTTSPFQIEKDIQKLLAA   233
                 *:    ::  :*.: :     ** . : :
```

Figure 5.11. Alignment of coding sequences for GPX. Sequences used belong to *Arabidopsis* (Ath), Jatropha Genome Database (Jcr), GenBank (NM), **F**ull **L**ength sequencing for own plasmid (FLseq). Yellow box indicate the signal peptide. In red are marked "H₂O₂" for the H_2O_2 binding site and "C" for the catalytic site. Important motives were marked in bold letters. (*) indicates aa identity, (:) is aa similarity in the same functional group, and (.) indicates similarity with bigger functional change.

Jcr4S05575.10 had one extra loop (marked in red) that was not present in any other alignment. A second loop of 13 amino acids (marked in blue) is present in both GPX sequences from the Database, without aa conservation. This loop was absent in the other sequences used to compare, from *Jatropha* and *Arabidopsis*.

Besides regular evaluation, conserved regions in GPX were observed. Pitsch *et al*. (2010) describe the conserved regions for chloroplast GPX. In Figure 5.11 the two regions considered important for effective glutation peroxidase (FPCNQFxxQEP *and WNFxKFL)* are marked. Catalytic sites were marked in red, corresponding to the sites determined in the same previous study. With the alignment it is possible to acknowledge that FL_JcGPX has homologies with both GPX1 and GPX7 from *Arabidopsis* but it is not possible to determine to which one of them it has more similarity. Conserved regions were identical in both isoforms.

- **Exon analysis**

Exons	Ath GPX1	Ath GPX7	FL_JcGPX	Jc_GPX.1
1	255	246	247	247
				40
2	77	77	78	77
3	62	62	61	61
4	119	119	119	119
5	168	168	170	170
6	30	30		
Total	**711**	**702**	**675**	**714**

Figure 5.12. Description of exon sizes for GPX sequences. Comparison of sizes for *Arabidopsis thaliana* (Ath) and *Jatropha curcas* (Jc). FL_JcAPX corresponds to self-sequence plasmid and NM_001306024.1 . Jc_GPX.1 corresponds to Jcr4S00052.210.

Gene composition of *Jatropha* GPX is similar to *Arabidopsis* isoforms. The length of the first exon was very similar in all compared sequences. The coding sequence proposed by the Jatropha Genome Database (JC_GPX.1) presents an exon of 40 aa that is not visible in other sequences with the alignments (Fig. 5.12 and 5.13) and does not affect the rest of the alignments. Exons 2, 3, 4 and 5 had almost the same size in both species. Still, the last exon in *A. thaliana* isoforms (30 bases) is missing for both *Jatropha* sequences.

- **Gene model**
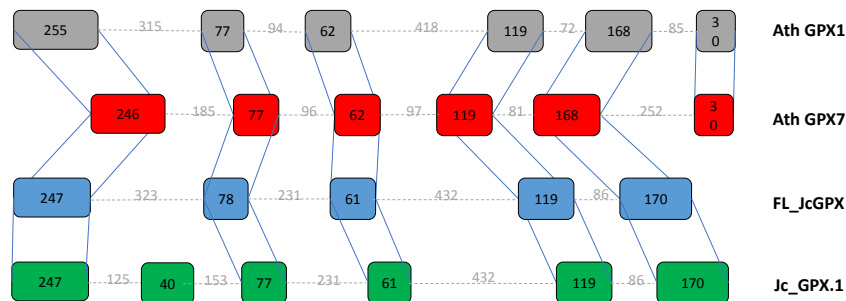
Figure 5.13. Gene model information for GPX in *J. curcas* and in *A. thaliana*. Comparison of exon and intron sizes based in the information collected for *A.thaliana* (Ath) from PeroxiBase. FL_JcGPX corresponds to self-sequence plasmid while Jc_GPX.1 corresponds to Jcr4S00052.210.

- **Chloroplast targeting**

For FL_JcGPX, ChloroP predicts chloroplast targeting peptide and describes it as 70 amino acids long peptide. TargetP confirms the chloroplast target with a score of 0.864, while it was 0.031 for mTP, 0.047 for SP and 0.086 for any other organelle, presenting strong results with a RC = 2 (high). Phobious and TMHMM did not predict any transmembrane helix for FL_JcGPX. No transmembrane helix was found in AT2G25080 (At GPX1) or AT4G31870 (At GPX7) either.

5.1.4. PrxQ

Two sequences were identified as Peroxiredoxin Q in Jatropha Genome Database (Jcr4S00136.50 and Jcr4s26102.10); both sequences were aligned and proved to be the same nucleotide sequence. Blasting was carried out to compare to all possible transcripts, which confirmed that the provided sequence was a peroxiredoxin Q. NCBI database has a sequence catalogued as "*Jatropha curcas* Peroxiredoxin Q" with another name (XP_012077107.1). After alignment to Jcr4S00136.50, it turned to be the same sequence, but XP 012077107.1 contains a pre- and post- sequence, assumable introns from other genes.

```
                      Signal Peptide
At3g26060     MAASSSSFTLCNHTTLRTLPLRKTL-VTKTQFSVPTKSSESNFFGSTLTHSSYISPVSSS   59
XP_012077107  ----MASISLPKHSLPSLLPTQKPRHSPSQNLPIFSKASQSQFYGLKLSNLSSLTIPSSS   56
Jcr4S00136    ----MASISLPKHSLPSLLPTQKPRHSPSQNLPIFSKASQSQFYGLKLSNLSSLTIPSSS   56
                 :*::* :*:      ** :*     . :: : :*:*:*:*:* .*:: * ::   ***

                                                                     C     C
At3g26060     SLKGLIFAKVNKGQAAPDFTLKDQNGKPVSLKKYKGKPVVLYFYPADETPGCTKQACAFR   119
XP_012077107  SLKTTIFAKVNKGQVPPSFTLKDQDGKNVSLSKFKGKPVVVYFYPADETPGCTKQACAFR   116
Jcr4S00136    SLKTTIFAKVNKGQVPPSFTLKDQDGKNVSLSKFKGKPVVVYFYPADETPGCTKQACAFR   116
                 ***    *********. *.******:** ***.*:******:*****************

At3g26060     DSYEKFKKAGAEVIGISGDDSASHKAFASKYKLPYTLLSDEGNKVRKDWGVPGDLFGALP   179
XP_012077107  DSYEKFKKAGAEVIGISGDDTSSHNAFAKKYRLPFTLLSDEGNKVRKEWGIPGDLFGALP   176
Jcr4S00136    DSYEKFKKAGAEVIGISGDDTSSHNAFAKKYRLPFTLLSDEGNKVRKEWGIPGDLFGALP   176
              ********************::**:***.**:**:*************:**:*********

At3g26060     GRQTYVLDKNGVVQLIYNNQFQPEKHIDETLKFLKAA     216
XP_012077107  GRQTYVLDKKGVVQLIYNNQFQPEKHIDETLKLLQSL     213
Jcr4S00136    GRQTYVLDKKGVVQLIYNNQFQPEKHIDETLKLLQSL     213
              *********:***********************:*::
```

Figure 5.14. Alignment of coding sequences for PrxQ. Sequences belong to *Arabidopsis* (Ath), Jatropha Genome Database (Jcr), GenBank (XP). Yellow box indicates the signal peptide. Changes in amino acids specific for *J. curcas* are marked in blue and catalytic site (C) are identified in red. (*) indicates aa identity, (:) is aa similarity in the same functional group, and (.) indicates similarity with bigger functional change.

Both sequences from *Jatropha* were compared to *Arabidopsis* PrxQ (AT3G26060) (Fig. 5.14). The characteristic and conserved C residues are located in the middle of the sequence, including two cysteins (peroxidatic and resolving cysteins). It presented a high level of amino acids conservation. This short gene has a long signaling peptide that is 40% the size of the complete peptide. The mature region has 14 modifications in the amino acid sequence specific to *Jatropha* when compared to *A. thaliana* (Fig. 5.15).

J. curcas	Group		A. thaliana	Group
Serine	Polar	S - K	Lysin	Basic
Phenylalanine	Non-Polar	F - Y	Tyrosine	Polar
Serine	Polar	S - A	Alanine	Non-Polar
Asparagine	Polar	N - K	Lysin	Basic
Phenylalanine	Non-Polar	F - Y	Tyrosine	Polar
Lysin	Basic	K - N	Asparagine	Polar
Serine	Polar	S - A	Alanine	Non-Polar

Figure 5.15. Variations in peptide sequences of *A. thaliana* and *J. curcas*. Groups were associated by color" sequences are from *Arabidopsis*. "Jcr" is the prefix from Jatropha Genome Database

Seven of these modifications were significant changes of amino acid group. There are four changes in polarity, two changes Polar-Basic and one change Basic-Polar. This part of the sequence is slightly more polar in *Jatropha* than in *Arabidopsis*, which may affect in protein structure.

- **Exon analysis**

Exon	Ath PrxQ	Jc PrxQ
1	204	195
2	138	138
3	90	90
4	219	219
Total	**651**	**642**

Figure 5.16. Exon sizes corresponding to peptide sequences of PrxQ , in *A. thaliana* and *J. curcas*. Exons are presented in the reading order. "Ath" corresponds to *Arabidopsis* and "Jcr" is the prefix for *Jatropha*.

PrxQ sequences for *Arabidopsis* (At3g26060) and *Jatropha* (Jcr4S00136.50), had the same number of exons with the exact same size (Fig. 5.16). Only the first one had a difference of nine extra amino acids in *Arabidopsis.* Evaluation about exact gene model was not possible because the gDNA sequence in the database for Jcr4S00136.50, do not corresponded to this gene nucleotide description (uploading error).

- **Chloroplast targeting**

PrxQ genes in *Arabidopsis* are situated in the chloroplast. Jcr4S00136.50 sequence has a chloroplastic peptide score of 0.958 and RC=1 in *TargetP*, while it has a low score for the other possibilities (0.033 for mitochondria, 0.006 for secretory pathway and 0.046 for any other place). *ChloroP* determines that the signal peptide is 86 amino acids long, with a score of 0.580. No transmembrane helix was detected with bioinformatic prediction tools.

5.1.5. CAT

Eight genes were tagged with catalase activity as molecular function inside Jatropha Genome Database. From these, three were considered partial sequences. NCBI database includes three sequences and a partial form. All sequences were aligned against each other and at the end, compared to three of *A. thaliana* CAT genes, AT1G20630 (CAT1), AT4G35090 (CAT2) and AT1G20620 (CAT3). Alignment of *Ath* CAT genes showed that all three sequences had the same size, contained highly conserved regions and only punctual variation in amino acids, which makes it difficult to establish comparative difference between them (Fig. 5.17).

One CAT1 gene homologue for *J. curcas* in GenBank was named XP_012073648.1. It showed almost 100% alignment with Jcr4S01159.40 from Jatropha Genome Database but differed in the first five amino acids after the starting Methionine. There were 84% identities and 91% similarities in the 888 amino acid aligned to *Arabidopsis*. Another sequence, XM_012218258.1 had a good alignment in nucleotide sequence when compared to Jcr4S01159.40 but had a loop of around 700nt in the middle and about 270 nt at the C-terminus that were not present in the others.

CAT2 genes homologues contained in NCBI database as XP_012065377.1 was exactly the same as Jcr4S1023.80 (100% amino acid similarity). They had 91% identity and 97% similarities in the 956 amino acid aligned to *Arabidopsis*. Another sequence in NCBI (XM_012209987.1) showed high similarity but had extra bases in the alignment (around 100 bases pre-sequence and 40 bases post-sequence).

NP_001292952.1 had a good alignment with Jcr4S001159.50 but had a loop at the N-terminal region. Last two sequences were not predicted by a BLAST as hypothetical versions of any specific CAT isoforms, therefore, discarded from further analysis. No sequence was specially predicted as homologues from Ath CAT3.

```
AT1G20620_CAT3    MDPYKYRPSSAYNAPFYTTNGGAPVSNNISSLTIGERGPVLLEDYHLIEKVANFTRERIP
AT1G20630_CAT1    MDPYRVRPSSAHDSPFFTTNSGAPVWNNNSSLTVGTRGPILLEDYHLLEKLANFDRERIP
AT4G35090_CAT2    MDPYKYRPASSYNSPFFTTNSGAPVWNNNSSMTVGPRGPILLEDYHLVEKLANFDRERIP
                  ****: **:*:::**:***.**** ** **:*:* ***:*******:**:*** *****

AT1G20620_CAT3    ERVVHARGISAKGFFEVTHDISNLTCADFLRAPGVQTPVIVRFSTVVHERASPETMRDIR
AT1G20630_CAT1    ERVVHARGASAKGFFEVTHDITQLTSADFLRGPGVQTPVIVRFSTVIHERGSPETLRDPR
AT4G35090_CAT2    ERVVHARGASAKGFFEVTHDISNLTCADFLRAPGVQTPVIVRFSTVIHERGSPETLRDPR
                  ******** *************::**.*****.**************:***.****:** *

AT1G20620_CAT3    GFAVKFYTREGNFDLVGNNTPVFFIRDGIQFPDVVHALKPNPKTNIQEYWRILDYMSHLP
AT1G20630_CAT1    GFAVKFYTREGNFDLVGNNFPVFFVRDGMKFPDMVHALKPNPKSHIQENWRILDFFSHHP
AT4G35090_CAT2    GFAVKFYTREGNFDLVGNNFPVFFIRDGMKFPDMVHALKPNPKSHIQENWRILDFFSHHP
                  ******************* ****:***::***:**********:*** *****:**:* *

AT1G20620_CAT3    ESLLTWCWMFDDVGIPQDYRHMEGFGVHTYTLIAKSGKVLFVKFHWKPTCGIKNLTDEEA
AT1G20630_CAT1    ESLHMFSFLFDDLGIPQDYRHMEGAGVNTYMLINKAGKAHYVKFHWKPTCGIKCLSDEEA
AT4G35090_CAT2    ESLNMFTFLFDDIGIPQDYRHMDGSGVNTYMLINKAGKAHYVKFHWKPTCGVKSLLEEDA
                  ***   : ::***:**********:* **:** ** *:**. :***********:* * :*:*

AT1G20620_CAT3    KVVGGANHSHATKDLHDAIASGNYPEWKLFIQTMDPADEDKFDFDPLDVTKIWPEDILPL
AT1G20630_CAT1    IRVGGANHSHATKDLYDSIAAGNYPQWNLFVQVMDPAHEDKFDFDPLDVTKIWPEDILPL
AT4G35090_CAT2    IRVGGTNHSHATQDLYDSIAAGNYPEWKLFIQIIDPADEDKFDFDPLDVTKTWPEDILPL
                   ***:******:**:*:**:****:.**:* :***.************* ********

AT1G20620_CAT3    QPVGRLVLNRTIDNFFNETEQLAFNPGLVVPGIYYSDDKLLQCRIFAYGDTQRHRLGPNY
AT1G20630_CAT1    QPVGRLVLNKNIDNFFNENEQIAFCPALVVPGIHYSDDKLLQTRIFSYADSQRHRLGPNY
AT4G35090_CAT2    QPVGRMVLNKNIDNFFAENEQLAFCPAIIVPGIHYSDDKLLQTRVFSYADTQRHRLGPNY
                  *****:***:.***** *.**:** *.::****:******* *:*:*.*:*********

AT1G20620_CAT3    LQLPVNAPKCAHHNNHHEGFMNFMHRDEEINYYPSKFDPVRCAEKVPTPTNSYTGIRTKC
AT1G20630_CAT1    LQLPVNAPKCAHHNNHHDGFMNFMHRDEEVNYFPSRLDPVRHAEKYPTTPIVCSGNREKC
AT4G35090_CAT2    LQLPVNAPKCAHHNNHHEGFMNFMHRDEEVNYFPSRYDQVRHAEKYPTPPAVCSGKRERC
                  *****************:************:**:**: * ** *** **    :* * :*

AT1G20620_CAT3    VIKKENNFKQAGDRYRSWAPDRQDRFVKRWVEILSEPRLTHEIRGIWISYWSQADRSLGQ
AT1G20630_CAT1    FIGKENNFKQPGERYRSWDSDRQERFVKRFVEALSEPRVTHEIRSIWISYWSQADKSLGQ
AT4G35090_CAT2    IIEKENNFKEPGERYRTFTPERQERFIQRWIDALSDPRITHEIRSIWISYWSQADKSLGQ
                  .* ******: *:***::  :**:**:*:*::: **:**:****.**********:****

AT1G20620_CAT3    KLASRLNVRPSI
AT1G20630_CAT1    KLATRLNVRPNF
AT4G35090_CAT2    KLASRLNVRPSI
                  ***:******.:
```

Figure 5.17. Alignment of CAT sequences for _Arabidopsis thaliana_. Peptide sequence browsed from Peroxibase, with the ID number.

```
Jcr4S01159.40    MLIIQNRPSSAFDTPFWTTNSGAPIWNNNSSMTVGQRGPILLEDYHLIEKLANFDRERIP    60
XP_012073648.1   MDPYKNRPSSAFDTPFWTTNSGAPIWNNNSSMTVGQRGPILLEDYHLIEKLANFDRERIP    60
AT1G20630_CAT1   MDPYRVRPSSAHDSPFFTTNSGAPVWNNNSSLTVGTRGPILLEDYHLLEKLANFDRERIP    60
Jcr4S01023.80    MDPYKFRPSSANNSPFFTTNSGAPVWNNNNSLTVGSRGPILLEDYHLVEKLANFDRERIP    60
XP_012065377.1   MDPYKFRPSSANNSPFFTTNSGAPVWNNNNSLTVGSRGPILLEDYHLVEKLANFDRERIP    60
AT4G35090_CAT2   MDPYKYRPASSYNSPFFTTNSGAPVWNNNSSMTVGPRGPILLEDYHLVEKLANFDRERIP    60
                 *    : **:*: ::**:*******:****.*:*** ***********:************

Jcr4S01159.40    ERVVHARGASAKGFFEVTHDISNLTCADFLRAPGVQTPVIVRFSTVIHERGSPETLRDPR   120
XP_012073648.1   ERVVHARGASAKGFFEVTHDISNLTCADFLRAPGVQTPVIVRFSTVIHERGSPETLRDPR   120
AT1G20630_CAT1   ERVVHARGASAKGFFEVTHDITQLTSADFLRGPGVQTPVIVRFSTVIHERGSPETLRDPR   120
Jcr4S01023.80    ERVVHARGASAKGFFEVTHDISHLTCADFLRAPGVQTPVIVRFSTVIHERGSPETLRDPR   120
XP_012065377.1   ERVVHARGASAKGFFEVTHDISHLTCADFLRAPGVQTPVIVRFSTVIHERGSPETLRDPR   120
AT4G35090_CAT2   ERVVHARGASAKGFFEVTHDISNLTCADFLRAPGVQTPVIVRFSTVIHERGSPETLRDPR   120
                 ********************::**.*****.*****************************

Jcr4S01159.40    GFAVKFYTREGNFDLVGNNFPVFFIRDGMKFPDLVHAFKPNPKSHIQESWRIFDFLSHVP   180
XP_012073648.1   GFAVKFYTREGNFDLVGNNFPVFFIRDGMKFPDLVHAFKPNPKSHIQESWRIFDFLSHVP   180
AT1G20630_CAT1   GFAVKFYTREGNFDLVGNNFPVFFVRDGMKFPDMVHALKPNPKSHIQENWRILDFFSHHP   180
Jcr4S01023.80    GFAVKFYTREGNFDLVGNNFPVFFIRDGMKFPDMVHALKPNPKSHIQESWRILDFFSHHP   180
XP_012065377.1   GFAVKFYTREGNFDLVGNNFPVFFIRDGMKFPDMVHALKPNPKSHIQESWRILDFFSHHP   180
AT4G35090_CAT2   GFAVKFYTREGNFDLVGNNFPVFFIRDGMKFPDMVHALKPNPKSHIQENWRILDFFSHHP   180
                 ************************:********:***:********** .***:**:** *

Jcr4S01159.40    ESLHMLTFLFDDLGIPQDYRHMEGSGVNTYTLINKAGKVHYVKFHWKPTCGVKCLLDEEA   240
XP_012073648.1   ESLHMLTFLFDDLGIPQDYRHMEGSGVNTYTLINKAGKVHYVKFHWKPTCGVKCLLDEEA   240
AT1G20630_CAT1   ESLHMFSFLFDDLGIPQDYRHMEGAGVNTYMLINKAGKAHYVKFHWKPTCGIKCLSDEEA   240
Jcr4S01023.80    ESLNMFTFLLDDIGIPQDYRHMDGSGVNTYTLINKAGKAHYVKFHWKPTCGVKSLLEDDA   240
XP_012065377.1   ESLNMFTFLLDDIGIPQDYRHMDGSGVNTYTLINKAGKAHYVKFHWKPTCGVKSLLEDDA   240
AT4G35090_CAT2   ESLNMFTFLFDDIGIPQDYRHMDGSGVNTYMLINKAGKAHYVKFHWKPTCGVKSLLEEDA   240
                 ***:*::**:**:**********:*:***** *******.*************:*.* ::*

Jcr4S01159.40    IKVGGTNHSHATQDLYDSIAAGNYPEWKLYIQTMDPDHEDRFDFDPLDVTKTWPEDILPL   300
XP_012073648.1   IKVGGTNHSHATQDLYDSIAAGNYPEWKLYIQTMDPDHEDRFDFDPLDVTKTWPEDILPL   300
AT1G20630_CAT1   IRVGGANHSHATKDLYDSIAAGNYPQWNLFVQVMDPAHEDKFDFDPLDVTKIWPEDILPL   300
Jcr4S01023.80    IRVGGANHSHATQDLYDSIAAGNYPEWKLFIQIIDPLDEDKFDFDPLDVTKTWPEDILPL   300
XP_012065377.1   IRVGGANHSHATQDLYDSIAAGNYPEWKLFIQIIDPLDEDKFDFDPLDVTKTWPEDILPL   300
AT4G35090_CAT2   IRVGGTNHSHATQDLYDSIAAGNYPEWKLFIQIIDPADEDKFDFDPLDVTKTWPEDILPL   300
                 *:***:******:*************:*:*::* :**.**:**********.********

Jcr4S01159.40    HPVGRLVLNKNIDNFFAENEMLAFCPGIVVPGVSYSEDKLLQTRIFSYSDTQRHRLGPNY   360
XP_012073648.1   HPVGRLVLNKNIDNFFAENEMLAFCPGIVVPGVSYSEDKLLQTRIFSYSDTQRHRLGPNY   360
AT1G20630_CAT1   QPVGRLVLNKNIDNFFNENEQIAFCPALVVPGIHYSDDKLLQTRIFSYADSQRHRLGPNY   360
Jcr4S01023.80    QPVGRMVLNRNIDNFFAENEQLAFCPAIIVPGVYYSDDKLLQTRIFSYSDTQRHRLGPNY   360
XP_012065377.1   QPVGRMVLNRNIDNFFAENEQLAFCPAIIVPGVYYSDDKLLQTRIFSYSDTQRHRLGPNY   360
AT4G35090_CAT2   QPVGRMVLNKNIDNFFAENEQLAFCPAIIVPGIHYSDDKLLQTRVFSYADTQRHRLGPNY   360
                 :****:***:****** *** :****.::;***: **:*******:***:*:********

Jcr4S01159.40    LQLPVNAPKCAHHNNHHEGFMNFMHRDEEVNYFPSRYDPVRHSEAFPFPATICSGKREKC   420
XP_012073648.1   LQLPVNAPKCAHHNNHHEGFMNFMHRDEEVNYFPSRYDPVRHSEAFPFPATICSGKREKC   420
AT1G20630_CAT1   LQLPVNAPKCAHHNNHHDGFMNFMHRDEEVNYFPSRLDPVRHAEKYPTTPIVCSGNREKC   420
Jcr4S01023.80    LQLPPNAPKCAHHNNHHEGFMNFMHRDEEVNYFPSRYDPVRHAEKYPIPPPVLTGRREKT   420
XP_012065377.1   LQLPPNAPKCAHHNNHHEGFMNFMHRDEEVNYFPSRYDPVRHAEKYPIPPPVLTGRREKT   420
AT4G35090_CAT2   LQLPVNAPKCAHHNNHHEGFMNFMHRDEEVNYFPSRYDQVRHAEKYPTPPAVCSGKRERC   420
                 **** *************:****************** * ***:* :*     : :*.**:

Jcr4S01159.40    VIPKENNFKQPGERYRSWAPDRQERFLNRIVGCLSDPRVTHEICSIWINYFTQCDRSLGQ   480
XP_012073648.1   VIPKENNFKQPGERYRSWAPDRQERFLNRIVGCLSDPRVTHEICSIWINYFTQCDRSLGQ   480
AT1G20630_CAT1   FIGKENNFKQPGERYRSWDSDRQERFVKRFVEALSEPRVTHEIRSIWISYWSQADKSLGQ   480
Jcr4S01023.80    VIPKENNFKQPGERYRSFTPDRKDRFIRRWVEALSDPRVTYEIRSIWISYWSQADKSLGQ   480
XP_012065377.1   VIPKENNFKQPGERYRSFTPDRKDRFIRRWVEALSDPRVTYEIRSIWISYWSQADKSLGQ   480
AT4G35090_CAT2   IIEKENNFKEPGERYRTFTPERQERFIQRWIDALSDPRITHEIRSIWISYWSQADKSLGQ   480
                 .* *****:*******::  :*::**:.* : .*:*:*:*:** ****.*::*.*:****

Jcr4S01159.40    KLASRLNVKPSI       492
XP_012073648.1   KLASRLNVKPSI       492
AT1G20630_CAT1   KLATRLNVRPNF       492
Jcr4S01023.80    KIASHLNMRPTM       492
XP_012065377.1   KIASHLNMRPTM       492
AT4G35090_CAT2   KLASRLNVRPSI       492
                 *:*::**::*.:
```

Figure 5.18. Alignment of coding sequences for CAT sequences. (*) indicates aa identity, (:) is aa similarity in the same functional group, and (.) indicates similarity with bigger functional change.

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Sequences considered homologues of CAT1 in *Jatropha curcas* (XP_012073648.1 and Jcr4S01159.40) as well as homologues for CAT2 (XP_012065377.1 and Jcr4S1023.80) where aligned with AT1G20630 (CAT1) and AT4G35090 (CAT2) to evaluate conserved regions and specific modifications for isoforms, plant species (Fig. 5.18). Inside the alignment, amino acids observed only in one of the isoforms, either for *Arabidopsis* or *Jatropha*, where marked in bold letters; the variation in amino acid that corresponded to the same option in At CAT1 was marked in red while the variation corresponding to CAT2 option was marked in blue. Besides, the variations found specifically for *Jatropha* sequences where marked in purple.

All this variation and specificities are summarized in Figure 5.19a. Besides the apparent error read in Jcr4S01159.40 (four amino acids at the beginning of the sequence, Fig. 5.18), the homologue sequences for CAT1 presented 30 positions with specific aminoacids, found only in this isoform (marked in bold letters); this value was similar to the 25 specific positions for amino acids found in At CAT1. Homologues of CAT2 in *Jatropha* had 14 specific amino acid variations, also similar to the 10 amino acid unique for At CAT1. Color-based differentiation allows to see that both isoforms in *Arabidopsis* shared similarities at some point with both *Jatropha* isoforms, though the proposed similarity to one or the other isoform was sustained in a major number of shared specific amino acids at punctual positions. Jc CAT1 homologue had 16 variations, from which 10 corresponded to similarity to At CAT1. In the same way, Jc CAT2 had 18 variations, from which 15 were similar to At CAT2. From these variations, there were 10 positions where the amino acid variation was similar to either one or the other isoform (Fig. 5.18) always to the corresponding one.

	Unique for this type	Similar to At CAT1	Similar to At CAT2	Unique for Jatropha		Error read
				Common Jatropha	Isoform difference	
Jc CAT1	30	10	6	6	5	4
Jc CAT2	14	3	15			
At CAT1	25					
At CAT2	10					

Position	Jc CAT1	Jc CAT2	At CAT1	At CAT2
277	D	L	A	A
334	S	Y	H	H
408	F	I	T	T
411	T	P	I	A
448	N	R	K	Q

Figure 5.19. Analysis of CAT sequence alignment. (a) indicates the number of amino acid variations in each isoform per species, (b) indicates the punctual variations, unique for *Jatropha*, where there is isoform specificity.

There were counted eleven positions were the amino acid variation was found only in *Jatropha* sequences (Fig. 5.18, marked in purple). From them, six variations were common for both isoforms (Fig. 5.19a), and five were unique for each isoform. The exact amino acid modification is summarized in Figure 5.19b.

- **Exon analysis**

For Catalase, gene model was designed by simply using gene description from the Jatropha Genome Database (Fig. 5.20). Though a very similar coding sequence, exon analysis demonstrated again some differences in the sequence proposed by the Jatropha Genome Database. Jcr4S01159.40, the hypothetical homologue of At CAT1, has a large first exon in an area that is apparently conserved (Fig. 5.20). Second exon is very similar in all sequences. The third exon is divided in two in this sequence, presenting one extra exon of 90 bp. 4th exon is highly conserved, 777 bp for all sequences. 5th and 6th exons in Jcr4S01159.40 are somehow different to the other options, since it has larger reads and larger introns. Suppressing the last exon (6th) is absent the *Jatropha* CAT sequence, but the total length of the coding sequence is the same as in *Arabidopsis*.

Exon	Ath CAT1	Ath CAT2	Jc CAT1	Jc CAT2
1	15	15	60	15
2	97	97	94	97
3	278	278	68	278
			90	
4	777	777	777	777
5	90	90	278	90
6	68	68	112	68
7		94	-	94
8	154	60	-	-
Total	**1479**	**1479**	**1479**	**1419**

Figure 5.20. Exon sizes corresponding to peptide sequences of CAT1 and CAT2 , in *A. thaliana* and *J. curcas*. Exons are presented in the reading order. "Ath" corresponds to *Arabidopsis* and "Jcr" is the prefix for *Jatropha.*

Jcr4S1023.80, identified as homologue sequence to At CAT2 shows large similarities in the exon composition with *A. thaliana* genes (Fig. 5.20). First seven exons are exactly in the same size but is missing the last exon (60 bases). Calculations reveal a gene with longer introns in general, including non-coding areas of 300-600 bp when in At CAT2 average intron size is around 90.

- **Chloroplast targeting**

Catalases are not typically targeted to the chloroplast and sequences from *Jatropha curcas* appear not to be the exception. Jcr4S01159.40 has a peptide score of 0.495 for chloroplasts,

while 0.578 for other compartments, with RC=5 (dubious classification). Jcr4S1023.80 has a peptide score of 0.444 for chloroplast, 0.126 for mitochondria and 0.508 for other organelle, with RC=5 (dubious classification). No transmembrane helix was detected with bioinformatics for any of these sequences.

5.1.6. SOD

Six sequences were described as superoxide dismutase in Jatropha database due to their activity, from them, three had a copper/zinc binding site, two used iron and one used manganese. Besides them, one chaperone was also identified (Fig. 5.21).

Sequence	Description	Score	cTP	SP lenght
Jcr4S00546.10	Cu/Zn SOD1	0.452	-	
Jcr4S02704.30	Cu/Zn SOD2	0.549	Y	73
Jcr4S00031.40	Cu/Zn SOD3	0.471	-	
Jcr4S00269.140	Fe SOD2	0.535	Y	38
Jcr4S09211.10	Fe SOD3	0.5	Y	33
Jcr4S00073.110	Mn SOD1	0.468	-	
Jcr4S10051.40	SOD1 chaperone	0.564	Y	75

Figure 5.21. Description of all superoxide dismutase genes found in the Jatropha Genome Database. Also includes the chloroplast prediction with ChloroP.

The chaperone and three of the SOD genes had chloroplast target. The gene with the highest prediction score was a copper/zinc superoxide dismutase 2 homologue (73 aa). The Cu/Zn SOD in *A. thaliana* were screened for their sub-cellular targeting (Fig. 5.22); only Cu/Zn SOD2 was targeted to the chloroplast with high score (0.960) and RC = 1.

Name		cTP	mTP	SP	other	Loc	RC
AT1G08830	Cu/Zn SOD1	0.118	0.100	0.203	0.692	_	3
AT2G28190.1	Cu/Zn SOD2	0.960	0.083	0.003	0.013	C	1
AT5G18100	Cu/Zn SOD3	0.069	0.318	0.053	0.445	_	5

Figure 5.22. Description of all superoxide dismutases found in the Jatropha Genome Database. Includes also the chloroplast prediction with ChloroP.

After blasting in GenBank, using the *Arabidopsis* Cu/Zn SOD2 (AT2G28190.1) as query, another sequence was found as homologue in *Jatropha* with a different name (NM_001308734.1). Nevertheless, the alignment of the two homologue sequences proved it to be exactly the same nucleotide sequence than Jcr4S02704.30. They had 74% identity and 81% similarity with a score of 304 bits when compared to *A. thaliana's* gene. Alignment of the three sequences (Fig. 5.23) show that there is 15 aa extra in *J. curcas* C- terminus.

```
                         Signal Peptide
AT2G28190.1_SOD2    --------MAATNTILAFSSPSRLLIPP-----SSNPSTLRSSFRGVSLNNNNLHRLQSV    47
Jcr4S02704.30       MQAAAAVAAMAAHTILAASPSSHPLLYPFPNPILSHSSSPLHSSFHGVSLKLPRQSL-PLS    59
NM_001308734.1      MQAAAAVAAMAAHTILAASPSSHPLLYPFPNPILSHSSSPLHSSFHGVSLKLPRQSL-PLS    59
                      *::****  *    *:  *:  *          *:  *  *:***:****:    .

AT2G28190.1_SOD2    SFAVKAPSKALTVVSAAKKAVAVLKGTSDVEGVVTLTQDDSGPTTVNVRITGLTPGPHGF   107
Jcr4S02704.30       LTTAAAPKKPLAVVAATKKAVAVLKGTSNVEGVVTLTQEDDGPTTVNVRVTGLTPGPHGF   119
NM_001308734.1      LTTAAAPKKPLAVVAATKKAVAVLKGTSNVEGVVTLTQEDDGPTTVNVRVTGLTPGPHGF   119
                     :. **.* *:**:*:*********** :*********:*.********:**********

AT2G28190.1_SOD2    HLHEFGDTTNGCISTGPHFNPNNMTHGAPEDECRHAGDLGNINANADGVAETTIVDNQIP   167
Jcr4S02704.30       HLHEYGDTTNGCISTGAHFNPNNKTHGAPEDEIRHAGDLGNIVANADGVAEATIVDNQIP   179
NM_001308734.1      HLHEYGDTTNGCISTGAHFNPNNKTHGAPEDEIRHAGDLGNIVANADGVAEATIVDNQIP   179
                    ****:*********** ****** ******** ********* ********:********

AT2G28190.1_SOD2    LTGPNSVVGRAFVVHELKDDLGKGGHELSLTTGNAGGRLACGVIGLTPL-----------   216
Jcr4S02704.30       LSGPNAVVGRALVVHELEDDLGKGGHELSLTTGNAGGRLACGLLAMCKAAWLSQCKEMGS   239
NM_001308734.1      LSGPNAVVGRALVVHELEDDLGKGGHELSLTTGNAGGRLACGLLAMCKAAWLSQCKEMGS   239
                    *:***:*****:*****:*********************:::.:

AT2G28190.1_SOD2    ----   216
Jcr4S02704.30       EVDT   243
NM_001308734.1      EVDT   243
                    ****
```

Figure 5.23. Alignment of coding sequences for Cu/Zn SOD2. Sequences belong to *Arabidopsis* (AT), Jatropha Genome Database (Jcr), GenBank (NM). (*) indicates aa identity, (:) is aa similarity in the same functional group, and (.) indicates similarity with bigger functional change. Signal peptide marked in a yellow box.

- **Exon analysis**

Exon	Ath Cu/Zn SOD2	Jc_SOD2 Jcr4S02704.30
1	265	301
2	62	62
3	40	40
4	96	96
5	32	32
6	76	76
7	54	54
8	26	71
Total	**651**	**732**

Figure 5.24. Exon sizes corresponding to peptide sequences of *J. curcas* Cu/Zn SOD2, Jcr4S02704.30. Exons are presented in the reading order.

For the superoxide dismutase, a gene model was designed using gene description from the Jatropha Genome Database (Fig. 5.24). Jcr4S02704.30, coding hypothetically as homologue of At Cu/Zn SOD2, has a large first exon in the signal peptide area (Fig. 35). From the second to the seventh, exons have the exact same size. Last exon is three times larger in *Jatropha* than in *Arabidopsis*.

5.2. Sub-cellular localization (Genetic approaches)

In order to confirm chloroplast target prediction from the sequences corresponding to the antioxidant system (Chapter 5.1), the predicted chloroplast targeting peptides were cloned into pCR8GW/TOPO and transferred into pMDC83, a vector that contained mGFP6 under a 35S promotor, by a Gateway reaction and cloned first in E. coli, then in *Agrobacterium tumefaciens.* The list of primer used for each construct is detailed in Table 5.1. After confirmation of the clones by sequencing, the strain was used for transient transformation of *Nicotiana benthamiana*. Each infiltration process covered the complete area of the leaf. After three days, infiltrated leaves of *N. benthamiana* were analyzed by excitation with 475 nm light to examine GFP excitation and localization. Each infiltration process covered the complete area of the leaf. Evaluations were done with a fluorescence imaging camera and afterwards confirmed by confocal microscopy.

Table 5.1 List of primers used to amplify signal peptides of 2CP, APX and GPX from *Jatropha curcas*. Primer pair includes a forward (f) and reverse (r) one. Position and amplicon size were based on the full-length sequence for each gene.

Primers	Sequence	Length	Tm °C	GC %
SP_Jc2CP_f	ATGGCTTGCTCCGCTACTTCAAC	23 nt	69,3°C	52.2%
SP_Jc2CP_r	ATCAGGTGCTGTATTTCCGACCAG	24 nt	69°C	50.0%
Amplicon size	249 nt			
SP_JcAPX_f	ATGGCAGACTCCTTGTTACACTTG	24nt	65,2°C	45.8%
SP_JcAPX_r	GATATCTTCTCTCGCACTCTTCAGC	25 nt	65,5°C	48.0%
Amplicon size	312 nt			
SP_JcGPX_f	ATGGCTTCTGTTCCTTTTCAGAG	23nt	64,5°C	43.5%
SP_JcGPX_r	GCTTGACAGTGTAGTCGTGTATGG	24 nt	64,6°C	50.0%
Amplicon size	250 nt			

5.2.1. 2CP

- **Construct activity confirmation**

After 3 days, plants were evaluated for construct activity. This was done to verify if the construct used for transformation could be expressed in a heterologous organism (from *Jatropha* in *Nicotiana*). Expression of the proposed *Jatropha* signal peptide was verified via the activation of the fused reporter gene (mGFP6) in *N. benthamiana* using an "In vivo Plant Imaging System" (Night Shade machine, Berthold Technologies), programmed to use excitation filter of 475 nm, emission filter of 520 mn, exposure time of 10 seconds and 50% illumination. The camera is set to maintain a fixed distance to its focal plane throughout all

images, in order to ensure a consistent efficiency in GFP excitation and detection between the different plants. Therefore, leaves had to be detached and arranged on a tray. GFP fluorescence images were captured after shutter was open for 0.1 seconds.

The construct carrying the signal peptide of 2CP confirmed its activity in *Nicotiana*. GFP fluorescence was detected in various leaves in each infiltrated plant, confirming that the construct was viable and expressing in the hosting material (Fig. 5.25).
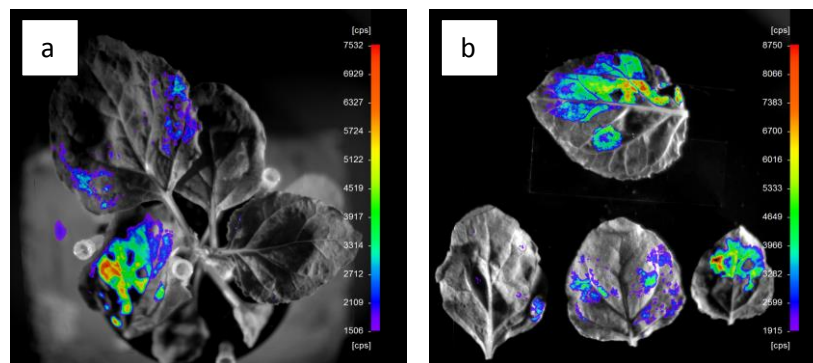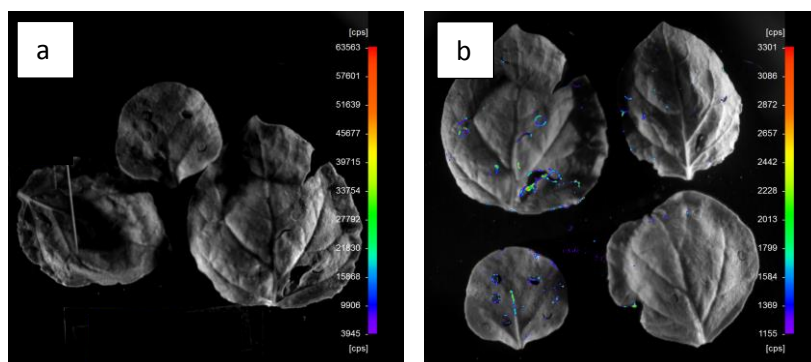

Figure 5.25. mGFP6 fluorescence in planta *(N. benthamiana*), **after 3 days infiltration with transformed** *Agrobacteria* **carring constructs expressing pMDC83 + SP_Jc2CP.** Displayed are merged images of the transfected plant material (grayscale) and the intensity of the resulting GFP signal (false color). (a) show complete plant in pots , while (b) show detached leaves. False colors ranging from purple to red represent an increasing intensity of GFP fluorescenc, as visualized by the lateral color scale. Gray zones are not transformed.

- **Analysis of subcellular targeting by CLSM**

Fresh cuts in transformed leaves were mounted on glass plates with 2-3 drops water, immediately before their observation. Leaves were scanned in the abaxial and adaxial side, from the epidermis to mesophyll cells, with the aid of 10X and 60X objectives and a manual zoom tool. Infiltration of *N. benthamiana* with SP Jc2CP+GFP construct resulted in a high transformation rate. Chlorophyll auto-fluorescence, visualized in red (Fig. 5.26 c,f), characterizes plastids and differentiates them from other cellular compartments. GFP fluorescence visualized in green, reflected the shape of chloroplast and was very strong. Merging those two channels together (Fig. 5.26 a, d), permitted to observe the location of each fluorescence signal at the same time, validating the co-localization of the sources emitting those wavelengths. There are areas in yellow (overlapping signals), only red (chlorophyll), and gray (bright field).
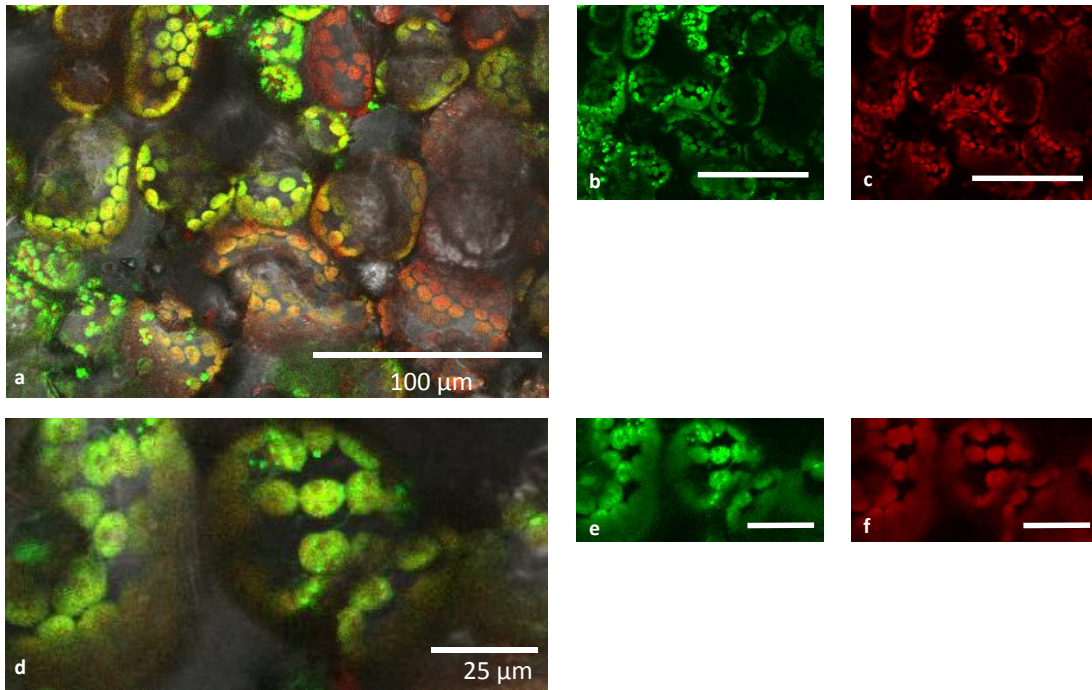
Figure 5.26. mGFP6 fluorescence under CLSM in leaves of *N. benthamiana*, after 3 days of infiltration with *J. curcas* 2CP signal peptide. Images (b) and (e) show GFP signal, while (c) and (f) display chlorophyll autofluorescence. (a) and (d) are the respective merged images, yellow colors correspond to exact co-localization of energy emission.

Displaying a higher level of magnification, Figure 5.26d shows round shapes like plastids with green and red channels activated at the same time. GFP was not detected in all chloroplasts observed, but it was only visible in this organelle. These results confirm that the construct used is targeted to chloroplast. In a broader extent, the sequence used as template for signal peptide analysis (FLseq_2CP) is confirmed to be a chloroplast isoform.

5.2.2. APX

- **Construct activity verification**

After 3 days, plants were evaluated for construct activity, to verify if the construct used for transformation could be expressed in a heterologous organism. *Jatropha* signal peptide expression was verified via the activation of the fused reporter gene (mGFP6) in the same way than for 2CP construct. The construct carrying the signal peptide of APX confirmed its activity in *Nicotiana*. GFP fluorescence was detected in various leaves in each infiltrated plant, confirming that the construct was viable and expressing in the hosting material (Fig. 5.27).
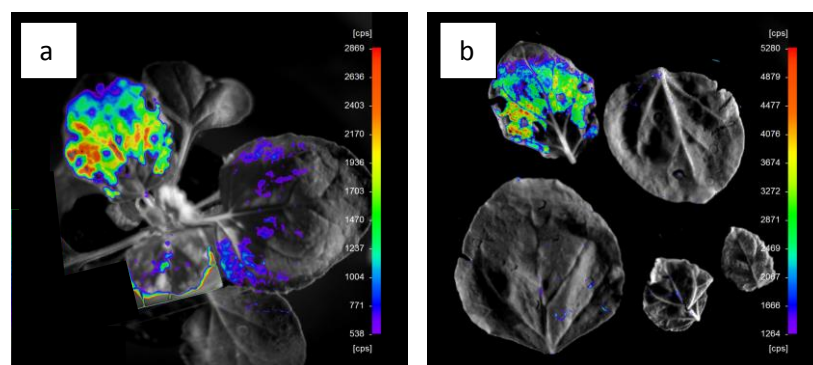
Figure 5.27. mGFP6 fluorescence in planta *(N. benthamiana)***, after 3 days infiltration with transformed** *Agrobacteria* **carrying constructs expressing pMDC83 + SP_JcAPX.** Displayed are merged images of the transfected plant material (grayscale) and the intensity of the resulting GFP signal (false color). (a) show complete plant in pots, while (b) show detached leaves. False colors ranging from purple to red represent an increasing intensity of GFP fluorescence, as visualized by the lateral color scale. Gray zones are not transformed.

- **Analysis of subcellular targeting by CLSM**

Infiltration of *N. benthamiana* with SP JcAPX+GFP construct resulted also in a high transformation rate. Surprisingly, GFP was only partly targeting chloroplast. Part of the GFP was localized in sinuous- line shape structures, that could be localized in the cytosol, the cell membrane, or even reflect the cell wall (Fig. 5.28).
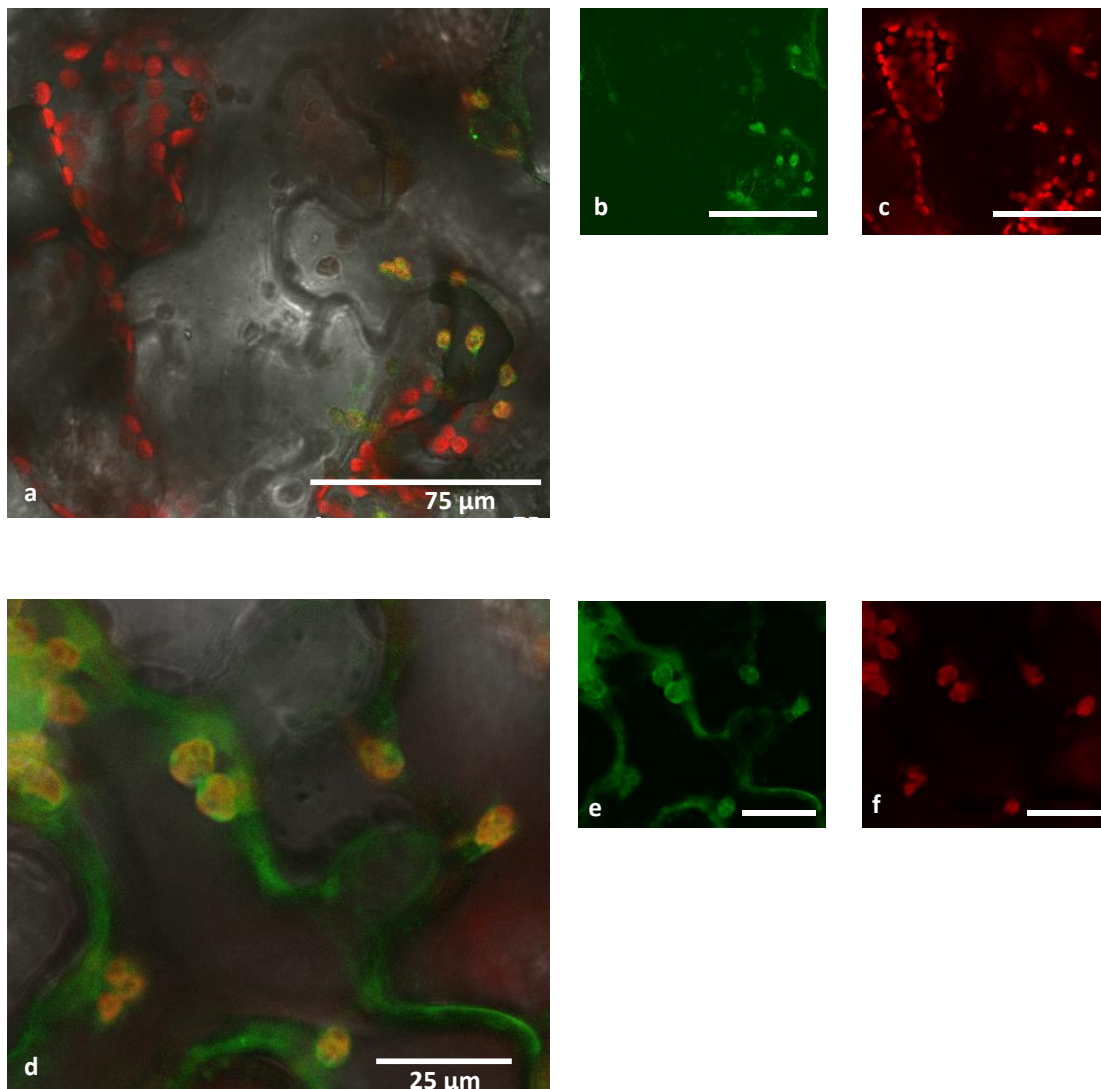
Figure 5.28. mGFP6 fluorescence under CLSM in leaves of *N. benthamiana*, after 3 days of infiltration with *J. curcas* APX signal peptide. Images (b) and (e) show GFP signal, while (c) and (f) display chlorophyll autofluorescence. (a) and (d) are the respective merged images, yellow colors correspond to exact co-localization of energy emission.

To analyze the nature of these sinuous lines glowing in green, plant material was treated with 1M NaCl, to induce plasmolysis. During this process, cell turgidity gets reduced and it is possible to identify visually the cell parts. Cell plasmolysis provokes retraction of the cell membrane and a separation from the rigid cell wall. Both structures slightly separated during plasmolysis. Cytosol is also easily distinctive and molecules in suspension would be congregated in a specific area.
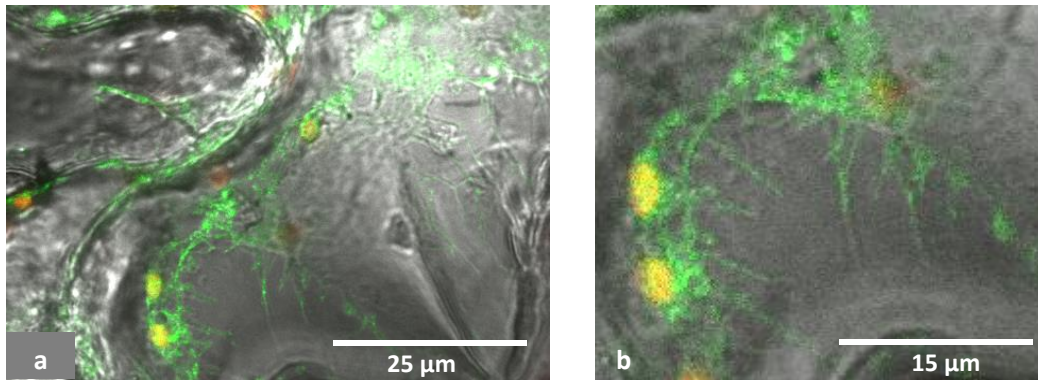
Figure 5.29. mGFP6 fluorescence under CLSM in *N. benthamiana*, after infiltration with *J. curcas* APX signal peptide. (a) and (b) correspond to merged images. Green color marks only GFP fluorescence, red color marks only chlorophyll fluorescence and yellow represent overlapped channels.

High magnification of the image allows to scrutinize GFP targeting. Besides in chloroplasts (yellow in Fig.5.29), it is also visible the location of the construct targeting what appears as "lines" in the cytosol. The length and direction pattern suggest ER (endoplasmic reticulum) localization.

To discard the possibility that this unexpected targeting was caused by degradation over time, plants were infiltrated and examined under a CLSM after 2, 3 and 4 days (Fig. 5.30); this pattern was observed during each examined time point. GFP signal was not monitored before 2 days post infiltration, in order to allow for the construct to be imported to its target. A period of 5 days is generally considered to be the maximal period for GFP expression after the transient transformation of *N. benthamiana*; therefore, no further observation was done after this point.
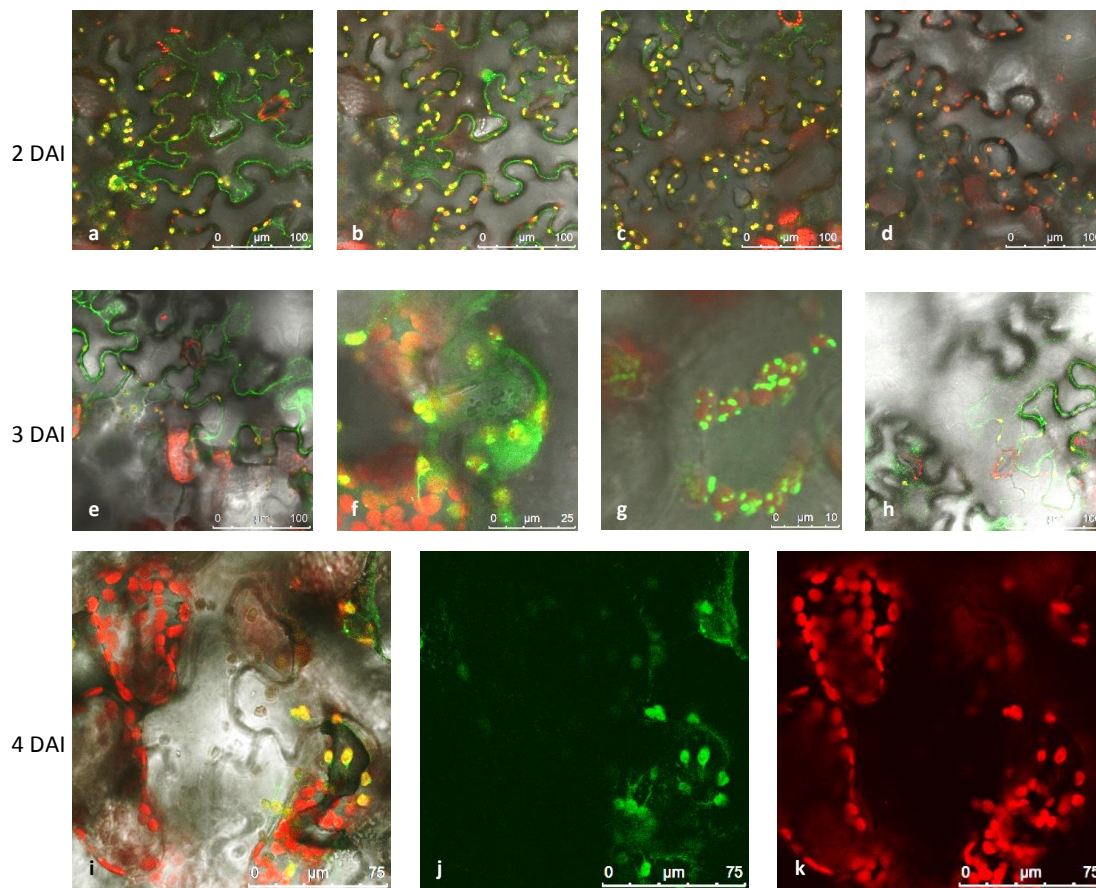
Figure 5.30. mGFP6 fluorescence under CLSM in *N. benthamiana*, after infiltration with *J. curcas* APX signal peptide, after 2-4 days. Almost all images correspond to a merged view of the fluorescence channels, GFP channel in green, chlorophyll in red and yellow shows overlap of both. In the first row (a-d) there are plants two **d**ays **a**fter **i**nfiltration (DAI), in the middle row (e-h) the samples displayed correspond to plants three days after infiltration, and last row (i-k) display one view four days after infiltration, with its respective emission channels.

- **Hydrophobicity test**

Since the construct was targeting most probably another membranous cell compartment, a test of hydrophobicity was performed on the amino acid sequence of the signal peptide. Hydrophobic scales are based on different chemical and physical properties of the amino acids included in the peptide sequence. When a peptide has a strong hydrophobic compound, it can remain attached to membranes. The analysis on hydrophobicity using the Expasy- Server online tool based on the algorithm by Kyte & Doolittle (1982) showed a preferentially hydrophobic region (Fig. 5.31).
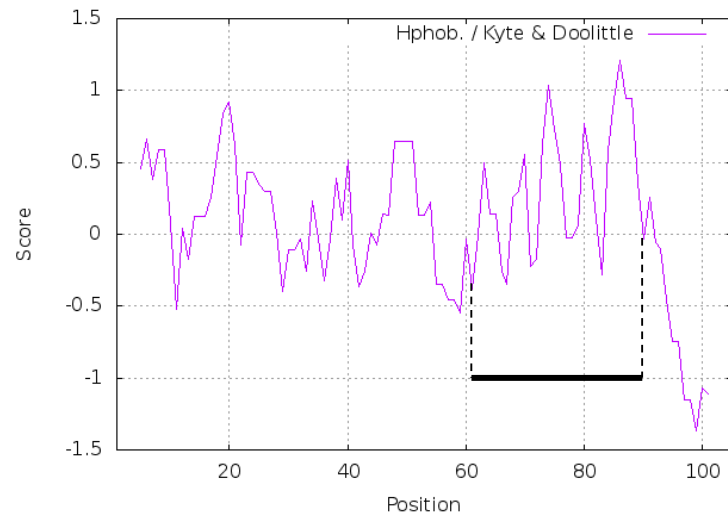
Figure 5.31. Evaluation of hydrophobicity in *J. curcas* APX signal peptide. The sequence evaluated was the predicted amplification of the forward and reverse primers in the FL_seq Jc APX.

Between aa 63 and aa 90 (Fig. 5.31, marked with a horizontal bar) were detected high scores of hydrophobicity in the signal peptide for APX. No α-helix was observed.
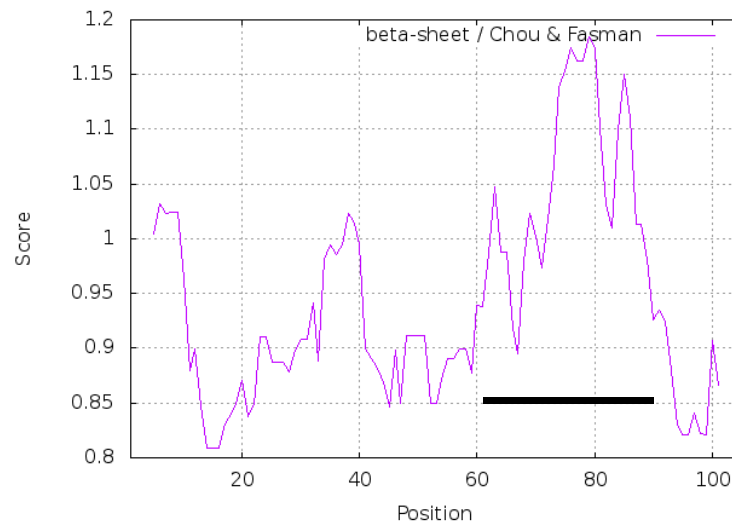
Figure 5.32. Evaluation of β-sheet formation in *J. curcas* APX signal peptide. The sequence evaluated was the predicted amplification of the forward and reverse primers in the FL_seq Jc APX.

In the scale of Chou & Fasman (1978), there is a high score for beta-sheet formation at the same region (Fig. 5.32). This type of component in the signal peptide might have caused integration or attachment of the expressed protein to endomembranes.

5.2.3. GPX

- **Construct activity verification**

After 3 days, plants were evaluated for construct activity, to verify if the construct used for transformation could be expressed in a heterologous organism, as done with the other two constructs. The GPX construct did not lead to GFP expression (Fig. 5.33). Despite a thorough search, no signal could be detected on the whole plant level or in individual detached leaves.

Figure 5.33. mGFP6 fluorescence in planta *(N. benthamiana)*, after 3 days infiltration with transformed *Agrobacteria* carring constructs expressing pMDC83 + SP_JcGPX. Displayed are merged images of the transfected plant material (grayscale) and the intensity of the resulting GFP signal (false color). (a) show complete plant in pots, while (b) show detached leaves. False colors ranging from purple to red represent an increasing intensity of GFP fluorescence, as visualized by the lateral color scale. Gray zones are not transformed.

- **Analysis of subcellular targeting by CLSM**

N. benthamiana transfected with pMDC83 + SP_JcGPX did not show any sign of cell transformation under CLSM analysis. Chlorophyll auto-fluorescence, visualized in red was the only signal detectable besides the bright field, in all samples evaluated. In merged images or independent channel view, it could not detect any trace of the green fluorescent protein (data not shown). In order to assess the possibility of protein or DNA construct degradation with time, two more time points were examined, just like they were for the APX construct without no success.

The reasons for the lack of fluorescence remain unclear and suggest an abnormal instability of the fusion protein. As reported, the primer design targeted an area of the signal peptide which was even larger than the predicted sequence. Hence, it is unlikely due to an incomplete signal peptide. In this case, GFP would be expressed throughout the cell, in a nonspecific manner. Sequenced plasmids and predicted construct sequences were translated and aligned (Fig. 5.34) showing perfect framing read. The starting Methionine was the first amino acid coded in the sequence.

a


```
LQVNMVEHDTLVYSKNIKDTVSEDQRAIETFQQRVISGNLLGFHCPAICHFIVKIVEKEGGSYKCHHCDK
GKAIVEDASADSGPKDGPPPTRSIVEKEDVPTTSSKQVD-CDNMVEHDTLVYSKNIKDTVSEDQRAIETF
QQRVISGNLLGFHCPAICHFIVKIVEKEGGSYKCHHCDKGKAIVEDASADSGPKDGPPPTRSIVEKEDVP
TTSSKQVD-DISTDVRDDAQSHYPSQDPSSI-GSSFHLERTSTLELVN-ELACMPAGRL-RIPGYRARII
TSLYKKAGSEFALMASVPFQSLKQFQINPKSCSFSPAMAFCIPSMKTSFGPSKSAFLQHGFSLQLPNFPG
FLSKTHSFAVYARAATEKTIHDYTVKGEFDPAFLYKVVIAWRASRGGPVPVEKMSKGEELFTGVVPILVE
LDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKRHDFFKSA
MPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQ
KNGIKANFKTRHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAA
ITHGMDELYKHHHHHH-
```

b

Figure 5.34. Product after Gateway cloning, displaying the info for the signal peptide (SP) of JcGPX in pMDC830.
(a) is the vector map predicted in Serial Cloner, (b) is the predicted translated sequence. Orange color corresponds
to p35S, pink corresponds to GPX signal peptide, and green corresponds to mGFP6.

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5.3. GFP detection via Inmuno-detection

To corroborate the results obtained with bioinformatic predictions and localization studies, immunoblot analysis was performed directly with chloroplast proteins. Plants transformed with pMDC83 constructs were used for chloroplast isolation; from them, the proteins were extracted. After differentiation into soluble and non-soluble proteins, 5 µg of soluble protein for each construct was used for SDS-PAGE. In parallel, protein extracted from *N. benthamiana* infiltrated only with water served as negative control, and raw GFP-ro protein served as positive control. After membrane blotting and hybridization with GFP antibodies, the Western Blots were examined chemiluminometrically.
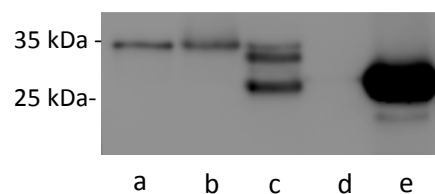
Figure 5.35. GFP detection via Western Blot. Electrophoresis was performed in a 12% SDS gel. Immunodetection was with α-GFP and α-rabbit antibodies. Samples correspond to the protein extraction out of (a) chloroplasts of plants infiltrated with 2CP-GFP construct, (b) chloroplasts of plants infiltrated with APX-GFP construct, (c) full protein extraction of plants infiltrated with 2CP-GFP, (d) full protein extraction of plants infiltrated with water, as negative control, and (e) raw GFP-ro protein, as positive control.

Non-fused GFP has a molecular weight of 27 Kilo Daltons (KDa). Complete protein extraction of plants infiltrated with 2CP-GFP construct presented two main bands (Fig. 5.35 c), corresponding to the fusion of GFP+ construct (35 KDa) and non-fused GFP (27 KDa). Protein obtained from chloroplast extraction (from plants infiltrated with 2CP and APX signal peptide constructs) showed only one band corresponding to the size of fused GFP to the signal peptide, 35.5 and 37.8 respectively (Fig. 5.35 a, b), suggesting that during protein import, chloroplasts in *Nicotiana* did not recognized cleavage site in fusion proteins. Negative control, with plants infiltrated with water showed no band for GFP as expected, and the positive control, containing GFP-ro, confirmed the expected size. The intactness of chloroplast after the extraction was proved, as suggested by Klinkenberg *et al*., (2014) with phase contrast microscopy.

The results of the immunodetection of GFP in chloroplast, confirm the chloroplast targeting of the signal peptides used, therefore, main sequence (FLseq_Jc2CP and FLseq_JcAPX) can be determined as chloroplast isoforms.

5.4.   Drought experiments

The focus for this part of the investigation was to evaluate the physiological parameters that correlate to drought stress. In this direction, a series of experiments were performed indoors and outdoors to investigate the drought responses in the ecotype found in Panama, including morphological and metabolic responses.

5.4.1. Experimental setup

The idea behind the experiments was to evaluate the capacity of the plants to resist drought stress and to recover after re-watering. As the conditions were not equal for all experiments, the total length variates. Each drought period was stopped only when stressed plants presented wilting leaves. After maximum stress, remaining plants were re-watered to maximum capacity daily, for up to 3-4 days. The experimental conditions are described as follows, while a detailed report of humidity, temperature and irradiance is found in Chapter 3.

Experiment 1

It was performed in an open shelter under a glass roof (to avoid any direct precipitation), in Gamboa, Panama, during March-April 2017 (refer to Material and Methods). The atmospheric conditions were the regulars for summer time in the country, with the highest irradiation (up to 2500 µmol photons $m^{-2}s^{-1}$), high temperatures (up to 36°C at midday) and low humidity during daytime.

From the start until the day determined as maximum stress passed 8 days, and then plants were re-watered for 3 days more, always reaching maximum water capacity of the soils, before last evaluation. Experiment had 8 days of drought and 3 days of re-watering. 45 seedlings were distributed, having sample number of n=5 at each measuring point, per group.

Experiment 2

It was performed in a closed shelter in Gamboa, Panama, covered on the roof and sides with a plastic mesh that reduces the entrance of light in 60%, working also to keep humidity. Atmospheric conditions were milder: irradiation (150-600 µmol photon $m^{-2}s^{-1}$ on daily basis) temperatures regularly 2-4° lower than outside and humidity of 55% during daytime. See details in Annex 1. From the start until the day determined as maximum stress passed 28 days, and then plants were re-watered for 3 days more to maximum capacity before last evaluation.

Experiment had 28 days of drought and 3 days of re-watering. 46 seedlings were distributed, having sample number of n=5 at each measuring point per group.

Experiment 3

It was performed in a greenhouse at the FU Berlin. Atmospheric conditions were controlled by a computer, keeping values of temperatures around 28-33°C during the day period. Irradiation was provided by lamps, controlled to 300-500 µmol photon $m^{-2}s^{-1}$ (measured on a highly illuminated area, under the lamps). The cabin did not have humidity control, but it was maintained artificially around 52% during daytime. From the start until the day determined as maximum stress, random seedlings from each treatment group were physically evaluated each 7 days. At the end of the 4th week, plants under stress were evaluated, collected, and the remaining ones were re-watered for 3 days more to maximum capacity before last evaluation. Experiment had 28 days of drought and 4 days of re-watering. 25 seedlings were distributed, having sample number of n=5 at each measuring point, per group.

Experiment 4

It was performed in the same greenhouse at the FU Berlin, but in a hand-made mini cabin. An area inside a growth cabin was closed at it sides with a black plastic foil, to control that indirect light from adjacent cabins affect plants. Seedlings were put on this zone, that had closed sides and open top. The temperature was around 28-33°C during the day period, the irradiation controlled to 100-300 µE $m^{-2}s^{-1}$ (measured on a highly illuminated area, under the lamps) and the humidity around 52% during daytime. From the start until the day determined as maximum stress, random seedlings from each treatment group were physically evaluated each 7 days. At the end of the 4th week, after sample evaluation and collection, the remaining seedlings were re-watered for 3 days more to maximum capacity before the last control. Experiment had 28 days of drought and 4 days of re-watering. 25 seedlings were distributed, having sample number of n=5 at each measuring point per group.

Experiment 5

It was performed in a greenhouse at the FU Berlin, at temperatures around 28-33°C during the day period. Irradiation controlled to 300-600 µmol photon $m^{-2}s^{-1}$ (measured on a highly illuminated area, under the lamps), and relative humidity of around 52% during daytime. From the start until the day determined as maximum stress, random seedlings from each treatment group were physically evaluated and plant material was collected for physical and molecular evaluation each two weeks. At the end of the 4th week, plants under stress were re-watered for 4 days more to maximum capacity before last evaluation. Experiment had 28 days of

drought and 4 days of re-watering. 28 seedlings were distributed, having sample number of n=4 at each measuring point per group.

Both experiments in Panama had multiple sampling points during mild stress. At each measuring date, physical parameters were evaluated before sample collection for molecular analysis. The experiments E3 and E4 in Berlin needed to be completed with a smaller total amount of samples (only the half). Non-invasive physical measurements were done each 7 days, while samples were collected only at the beginning, at the maximum stress point and after re-watering. Experiment 5 in Berlin was conducted as those experiments in Panama but included less parameters. Every measuring point included some physiological parameters and sample collection.

Based on illumination, we could group Experiment 1 (sun in Panama) with Experiment 3 and 5 (high light in Berlin), and in another group Experiment 2 (shadow in Panama) with Experiment 4 (low light in Berlin). This semi-classification is suggested based on the reported demand for high illumination in *Jatropha*, that could affect photosynthesis activity.

Based on general control in the rest of the environment, we could compare both experiments in Panama, relaying on open atmospheric conditions (E1 and E2), and the three experiments in Berlin, with stable conditions (E3, E4 and E5).

5.4.2. Soil description

5.4.2.1.   Soil Water Content

As a measure of water balance in the soil at each measuring time, soil water content was calculated. In all experiments is observed the expected reduction in total water content (Fig. 5.36).

Experiment 1, with plants under full sun light in a tropical country, started with seedlings at a soil water content of 650 ml H_2O per pot. Three days later, the water content was significantly reduced to 554 ml H_2O per pot (p= 0.0115). Drought stress made water soil content in seedlings pot decrease to 204 ml on day 8, which was highly different to the control pots on this day (p = 8.3 E^{-6}). After the 3 days re-watering, the water amount in soil was comparable to the one in control seedlings.
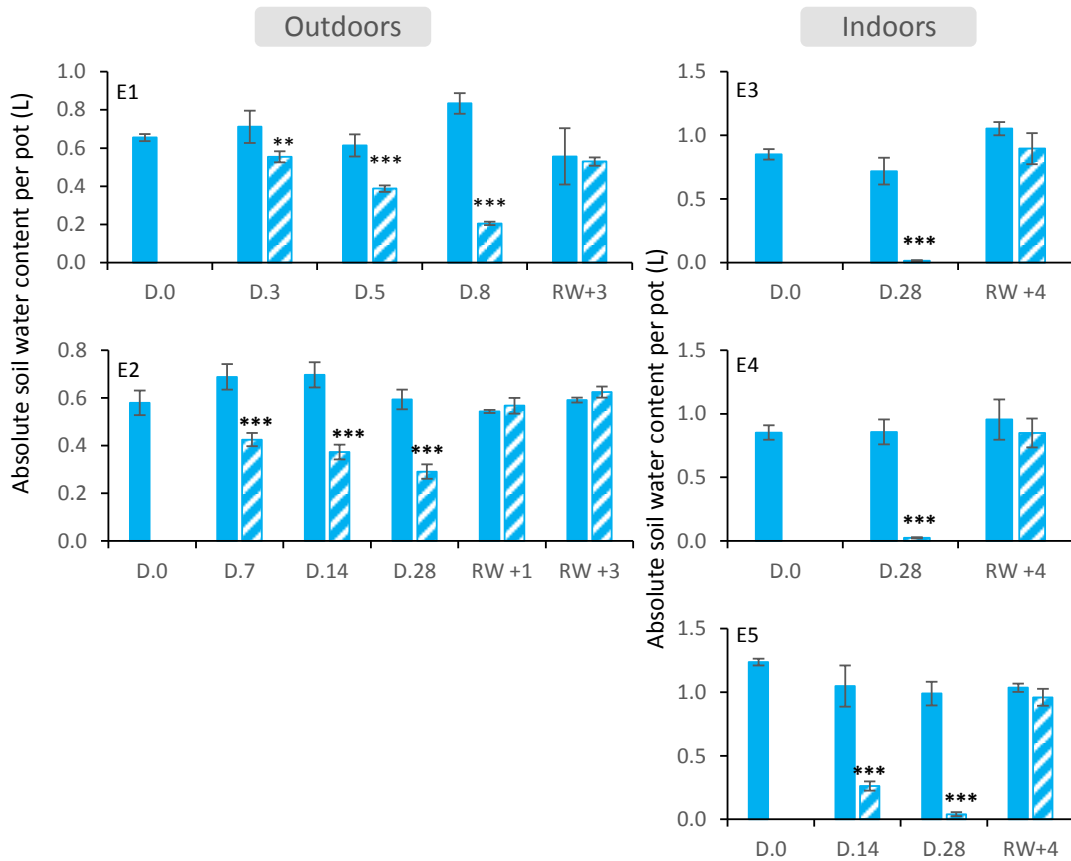
Figure 5.36. Average water content (WC) in seedlings pot for each experiment (n=5). Calculated [Soil Fresh Weight] – [Soil Dry Weight]. In **E1** plants had sunny conditions in Panama, in **E2** plants were under shadow conditions, in Panama. **E3** was in high light conditions, in Berlin (greenhouse); **E4,** in low light conditions in Berlin (greenhouse) and **E5**, in high light conditions + fertilization, in Berlin (greenhouse). Statistic significance corresponds to Student's T-Test: $p < 0.1$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)

In **Experiment 2**, plants were protected from direct light with the mesh which helped also to keep humidity inside as wind was not just blowing it off. It started with seedlings soil water content of 580 ml H_2O per pot, and after seven days, stressed plants had significantly less water than the controls ($p= 0.001$). Due to non-irrigation, the water soil content in seedlings went down up to 290 ml H_2O on day 28 ($p=0,000005$). After 1-day irrigation, soil humidity recovers to similar values as control, with similar trend for 3 days re-watering.

Seedlings under treatment in full sun lost the double amount of water than the ones under shadow after one week of stress. Moreover, the amount of water lost happened in 8 days of full sun exposure (204ml H_2O) was higher than the water lost after four weeks drought under shadow (290ml H_2O).

Experiment 3, under high light inside a greenhouse with controlled conditions, started with pots containing 850 ml H_2O in average. The next soil evaluation was at maximum stress, after

seeing leaves wilting. After 28 days of non-watering, pots of seedlings under treatment had a very small amount of water (15 ml), posing a high significance in the difference with the controls at this moment, p= 0.00011. Treatment was affecting soil properties, to the point that after four days re-watering, it was not possible to bring soil moisture to similar values to controls (p= 0.0418).

For **Experiment 4**, where direct and indirect light was reduced, soil water content has very similar to the one under high light at the beginning of the experiment. Control plants on day 0 had pots with the same amount of water per pot than in E3 (p=0.9). At the end of the experiment, stressed plants were in soils that contained an average of only 24 ml H_2O, while controls had 857 ml H_2O (p= 0,00004). Re-watering was bringing soil water content to similar values to the controls (p= 0.26).

In **Experiment 5,** soil from stressed plants had significantly less water than the controls (p= 0.001) after 14 days of drought. At the end of the experiment, the water soil content in soil was reduced to 75 ml H_2O on day 28 (p=0.000005). After 3-day irrigation, soil humidity recovers to similar values as control.

The effect of atmospheric condition over the water content in soil (soil transpiration, Fig. 5.37) was assessed by control pots filled with soil, not having any growing plant. Those pots were irrigated at the same time and with the same intensity as pot with seedlings. In this way, it was evaluated water lost in soil just due to transpiration, in other words, just because air surrounding is dry and hot.
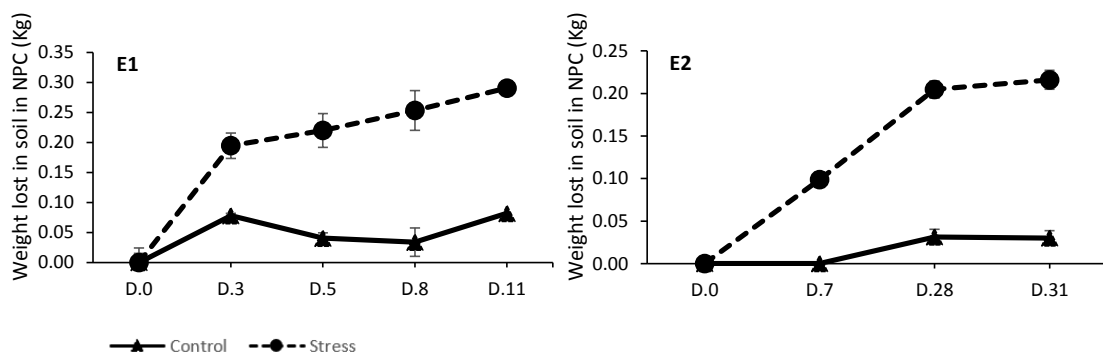
Figure 5.37. Soil transpiration. Water lost from control pots without seedlings can be only due to effect of the environmental conditions. **N**o-**P**lant-**C**ontrol (NPC) pots were irrigated (control) or not (stressed), following the same irrigation pattern as pots containing experimental seedlings.

The soil from seedlings in experiment 1 were losing up to 250 ml water in 8 days with a statistical difference against control of p= 0.021 (Fig.5.37). In the case of the experiment 2, reduction was up to 200 ml water in 28 days, with a difference of p= 0.00003 from the control. Transpiration at the end of the experiments was higher in experiments in Berlin due to soil composition and probable easier leakage. Control pots lost around 150 ml more in E3 (170 in dry s´pots) and 70 ml more in E4 (90 ml in dry pots) in 28 days of drought stress; this calculation was made based on the weight difference of control pots only at the end of the drought period. Transpiration on E5 was not evaluated.

5.4.2.2.   Soil Composition

Soil in experiments in Panama (E1 and E2) were mostly of clay type. This soil was not extra fertilized and used only once, so it contains the regular amounts of nutrients that plants find in natural conditions (Table 5.1). Soil for experiments in Berlin (E3, E4 and E5) was the typical soil used for *Arabidopsis* growth (refer to Chapter 3 for detail), consisting in a fibrous material similar to coconut skin, fertilized once when seedlings were developing.

In soil analysis for *Jatropha* nutrient use (Table 5.1), it is visible that all values were smaller for the starting soil compared with samples of pots that contained seedlings. That might be caused by the irrigation with tap water.

Table 5.1. Soil nutrient composition in Panama natural conditions. Starting soil represent the soil used to fill all pots at the beginning of the experiment. At the end of the drought phase, representative samples were taken for both control and stressed seedlings. Nutrient content values are expressed as (mg/ g soil) some elements had **u**nder-**d**etection **v**alues (UDV). Total difference refers to the value in stress minus in control, and relative values are compared to control values.

		Al	B	B	Ca	Cr	Cu	Fe	K	Mg	Mn	Na	Ni	P	Zn
		Soil nutrients for *Jatropha curcas*, in shadow experiment (E2) - (mg/g soil)
	Standard	66.72	UDV	0.11	3.43	UDV	0.02	43.40	5.61	5.86	0.94	0.34	UDV	0.48	0.11
	Starting Soil	72.54	UDV	0.14	7.34	UDV	0.05	58.55	4.77	13.99	0.67	0.26	UDV	0.85	0.18
	D.28 Control	74.98	UDV	0.15	8.46	UDV	0.07	61.91	4.75	14.87	0.65	0.54	UDV	0.94	0.25
	D.28 Stress	76.89	UDV	0.15	8.80	UDV	0.07	62.60	5.07	15.15	0.70	0.38	UDV	1.00	0.22
Nutrient	Total Diff.	1.92		0.01	0.33		0.00	0.69	0.33	0.28	0.05	-0.16		0.06	-0.03
use	Rel. Diff.	**2.6%**		**5.4%**	**3.9%**		**2.7%**	**1.1%**	**6.9%**	**1.9%**	**8.0%**	**-29.3%**		**6.3%**	**-12.3%**

Plants under stress are supposed to arrest growth, therefore the intake of nutrients might be reduced. We can see that the values of some minerals are higher in stress seedling's soil, meaning that it was not taken for plant growth, remaining available. That is the case of aluminum, boron, calcium, copper, iron, potassium, magnesium, manganese, and phosphorus; having respectively 2.6%, 5.4%, 3.9%, 2.7%, 1.1%, 6.9%, 1.9, 8.0% and 6.3% more than control soil. The only elements decreasing where sodium (-29.3%) and zinc (-12.3%).

5.4.3. Eco-Physiological Measurements

5.4.3.1.   Shoot size

One of the physical measurements done directly on plants was the apical shoot size. Thinking that *Jatropha* has a constant growth, it was investigated how much effort was put into vertical growth. Fig. 5.38 shows how control plants were having a stable rate of vertical growth, while stressed plants stopped drastically their growth at one point, measured as absolute size of the seedling. Same behavior was observed in plants growing in natural conditions and in the greenhouse, when the experiment had an extended drought period. This point could represent the exact moment when the balance of "regular conditions" and stress is been sensed by the plant.

In **experiment 1**, at the end of drought treatment, even when there was a reduction in vertical growth of 3.2 cm in average, there is not statistically different between controls and drought stressed plants (p=0.131). This difference meant reduction in 16 % of its size, but the observed difference could be based in starting sizes of plantlets. The actual increment in the starting and final size for each plant appear to be statistically different at the end of drought period, but three days after it was having the same increment. This are contradictory results.

For **E2**, size in stressed seedlings had statistical difference to controls after seven days of drought (25.5 cm for controls and 23.6 cm, p= 0.03). This difference becomes more significant at the end of the dry period when stressed plants were 33% shorter than controls (p=0.000002). Compared with the initial size (24.8 cm), stressed plants, seedlings grew only 0.9 cm in the whole dry period (28 days), which could indicate that plants could detect drought stress from the first week on and engaged in growth arrest to protect the plant. The difference between the two groups in the increase of individual plant size was significant from 14 days on, when average size increase in controls was 7.9 cm, while stressed plants grew only 1 cm. Also in E1, control plants grew less after 8 days, than in the same period in the experiment under shadow.
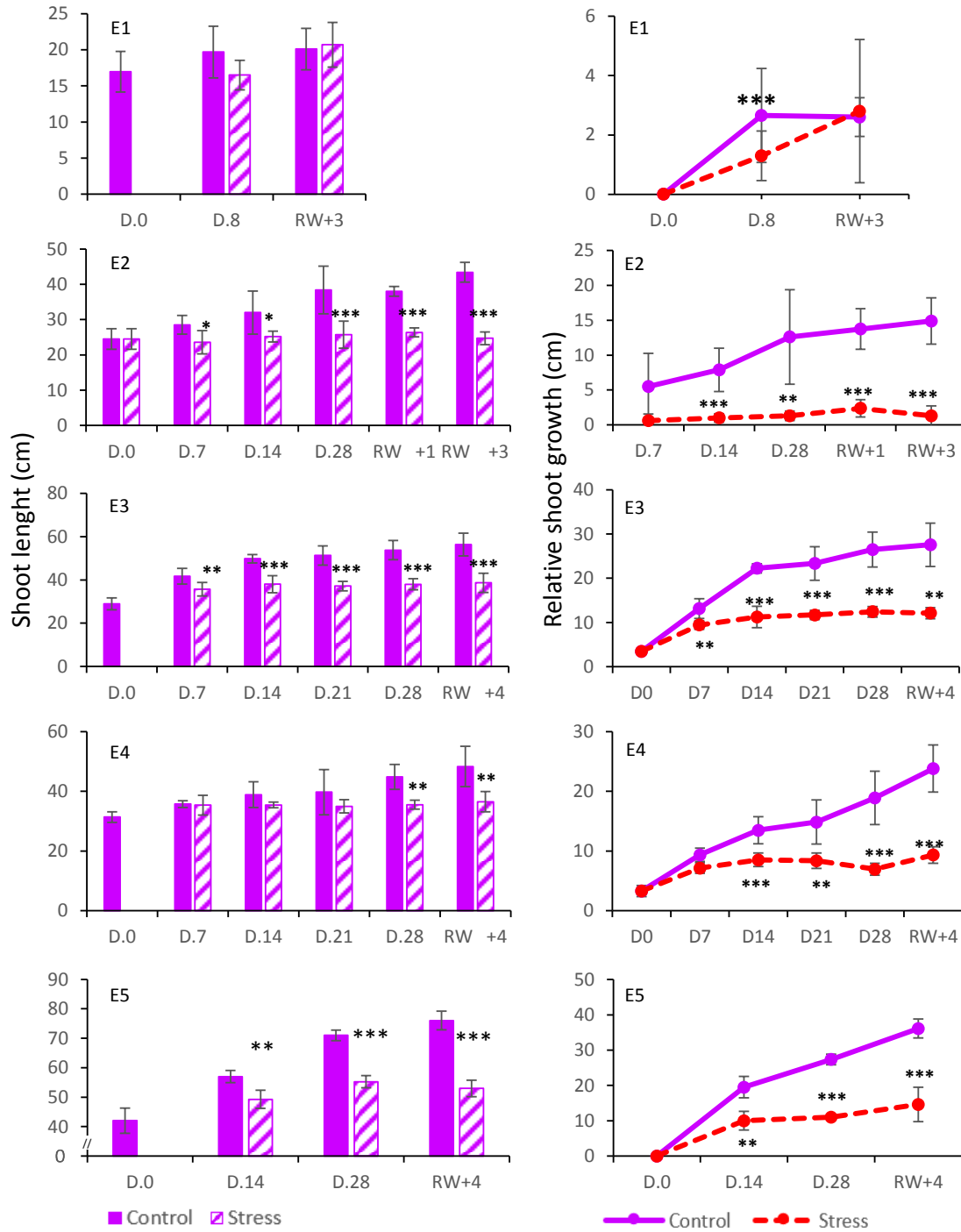
Fig 5.38. Apical shoot size. Vertical measured from the soil until the last bud in the stem, for each plant. The relative shoot length correspond to the difference of sizes in plants at the beginning and at the collecting point.

For Berlin's greenhouse experiments, the difference between control and stressed plants was significant after seven days in **E3**, after 14 days in **E5** and only after 28 days in **E4**. Two weeks after start of E3, difference in size was 11 cm (p=0.001); from this moment on, there was completely no growth in stressed seedling. In **E4**, shadow seems to have induced growth of seedlings at the start of the experiment, because after 7 days there was no difference in total size or increase. Afterwards, even when values were similar for total size, the increment in seedlings shoot size was statistically lower in stressed plants. At the end of E4 drought treatment, stressed plants were having a size of 38 cm, about 15.8 cm smaller than controls (p=0.00004). In E5, stressed seedlings presented smaller sizes and less growth after 14 days of drought until the end of the experiment than the controls.

Control plants absolute size and growth (increment in size) was affected by the environment visibly, presenting higher values in greenhouse experiments. After seven days of drought, control plants in **E3** were 22 cm taller than in E1 and 13.2 cm taller than in E2. Control plants after 31 days reach a size of 43.4 cm in E2, similar to the final size in E3 (48.3 cm). Nonetheless, the increase on shoot size was 27.6, 23.8 and 36.1 cm for E3, E4 and E5 respectively, while it was of 14.9 cm in E2, after the same period length.

5.4.3.2.   Total biomass above ground

Plant growth was evaluated also based on the shoot biomass production; details are presented in Fig. 5.39. Fresh biomass (FW) was significantly less in drought stressed plants in **E1** at the end of the experiment, but the dry biomass (DW) did not present any difference to controls, or to the starting values. In this case, the only possible difference might correspond to water content variation in the samples.

Seedlings in **E2** had 24 g of starting fresh biomass production, comparable with E1 seedlings, but after one-week stressed plants (23.8 g) produced almost 2X more FW under shadow than plants under full sun experiment (E1). After one-week drought, stressed seedlings had a considerably smaller FW biomass than their controls (-11 g, p= 0.014), but dry biomass values indicate that this difference in weight was not product of organic accumulation. At the end of the stress period, difference in fresh biomass production between stressed plants (21.6 g) and their controls (63.7 g) was p < 0.0001. This difference is supported by dry biomass values, which show that stressed seedlings stopped organic growth after one week, and after 31 days treatment their DW values represented a reduction of 7.1 g (p < 0.0001) to controls.

For E3 and E4 it was not possible to collect material during mild stress points. Stressed plants in **experiment 3** reached 30.2 g F.W. biomass after 28 days of drought, significantly less (-75.5 g) than controls. This difference was also found in dry weight of biomass: controls produced 24.7 g, corresponding to 19.5g more than in stressed seedlings (p < 0.0001). After re-watering, there was 87.9 g FW more biomass in controls than in stressed plants (p < 0.0001) and a total accumulation of 2.9 g DW in seedlings under treatments, not comparable to the 26.1 g DW accumulation of controls in the same time. Stressed seedlings presented a very small biomass accumulation, changing from 2.6 g dry weight at day 0 to 5.5 g at the end of the experiment, which was not statistically different.

For **E4**, all seedlings started with 32.8 g of FW biomass, not different from E3, and after 28 of treatment the values in stressed plants from E4 were also not different from E3 values. Stressed seedlings had significantly lower FW biomass, corresponding to a reduction of 2.6X from the controls. DW biomass suggests that the detection of stress happened early since there is only an increment of 1 g biomass in one month, compared to the starting value, while control plants accumulated 8 g in the same period.
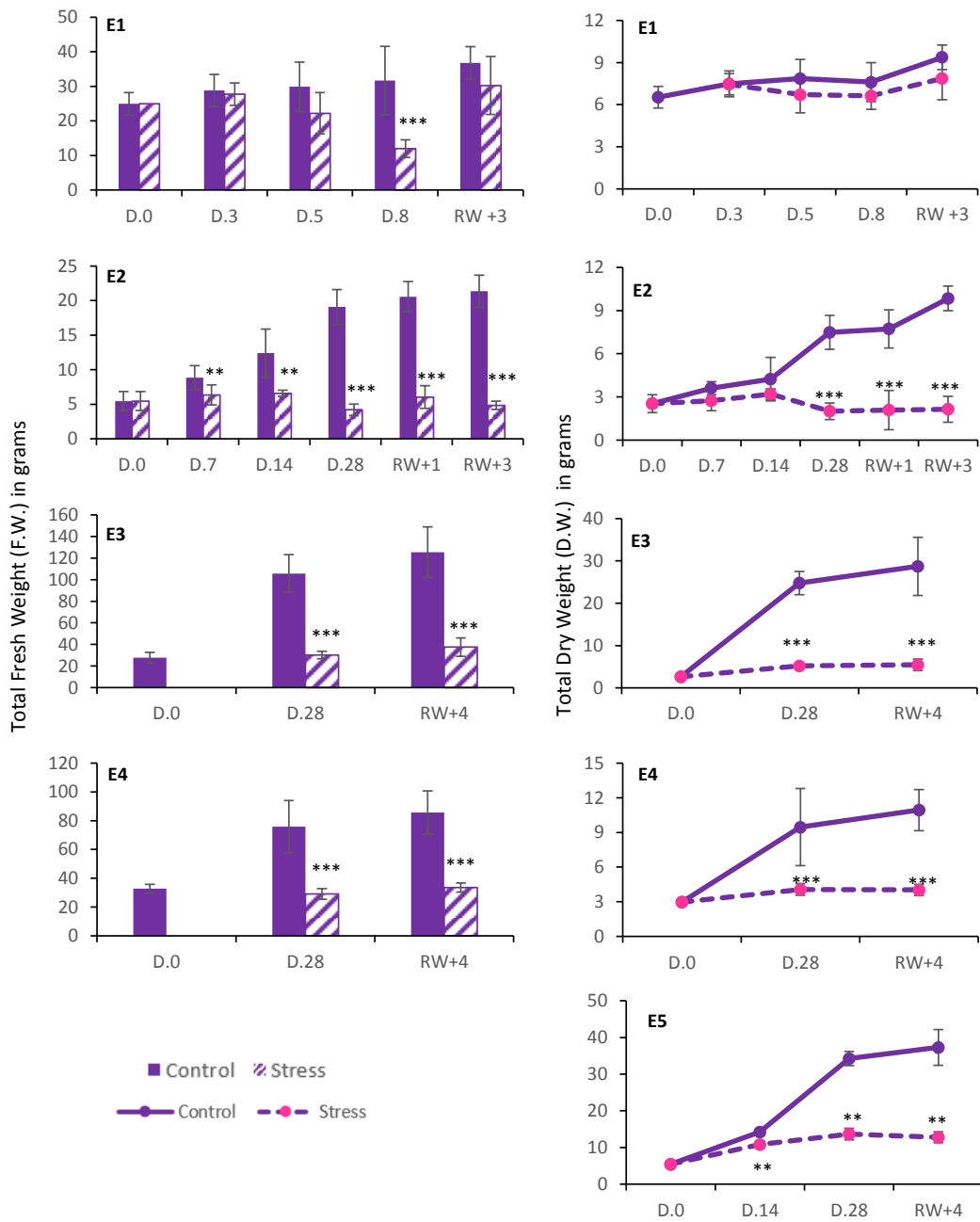
Figure 5.39. Total fresh and dry biomass production. Fresh weights of leaves and stems summed up, were weighed at measuring point and after oven drying. Statistics correspond to t-test between control and stressed plants.

5.4.3.3.   Water content in biomass

With increased water limitation, first expected response from plants is to regulate photosynthesis and to close stomata. In order to analyze the water status in plants, water content in above ground biomass was calculated, making distinction between leaves (& cotyledons) and stems (& petioles). Plant material was weighed after photosynthesis measurements (FW), trying to collect and evaluate samples as fast as possible to avoid loose of latex. Afterwards, it was dried out completely and re-weighed (DW) this was done for E1, E2, E3 and E4. Water content (WC) was calculated as the absolute difference in weight between FW and DW (**WC**= FW – DW), while the relative water content (RWC) corresponded to the ratio of absolute loose of water by it corresponding fresh weight (**RWC** = WC/FW * 100).

5.4.3.4.   WC in Leaves & Cotyledons

Leaves and cotyledon represent the most specialized photosynthetic surfaces, and the values of water content depended on the overall production amount and the atmospheric conditions. The values from control plants displayed variable values at the beginning and the end, for all experiments (Fig. 5.46). Outdoor experiments started with different WC (2.6 ml in E1 and 4.6 ml in E2; p=0.015), and while on full sun (E1) it corresponded to close to 50%, under shadow (E2) it represented > 80%. Indoors experiments seedlings started with twice the WC, E3 with 7.8 ml and E4 with 9.1 ml, that represented 86% for both. At the end of one week of drought stress in E1, WC was 4.3 ml while samples under shadow had 7.6 ml after the same period. For the experiments taking one month of drought stress, the WC was 17.9, 27.3 and 18.5 ml, for E2, E3 and E3, respectively, that corresponded to 84%, 77% and 51%, respectively.

For **Experiment 1**, the absolute (WC) and relative water content (RWC) differed between droughted plants and controls at the end of the stress period, after 8 days (Fig. 5.40). In **experiment 2**, seven days after starting drought treatment there was no statistical difference in the absolute WC but was observed a reduction in growth. Stressed plants had an average of 5.3 ml water accumulated in their leaves, increasing just 0.72 mg from the values at day 0, while controls reached values of 7.6 ml, corresponding to an increase of 3 ml WC, almost doubling in one week the starting value. After this point, stressed seedlings did not modify their WC, keeping very similar values for two weeks more. In the measurement on day 14 until the end of experiments, there were significant difference between stressed and control plants. RWC in E2 was highly different after 7 days stress (p < 0.01), but this difference was getting reduce afterwards (p < 0.1). At the end of the experiment, we have a big difference in

water accumulation between groups, with controls having 5X the amount of water than in stressed plants. Re-watering did not affect the final WC values, but the RWC showed that the percentage of water in each fresh biomass was similar in controls and stressed plants, after one day.
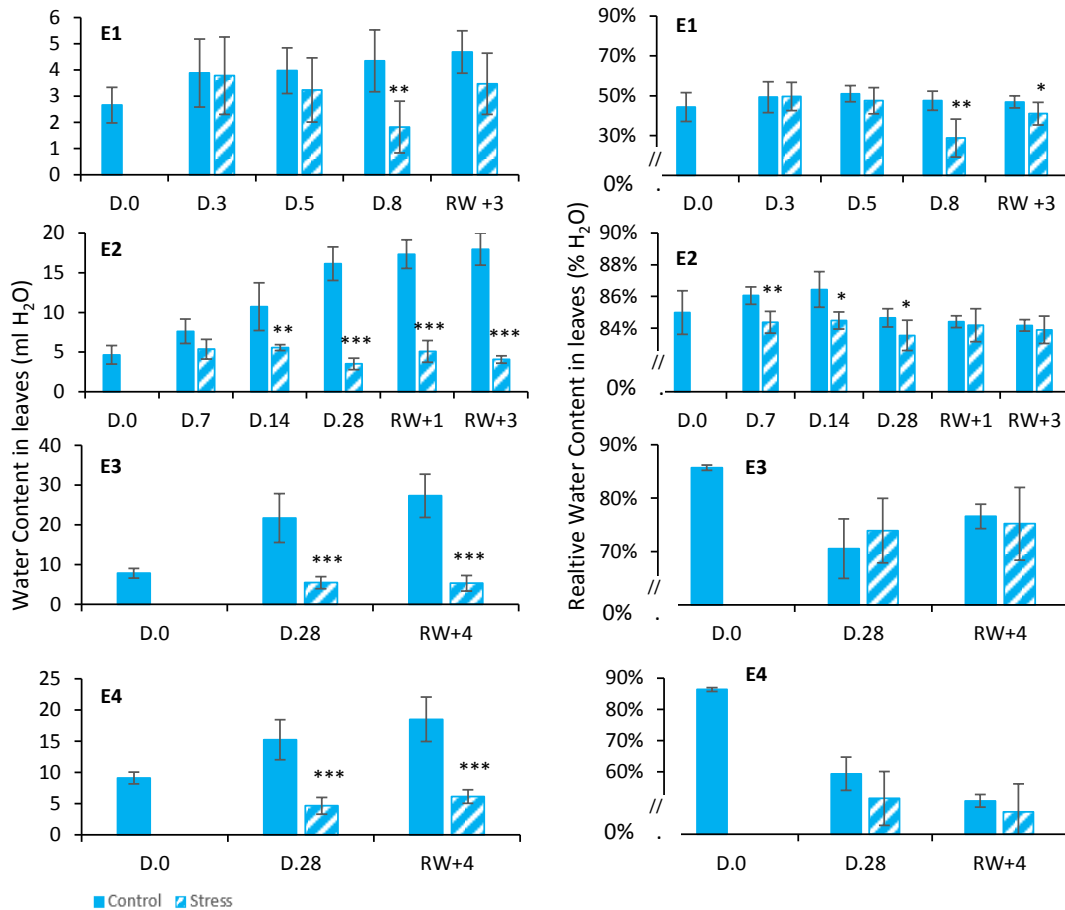
Figure 5.40. Water content in leaves and cotyledons biomass. Total water (WC) calculated as FW-DW after sample drying, while the relative water content (RWC) was calculated as WC/ FW.

Plants in E3 and E4 were too few to have also sampling collection every week, so water accumulation during middle stress was not recorded, nor the probable moment when plants decide to stop growth (Fig. 5.40). **Experiment 3** started with seedlings having an accumulation of 7.8 ml water, already 66% more than seedling in E2 and 123% more than in E1. At the end of the dry period, stressed plants accounted for 5.5 ml water while controls had 21.7 ml (p=0.003), having stressed plants with smaller values than on day 0, with a statistical significance (p=0.02). Water content measurements ended with a difference of 5X more for controls than stress, even after re-hydration of soils. WC for controls after 32 days was 27.3 ml, that represent an increase of 19.5 ml from the values on day 0.

In **E4**, seedling started the experiment with 9.10 ml water, even higher than in E3 (p=0.004). After 28 days of no irrigation, stressed plants had a water content of 4.7 ml (lower than at the beginning, p=0.0004) while controls had 15.3 ml. Control value at this point was significantly higher (p=0.01) than day 0, and than stressed plants (p=0.0007) at this day. Still, it was 6.4 ml water less than in E3 (p=0.08), confirming the idea of growth arrest due do less light intensity observed also in apical size (Fig. 5.45). Re-watering made stressed plants increase in 1.5 ml their WC, but it was 3X less than in controls (p=0.0009), still a loose of 2.9 ml from starting point. Control plants after 32 days had 18.5 ml WC, which corresponds to an increase of 10.4 ml from the starting point.

5.4.3.5.   WC in stems and petioles

Taking in account that *Jatropha curcas,* as *Euphorbiaceae* contains a sap and has a sort of green succulent stem, it was also evaluated. Fig. 5.41 summarizes the values of water content for stems and petioles of seedlings in each experiment and collect day.

WC in stems and petioles was apparently affected by the environmental conditions as occurred to leaves. Control seedlings started the experiment with different WC in all experiments, been 13.7, 4.6, 17.2 and 20.7 ml water for E1, E2, E3 and E4, respectively, which corresponded to 44%, 84%, 93% and 93% RWC, respectively. After one-week drought, controls in E1 had an increase of 6.7 ml while E2 had only 3 ml water extra accumulation in stems. Stressed plants after 7 days drought had an increase of 2.3 ml in E2 reaching 5.3 ml WC, while in E1, stressed plants suffered a reduction of -8.8 ml water, for an absolute WC of 4.9 ml water.

After 28 days of drought, control plants achieved different degrees of increase in their WC but maintained similar values of RWC. The increment in WC was 11.6, 42 and 22.8 ml water for E2, E3 and E4, respectively; in all cases highly different from stressed seedlings. After 32 days of experiment, control plants had WC of 17.9 (increase of 13.3 ml), 69.4 (increase of 52.2 ml) and 56.1 ml (increase of 35.4 ml) for E2, E3 and E4, respectively. Re-hydration recovered completely stressed plants as they presented a WC of 20.7 ml (gain of 15.8 ml water in three days) and controls, 25.0 ml water (p=0.27). RWC was around 45% for both groups. RWC for E2 and E4 was similar between controls and stressed plants, while in E3 it presented an increase on stressed plants (88%) compared to controls (77%).
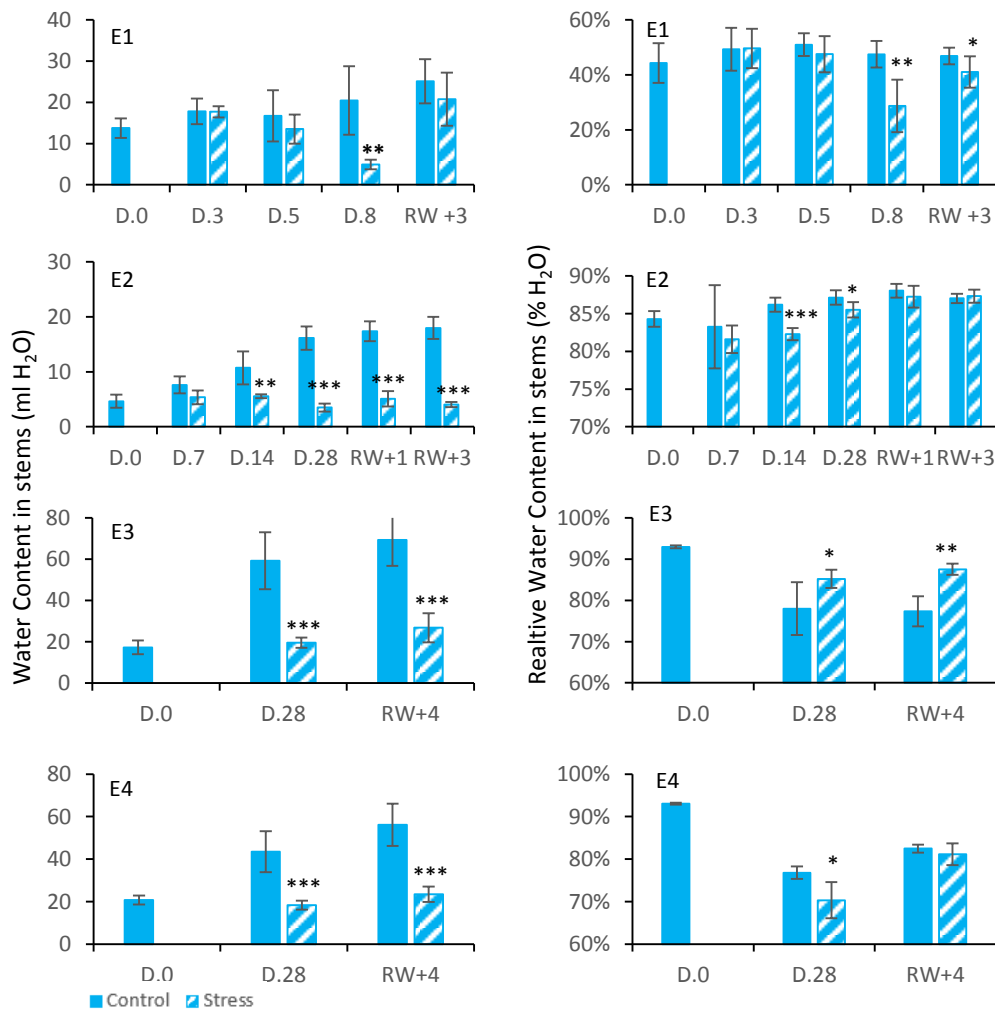
Figure 5.41. Water content in leaves and cotyledons biomass. Total water (WC) calculated as FW-DW after sample drying, while the relative water content (RWC) was calculated as WC/ FW.

Each individual experiment had also its highlights. The short period in **experiment 1** does not allow to see statistical difference in WC during the experiments in control plants, but the trend of diminution in stressed plants is obvious. Seedlings start with 13.7 mg water accumulated in their stems, and after three days, values for control and stressed plants were similar (15.2 mg for both groups). Five days after starting the stress period, the controls plants keep stems with the same WC values, but stressed plants had 5 ml less water (p=0.10); this difference becomes significant after eight days of stress (p=0.007) when controls reached 20.4 ml of water content (RWC = 47%) while stressed plants reduced their WC until 4.9 ml (RWC = 29%), meaning drier stems.

Experiment 2 started with 4.6 ml WC, a lot less than in E1, and after the first week, stressed plants behaved similarly as in E1, presenting a significant difference from their controls, with 5.3 ml water in stressed plants (RWC = 82%) and 7.6 ml for controls (RWC= 83%), p=0.008.

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Control plants kept almost a linear increase in their water content of around 3 ml water per week until the end of the experiment, reaching a maximum of 17.9 ml in 31 days (RWC= 87%). Stressed plants kept the same content of day 7 for two weeks, before drying even more, reaching 3.5 ml WC (RWC = 85%) at the end of dry period, with a statistical difference from controls of $p < 0.0001$. Rehydration of soil helped plants under treatment to gain 60 ml in WC in three days (RWC = 87.3%); the difference in absolute WC to controls stayed abysmal, but the RWC was similar (control had 87%).

In **experiment 3** seedling started with 17.2 mg of water content, 12.6 mg more than in E2 ($p < 0.0001$). After 28 days of drought, stressed plants had 19.5 ml water (RWC = 85%), not significantly different than at the start of the experiment (p=0.26), but highly different to controls (p=0.002) that reached 59.2ml (RWC= 78%). After re-watering, the seedlings under treatment had 26.7 ml WC (RWC= 88%), but difference from controls was not overcome (p=0.003); control plants experienced increase of 52.2 ml while stressed plants increased only 9.5 ml.

Seedlings in **experiment 4** behave similarly as on E3 (Fig. 5.41). At the end of the drought treatment, stems of stressed plants had 18.3 ml WC (RWC= 70%) which was a significantly drier version of the 43.5 ml control WC (RWC= 77%) at day 0 (p=0.006), same as compared to the controls on the same day (p= 0.004). Re-watering allowed seedlings under treatment to allocate 5.1 ml water more in four days, but difference with control plants at this point (56.1mg) was impossible to equate; RWC for both groups at the end was 82%.

5.4.3.6.　　　　　　Leaf production

One of the classical ways to evaluate plants growth effort is through leaf area quantification.

With the help of fig. 5.42 it is possible to observe trends.
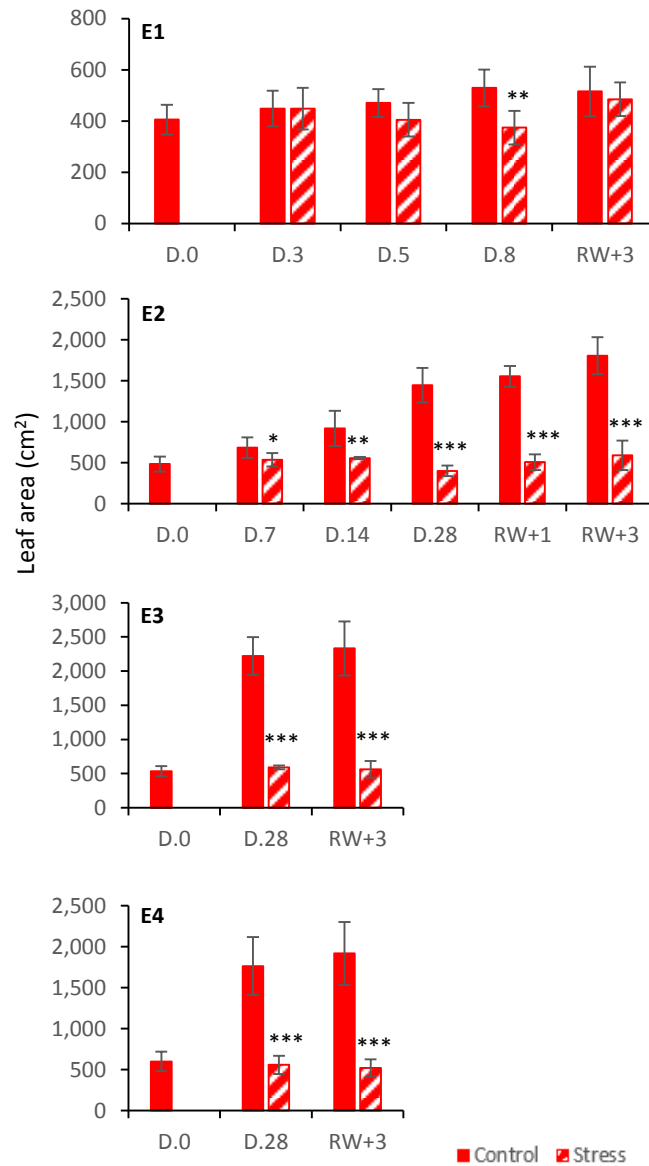
Figure 5.42. Total leaf area production. Including all leaves and green cotyledons at the moment of collect, with minimum 1 cm length.

For **experiment 1**, there was a high difference between controls and stressed plants at the end of the experiment, and somehow, leaves appear to have gained in size after three days of rehydration, probably achieved with the expansion of smaller leaves.

Leaf area in **E2** presents control seedlings with a steady increase, directly correlated to water content in leaves (Fig. 5.42). After one week of drought, stressed plants in E2 reached 535 cm^2 ($p < 0.1$ to controls), while on full sun stressed plants reached 374 cm^2. The modification in

leaf area from this moment on was almost imperceptible, indicating that after 7 days of drought treatment, plants do not invest in leaf expansion. At the end of the dry period (28 days), stressed plants had substantially smaller leaf areas than the controls. The same occurred with **E3** and **E4**. Understandably, after re-watering, difference was not possible to overcome.

Almost all experiments started with seedlings having relatively the same leaf area: 405 cm^2, 483 cm^2, 533 cm^2 and 599 cm^2 for E1, E2, E3 and E4 respectively. But expansion in leaf area occurred differently and based on environmental parameters. For the experiments in Panama, seedlings on control conditions after seven days had increased in their leaf area of 124 cm^2 when exposed to full light, and an increase of 201 cm^2 when kept under shadow. After 31-32 days, controls of E2 reached 1,805 cm^2, but the unstable environment limited their growth compared to indoor experiments. E3 controls reached a maximum average of 2,331 cm^2 appearing to have best conditions, while in E4 they had 1,916 cm^2, 415 cm^2 less than in E3, confirming the unsuitability of conditions for optimal growth of *Jatropha curcas*. Stressed plants had also different growth depending on environmental conditions: 562 cm^2 (E3), 589 cm^2 (E2) and 520 cm^2 (E4).

5.4.3.7.   Petiole analysis

During the test to determine growth conditions in *Jatropha* inside of our greenhouse, it was remarkable the differences in sizes of petioles when growing in different environment, so this parameter was considered for the experiments to compare.

Experiment 1 was so short that it was not possible to determine difference in petiole size of stressed or control plants. Values after 8 days of drought and three days re-water did not show any significance; at the end of the experiment, control plants had 9 cm^2 while stressed plants had 8.6 cm^2 (Fig. 5.43). High irradiance and volatile atmospheric conditions produced the shortest petioles from the four experiments. When comparing both outdoor experiments, control plants in E1 had 9.4 cm length petioles after 8 days of drought, while E2 control plants had petioles of 14.2 cm, suggesting that the amount of light in each experiment affected petiole length.
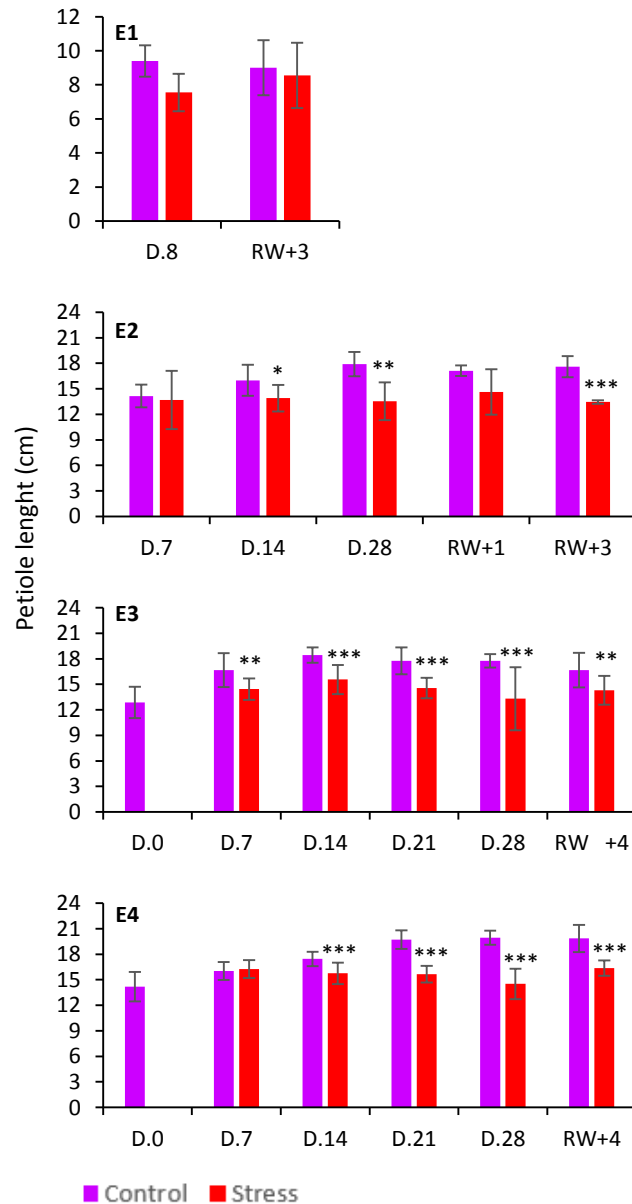
Figure 5.43. Petiole size. Average of size of the two longest petioles per plant at each measuring time (n=10).

In **experiment 2**, there was no difference between controls and stressed plants in petiole length after seven days of stress either. Two weeks were necessary for observing significant difference, with controls having petioles of 16 cm and stressed plants, of 13.9 cm. At this point, these results suggest that under a well-irrigated condition, plants would put more effort into reaching the sun by elongating petioles (increase of almost 2 cm in average) while stressed plants did not spend energy on this aspect, growing only 2 millimeters (p=0.06). After 28 days of drought, values in petiole length in stressed plants did not changed substantially from the ones observed after 14 days (13.6 cm petioles) while controls increased almost 2 cm,

presenting plants bearing 17.9 cm long petioles. Re-watering did not increase petiole size, maybe 3 days is too short for such a response.

In the indoor experiments, sizes in petiole length variated also based on the light intensity. Controls in E3 (higher irradiation) had petioles of 17.8 cm after 28 days, while in E4 (with lower light irradiation) petioles were 19.9 cm long in average.

Stressed seedlings in **E3** had petioles significantly shorter than their controls (p=0.008) after seven days, and this difference stayed until the end of the experiment. Control seedlings got a maximum size of 17.7 cm after 4 weeks, while the size of drought treated seedlings at this moment was 4.4 cm shorter.

In the low irradiance conditions of **E4**, stressed seedlings tried to take as much advantage from available light as possible during the first seven days, by increasing their petioles in the same range as controls (2 cm growth for both groups). But after the first week, probably the plants changed the strategy and controls the use of supplies, to protect from drought, instead of reaching for more light. Control plants keep their pace elongating their petioles (mostly vertically) 1,5-2 cm per week, reaching a maximum size of 19.9 cm after 4 weeks. Seedlings under drought treatment stopped the elongation of petioles after the first week, and at the end of the experiment had average 14.5 cm petioles (5.4 cm shorter than controls). Re-watering for three days increased the size in almost 2 cm but did not get even close to the values of controls (p < 0.0001).

5.4.3.8.   PSII quantum yield measurements

Quantum yield analysis presented the first overview of the photosynthetic status of plants. In this study, the maximum and effective quantum yield of PS II (Fv/Fm and ΔF/Fm, also called Φ_{PS-II}), where calculated as indicators of damage and excitation potential of photoreaction centers. Quantum yield analysis allow the possibility of measuring in a non-invasive way, based on chlorophyll-a fluorescence levels. In other species, for example *Arabidopsis*, the values of maximum quantum yield (Fv/Fm) in control plants were around 0.83 and during stress, around 0.400 (van Buer *et al.*, 2016). The unity used to measure electron quantum yield are ratios of chlorophyll fluorescence at different stages of excitation.

For the experiments in Panama (E1 and E2), it was measured only at the beginning, at the end of the stress period and after re-watering. The experiments indoors in Berlin had measurements along the drying process of the soil also. In general, it was possible to observe that in all experiments, effective quantum yield was reduced drastically for plantlets under stress at the end of drought period (Fig. 5.44). But this reduction was actually not meaning the degradation of chlorophyll, because after dark adapting the same leave presented higher values, and after re-watering, there was no significant difference between treated plants and controls.

In **E1**, ΔF/Fm' for stressed plants was 0.485 after 8 days of treatments, while for controls was 0.666 (p= 0.002), indicating a considerably reduction of the photosynthesis activity in controls under full sun conditions, when compared to the maximum yield. After the re-watering difference in effective quantum yield was not significant, being 0.523 for controls and 0.564 for stressed plants. In the same experiment, when leaves were dark adapted the values for maximum quantum yield (Fv/Fm) were higher, indicating that the amount of light was in excess for the plant in regular conditions. After 8 days of drought, stressed plants had in average Fv/Fm values of 0.649, while controls had 0.755, with statistical difference of p=0.02. After re-watering, values for stressed and control plants were 0.74 and 0.75, respectively, without significant difference.
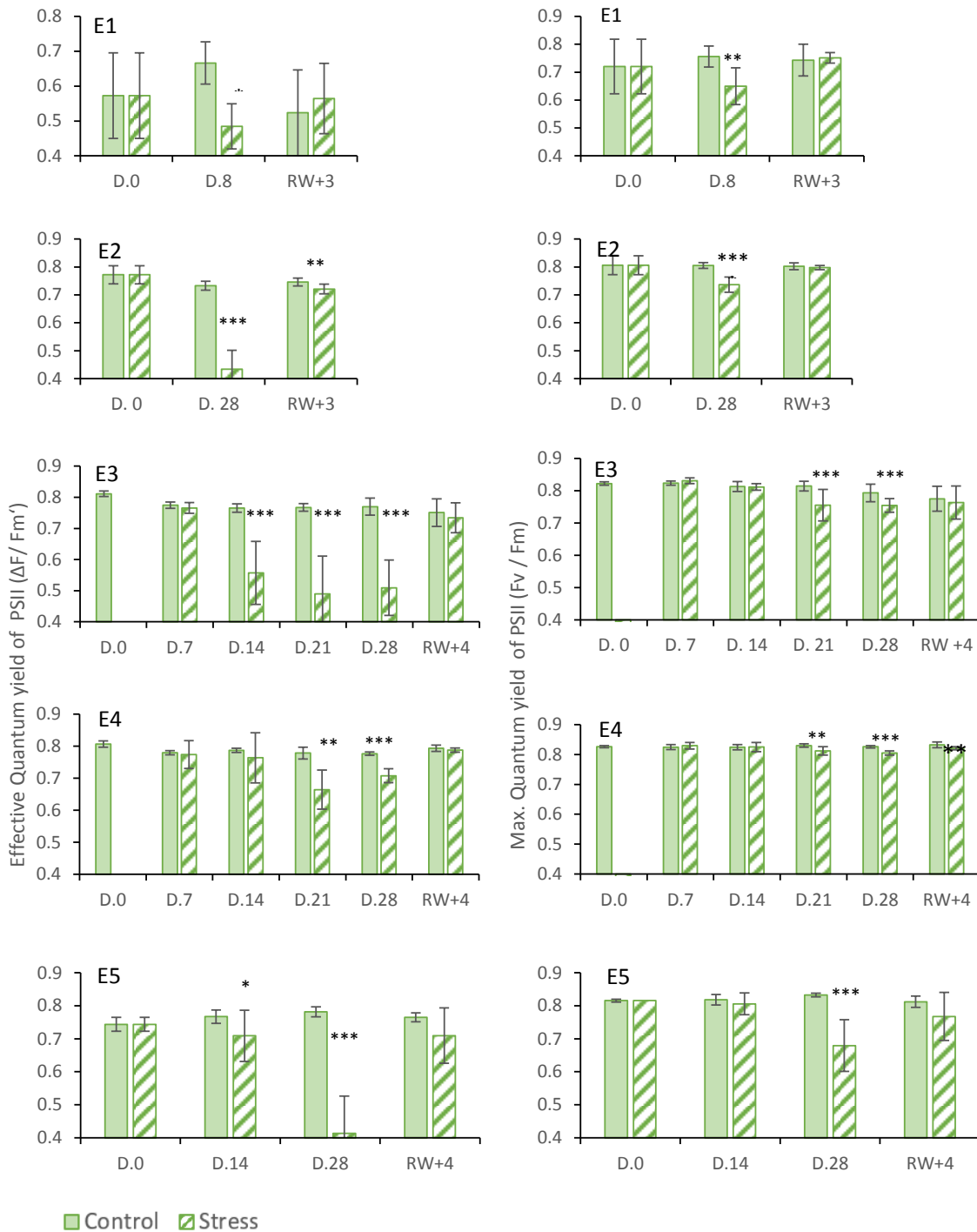
Figure. 5.44. Photosynthetic quantum yield in PS II. To the left, all graphs represent "Maximum quantum yield" and was measured with leaves dark adapted with a clip, for 30 minutes. The column on the right represent the "Effective quantum yield", measured with light adapted leaves.

In the **Experiment 2**, the effective quantum yield after 28 days of drought was 0.434 for stressed plants, while it was 0.733 for controls (p= 0.0003), showing that plants under drought treatment were stressed and had a reduced photosynthetic capacity. 3 days after re-watering, the effective quantum yield recovered to control values (0.72 and 0.74 respectively),

indicating that the reduction observed during the drought period was reversible. In the same experiment after dark adaptation of leaves, at the maximum drought point, there is a statistical difference of p = 0.003, between stressed plants (0.736) and controls (0.805). After re-watering, there is no difference, with values of 0.802 for controls and 0.797 for plants under treatment.

Experiment 3 and 4, as were performed in controlled conditions, can indicate the type of response of *Jatropha* to drought, based on its PS II status. In **E3** no difference in quantum yield was observed after 7 days of drought (light or dark adapted). After 14 days, it was possible to observe difference in the effective quantum yield, with high significance between stressed plants and control (p < 0.0001), and values of 0.557, 0.490 and 0.509 for day 14, 21 and 28, respectively. But after 4 days re-watering, stressed plants showed similar values to controls (0.734 and 0.750, respectively). There is high significance in the difference between controls and stressed plants in the maximum quantum yield from day 21 on. On the last day of drought, stressed plants had a maximum quantum yield of 0.754, while controls were on 0.793 (p= 0.002). Re-watering dissolved the statistical significance, while values were of 0.736 and 0.775 for plants under treatment and controls, respectively. Values after re-watering were almost the same for effective and maximum quantum yield in control plants and in plants under stress.

In **Experiment 4**, plants had the same watering situation as in E3, but had less light available for photosynthesis. Effective quantum yield presents statistical differences after 21 days of drought, meaning a delay in one week compared to E3. Values for day 28 were of 0.708 and 0.776, for stressed plants and controls, respectively (p= 1.7 E-06). That means that both groups are highly different even when the value for treated plants is hard to situate as one for a stressed plant. And after watering all plants again, there is no statistical difference anymore (0.793 for controls and 0.757 for treated plants; p= 0.11). With dark adapted leaves, differences were even harder to determine. Statistical difference was stablished also after 21 days of drought, but all values are hardly representing a stressful situation. The minimum value on drought stressed plants was after 28 days treatment, but the average value was 0.805; with p= 0.00000013, smaller than controls. In general, experiment 4 appeared to not have experienced real stress, or plant is quite adapted to such situations and able to recover widely.

Plants in **experiment 5** had a small difference in ΔF/Fm' after 14 days, that was not significant in terms of Fv/Fm. After 28 days, plants under drought treatment had a highly significant smaller ΔF/Fm' than their controls, 0.413 and 0.781, respectively; the Fv/Fm was also very different ($p < 0.0001$) with 0.832 for controls and 0.679 for stressed plants. Again, at maximum stress, still indicate a good condition level for the PS II, which is not degraded so far. After re-watering, values of both groups appear statistically similar; with values for ΔF/Fm' of 0.764 and 0.709, and for Fv/Fm of 0.811 and 0.767, for controls and stressed plants respectively, in both cases.

5.4.3.9.   CO$_2$ assimilation and Water conductance

In Panama, it was possible to measure with an Infra-Red Gas Analyzer (IRGA) the values for photosynthesis activity (by CO$_2$ assimilation rate) and water conductance. This type of device measures gas exchange of leaves; when stomata are open, water vapor can diffuse out of plant tissues to allow the entrance of CO$_2$ from the air. In a closed system, with a known amount of water and CO$_2$ in the incoming air flux, it is possible to measure the concentration in those molecules present in the outcoming air flux. In this way, gas exchange measurements provide a direct measure of the net rate of photosynthetic carbon assimilation.

Values for CO$_2$ assimilation correspond to the amount of this molecule taken from the air for photosynthesis, while the values for water conductance remit to the degree of openness of stomata, allowing water to go outside of leaves.

In both experiments, net photosynthesis and water conductance had similar patterns (Fig. 5.45). For **E1**, controls showed a reduced photosynthesis, probably for a less sunny day or too high temperatures, stomata closed to protect the plant leading to a lower water conductance, but on days with high net photosynthesis like day 1 or day 8, water conductance was high. For stressed plants, net photosynthesis was drastically reduced after three days due to atmospheric conditions reaching incipient values of CO$_2$ fixation. These minimal values were maintained for at least two days more, with almost complete closed stomata. Carbon assimilation and stomata conductance had values of zero after eight days, but the period of re-watering allowed plants to increase their photosynthetic values to similar ones to the control, while the conductance was increasing up to 72% of control value.
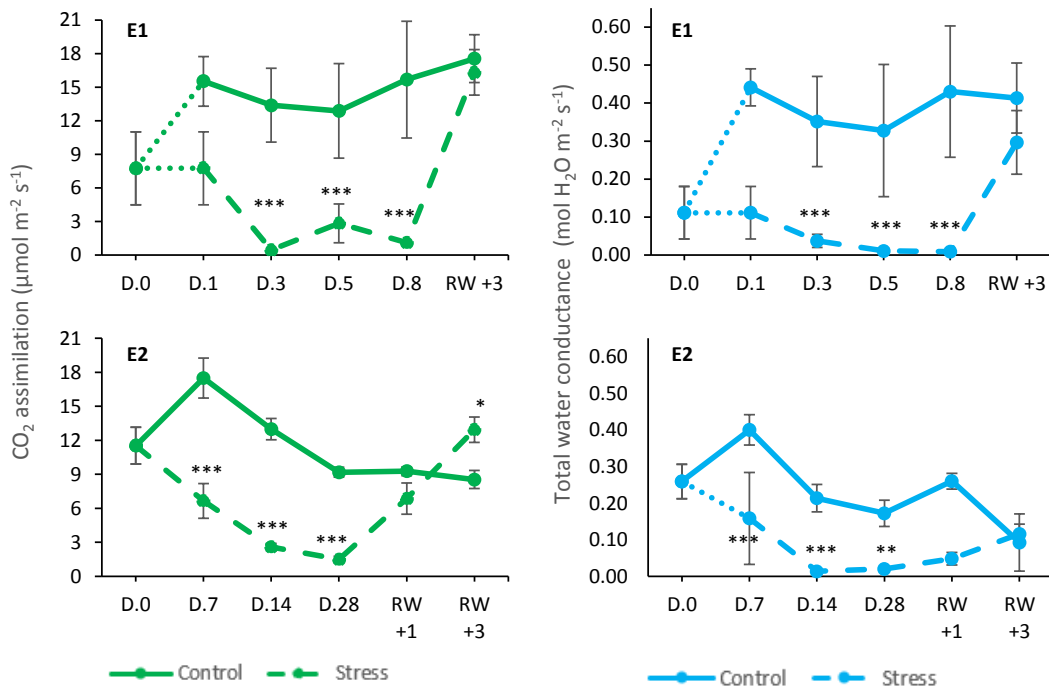
Figure 5.45. Net photosynthesis and water conductance in experiments in Panama. Net photosynthesis was calculated as μmol CO_2 assimilation per m^2 s^{-1}, and water conductance, on the stomata opening allowing the pass of water molecules per m^2 s^{-1}.

In the experiment under shadow in Panama, **E2**, after 14 days there is a decrease in net photosynthesis in control plants, which corresponds to a reduction in water conduction during the same time. There is no other explanation for this behavior than the response of plants to higher values of light or temperature, which was not optimal or represented a substantial change from previous (shadow) conditions.

Stressed plants in E2 present a slow and constant reduction in the net photosynthesis which reaches the minimum after 28 days of drought ($p=9.2$ E^{-9}). Water conductance was close to zero after the second week, so these plants had a way to continue photosynthesizing with very small intake of CO_2 from the air, during day time, for around two weeks. After one day re-hydration, net photosynthesis was quite similar to the controls ($p=0.03$), while water conductance was still low ($p=7.1$ E^{-6}); but after three days watering, values for water conductance were not different from controls ($p=0.55$), while photosynthesis was unexpectedly higher ($p = 0.07$) than controls.

5.5. Molecular analysis of plant material

After the elucidation of each sequence for the genes in the chloroplast antioxidant system and performing experiments with *Jatropha curcas* in different conditions, it was finally possible to observe gene regulation under drought stress in this plant.

5.5.1. RNA Extraction

For *Jatropha curcas*, there is also no protocol established for RNA extraction protocol. In this study, RNA was extracted using the Roboklon Universal RNA extraction kit according to manufacturer's guidelines, except from minor changes. It was tested the effect of a pre-pulverization of the material with an electric bead mill, effects of extra phenolics extraction and effects of extra RNA precipitation with NaAc (Fig. 5.46). The pre-pulverization of plant material proved to be necessary (Fig. 5.46a) and the best way was a dry shaking process, with micro tubes containing (50-100mg) powder sample material directly from liquid nitrogen into the mill. If samples were thawed to add the extraction buffer, results were not favorable.
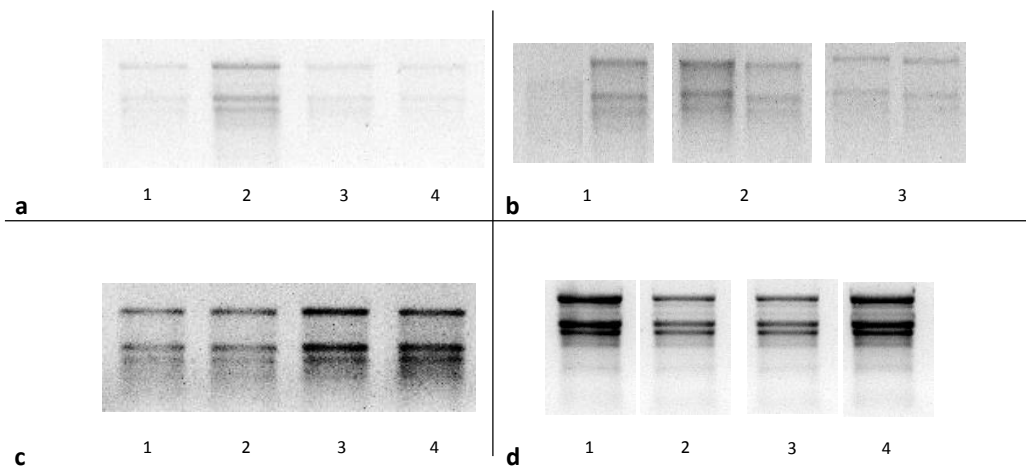
Figure 5.46. RNA extraction for *Jatropha curcas* leaf material under different conditions. Same kit was used with small modifications: (a) difference in extra pulverization with a beat mill, 1= regular extraction with no use of mill, 2= dry mill, 3= wet mill, 4= dry and wet mill together. (b) tests with extra phenol extraction step, 1= two samples with phenol extraction and dry mill, 2= two samples with manual grinding inc. phenol mix, 3= no phenol step, regular extraction. (c) samples precipitation after double elution of membranes. (d) extraction on fresh samples from the greenhouse. Each number represent a different sample material in c & d.

Euphorbiaceae are known for containing high levels of phenols, therefore an extra step for phenolics extraction was administered (Fig. 5.46b). This option improved results, but even in the best samples the RNA concentrations were in average 30-60 ng/µl and consequently, too low for quality cDNA synthesis. The best results for samples with some degradation was obtained after RNA precipitation (Fig. 5.46c): at the end of the regular extraction protocol,

RNA-binding membranes were washed twice; this contributed to getting concentrations of 4-10 µg in 20 µl.

Greenhouse samples after regular extraction protocol (Fig. 546d) produced concentrations of 200 - 350 ng/µl diluted in 40 µl water for each sample, which corresponds to a total of 8-14 µg RNA. Moreover, when a mill was used, the membranes in columns from the extraction kit were clogged and broken. The samples in Panama apparently differed in chemical composition and had several critical moments that compromised their quality. They needed to be kept ultra-frozen since collection, but the transportation in a long-haul flight did not guarantee this requirement for all samples. Samples that were degraded to some degree did not produced sufficient cDNA for qPCR thus could not be included in the analysis.

5.5.2. Reference genes

Previous studies (Zhang *et al.,* 2013; Rocha *et al.,* 2016) evaluated the stability of several genes in *Jatropha curcas* when seedlings were submitted to various abiotic stresses, though in these studies, drought was never assessed. The suggested genes to be used as reference under similar conditions (e.g. salinity, desiccation and vegetative stage of plants) were analyzed, using the same primer pair sequences in plant material from seedlings during a drought process.

A total of six genes were evaluated: *ACT, GAPDH, EF1α, TUB5, TUB8* and *18S*. To control the specificity, the products were separated by electrophoresis in a 3% gel (Fig. 5.47). Two primer pairs (*EF1α* and *18S*) had very unspecific binding capacities and were therefore discarded from further analysis. Primers for **ACTIN**, **GAPD**, **TUBULIN 5** and **TUBULIN 8** showed one main band at ~150bp, the expected size. Any extra band was faint and about 500 bp.
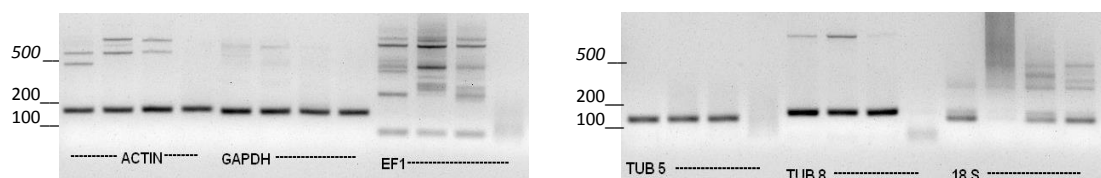
Figure 5.47. qPCR Primer test for possible reference genes. PCR product electrophoresis for six genes in gel 3% agarose+ 0.02% ethidium bromide; each primer pair was tried at 60°C using 3-4 different biological samples. Band sizes were compared to a 50 base ladder (Applied Biosystems, California, USA).

The four primer sets with specific binding were further evaluated with a quantitative RT-PCR (Fig. 5.48), using cDNA from four different plants. Melt curve for all primer pairs had one single peak, but based on the quality of the curve, *TUB8* was excluded from further analysis.
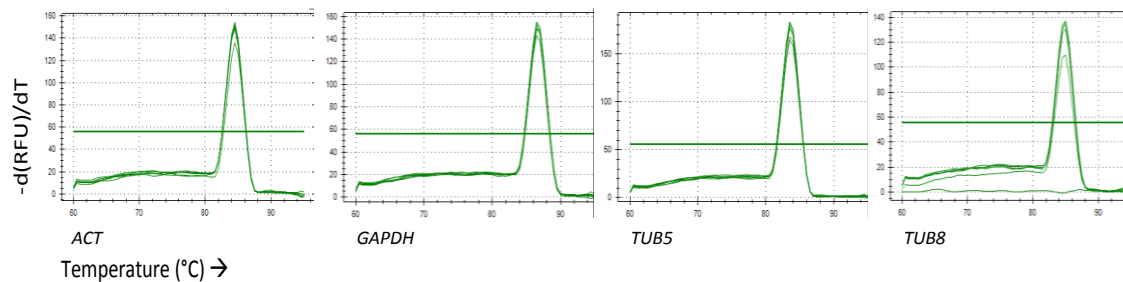
Figure 5.48. qPCR Primer test for possible reference genes. Graphs correspond to melt peaks in qPCR reads for *ACT*, *GAPDH*, *TUB5* and *TUB8*. Each gene was tested in four samples from different experiments and conditions.

Stability of ACT, GAPDH and TUB8 expression in control and stressed plants was evaluated in a series of cDNAs from sun and shadow experiments in Panama, to select the best pair. Average Ct values for each gene and sample, was compared with the other two primer pairs. Ratio of two primer pairs was calculated for each sample (Fig. 5.49); the averages and standard deviations for the Ct value of all three combinations were calculated. In both experiments, the pair with the most stable expression was found to be *GAPDH* and *TUB5*, since the standard deviation for this gene couple was always the smallest one between the three options.

SUN					Shadow			
Sample #	Actin/ GAPDH	Actin/ Tub 5	GAPDH/ Tub 5		Sample #	Actin/ GAPDH	Actin/ Tub 5	GAPDH/ Tub 5
1	1.20	0.93	0.78		1	1.08	0.93	0.86
2	1.54	1.25	0.81		2	1.07	0.92	0.86
3	1.38	1.17	0.85		3	1.06	0.92	0.86
4	1.36				4	0.91	0.87	0.96
5	1.08				5	1.13	0.87	0.77
6	1.18				6	1.08	0.92	0.86
7	1.04	0.90	0.86		7	1.12	0.96	0.86
8	1.09	0.92	0.85		8	1.24	1.02	0.83
9	1.07	0.93	0.87		9	1.11	0.97	0.88
10	1.18	0.86	0.73		10	1.05	0.88	0.83
11	1.12	0.86	0.77		11	1.07	0.91	0.85
12	1.07	0.91	0.85		12	1.08	0.91	0.84
					13	1.23	1.04	0.85
Average=	1.20	0.97	0.82		14	0.96	0.70	0.73
ST. Dev	0.16	0.15	0.05		15	1.09	0.89	0.82
					16	1.08	1.03	0.95
					17	1.10	0.92	0.84
					18	1.08	0.82	0.76
					19	1.63	1.43	0.87
					20	1.10	0.92	0.84
					21	1.09	0.99	0.90
					22	1.09	0.98	0.91
					23	1.13	1.00	0.88
					Average=	1.14	0.98	0.84
					ST. Dev	0.18	0.21	0.06

Figure 5.49. qPCR Primer test for possible reference genes. (a) Corresponds to PCR product after electrophoresis for six genes in gel 3% agarose+ 0.02% ethidium bromide; (b) correspond to melting peak in qPCR for ACT, GAPDH, TUB5 and TUB8. Each gene was tested in four samples from different experiments and conditions.

5.5.3. qPCR analysis for 2CP

The information produced during gDNA alignment to FLseq_2CP and gene model was used to evaluate the best regions to situate the primer pair. Several options were produced and after their evaluation, based on similarity on melting temperature and GC content, the qPCR primers were selected. Forward primer for 2CP is situated (Fig. 5.50) spans exons 7 and 8 (starting at position 738), while reverse primer is located at exon 8, including positions 829-810.

```
TTCTCCCACCTTGCATGGGTTCAAACTGATAGGAAGTCAGGTGGTCTTGGAGATTTGAACTATCCCTTGA
TATCTGATGTCACCAAGTCAATTTCAAAATCTTACGGTGTGCTAATCCCTGATCAGgtataaatatcc
atatttgtctaatcttgttcttaatagcataatggttttttgtttattttcctttctatcttttccttgtt
tagttcatttgataaatttgcaaggacacatgcgtgcacgtgcccacacacaaaatgtgcttttcatgca
ttaacagtcttattgtttgatactttgttttgcactatccaattatgtagCCTGTGCATGTTGTCCTCT
CCGGCAATATTGTTAAGTCGAGCCAGAATGCTGTTCTAGttaatgtgttggatccatgttctcatatttt
atcacagctgtccctttactgctatggagtttataacacctcttttcatgcttctcttttgtgacatttg
gtattatgtcaggGAATCGCTTTGAGAGGACTTTTCATTATTGACAAGGAAGGAGTCATCCAACACTCCA
CCATTAACAATCTTGCTATTGGGCGTAGTGTTGATGAGACATTGAGAACACTCCAGGtgattgtgataaa
aaaacacatgataaatattatctaaaattgaaagatattattgacaaagcttgactcagtttgtagaact
ttagaaatgctatttaggatacaactggttatgatctaatacattcttgttcgttgcttttgcgtttgca
tcaggCACTGCAATATGTGCAAGAAAATCCAGATGAGGTTTGCCCAGCAGGGTGGAAGCCTGGTGAGAAG
TCGATGAAGCCAGACCCAAAGCTGAGCAAGGAGTACTTTGCGGCAATATAG
```

Figure 5.50. Fragment of gDNA from Jc_2CP, where qPCR primers are located. Detail of sequence, starting at the 5th exon in the genomic DNA sequence. Exons are highlighted in yellow and primer positions is marked in **red letters**.

This primer pair was tested with three different samples by RT- PCR and with qRT-PCR (Fig. 5.51). The results of this test confirm that the designed primer pair has a high specificity for the targeted area; the separation of RT-PCR in gel shows a main band at the correct size, and the other bands are faint and of bigger sizes that might not be amplified during the qPCR program. The Melting Curve obtained for the qPCR product supports this evaluation.
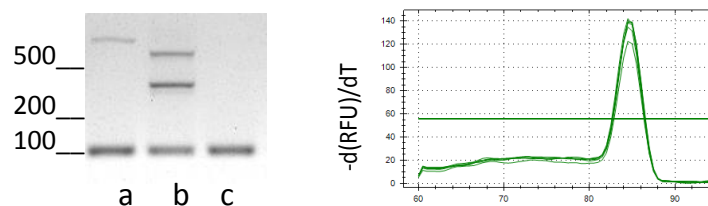
Figure 5.51. qPCR primer test for Jc_2CP. (a, b, c) represent different samples after amplification by RT-PCR and electrophoresis on agarose gel 3% (left). Samples were also amplified by qPCR to observe it melt curve (right).

This primer pair was then used to evaluate 2CP expression levels in the samples from the drought experiments performed, normalized with the expression of GAPDH and TUB5 (Fig. 5.52).
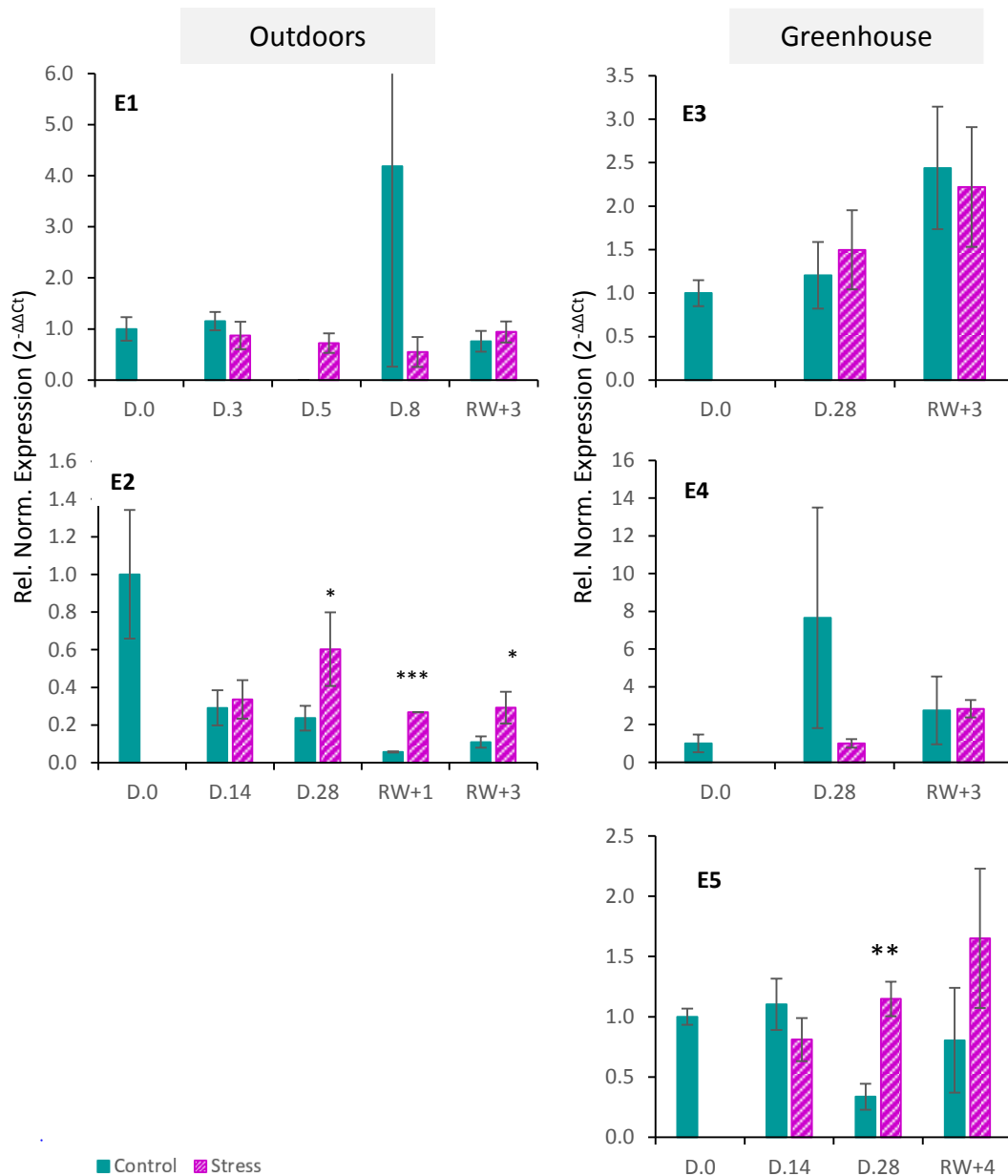
Figure 5.52. Gene expression for the typical 2-Cys-peroxiredoxin (*2CP*), during drought conditions. Displayed values represent the relative normalized expression of the gene ($2^{-\Delta\Delta Ct}$) for each experiment. E1= sunny conditions, E2= shadow conditions, E3= high light chamber, E4= low light chamber, E5= high light chamber + fertilization. Student T-test significance of $p < 0.05$ (*), $p < 0.001$ (**) and $p < 0.0001$ (***).

There is not a stable regulation on 2CP in all experiments. During mild stress, the experiments evaluated showed the same expression in treated and control plants. Most remarkable variations are the upregulation in stressed plants at maximum stress, being significant in E2 and E5 (the experiments considered under stable and positive environmental levels), and a slight trend to the upregulation in E3 (also in positive light conditions but with lack in nutrients). After Re-hydration, E2 showed significant upregulation; E5 has the same trend but does not reach significance due to the standard deviation in both control and stressed plants.

Results can be also analyzed by experiment. Short experimental period in experiment 1 does not show modification on its regulation. Control value for measurements on day 8 present a high difference together with high standard deviation, that was not possible to avoid; even when it consisted on 5 biological replicates, values were distributed in extreme values, with two values below 1.2, two values between 6 and 8, and one extreme value of 123 (discarded). The other outdoor experiment (E2, Fig. 5.52) presents a gradual response. Already after 14 days of stress, there was a trend in the increase, that became significant at the maximal stress (p= 0.02). Compared with their respective controls, both after one or three re-watering days, plants kept values significantly higher (p=0.0001 and p=0.05, respectively).

The experiment performed indoors with high illumination (E3, Fig. 5.52) presented the same trend of an increased expression of 2CP under high stress, lowering a little after re-watering, but with no statistical significance. Experiment 5 shows the same trend than E2, and the difference from treatments was significant to p=0.001 after 28 days of stress. As the main difference between these two similar experiments was the fertilization, it could indicate that nutrition levels are important to achieve higher 2CP expression during stress. In experiment 4, with plants growing inside a greenhouse but with reduced light available, 2CP shows less differential response. Samples collected for day 28 of drought treatment were extremely variable, with average values between about 2, 6 and 14 Ct unities, that created big standard error and produce a p=0.18, even when average from stressed plants was 7.5X smaller than controls.

5.5.4. qPCR analysis for APX

Analysis of the gene model for *Jatropha* and *Arabidopsis* was made, using the information for FL_JcAPX. Primers to evaluate APX transcripts were attempted in different regions inside the mature peptide area. Ten possible primer pairs that span an exon-exon junction were designed to analyse APX expression. Among them, the first primer pair didn't give any amplification in the expected region. Therefore, three other primer pairs were tested subsequently. Gel electrophoresis of amplification products using the four APX primer pairs are shown in Fig. 5.53, showing bands in different sizes for the three first primer pairs.

This is inconsistent to the expectations for quantitative PCR primers, which should target a single zone with homogenous expression. The best result corresponded to the primer pair #4,

with clear single bands for all samples. This primer was also tested in three temperatures (60, 61 and 62 °C) and was stable always.
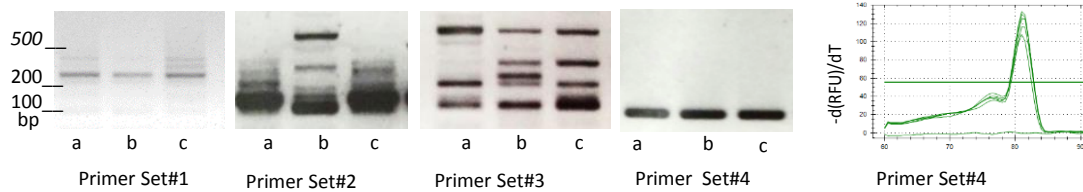
Figure 5.53. Electrophoresis of PCR products with four *APX* primers sets (for qPCR). Agarose gel 3% + 0.02% ethidium bromide. Expected amplification size was about 110 for all primer sets. (a), (b) and (c) represent different samples, all with annealing temperature of 61°C. Set #4 was tested with qPCR and the melt curve is presented

The forward primer from the Set #4 is starting at position 376 and the reverse primer in position 450 - 469 in the coding sequence of APX. As can be observed in the gDNA fragment where primers are located (Fig. 5.54), both forward and reverse are situated in the same exon.

```
...ATGGCAGACTCCTTGTTACACTTGCAACTATCACCATCGTCGCCAATGGCTTCGCTTAGTGGCGCCG
CCTCTTCTCGCCTTCTCCCTTCCGCTTCTCGGGCCCGAATCGCATTCCACTCAGCCACCTCTTCCTCCTC
TCTCTCTCCTCTATCTCTCTCCTCCTCCTCCTCCTCTTACTCTTTTTCTCCTCTCAAATGTCTCCGATCT
TCTCCTCTCGTACCTCGTGTCTTCTTCAATAaggtatgtacacttgtagtgaagcatctttgttttagtg
gatttttatggctgtttatctgatagttttggttttctgttgtgtgtAGACGGTATCGGCGATGAGCAC
GGTGACTGCAGCGTCGGATCCTGCTCAGCTGAAGAGTGCGAGAGAAGATATCAAAGAGCTTCTTAAATCT
ACGTTTTGTCATCCTATTTTGgtatgttttcttttactattacacttaaatttgtatagttgcttcatga
tttgatctgtgtgttaattttgctgttgccttcttcgagttcgattttttcttgattgaatctttgaatgg
atgtgctctgtgagagtgtggttatgttggatcatagtcgttctgcacttcaattctcaacaagcacttc
ttatatgcatatataaaattcctagatgaagaattttttggaatatttcgtatgtttcttgcaactgagg
acacacacagaatatttcttaaaattcattttttaagtgcatgaaaatagagttacaaaaaaagaaaaaa
aaaaaaaacgtgaatattattttttatcctatgtatagaacatgatttgactaaatcgttaatgcgttaa
gaccaattggtttgcgctggataaatggaaatgtaatgttggcaacttgaacataaaatctgtaatatgt
ataagaatatatgtttctttatttaacagtggaactttcctgtattatatctcatttgctgtttgcgagt
atcttataattatttcattttcaagaatactgatgctcaagccattaagctgaattacttgttgccgcac
agcattgatgctcaagcccactaaaatgcttataattacaGGTTCGACTGGGATGGCATGATGCTGGTAC
ATACAACAAGAACATAGAGGAGTGGCCAAAANNNNNNNNNNNNNNNNNGATTTGAAATTGAGCTAAAACA
TGCAGCGAATGcaggtagttatttcatatcatgtgtaactttagcttggtttataatatttcttcctgac
gagctggcgtttcaGGGCTGGTAAATGCTTTGAAACTCCTTCAGCCAATCAAGGACAAGTATTCTGGTGT
GACATATGCAGATTTGTTTCAGTTGGCTAGTGCCACTGCTATAGaggtctttatttctcttaatatggag
atagtatgatgtttcaattgatcgcacaaattattctattgaattgctcttgcaatttaGGAAGCTGGG
GGCCCCAAACTCCCCATGAAGTATGGAAGGGTGGATGTTTCAGCACCTGAAGACTGTCCCGAAGAGGGAA
GGCTTCCCAGtgagtaaattaattttgcatttttttttgtgttatttctgtacaaaaaagagcatgttcc
tctgttgttgcctcct...
```

Figure 5.54. Fragment of gDNA from *Jc_APX*, where qPCR primers are located. Detail of sequence, starting at the 5th exon in the genomic DNA sequence. Exons are highlighted in yellow, and primer positions is marked in **red letters**.

Some of the samples from the experiments performed in Panama were degrading to a point that it was not possible to extract RNA in good quality, therefore gene expression analysis was not done.
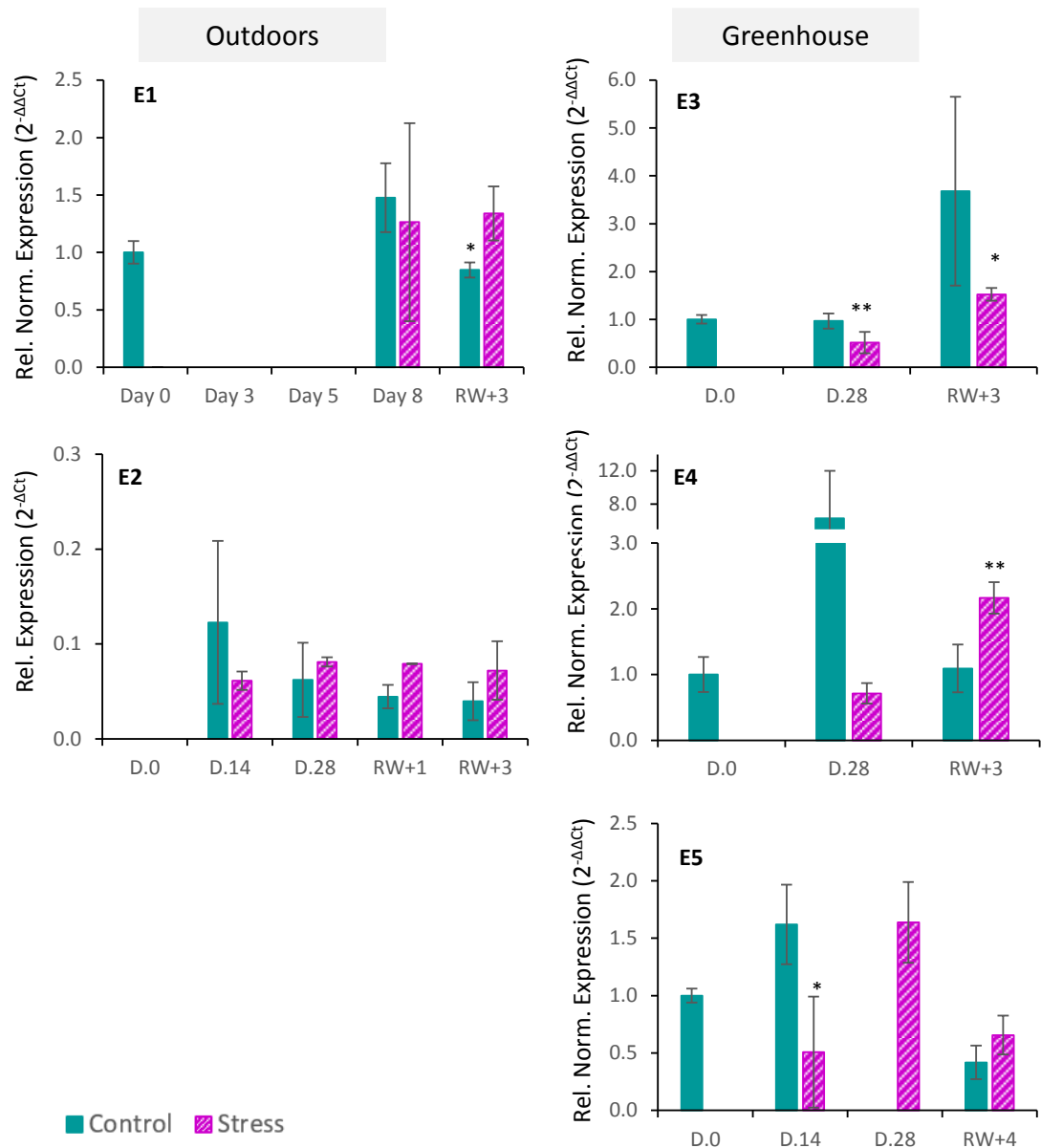
Figure 5.55. Gene expression for the ascorbate peroxidase (*APX*), during drought conditions. Displayed values represent the relative normalized expression of the gene ($2^{-\Delta\Delta Ct}$) for each experiment, except for E2, which is expressed as $2^{-\Delta Ct}$. E1= sunny conditions, E2= shadow conditions, E3= high light chamber, E4= low light chamber, E5= high light chamber + fertilization. Student T-test significance of p <0.05 (*), p< 0.001 (**) and p < 0.0001 (***).

Transcript levels for APX show a trend of downregulation in stressed plants during stress, being only a slight difference in E2 and significant in E5 during mild stress; for high stress measurements, its only significant for E3, but slight for E1 and E4 (Fig. 5.55). During re-watering, there was upregulation, significant in the experiments with the hardest conditions (E1 and E4), and slight in the most favorable conditions, E2 and E5, but it was also observed an unexpected downregulation in E3.

For E1, the value of *APX* expression at the maximum stress point (8 days) was similar to controls, including a big variation. After three days re-watering, the there was higher levels of

transcripts for treated plants than in controls (p=0.02). Samples for the starting point in E2 were not possible to analyze (Fig. 5.55), which eliminates the possibility to calculate $2^{\Delta\Delta Ct}$ (evaluate the samples in relation to control on day 0). This is the only experiment that was evaluated as $2^{\Delta Ct}$, but the outcome is still attractive. During mild stress (day 14) there is an apparent reduction in *APX* transcripts in treated plants, but the high variation in controls does not allow statistical approval. At the end of the dry period, stressed plants had a slight higher expression of APX than in controls, maintained for the next three days of re-watering. Experiment 5 does not have a control for day 28 but results could be comparable to those of E2. There is a reduction on APX expression during mild stress (p= 0.04), and the value for stressed plants after 28 days drought was increased in comparison to the values two weeks before. With the re-watering, there is a reduction in total transcript abundance, but values are slightly higher to those of controls. Plants of indoor experiments without fertilization (E3 and E4) present a different behavior than under soil fertilization (E5). In both cases there is a reduction in *APX* transcripts in stressed plants after 28 days of drought, that is significant for E3 (p= 0.007). For E3, reduced expression continued after three days of rewatering (p=0.05) but it was increased in E4 (p=0.003).

5.5.5. qPCR analysis for *GPX*

The set of primers for targeting *GPX* homologue in *Jatropha* was designed using the exon information and the gene model as reference. Using the gDNA data provided by Jatropha Database, intron-exon position was determined in gDNA; this was used to search for a place of around 100 bp that included two exons position. Several options were designed; the selected pair is formed by a forward primer in position 464-483 and reverse primer in position 544-563, from its coding sequence (Fig. 5.56).

```
…tatgatatttggaaacaattaatgcacttgatataaatatagttcagttatcctggggagcttagtaaa
gctatcgacattcatgaacctattttgaacattttgaagcttcacaaaagaatattcttttccttattgt
tcgtatgatttgcatctcaGGATTTGAAATTCTAGCATTCCCTTGCAATCAATTTGGAGGACAAGAACCT
GGGTCAAATCCTGAGATCAAACAATTTGCTTGTACCAGGTACAAAGCAGAATTCCCAATATTTGATAAgg
tatgcagcatgttcccttttccttggcggaagatgtattttagagagggaaaaataagtcaggttcctgt
gatcttgcacgacaGGTGGATGTGAATGGACCAAATACAGCTCCAGTTTACCAATTCCTGAAGTCAAGTG
CTGGAGGATTTTTAGGTGACCTGATCAAGTGGAATTTTGAGAAGTTCTTAGTGGACAAGAATGGTAAGGT
TGTGGAGAGGTATCCACCAACAACATCACCTTTCCAAATCGAGgtatatttctttgttcatttaaattac
ttcgacatccttgaatcattaagaattgtaatgaactatagaaggtggtcatgtctttttttcagaaacca
ttccaaatcaatgcatgaaaaatcttgtcttcacaatttacttttcatgatgtacaacaaagatgggtcc
atactacaagtttaaaagaatttttgcgttttttagaaaaactaaggattttctggtcagtccattgtcg
atgtttagaaacagtttgaagagtaaaagaaaatgtaagctctaccatatgtattttggaaagaccagtg
tctttatccttctaagcaagcagtagcttcaaagaataaattttcttgaagattttattaatccgctgaa
tctaattctcctcattcttgttgcagAAGGACATTCAGAAGCTTCTTGCAGCATGA
```

Figure 5.56. Fragment of gDNA sequence from *Jc_GPX*. Detail of sequence, starting at the 5th exon in the genomic DNA sequence. Exons are highlighted in yellow, and primer positions is marked in **red letters**.

This primer pair was tested with three different samples with Reverse Transcription PCR and with qPCR. The results of this test confirm that the designed primer pair has a high specificity for the targeted area (Fig. 5.57); the separation of RT-PCR in gel shows a main band at the correct size, and the other bands are faint and of bigger sizes that might not be amplified during the qPCR program. Melt curve during qPCR support this evaluation.
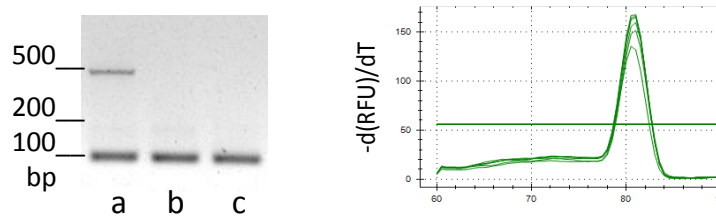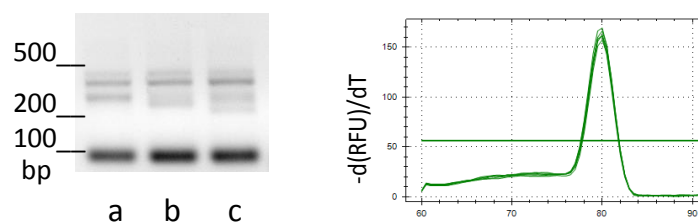

Figure 5.57. qPCR primer test for *Jc_GPX*. (a, b, c) represent different samples after amplification by RT-PCR and electrophoresis on agarose gel 3% (left). Samples were also amplified by qPCR to observe it melt curve (right).

Transcript levels for *GPX* in the analyzed samples present a trend of increase in stressed plants during mild stress (mostly visible in E2 and E5), that amplifies and becomes significant towards maximum stress point in E2 and E3 (Fig. 5.58). At this point, E1 and E5 had a small difference towards the increase, while E4 had less transcripts than controls, though not statistically different. After Re-watering, there is a significant reduction of gene expression in stressed plants in E1 and E4, though in E5 there is increase in transcript levels.

In the outdoor experiments, there is only a shared trend on upregulation during mild and maximum stress. For indoor experiments, the upregulation trend along the stress is shared in the experiments with high irradiance, where it is maintained even (in E5) after re-watering; in low-light E4, there is even a significant downregulation after re-watering.
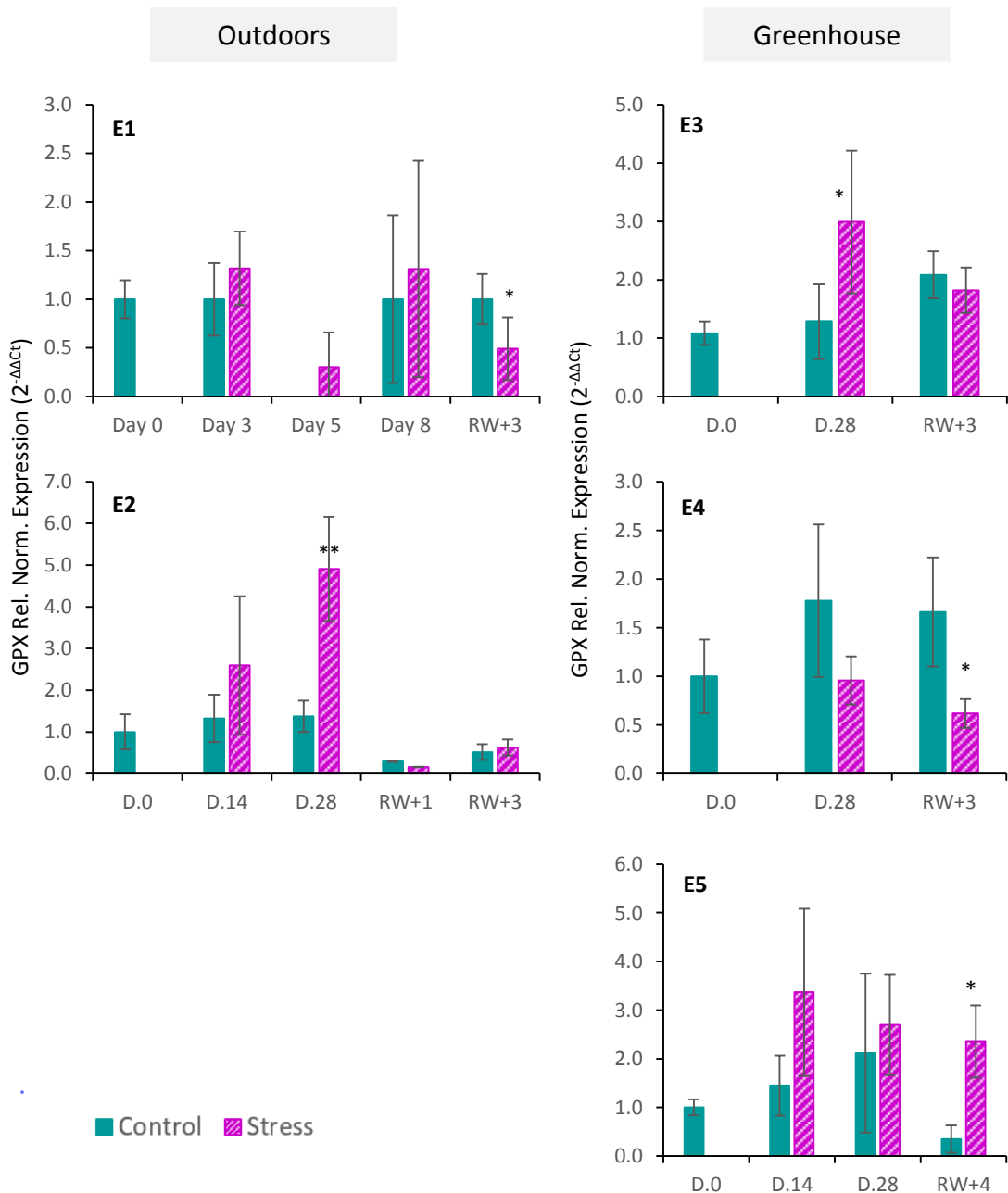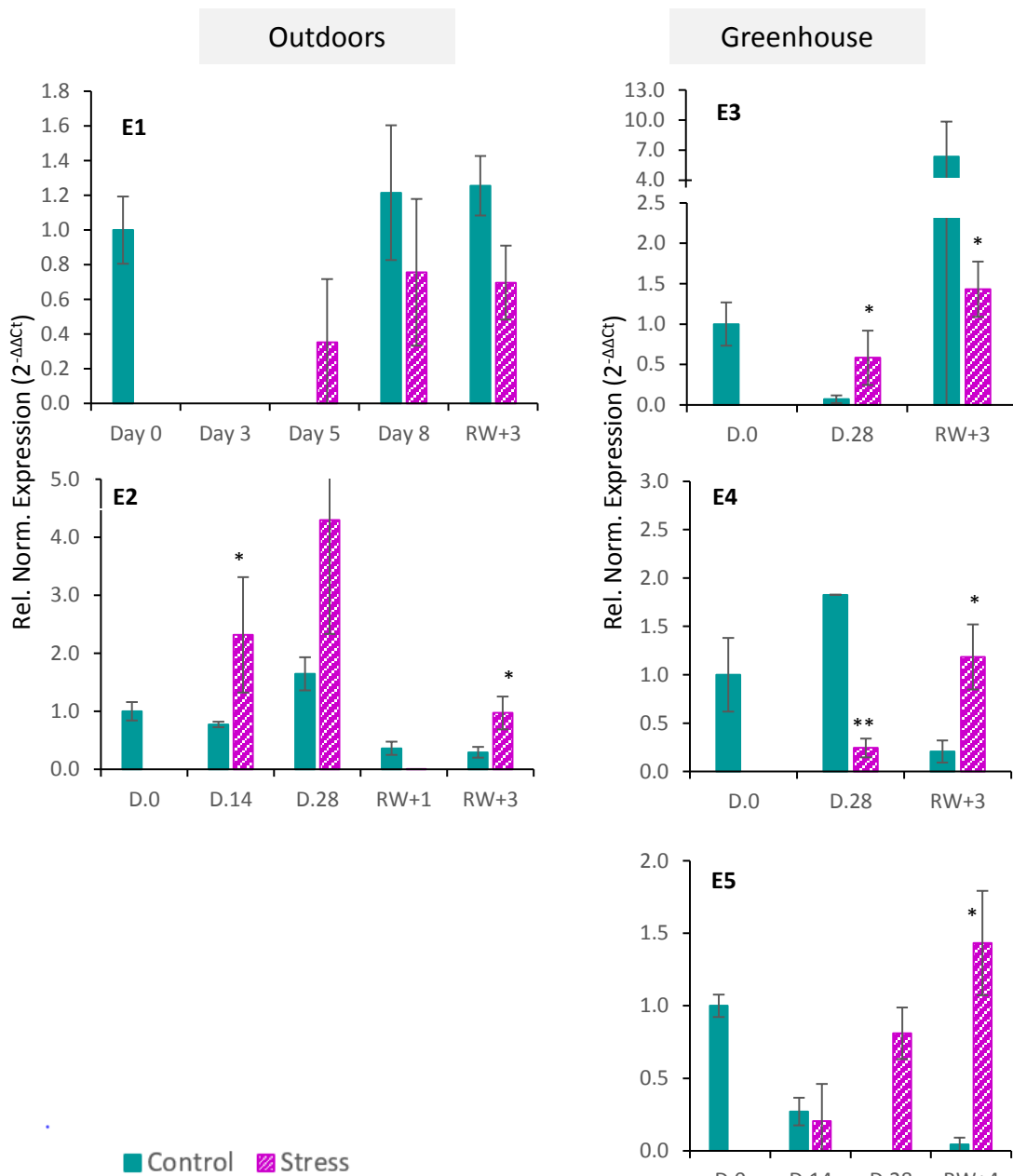
Figure 5.58. Gene expression for the glutathione peroxidase (*GPX*), during drought conditions. Displayed values represent the relative normalized expression of the gene ($2^{-\Delta\Delta Ct}$) for each experiment. E1= sunny conditions, E2= shadow conditions, E3= high light chamber, E4= low light chamber, E5= high light chamber + fertilization. Student T-test significance of $p < 0.05$ (*), $p < 0.001$ (**) and $p < 0.0001$ (***).

5.5.6. qPCR analysis for *PrxQ*

The gene consist only in 642 amino acids coded in 4 exons. Exact evaluation of the gene was not possible because the gDNA sequence in the database for Jcr4S00136.50, do not corresponded to its reported coding sequence; verification was done with sequence alignment (data not shown). Taking the information of nucleotide sequence and gDNA, a pre-model was constructed (Fig. 5.59). Several options for primers were identified, and after evaluations based on GC content and melting temperature difference, the most similar pair was selected. For this analysis, primers targeting *PrxQ* were placed in a way to amplify two consecutive exons. Forward primer starts in position 434 and is sequenced between two exons (3rd and 4th), while the reverse primer is in the 4th exon in position 528- 509.

```
ATGGCTTCCATTTCTCTACCTAAGCATTCTCTACCTTCTCTCCTTCCTACTCAGAAACCCAGACACTCTCCTTCTC
AAAATCTCCCAATTTTCTCCAAAGCTTCACAATCCCAATTTTATGGCCTCAAGCTCTCTAATTTATCTTCTCTCAC
AATTCCATCTTCTTCTTCTTTGAAGACTACCATTTTTGAAGACTACCATTTTTGCTAAGTTTGCTAAG--------
-------------------- Intron 1-----------------------------------------------
GTGAATAAAGGACAGGTGCCTCCGTCTTTCACATTGAAAGATCAAGATGGGAAAAATGTGAGCCTTTCTAAATTCA
AAGGGAAGCCTGTAGTTGTTTATTTCTACCCAGCTGATGAGACCCCTGGCTGCACCAAACAG-------------
------------------- Intron 2-----------------------------------------------
GCCTGTGCTTTCAGGGATTCTTATGAGAAGTTTAAGAAAGCAGGAGCAGAGGTTATTGGAATCAGTGGTGATGATA
CATCGTCGCACAAT------ Intron 3-----------------------------------------------
-----------------------------------------------------------------------------
GCTTTTGCGAAAAAATATAGACTTCCATTTACATTGCTGAGTGACGAAGGAAATAAAGTAAGAAAAGAGTGGGGCA
TTCCAGGAGATTTATTTGGAGCATTACCTGGAAGACAGACGTATGTTCTTGACAAGAAAGGCGTGGTTCAACTCAT
CTACAACAACCAGTTCCAACCCGAAAAGCATATTGATGAAACCCTGAAACTACTTCAAAGCCTTTGA
```

Figure 5.59. Pre-gene model for *Jc_PrxQ* and qPCR primers location. Detail of gDNA sequence, including all exons There was no gDNA sequence to compare, therefore intron sequences are unknown.

This primer pair was tested RT- PCR and qRT-PCR. The results of this test confirm that the designed primer pair has a high specificity for the targeted area (Fig. 5.60). The electrophoresis shows a main band at the correct size, and the other bands are faint and of bigger sizes that might not be amplified during the qPCR program. Melt curve during qPCR support this evaluation.

Figure 5.60. qPCR primer test for *Jc_PrxQ*. (a, b, c) represent different samples after amplification by RT-PCR and electrophoresis on agarose gel 3% (left). Samples were amplified by qPCR to observe it melt curve (right).

Transcript levels on stressed plants for *PrxQ* during mild stress were assessed for E2, which shows a small but statistically significant upregulation while in E5 remains equal to controls.

At maximum stress, there is a trend in upregulation in "stable and control environments" (E2 and E3), while there was downregulation under highly stressed conditions (E1 and E4). With the re-watering, stressed plants appear to upregulate *PrxQ* in E2, E4 and E5, though in E1 and E3 (with the highest irradiance) was downregulated (Fig. 5.61).

Figure 5.61. Gene expression for the peroxiredoxin Q (*PrxQ*), during drought conditions. Displayed values represent the relative normalized expression of the gene ($2^{-\Delta\Delta Ct}$) for each experiment. E1= sunny conditions, E2= shadow conditions, E3= high light chamber, E4= low light chamber, E5= high light chamber + fertilization. Student T-test significance of $p < 0.05$ (*), $p < 0.001$ (**) and $p < 0.0001$ (***).

Samples for day three E1, suffered degradation and were not possible to analyze. After five days of stress, the total transcript level for *PrxQ* was reduced from day zero (Fig. 5.61). At maximum stress and after re-watering, stressed plants had a lower total expression than in controls, but it was never significant. Experiment 2 presents a steady increase in *PrxQ* transcript level through dry period. After 14 days, stressed plants had significantly higher transcript levels than controls p= (0.05); this increase reached an average of 4.30 at maximum stress, but difference between samples mask the significance of the difference to control group. Re-hydratation reduced total expression values, but after three days, values of pre-stressed plants were still higher than controls (p=0.04). Under mild stress, stressed plants of experiment 5 did not show different gene expression compared to controls on same day. At the end of dry period, is not possible to compare expression of *PrxQ* to controls of the same day, but when compared to previous total values, there was an increase. If compared values of stressed plants on day 28 with day 0, there was a maintenance in total values, assuming stability. Re-watering appears to present higher values for treated plants (p=0.016), though control values were strangely low at this point.

Experiment 3 shows increased transcript levels of *PrxQ* for plants under drought stress after 28 days (p=0.03), coinciding with behavior of stressed plants in E2 and E5. Values after re-watering appear to present a drastic reduction in transcript levels (p=0.07), but levels for control are unexpectedly high and included a large internal variation. Both groups had n=5 at RW+3, but while values of treated plants were in the range of 0.8-1.7, those for controls included two low values (0.4-1.1) and three times values around 8, which is highly unlikely.

Experiment 4 presents an opposite behavior to E3, with very low transcript levels after 28 days of drought (p=0.001) and a drastic increase after three days of re-hydration (p=0.03). Representativity of these values can found objection on the sample number for each point, since control group is represented only by two biological replicates. *PrxQ* expression during high levels of stress in E4 could also be compared to the one observed in E1, and since those experiments had the hardest condition, these results might indicate that under high levels of stress and unfavorable conditions, there is a different regulation in gene expression.

5.5.7. qPCR analysis for *CAT*

After the evaluation of all predicted sequences in databases, two sequences were selected to continue working with. Nucleotide sequence reported for each gene was divided using the model prediction. One set of qPCR primers was selected for CAT1 (Fig. 5.62) and CAT2 (Fig. 5.63), using Jcr4S01159.40 and Jcr4S01023.80 as models, respectively.

```
ATGCTGATCATACAGAACCGTCCTTCAAGCGCTTTTGATACGCCCTTTTGGACCACGAAT----------------
---------------------- Intron 1-------------------------------------------
TCCGGTGCTCCAATATGGAACAATAACTCTTCCATGACTGTTGGACAAAGAGGTCCAATCCTTCTTGAGGACTATC
ATTTGATAGAGAAACTTG----- Intron 2-------------------------------------------
-------------------------------------------------------------------------
CCAACTTTGATAGGGAGCGTATTCCAGAGCGTGTTGTCCATGCTAGGGGAGCAAGTGCAAAGGGTTTC--------
---------------------- Intron 3-------------------------------------------
TTTGAGGTGACCCATGATATCTCTAACCTAACATGTGCTGATTTCCTTCGAGCTCCTGGAGTTCAGACGCCTGTCA
TTGTCCGTTTCTCC--------- Intron 4-------------------------------------------
ACTGTTATTCATGAGCGTGGCAGTCCAGAAACCCTGAGGGATCCTCGAGGTTTTGCAGTGAAGTTTTACACCAGAG
AGGGCAACTTTGATCTCGTGGGAAATAATTTCCCTGTATTTTTCATCCGTGATGGAATGAAATTCCCAGATTTGGT
ACATGCTTTTAAGCCCAACCCTAAATCTCACATCCAAGAGAGCTGGAGGATCTTTGACTTCTTATCCCATGTCCCT
GAGAGCTTGCACATGCTTACCTTCCTCTTTGATGATTTGGGTATTCCACAAGATTACAGGCACATGGAAGGCTCAG
GTGTTAACACCTATACTCTAATAAACAAGGCCGGAAAGGTACACTATGTGAAGTTTCACTGGAAACCGACTTGTGG
TGTGAAGTGTCTGTTGGATGAAGAGGCAATCAAGGTTGGAGGAACCAATCATAGCCACGCAACCCAGGATCTATAT
GATTCAATTGCAGCTGGAAATTATCCAGAGTGGAAGCTTTATATCCAGACCATGGATCCAGATCATGAAGACAGAT
TTGACTTTGATCCACTTGATGTGACCAAGACCTGGCCTGAGGTATCCTGCCCCTGCATCCAGTTGGTCGCTTGGT
CTTGAATAAGAACATAGATAATTTCTTTGCTGAGAATGAGATGCTTGCATTCTGCCCTGGT**ATTGTTGTTCCTGGT**
**GTTTC**ATATTCAGAGGATAAGTTACTCCAGACTAGGATCTTTTCCTACTCTGATACTCAGAGGCACCGTCTTGGAC
CTAACTAT**CTACAGCTC**------------------------------------------------------
--------------------- Intron 5-------------------------------------------
**CCTGTTAATG**CTCCCAAGTGTGCTCATCATAACAATCACCATGAAGGTTTCATGAATTTCATGCATAGGGATGAGG
AGGTCAATTACTTCCCGTCCAGATACGATCCGGTTCGCCATTCTGAGGCGTTCCCCTTTCCGGCCACTATATGCAG
TGGAAAGCGTGAGAAGTGTGTGATACCAAAGGAGAACAATTTCAAGCAACCTGGAGAGAGATATCGATCCTGGGCT
CCTGACAGGCAAGAGCGATTCCTCAATCGGATTGTTGGTTGCTTATCTGATCCTCGTGTCACACATGAGATTTGTA
GTATTTGGATCAACTACTTTACTCAGTGCGACAGATCTCTGGGTCAGAAGCTAGCGTCTCGTCTGAACGTGAAGCC
AAGCATTTGA
```

Figure 5.62. Pre-gene model for Jc_CAT1 and qPCR primers location. Detail of all exons with primer positions marked in **red letters**. There was no detail on intron sequences for this gene.

For CAT1 homologue (Jcr4S01159.40), primers were situated at the end of the 5th exon. Forward primer start at position 982 and reverse primer is situated in position 1099-1081 in the cDNA, but its separated by one intron.

Both primer sets were tested with a regular PCR first (Fig 5.64), and product was loaded in a gel and separated through electrophoresis. Primers for gene CAT1 (Jcr4S01159.40) produced three very distinctive bands at the same size for three samples out of four. Those primers were designed to amplify a region of 120 base pairs, but the band at this position was the faintest one; strongest band for those primers was over 500 bp.

```
ATGGATCCTTACAAG------------------------------------------------------------
------------------------------------- Intron 1-------------------------------
TTTCGTCCATCAAGTGCTAACAATTCTCCTTTCTTCACCACAAACTCTGGTGCTCCTGTATGGAACAACAACAATT
CCCTCACTGTTGGATCCAGAG----------------- Intron 2-------------------------------
GTCCAATTCTGCTTGAGGACTATCATTTGGTGGAAAAGCTAGCAAATTTTGACAGAGAGCGAATTCCAGAGCGTGT
AGTTCATGCTAGAGGAGCCAGTGCTAAAGGGTTCTTTGAAGTTACTCATGACATCTCTCATCTCACTTGTGCGGAT
TTTCTTCGAGCACCTGGTGTTCAGACTCCTGTTATTGTCAGGTTTTCTACTGTTATTCATGAGCGTGGCAGTCCTG
AAACTCTTAGAGACCCTCGTGGTTTTGCTGTCAAGTTTTACACTAGAGAG
------------------------------------- Intron 3-------------------------------
GGTAATTTTGATCTCGTGGGCAACAATTTCCCAGTCTTTTTCATTCGTGATGGAATGAAGTTTCCTGATATGGTTC
ATGCTTTGAAACCAAACCCCAAGTCACACATTCAAGAAAGCTGGAGGATACTTGACTTCTTCTCACACCACCCCGA
AAGTTTGAACATGTTCACCTTTCTCCTTGATGATATTGGTATTCCTCAGGATTACAGGCACATGGATGGTTCTGGT
GTTAACACGTACACTTTAATCAACAAGGCTGGAAAAGCACATTATGTCAAATTCCATTGGAAACCAACTTGTGGGG
TTAAGAGTTTGTTGGAAGATGATGCCATAAGAGTTGGTGGCGCAAACCACAGTCACGCCACTCAGGATCTCTATGA
CTCAATTGCTGCTGGAAACTACCCTGAATGGAAGCTCTTTATTCAGATAATTGATCCTCTTGATGAAGATAAGTTT
GACTTTGATCCACTTGATGTGACTAAGACTTGGCCTGAAGATATCTTGCCATTGCAGCCTGTTGGTCGGATGGTTT
TGAATAGGAATATTGATAACTTCTTTGCTGAGAATGAACAACTTGCATTTTGTCCTGCTATTATTGTGCCTGGTGT
CTATTATTCAGATGATAAGTTGTTGCAGACTAGAATTTTCTCTTATTCTGATACCCAAAGGCACCGTCTTGGACCA
AATTATTTGCAACTTCCACCAAATGCACCCAAATGTGCTCATCACAACAATCACCATGAAGGTTTCATGAACTTTA
TGCATAGGGATGAGGAG-------------------- Intron 4-------------------------------
GTCAATTACTTCCCTTCGAGGTATGATCCTGTTCGTCATGCTGAGAAGTATCCTATCCCTCCTCCTGTTTTAACTG
GAAGGCGTGAAAAG---------------------- Intron 5-------------------------------
ACTGTAATTCCAAAGGAGAACAACTTCAAACAACCTGGAGAAAGATACCGCTCCTTTACACCTGACAG--------
------------------------------------- Intron 6-------------------------------
GAAAGACCGGTTTATACGACGTTGGGTTGAGGCCTTGTCTGACCCAAGAGTCACCTATGAAATTCGCAGCATTTGG
ATCTCATACTGGTCTCAGGCTGACAAATCTTTGGGTCAGAAAATTGCCTCTCACCTCAACATGAGGCCAACCATGT
GA
```

Figure 5.63. Pre-gene model for Jc_CAT2 and qPCR primers location. Detail of all exons with primer positions marked in **red letters**. There was no detail on intron sequences for this gene.

Primers for gene CAT2 (Jcr4S01023.80) amplified one main band at the expected size (119bp). In two samples it was possible to observe a band at about 300bp and some more over 600bp, but they were faint when compared to the first band. It is not possible to determine the reason behind those un-specificity.
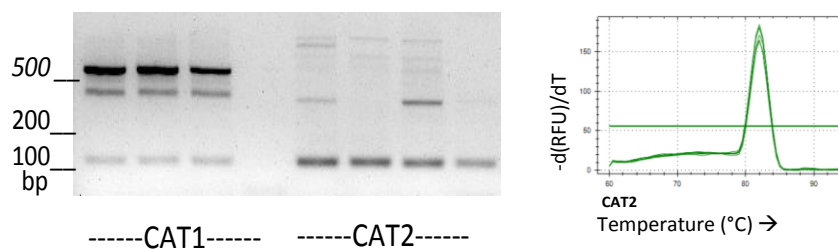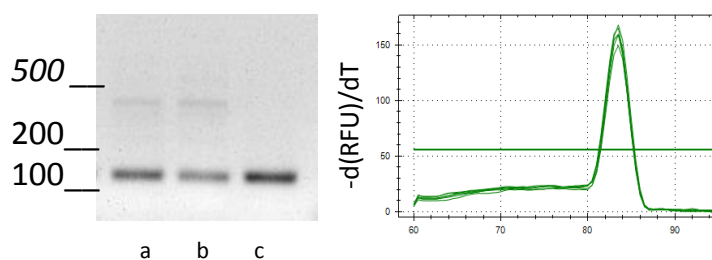

Figure 5.64. qPCR Primer test for catalase genes. (a) Agarose gel 3% + 0.02% ethidium bromide. Numbers on top represent different sample number. (b) melting curve in qPCR.

Melting curve showed that CAT2 primers had stable amplification (Fig. 5.64). Decision was to keep primers for CAT2, discarding the other primer set to avoid unspecific binding.

Transcript levels for catalase appeared in general to get reduced through the dry period, been statistically less in stressed plants at maximum stress in both outdoor experiments and keeping the trend in E3 and E4. After re-watering, there is upregulation in E3 and similar trend in E5, while the other experiments had similar values to control plants, even a reduction trend in E4 (Fig. 5.65).

For experiment 1, there was a reduction in values after three days of drought, that was shaded by the high variation in treated group: two values were lower than controls, and one was higher; for this sampling moment there were used n=3. At the maximum stress point, treated plants had significantly less transcripts than controls (p= 0.01), and after re-watering, both groups had similar values. Experiment 2 shows similar values for control and stressed plants after 14 days drought, with total values higher than on day 0. After 28 days drought, *CAT* expression in stressed plants was significantly lower than in controls (p=0.02). This regulation was erased after three days of re-hydration.

For the experiments indoors without fertilization, behavior under stress was similar. E3 presents reduction in transcript levels after 4 weeks of drought, but significance got shaded by the high variation between samples in both groups. After soil re-hydration, transcript levels for treated plants increased to values similar to controls on day 0 and day 14, but since control on the same day was very low, there is a significance of p=0.004. In experiment 4, there was a difference of 10X between values of controls and stressed plants, but it did not reach statistical significance due to high variability between samples in the control group. Soil re-hydration did not modify the regulation of previously drought stressed plants, while the variation in control group was still large. High variation in control group might indicate that not all the individuals are experiencing the atmospheric conditions in the same way, and that probably, some of them are also facing some sort of stress.
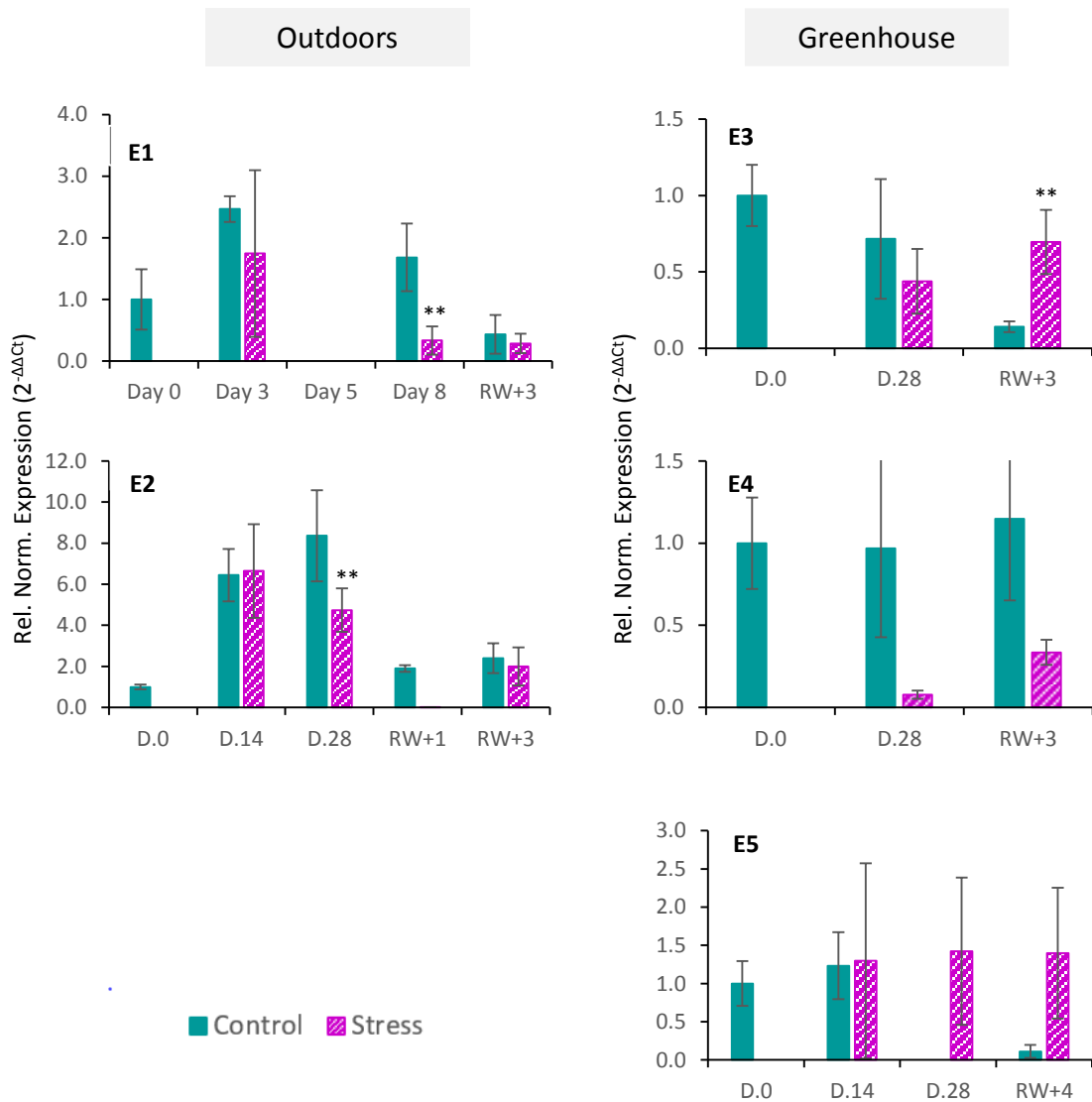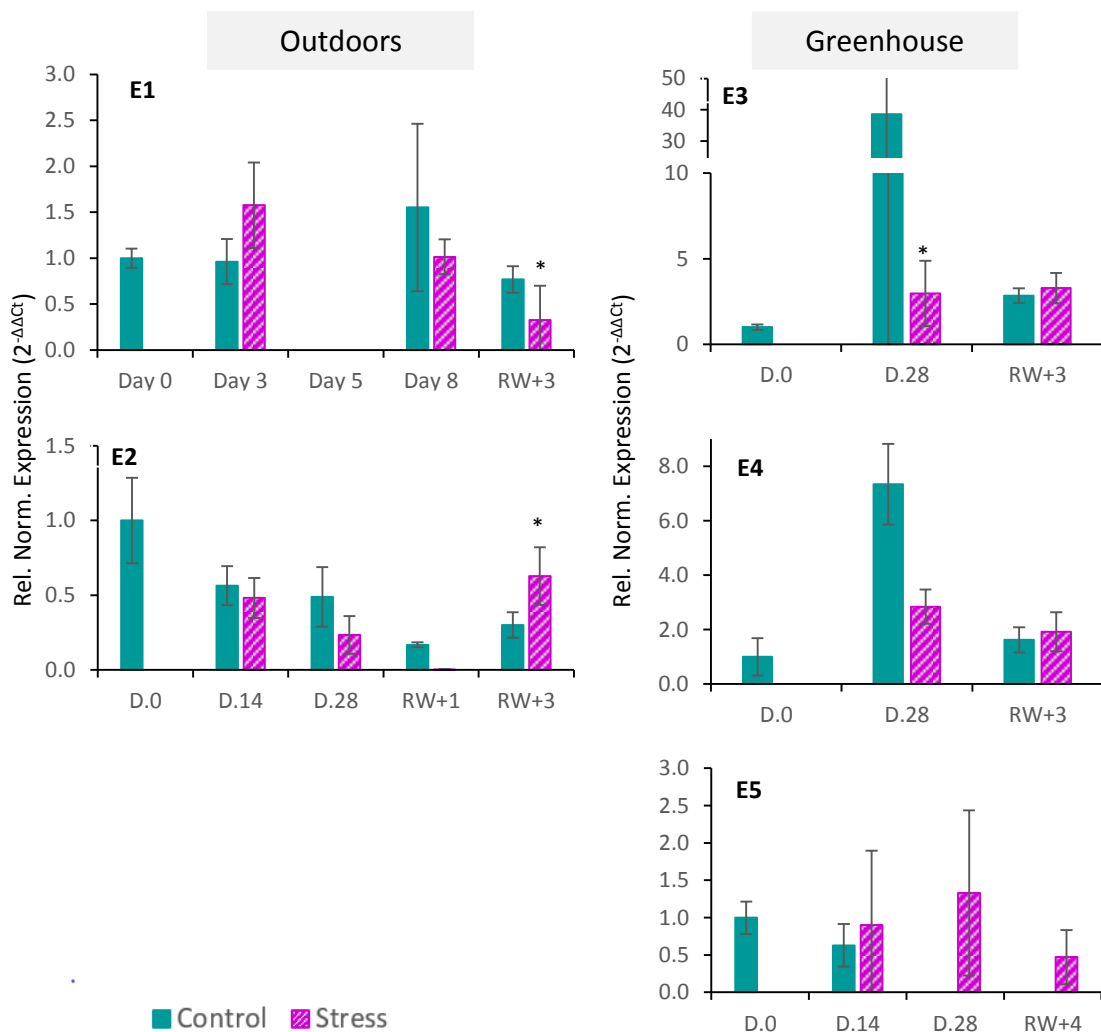
Figure 5.65. Gene expression for the catalase gene (*CAT*), during drought conditions. Displayed values represent the relative normalized expression of the gene (2 $^{-\Delta\Delta Ct}$) for each experiment. E1= sunny conditions, E2= shadow conditions, E3= high light chamber, E4= low light chamber, E5= high light chamber + fertilization. Student T-test significance of p <0.05 (*), p< 0.001 (**) and p < 0.0001 (***).

Evaluation of results for experiment 5 (E5, Fig. 5.65) are harder to decipher. After two weeks of drought, there is no difference in *CAT* transcription levels between treatments, but in detail, high variation in stressed plants cover lower values than the control (0.4- 0.7), and one sample with a value of 2.76. At the end of the dry period, transcript levels for stressed plants cannot be compared to controls on the same day due to sample degradation; however, if compared to previous values of stressed plants or to day zero, values appear to stay stable. After 4 days of re-watering there is no modification in gene regulation from the beginning, though expression in stressed plants is highly variable. This is the only case where there is no difference in CAT regulation through a dry period.

5.5.8. qPCR analysis for *SOD*

For comparison of gene expression, qPCR primers were designed using the nucleotide sequence reported for Jcr4S02704.30 as reference. Using the gene model proposed in the database, a pre-model for this coding sequence was designed (Fig. 5.66). One pair of primers was selected out of ten options. Forward primer starts at position 308, at the beginning of exon 2, and the reverse primer is situated covering positions 407-425, in exon 4. The correct amplification will be 119 bp.

```
>Jcr4S02704.30_SOD2
ATGCAAGCAGCAGCTGCTGTAGCAGCCATGGCTGCTCATACAATCCTTGCAGCCTCTCCATCCTCTCATCCTCTCC
TCTATCCATTCCCAAACCCTATCCTTTCCCACTCTTCACCTCTGCATTCCTCATTCCATGGTGTTTCTCTCAAACT
TCCTCGTCAATCGCTACCTCTTTCTCTTACCACCGCCGCCGCCCCAAAAAAACCCCTTGCTGTCGTTGCTGCTACC
AAAAAGGCCGTCGCTGTCCTTAAGGGTACCTCCAATGTTGAAGGCGTTGTCACTTTGACCCAGGAAGACGATG---
----------------------------------- Intron 1----------------------------
GTCCCACAACAGTGAATGTTCGTGTCACTGGCCTTACTCCAGGGCCTCATGGATTCCACCTA-------------
----------------------------------- Intron 2----------------------------
CATGAGTATGGTGACACAACAAATGGATGCATTTCTACAG--------------------------------
----------------------------------- Intron 3----------------------------
GAGCTCATTTCAATCCGAACAACAAGACACATGGTGCTCCTGAAGATGAAATCCGTCATGCGGGTGACCTGGGAAA
CATAGTTGCTAACGCTGATG----------------- Intron 4----------------------------
GGGTGGCAGAAGCAACAATTGTGGATAACCAG------ Intron 5----------------------------
----------------------------------------------------------------------
ATACCATTGAGTGGTCCAAATGCAGTAGTCGGAAGAGCACTTGTTGTCCATGAGCTTGAAGATGACCTTGGAAAGG
----------------------------------- Intron 6----------------------------
GTGGGCATGAACTTAGTTTGACCACTGGCAATGCAGGTGGAAGATTGGCTTGTG--------------------
----------------------------------- Intron 7----------------------------
GGTTGTTGGCAATGTGTAAAGCGGCTTGGTTATCACAATGCAAGGAAATGGGAAGTGAAGTAGATACCTAA---
```

Figure 5.66. Pre-gene model for Jc_SOD Cu/Zn, including qPCR primer location. Detail of all exons with primer positions marked in **red letters**. There was no detail on intron sequences for this gene.

Primers were tested with three samples from the same experiment and presented similar results (Fig. 5.67). There is one main band at the expected size (119 bp). One slightly visible band appears close to 500 bp, but as it represents such a small amount of fragment amplification, this fact was ignored. Melting curve showed a constant amplification, with only one peak, validating the quality of the primer.

Figure 5.67. qPCR primer test for superoxide dismutase gene. Agarose gel 3% + 0.02% ethidium bromide. Numbers on top represent different sample number.

Transcript levels of SOD in the experiments performed showed similar values in during mild stress with a small trend in the increase in E1 and E5, while on maximum stress the trend was mostly to the reduction, with E3 with a significant reduction when compared to controls. Re-watering produced downregulation in previously stressed plants in E1, but upregulation in E2; the other experiments presented no difference from controls, without even a trend (Fig. 5.68).

Figure 5.68. Gene expression for the superoxide dismutase (*SOD*), during drought conditions. Displayed values represent the relative normalized expression of the gene ($2^{-\Delta\Delta Ct}$) for each experiment. E1= sunny conditions, E2= shadow conditions, E3= high light chamber, E4= low light chamber, E5= high light chamber + fertilization. Student T-test significance of $p < 0.05$ (*), $p < 0.001$ (**) and $p < 0.0001$ (***).

For experiment 1, there was an increase in transcript levels for stressed plants after three days. It was not possible to evaluate what happens after 5 days, but after 8 days there was a reduction in SOD values for stressed plants. Re-watering does not modify the regulation way selected during stress, spotting significantly lower levels for treated plants (p=0.09). It is not sure if the result for 3 days of stress in experiment 1 was possible to observe due to timing,

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but in E2, plants under mild stress (day 14) showed a reduction in transcript levels compared with controls. This reduction was maintained all along the dry period, but never reaching statistical significance. With three days of soil re-watering, treated plants presented more transcripts than controls (p=0.08). For experiment 3, regulation was similar to the one observed in E1. After 28 drought stress, treated plants had lower transcript levels than controls (p=0.03), even after the incredibly high variation in control group. This variation included 2x values around 0.6 and 2x values of 80, so it was impossible to determine which direction was correct. Re-watering changed the regulation of the gene, causing similar transcript levels for treated plants and controls. Statistical similarity was again a product of variation in control group.

For indoor experiment under shadow, gene regulation was alike to E3. Without so high variation in control groups, it is possible to observe a trend in stressed plants of transcript level reduction at the end of dry period. Re-watering rehearse plants, and both control and treated plants had comparable levels for SOD.

In the case of E5, an experiment inside a greenhouse, with enough light available and soil fertilization, gene regulation was not possible to asses due to degradation of control samples. During mild stress (day 14), there is a slight increase in SOD values for plants under stress. Stressed samples on day 28 could not be compared to a control but are similar to those of day 14 and day 0. After three days of soil re-watering, treated plants appear to continue having same levels of SOD.

Chapter 6
Final Discussion

Drought experiments performed in Panama for *Swietenia* and *Jatropha*, allowed to observe the responses of those species to the natural conditions. The experiments in the greenhouse in Berlin for *Jatropha* permitted to differentiate under controlled conditions, the response of plants to drought under various settings including light intensity and fertilization.

6.1 Physiological parameters

The physiological parameters used to determine drought avoidance methods show high similarity in both species under the same conditions, based on the general trends, adapted to the size and biomass of each plant. *Swietenia macrophylla* lost 57 – 62% of the soil water content (WC) during sunny experiments and 54% under shadow (Fig. 4.11); this difference indicates that there were different environmental conditions.

At maximum stress, *Jatropha* lost in outdoor experiments 68% of soil the WC in full sun, but only 50% under shadow (Fig. 5.36). The comparison with *S. macrophylla* indicates that *Jatropha* used less water during shadow conditions, using probably the canopy to protect soil from direct light incidence and evaporation, while *Swietenia* was using the same amount of water under all conditions.

Roman *et al.* (2012) reported that *Swietenia* has a small growth rate during the juvenile phase in natural conditions and Hall & Ashton (2016) described young trees of *Swietenia* with smaller trunks and slimmer canopy diameter than other species. In this study, it was not possible to detect differences in seedling size after any drought experiment. The seedling never stopped vertical growth: stressed ones decrease the pace slightly. *Jatropha*, in contrary, a reduction in total shoot size was observed in all experiments and growth arrest was determined after seven days in high light experiments, or after 14 days in the lower light experiment. The growth of *Jatropha* was limited by low, stable environments (outdoor experiments) and by light intensity; the most limiting conditions were found in E1 and E4. Arrest in stem growth during drought was also observed by Sapeta *et al.* (2013) in seedlings of the same age of two different accessions submitted to drought, by Maes *et al.* (2009) and Aachten *et al.* (2010) analyzing 9-weeks old seedlings in greenhouse conditions under elevated CO_2 and drought, and also Pompelli *et al.* (2010), working with11-month- old plants in a glasshouse with solar

illumination in Brazil. Transcriptomic analysis (Sapeta *et al.*, 2016) revealed that the GO terms associated to growth were downregulated in leaves and roots under severe drought stress, including genes for cell wall organization and biosynthesis, and also genes for the cellulose biosynthesis and metabolism.

Changes in fresh biomass were also difficult to observe in *Swietenia* (Fig. 4.15). In *Jatropha*, the fresh biomass differed from the first week onwards in all experiments that included this measurement (Fig. 5.39), also in agreement with Sapeta *et al.* (2013). The difference in FW was due to water content, since there was no difference during mild stress in biomass DW for most of the experiments, only at maximum stress. The results of WC in leaves and stems in both species indicates diverse mechanisms (Fig. 4.16 and 5.40). In *Swietenia* and *Jatropha* experiments under the hardest conditions (E1 for both), no difference in leaf WC was found between control and stressed plants. But while for the most extended experiments in *Swietenia* a significant reduction was observed in the relative WC in leaves at the maximum stress stage, in *Jatropha* was observed a reduction during mild stress that presented a trend in the increase in E2, and that evidenced plants at maximum stress point in E3 and E4 with similar WC than in controls. This result coincided with Maes et al. (2009), Silva *et al.* (2010a) and Sapeta *et al.* (2013 and 2016), which suggest either an active accumulation of water due to solute concentration, or a redistribution of the existing water in different organs, shading some leaves (e.g.) to decrease the total area of biomass.

For *Swietenia*, the WC in stems turned out to be a better indicator of stress than that of leaves. In this study (Fig. 4.17), a significant reduction in relative WC in stems was found at maximum stress in E1 and E3 (full sun) and since mild stress in E2 and E4 (lower light levels). In *Jatropha* (Fig. 5.41), a similar trend as for leaves is observed in E2 and E3 (probably enough light intensity and more stable conditions): there is a significant reduction in the relative WC for stems and petioles during mild stress that progressively increased, finishing in even higher values in stressed plants than in controls. A reduction in the relative water content in stems was found only on stressed plants in E1 and E4, that is related to the degree of fresh and dry biomass. While in E2 and E3 there was about 85 – 90% stem WC for controls, controls in E1 and E4 had 50% and 80% stem WC, respectively. Similar behavior in water accumulation was observed by Maes *et al.* (2009) while Aachten *et al.* (2010) found no difference in stem density after drought stress.

The determination of leaf area is one of the most prominent evaluations in many stress experiments because it is directly related to photosynthesis capacity and stomata conductance (Wu *et al.*, 2018; Lindroth *et al.*, 2008; Doughty and Goulden, 2008). In *Swietenia* (Fig. 4.18), there was not found any statistical difference or trend at the end of experiments, in any of the experiments performed. This apparent insensitivity of leaf area regulation lies mostly on the standard growth rate, very small even in control plants as observed in all experiments. In *Jatropha*, contrarily, there is a visible arrest in leaf expansion due to drought directly after the first week (Fig. 5.42); the degree of growth arrest in treated plants was affected in general by environmental conditions and stability. In outdoor experiments, control plants in E2 (under shadow) produced almost 2X more leaf area than E1 (at the full sun) after one week, indicating plant's effort for access to more light.

In one-month-long experiments, controls in E3 (indoor and high light) produced 2331 cm^2 of leaves; in the same time, leaf areas for control plants in not so optimal conditions produced less leaf area, -18% in E4 (lower light intensity) and -23% in E2 (outdoors). Stressed plants had significantly less leaf area than controls from the first week until the end of measurements, corresponding to a reduction of -30%, -72%, -73% and -68%, for E1, E2 E3 and E4 respectively, at the end of the experiments. This difference corresponded to approx. 3.5X more production in controls than in stressed plants. Silva et al. (2010a) found similar results in *Jatropha* seedlings under salinity and PEG treatments, while Aachten *et al.* (2010) and Maes *et al.* (2009) found 7X less leaf area in 9 weeks-old stressed plants under higher CO_2 concentration and similar temperature and humidity, using a model for canopy estimation. Stressed plants were affected more by the variable conditions in E2 than by the extremely reduced light availability in E4, with -32% and 5% less leaf area (respectively) than in the stressed plants of E3.

One parameter that has not been evaluated so far in other studies is the petiole length in *Jatropha*. Petiole length variations in response of light intensity is a well described mechanism in plants, proving a worthy shade-avoidance strategy in *Arabidopsis* (Robson et al, 1993 and red mangrove (Sinoquet *et al.,* 2005), *Liriodendrom* (Niinemets *et al.*, 2002) and Adenocaulon (Pearcy and Yang, 1998), though Niklas (1992) considered it metabolically a non-economical option for growth in *Aesculus*. During the establishment tests in the greenhouse in Berlin, it was observed that the petiole sizes differed with the light intensity that the plant received; therefore, this parameter was used in *Jatropha* as an indicator of light intensity effects.

In the experiments performed (Fig. 5.43), it was possible to observe a variation after seven days in E3, while under reduced light experiments E2 and E4, petioles of stressed plants grew similarly to controls until shortly before day 14, assumingly competing for light. Petioles were steadily elongating in control plants during the experiments, reaching the longest ones in E4 (19.9 cm), apparently reaching for more light to cope with the photosynthesis without water restriction, followed by E2 (17.9 cm) and E3 (17.7cm). Stressed plants presented petiole length in the same order as controls with 14.5 cm, 13.6 cm and 13.3 cm, respectively.

- **Photosystem II activity**

All these physical evaluations showing the effects of drought on *Swietenia* and *Jatropha* plants, and the different mechanism to survive under these circumstances are closely related to the electron transport inside the chloroplasts. Their photosynthesis activity can assess the level of stress; this activity can be measured in the form of quantum yield of the photosystem II (PSII) with a controlled excitation of the chlorophylls. This type of measurement is a non-invasive technique commonly used in plant physiology (Murchie and Lawson, 2013). During favorable conditions and an intact photosystem, the quantum yield will be high due to the excitation of the centers, but under severe drought stress the photosynthesis is inhibited due to (e.g.) protein degradation, quantified as a low quantum yield.

The values for quantum yield in the Photosystem II were included since they denote the electron transport and can indicate the level of degradation in the proteins with a central role in the photosystems, and this degradation might happen in the presence of elevated ROS concentrations in the chloroplast.

The status of the photosystem II (PSII) was evaluated by the effective and maximum quantum yield in both species. *Swietenia* presented astonishing high levels for both parameters along the drought period and re-watering, with no differences between control and stressed plants (Fig. 4.19). These levels were unexpected for a species considered not tolerant to drought, demonstrating that there is another type of mechanism protecting its photosynthetic activity. *Jatropha* had a significant reduction in both parameters at maximum stress point for all experiments (Fig. 5.44); though ΔF/Fm' was already reduced at mild stress (d.14 in E3 and E5 -high light intensity, and d.21 in E4 -low light intensity). Fv/Fm was reduced even after 21 days in E3 and E4, the only experiments that included this measurement point. These results are in concordance with Sapeta et al. (2013 and 2016), though the values of fluorescence that they

present (0.6 for controls and 0.15 at maximum stress) were a lot lower than the ones observed in this study, where the minimum average value recorded was > 0.4. Silva et al. (2010a) in the other hand, reported no difference in Fv/Fm or ΔF/Fm' after salinity or PEG stress (0.79), but plants on the 3-weeks-old seedlings submitted to 5 days drought, values for effective quantum yield were reduced from 0.7 in controls to 0.2 in treated plants (Silva et al., 2010b). In another study with two species with different tolerances to drought (*Jatropha* and *Ricinus*), Lima Neto et al. (2017) also used 6-weeks old plants and submitted them to 6 days of drought, and while finding no difference in Fv/Fm after six days of stress in *Jatropha* (values of 0.8), ΔF/Fm' was reduced in both species at the end of the experiment. At this point, control plants had values of 0.3 while stressed ones were about 0.12. These shallow values of effective quantum yield compared to the ones of this study might be related to the high irradiation program (1800 μmol photon m^{-2} s^{-1}) used in the evaluation of Lima Neto et al. (2017).

In general, its observed that during the entire drought process, both plants maintained the PSII protected, since high levels of maximum quantum yield were observed in almost all experiments. Only in *Jatropha*, one experiment presented a significant reduction of PSII quantum yield during light and dark-adapted leaves, while in all the other experiments, even at high stress levels, the quantum yields for drought treated plants maintained incredibly high values.

- **Stomatal activity**

The measurement of PSI integrity by chlorophyll fluorescence calculations can be compared with the levels of CO_2 assimilation in plants, to indicate the real activity of photosynthesis during the droughting process. Photosynthesis also needs to be evaluated in function of the stomatal water conductance, to observe the balance of CO_2 intake (by stomata opening) and assimilation.

Net photosynthesis (CO_2 assimilation rate) in *Jatropha* was monitored only for the experiments performed in Panama (Fig. 5.45). In them, it was observed a rapid reduction of this parameter in E1, from control values of 7-15 μmol CO_2 m^{-2} s^{-1}, until stressed values of 0.1 μmol CO_2 m^{-2} s^{-1} in eight days of drought. Stomatal conductance was maintained very low during the entire period, considering probably the starting value as "mild stress" . A fast response in the reduction of net photosynthesis was also observed in plants of the same age under elevated irradiance and CO_2 concentration (Lima Neto et al., 2017) and under drought,

heat and combined stress (Silva et al., 2010b) though in this last case, seedlings were 3 weeks old. Silva 2010b also reported a reduction in stomata conductance from 0.14 (controls) to 0.04 (stress).

Under shadow (E2), experiment starts with an assimilation rate of aprox. 12 CO_2 m^{-2} s^{-1}. Due to environmental conditions, controls increase after seven days to 18 while stressed plants regulate the process to 6 CO_2 m^{-2} s^{-1}. Stomata appeared closed after 14 days according to the transpiration measurements, but incipient photosynthesis was maintained for two weeks more before reaching values of 0.05 CO_2 m^{-2} s^{-1}. One day after re-hydration of soils, water conductance was slightly increased while the photosynthesis rate reaches control values already, and three days after, while stomata regulation was similar to controls, the CO_2 assimilation of previously stressed plants outdoes that of controls. Pompelli et al., (2010) also reported such a fast response (after four days) in 11- month-old plants in a 28-days drought experiment. Sapeta et al. (2013) present similar results, though in their experiments under controlled conditions, the water conductance decrease is progressively lessening while the photosynthetic activity is drastically turned off at maximum stress. The recovery phase with outdoing photosynthesis in previously stressed plants in also observed in seedlings of the same age in after 49 days of drought stress (Sapeta et al., 2016) and in 11-month-old seedlings after 28 days of drought (Pompelli et al., 2010).

In *Swietenia*, photosynthesis (Fig. 4.20) and water conductance (Fig. 4.21) have similar behavior to *Jatropha*, with reduction of both towards maximum stress. In E3, for example, stomatal conductance is virtually stopped, but there is still incipient photosynthesis. Stomata and photosynthesis regulation are coordinated, though it was possible to spot points with very low water conductance and considerable photosynthetic levels (e.g., E3, D.14, 15, 16; E4, D.14). After re-watering, photosynthesis levels returned to average values.

6.2 Gene regulation

As discussed before, under drought stress the CO_2 uptake from the air is restricted during the stomatal closure, which causes increased accumulation of electrons; they cannot be transported and used in the Calvin cycle. The increased accumulation of electrons promotes ROS formation. In the whole cell, but specifically in the chloroplast, there is a system of antioxidant enzymes, that scavenge ROS, and have high regulation to keep ROS levels at non-toxic concentrations. To evaluate how the photosynthetic machinery is kept protected, the transcriptional regulation of the genes from the antioxidant system in the chloroplast were evaluated.

6.2.1.   2-cys-peroxiredoxin (2CP) in both plants

Since the discovery of this gene in the plant kingdom (Baier *et al.*, 1997), the 2-cys-peroxiredoxin enzyme has been reported in the chloroplast and that its regulation, at mRNA and protein level, responds to signals from the photosynthetic electron transport (PET), downstream of PSI (Baier *et al.*, 2004). Shakhali *et al.* (2008) proved that 2CP is regulated by Rap2.4a, a redox-sensitive transcription factor, which binds to the 2CPa promoter region and activates the transcription, conferring redox responsiveness to the gene. The antioxidant activity of 2CP was described in *Arabidopsis*, increasing during moderate stress and decreasing under severe stress conditions, due to this sensitivity to redox balance (Shaikhali *et al.*, 2008); it was suggested that this regulation should be the same in all plants (Baier *et al.*, 2004).

Even though not statistical differences were found in any of the experiments, it is possible to affirm that *Swietenia* showed a "normal" regulation for 2CP (Fig. 4.26). It was observed a trend in upregulation (of up to 3X) for E1, E2 and E3 during mild stress; then, a down regulation during maximum stress in the same three experiments with high levels of irradiance. After one day re-watering, treated plants had similar values to controls and after three days, E1 had an increasing number of transcripts, while E3 kept similar values to controls. In experiment 4 (under shadow), no regulation was observed at any moment.

The activation of 2CP was reported as light-sensitive, with reduced sensitivity in the dark (Shaikhali and Baier, 2010). Since the main difference of E4 with the rest of the experiment was the degree on light intensity, the results of this study indicate that this light-regulation effect might also occur in *Swietenia*. It general, it can be pointed out that under higher light intensities, 2CP regulation in *Swietenia* is very similar to the one reported previously for other

drought-sensitive species, but at lower light intensities, expression of 2CP is not regulated in the same way.

2CP transcript levels in stressed plants of *Jatropha* were similar to controls in all experiments during mild stress (Fig. 5.52). At maximum stress, it was instead observed a significant upregulation in E2 and E5, also found in E3 (n.s.), while it was downregulated in E1 and E4 (including a high standard deviation). Downregulation was associated with the most unfavorable conditions for growth, including the extreme light intensity (E1, the highest and E4, the lowest). Re-hydration showed equal values to control for E1, E3 and E4 with values for treated plants equal to controls, somehow as a product of increase from previous values in E4 (n.s.) but presented significant upregulation in E2 (RW+1 and RW+3), and also in and E5 (n.s.).

During mild stress, only one strategy was observed in stressed *Jatropha* seedlings, but during severe stress, two options could be spotted. The normal downregulation was only observed in E1 and E4 (experiments under the highest and the lowest light intensity, respectively). In experiments with light intensity levels that promoted the growth, 2CP was kept continuously upregulated. This variable regulation could indicate that in favorable conditions, 2CP is expected to engage in ROS detoxification even at high stress levels, but under unfavorable conditions, this task is transferred to another mechanism.

2CP		Mild	Severe	RW
Swietenia	E1	↑	↓	= C
	E2	↑	↓	n.m.
	E3	↑	= (↓)	= C
	E4	= C	=	= C
Jatropha	E1	= C	↓	= C
	E2	= C	↑	↑
	E3	n.m.	= C	= C
	E4	n.m.	↓	= C
	E5	= C	↑	↑

Figure 6.1 Regulation of Jc 2CP during drought. Arrows indicate if the trend indicated upregulation or downregulation. (=C) was the annotation when stressed and control plants had similar transcript levels. (n.m.) indicate not measured for this experiment.

2CP appears to be upregulated during mild stress in only in *Swietenia* (Fig. 6.1). The standard down regulation expected at maximum stress was observed in *Swietenia* under high light intensities, and in *Jatropha* under hostile conditions. *Jatropha* stressed seedlings that were

grown in conditions that promoted its growth maintained 2CP gene upregulated even at maximum stress, which suggest a significant involvement of this enzyme in the redox balance during drought, or that the redox balance in this species, at this point, was not enough for de-activation.

After re-watering, *Swietenia* treated plants met the described regulation, with similar transcript levels to controls, while *Jatropha* experiments with the same behavior were E1, E3 and E4. Surprisingly, there was an active upregulation observed in E2 and E5, which can be related again, to the degree of plant recuperation. E2 was the outdoor experiment with the most stable humidity level and with less direct light incidence, which might have helped in a gradual increment of photosynthesis with more controlled electron transport. E5 had no handicap due to light intensity, and in difference from the other indoor experiments, the soils were fertilized. Including this information, the results suggest that independently for the maximum drought sensed, 2CP is supposed to continue ROS detoxification produced by the previous stress and the increment of photosynthesis after re-water when the recuperation can be guarantied by a controlled electron transport level and an available pool of nutrients.

In summary, these results indicate that *Swietenia*'s regulation for 2CP count as the one described for other species in the complete drought process and that the gene regulation might be light dependent. For *Jatropha*, 2CP presents a higher engagement level in ROS detoxification; its downregulation at severe stress appeared only in the most stressing conditions, but in favorable conditions, it is upregulated even after re-water. This work represents the first information about 2CP regulation in both species to date; moreover, it is the first one addressing gene structure and characterization in *Jatropha*.

6.2.2. Thylakoidal ascorbate peroxidase (*tAPX*) in both plants

The peroxidases are one of the main group of enzymes I charge of the scavenging of H_2O_2 in the cells. In plants, the primary electron donor for the peroxidase activity is ascorbate. Hydrogen peroxide is produced by dismutation of the superoxide radical O_2^- in a reaction mostly catalyzed by Cu/Zn SOD, localized in the stroma. Since catalase (which has the same function) is not found inside the chloroplast, H_2O_2 scavenging is depending on chloroplast (stromal and thylakoid bound) APX (Asada, 1992) and 2CP (Kangasjarvi *et al.*, 2008). Despite its functional role, chloroplast APX activity has been proved that under low levels of ascorbate, the enzyme has a very short life time while its activity is rapidly reduced (Kangasjarvi *et al.*,

2008; Asada, 1992). The ascorbate levels also depend on the redox balance; therefore, there is a cyclic regulation under stable conditions and inactivation under imbalances. tAPX is also reported as light-dependent (Kangasjarvi *et al.*, 2008).

APX regulation in *Swietenia* was similar than the one of 2CP, with a trend in up regulation during mild stress and down regulation during severe stress for all four experiments (Fig. 4.27). After one day re-watering, transcript levels were found to be similar to controls (E1 and E3); after three days, E3 remains similar while E1 had an increasing trend. These results suggest that APX regulation in *Swietenia* follows the same pattern described (above) for drought sensitive plants. Chloroplast programs to maintain the redox balance during mild stress by increasing the transcript levels of APX, but when the stress is too severe, chloroplast APX is inactivated by the low concentrations of the donor. Photosynthetic activity was severely reduced during high levels of stress, due to stomata closure; therefore electron transport is reduced. The elevated levels of hydrogen peroxide that are not scavenged inside the chloroplast might migrate through the membranes as explained by Asada (1999) and have a signaling role for the nucleus and the rest of the cell.

For *Jatropha*, during mild stress it was observed a trend in reduction of APX transcript levels, significant in E5 and non-significant in E2, the only two experiment measured (Fig. 5.55). This response was not expected since at this point it could still not occur such a significant modification in the redox balance; therefore, this behavior might indicate a signaling role of APX. The hypothesis at this point is that while tAPX is downregulated, the excess of hydrogen peroxide molecules from the chloroplast will difuse towards the cytosol and serve as a signal of small imbalance; this could then prepare the future responses in case of higher stress levels.

During severe stress, treated plants and controls had similar amounts of transcripts in E1, E2, and E5, while in indoor experiments was observed a downregulation, significant for E3, and 8X reduction (n.s.) in E4. The experiments that presented downregulation during mild stress were not downregulated during severe stress; instead, they maintained similar levels as controls. If the regulation during mild stress served as a signal, it is possible that it would have affected active ascorbate accumulation. As described for *Swietenia* (Kangasjarvi *et al.*, 2008 and Asada 1992), under low levels of ascorbate, the enzyme loses its activity rapidly; if at higher stress the transcript levels for tAPX were similar in treated and control plants, it means

that the increased levels of hydrogen peroxide, produced in the chloroplast due to drought stress, did not affect it.

After re-watering, an upregulation trend was observed in almost all experiments, significant for E1 and E4; the only experiment with downregulation after re-water was E3. The relative increase in transcript levels might indicate a conservative role in the recuperation of the homeostasis. The only experiment with still down regulation after re-water could have experienced probably a depletion in ascorbate since it was one of the non-fertilized experiments under high illumination.

In younger seedlings, Silva *et al.* (2010b) observed an increased enzymatic activity of APX during drought treatment, though it did not wholly compensate lipid peroxidation in their study. Silva *et al.* (2012 and 2015) also observed an increase in enzyme activity during drought stress in plants of unknown age and Lima Neto *et al.* (2017) confirmed high APX enzyme activity product of drought treatment, under elevated CO_2 concentration and irradiance of 1800 µmol photon m^{-2} s^{-1}. In older plants (11-month-old), Pompelli *et al.* (2010) found the exact proposed trend for enzyme concentration after mild and severe drought stress: at mild stress there was a reduction of APX concentration in treated samples, but in two different measuring points in severe stress, there were higher enzyme concentrations than in controls. The increased activity reported in those studies partially support the idea of the transcriptional stress signaling during mild stress because for the enzyme activity is necessary to have enough ascorbate as a substrate. However, enzyme activity levels do not depend uniquely on gene regulation at transcript levels. Other regulatory mechanisms could affect the final total concentration and activity of the enzyme. Lima Neto *et al.* (2017) also reported that tAPX activity was higher than total APX and that there was a 3-fold increase in the activity of stressed seedlings than in controls. This results then, together with our evaluation of gene regulation, point out an effectively modulated protection level in the chloroplast.

Though these results are in contradiction with the ones of Sapeta *et al.* (2016) who found that after 49 days of drought in seedlings of the same age with high fertilization, APX was strongly upregulated in their transcriptomic analysis; they also confirmed those results with qPCR. Cartagena *et al.* (2014) also made a transcriptomic analysis on drought stress with seedlings in similar age but did not report any differential regulation in APX or any other gene in the chloroplast antioxidant system.

While outdoor experiments were able to send stress warning signals and to hold transcript levels during the maximum stress, the indoor experiments E3 and E4 had lower transcript levels in treated plants at the same point. The reason behind, based on the hypothesis of accumulation, is that the lack of nutrients in the soil hindered the required uptake for the program, thus were not able to engage in the same response mechanism at higher stress as the one observed in the other experiments.

APX		Mild	Severe	RW
Swietenia	E1	↑	↓	= C
	E2	↑	↓	n.m.
	E3	↑	↓	= C
	E4	↑	↓	= C
Jatropha	E1	n.m.	= C	↑
	E2	↓	= C	= C
	E3	n.m.	↓	↓
	E4	n.m.	↓	↑
	E5	↓	= C	=C

Figure 6.2 Regulation of Jc *tAPX* during drought. Arrows indicate if the trend indicated upregulation or downregulation. (=C) was the annotation when stressed and control plants had similar transcript levels. (n.m.) indicate not measured for this experiment.

In summary, tAPX gene regulation in *Swietenia* and *Jatropha* present different mechanisms (Fig. 6.2). While in *Swietenia*, plants respond to mild stress increasing transcript levels to protect the chloroplast and suffer downregulation at maximum stress, *Jatropha* gets downregulated at mild stress, hypothetically as stress signaling for adjustments, that allow them to pair control transcripts levels at maximum stress.

6.2.3. Chloroplastic glutathione peroxidase (*GPX*) in both plants

In the broader definition, Milla *et al.* (2003) described GPX genes in *Arabidopsis* as ubiquitous and observed that they are regulated by various abiotic stressors, through diverse signaling pathways. In 2006, Navrot *et al.* defined GPXs as functional peroxiredoxins distributed in several compartments. Chang *et al.* (2009) demonstrated that chloroplastic isoforms play a role in the cross-talk for photooxidative stress, Faltin *et al.* (2010) considered that GPX enzyme activity regulation was crucial for in vitro regeneration in *Citrus*, suggesting the involvement of reactive oxygen species (ROS) in this process and Passaia *et al.* (2015) determined them as redox sensor proteins.

GPX regulation in *Swietenia* was showed different patterns (Fig. 4.28). E1 and E4 presented increased transcript levels during mild stress and downregulation at maximum stress, similar to the pattern observed for 2CP and APX. In E2 there was also observed upregulation during mild stress, like in the other experiments, but in severe stress points, the transcript levels increase even more. In E3 (which differs from the others in the duration of the droughting process) no regulation was observed during mild stress but at maximum stress, presented higher transcript levels. After re-watering, E1 and E4 still keep upregulated expression, while in E3 the trend appears to increase after three days.

During mild stress, GPX transcript levels in *Swietenia* have a trend in the increase in almost all experiments that was maintained until the end of the stress period in the two experiments at full sun conditions with slower photosynthesis inhibition. In maximum stress, GPX levels were downregulated in the experiment with fast drying (E1) and the one in the shadow (E4). This result suggests that under fast drying or low-level conditions, GPX is not that engaged in ROS detox, but that in more normal conditions, at full light, GPX function is still required at maximum stress. After the re-watering, GPX is not regulated in any of the conditions, which suggests that GPX protection is useful during the drought period and not during the re-watering.

In *Jatropha*, GPX appears to be upregulated during mild stress in all evaluated experiments (E1, E2, and E5); this accounts for a very sensitive response to the first drought signals (Fig. 5.58). At maximum stress point, there are three possibilities observed: a significant upregulation was observed in E2 and E3; similar transcript levels in stressed and control plants in E1 and E5 with a small increase trend but with very high biological variation; and downregulation under too less light (E4). The trend points out that there is an upregulation maintained at severe stress, that is more visible in high irradiation under the most stable conditions outdoors (E2) and indoors (E3).

Re-watering produced a significant down regulation in E1 and E4, similar values to controls in E2 and E3, and a significant up regulation in E5. At this point, the regulation kept the same pattern for the previous classification, the experiments that had a significative increase, presented values similar to controls product of a reduce in the total transcript levels. E1, that only achieved to maintain transcript levels of stressed plants similar to controls, presents a downregulation after re-hydration, also, a product of a decrease in total transcript levels. Only

E5 had upregulated transcription after re-watered, indicating a completely different mechanism of recovery in indoor conditions with fertilized soils. The downregulation trend in E4 all along the experiment can be interpreted as a light-depending response, that modifies the gene transcription under low light.

In general, it was observed a highly sensitive reaction in GPX present in *Jatropha*, with a trend of increase for both species (Fig. 6.3). During severe stress, *Swietenia* keeps relying on GPX for scavenging only on favorable and controlled conditions, but its downregulated when there is too much stress. *Jatropha* in the other hand, present significant upregulation during severe stress in favorable conditions, or at least maintains the same levels as in controls; for this species, GPX plays a more active role in ROS scavenging during stress. After re-hydration, there is a trend in decrease which depends on the actual transcript level at maximum stress: either from higher values to control-comparable values or from control values to lower values. This analysis suggests that the plant's response to re-water engage in another physiology, which corresponds to the high CO_2 assimilation rates observed at this point (Fig. 5.45).

GPX		Mild	Severe	RW
Swietenia	E1	↑	↓	=C
	E2	↑	↑	n.m.
	E3	=C	↑	=C
	E4	↑	↓	=C
Jatropha	E1	↑	=C	↓
	E2	↑	↑	=C
	E3	n.m.	↑	=C
	E4	n.m.	↓	↓
	E5	↑	=C	↑

Figure 6.3. Regulation of Jc t*GPX* during drought. Arrows indicate if the trend indicated upregulation or downregulation. (=C) was the annotation when stressed and control plants had similar transcript levels. (n.m) indicate not measured for this experiment.

On the activity level, Soares *et al.* (2016) found a substantial activity of GPX in seedlings during salicylic acid (SA) and methyl jasmonate (MeJA) treatments and Verma *et al.* (2014) found increased activities for GPX in *Jatropha* seedlings under soil flooding. Our results could be explained with the activity reported by Soares *et al.* (2016) and Verma *et al.* (2014), assuming that an apparent upregulation in transcript levels might impact significantly in the elevated activity they observed. Li *et al.* (2013) found decreased activities during chilling stress but

remarked that the values were still very high, and in this direction, Tian *et al.* (2017) considered *Jatropha* as a cold sensitive tropical plant, therefore a different protection mechanism under lower temperatures is understandable.

The analysis in the regulation of the enzymatic antioxidant system was further enhanced for *Jatropha curcas*, including other chloroplast enzymes (PrxQ, and Cu/Zn SOD2) and CAT, due to the availability of gene sequences.

6.2.4.   Catalase (*CAT*) in *Jatropha*

CAT enzyme extraction and determination were described for plants in 1955 by Galston, and since then, the evaluation of this enzyme is regularly included in stress response experiments. The catalase multi-gene family in *Arabidopsis* includes three genes encoding individual subunits which associate to form at least six isozymes that are readily resolved by non-denaturing gel electrophoresis (McClung, 1997). Catalases are mainly associated with H_2O_2 removal in peroxisomes, and its activity is considered determinant in the protection of photosynthesis against oxidative stress (Willekens *et al.*, 1995). All three CAT genes activity is light-dependent, and from them, *CAT2* and *CAT3* are highly expressed in leaves (McClung, 1997). CAT 2 and CAT3 mRNA abundance is controlled by the circadian clock: while CAT2 mRNA abundance peak occurs in the early morning (induced by light), CAT3 is negatively responsive to light, showing mRNA abundance peak in the evening (McClung, 1997).

Zhang *et al*. (1994) observed that in *Triticum* CAT enzyme activity increased during the early phase of droughting, and then decreased along significant stress levels; this was an indication that water balance alters the equilibrium between free radical production and enzyme defense reaction. Luna *et al*. (2004) working also in *Triticum*, observed that H_2O_2 accumulation correlates to the soil water content and that it increases after re-watering, even when CAT activity doubled under severe stress.

In the present study with *Jatropha* (Fig. 5.65), CAT2 transcription level was not regulated during mild stress in any of the evaluated experiments (E1, E2, and E5). Under severe stress, it was observed a down regulation, significant for E1 and E2, while non- significant for E3 and E4. After re-watering, three options were distinguished: in outdoor experiments (E1 and E2) pre-stressed plants and controls had same transcript levels, product of an increase from

previous values; indoor experiments with high light regime (E3 and E5) observed upregulation in their transcripts, significant for E3; and under low-light regime indoors (E4) it was observed that the previous downregulation in maximum stress stayed unchanged. During a drought period, regulation was the same for all experiments, but at the end of the stress period, CAT regulation appears to be light-dependent.

CAT enzyme activity was reportedly reduced in drought stressed seedlings (Silva *et al.*, 2010b, 2014; Lima Neto *et al.*, 2017); besides, Hertwig (1992) reported a light-dependent decrease of CAT activity during salinity treatments using *Secale cereal* L. and Feierabend *et al*. (1992) showed how CAT was inactivated by temperatures of 40 °C and in chilling temperatures, with a decisive sensitivity towards photoinactivation.

CAT		Mild	Severe	RW
Jatropha	E1	=C	↓	=C
	E2	=C	↓	=C
	E3	n.m.	= C (↓)	↑
	E4	n.m.	↓	↓
	E5	=C	=C	↑

Figure 6.4. Regulation of Jc *CAT* during drought. Arrows indicate if the trend indicated upregulation or downregulation. (=C) was the annotation when stressed and control plants had similar transcript levels. (n.m) indicate not measured for this experiment.

Luna *et al.* (2004) reported a complex regulation of CAT2 mRNA in *Triticum*, particularly at the transcriptional level, for the control of H_2O_2 accumulation. While enzyme activity stayed mostly stable along the day, the mRNA abundance during mild stress (activation observed between 9:00 and 17:00) included a notorious inhibition during midday. Our results showed no regulation of transcript levels during mild stress, but the results could fit in the curve of deactivation towards midday inhibition (Fig. 6.4). Sample collection for RNA extraction occurred between 10:30 and 12:00, after all the physiological measurements, explicitly waiting after one hour of direct sun irradiance for the activation of PSII activity. Even if there was an upregulation in the early morning also in *Jatropha*, it is possible to have missed the maximum abundance peak due to the reported CAT light induction due to the collection time.

At severe stress, Luna *et al*. (2004) determined no midday inhibition, but there was also no regulation during the day, with lower transcript abundance than during mild stress. In the experiments with *Jatropha*, there was also a trend in reduction of CAT2 transcript levels. These

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two results suggest that at maximum stress, downregulation in *Jatropha* was consistent with the gene expression pattern in *Triticum*, a C4 plant, therefore with higher drought tolerant adaptations.

After re-watering, no difference in transcript levels for outdoor experiments was observed, though, in indoors experiments an upregulation was detected. This difference could indicate that the sensitivity of CAT regulation is affected by other environmental parameters that need to be met before the plant goes back to "normal" regulation levels.

The downregulation pattern observed in E4 (indoor experiment under shadow), confirms the general description of CAT2 as light-sensitive. It can indicate that the levels of irradiance for this experiment did not reach the minimum for CAT2 activity and that in this case, the photooxidative protection was under another isoform, probably under a CAT3 homologue. The results found in this study is partially antagonist to the results of Sapeta *et al.* (2016) who reported a strong transcript upregulation for catalase in leaves. That can imply that the post translational regulation is not the same as for the transcript levels.

6.2.5. Cu/Zn superoxide dismutase (*Cu/Zn SOD*) in *Jatropha*

Molecular oxygen is reduced in the thylakoids forming superoxide (O_2^-) by the excess of electrons in the area; this superoxide is disproportionated to hydrogen peroxide (H_2O_2) and O_2 by SOD enzymes, to avoid the danger posed over membrane destruction (Asada, 1999). This enzyme type is unique because its activity determines the concentrations of O_2^- and H_2O_2, the two Haber-Weiss reaction substrates, and it is therefore considered as a central factor in the defense mechanism (Bowler *et al.*, 1992). The three known types of SOD are classified by their metal cofactor: the copper/zinc (Cu/ZnSOD), manganese (MnSOD), and iron (FeSOD) ones. Subcellular studies have determined Fe SOD and/ or Cu/Zn SOD enzymes present in the chloroplast stroma, and under drought stress, both enzymes activities were increased in drought tolerant species (Bowler *et al.*, 1992).

In experiments with *Jatropha* seedlings, the chloroplast copper/zinc superoxide dismutase (Cu/Zn SOD) was not regulated during mild stress (Fig. 5.68), though had a small increasing trend in E1 and E5. During maximum stress, it was statistically downregulated in E4, with the same trend in all other experiments (E1, E2, and E4; not significant). After re-watering, experiments indoors (E3 and E4) had similar values to controls, a product of an increase from

previous values; this trend was evaluated statistically significant in E2, with an evident gene upregulation. Only in E1 the transcript level of treated plants remained low, not changing from previous values; maybe the response period of this experiment was too short (or the stress level, too high) for a modification in SOD regulation towards control values.

Enzyme activity was reportedly not modified in *Jatropha* seedlings submitted to different oxidative stress, including soil flooding (Verma *et al.*, 2014), chilling temperatures (Li *et al.*, 2013), Methyl jasmonate (MeJA) and salicylic acid spray (Soares *et al.* 2016) or under drought treatment (Silva *et al*., 2010b), when using younger plants; but in this last case, SOD activity increased in combined stress (drought heat). Two years later the same last group (Silva *et al*., 2012) reported increased SOD activity after ten days drought, based on the soil water availability. Lima Neto *et al*. (2017) also found increased values of SOD in drought stressed seedlings, under elevated CO_2 and irradiance.

Enzyme activity is, as also observed for other genes, not always directly related to the transcript levels. In the case of SOD, we found minimal responsiveness at transcription level under mild stress and a slight tendency in decrease during severe drought (Fig. 6.5), which corresponds to the enzyme activity reported by Verma et a. (2014), Li *et al.* (2013), Soares *et al*. (2016) and Silva *et al*. (2010b), but contrary to the one reported by Silva *et al.* (2012) and Lima Neto *et al.* (2017).

SOD		Mild	Severe	RW
Jatropha	E1	↑	=C (↓)	↓
	E2	=C	=C	↑
	E3	n.m.	↓	=C
	E4	n.m.	↓	=C
	E5	=C	=C	n/a

Figure 6.5. Regulation of Jc *SOD* during drought. Arrows indicate if the trend indicated upregulation or downregulation. (=C) was the annotation when stressed and control plants had similar transcript levels. (n.m.) indicate not measured for this experiment and (n/a) indicates that control values were not available.

One of the possible explanations in the reduction in enzyme activity and transcript abundance for SOD during drought stress in most of the experiments can be related to the availability of copper (the enzyme's cofactor) in the soil. Cohu and Pilon (2007) proved that in several species including *Arabidopsis*, *Brassica*, *Lycopersicum, Zea,* and *Oryza*, chloroplastic Cu/Zn SOD was

downregulated in response to copper limitation. Under this condition, they reported that Fe SOD expression was upregulated to replace Cu/Zn SOD in the stroma in almost all species. Cohu and Pilon (2007) conclude that plants that downregulate Cu/Zn SOD and upregulate Fe SOD under copper limitation can maintain superoxide scavenging and save copper for use in plastocyanin, which is essential for photosynthesis. As the experiments conducted in this study presented a strong tendency of downregulation, the scavenging activity of Cu/Zn SOD could have been replaced by Fe SOD.

Another explanation could be the actual level of energy dissipation that occurs in the plant. Silva *et al.*, (2010b), Sapeta *et al.*, (2013) and Lima Neto *et al.*, (2017) found increased levels of in NPQ levels; besides, Lima Neto (2017) found increased activities in the cyclic electron transport (CEF) and in ETR I. All these authors explain their results of stress responses of *Jatropha*, claiming that drought tolerance in *Jatropha* is related to antioxidant protection, CEF and NPQ activity.

Further analysis on Cu/ZnSOD response towards stress in *J. curcas* must include information about copper content in soil and leaves, to understand the mechanism under non-limiting conditions; this also will allow comparing results between the few researchers in this area.

6.2.6.   Peroxiredoxin Q (*PrxQ*) in *Jatropha*

Peroxiredoxin Q (PrxQ) is one out of ten peroxiredoxins encoded in the genome of *Arabidopsis thaliana*, and one out of four that are targeted to plastids; represents about 0.3% of chloroplast proteins and decomposes peroxides using mostly thioredoxin as electron donor but can accept a wide range of electron donors and substrates (Lamkemeyer *et al.*, 2006). It was localized in the chloroplasts and especially to those of the guard cells (Rouhier *et al.*, 2004) but has a specific function distinct from 2-Cys peroxiredoxin in protecting photosynthesis. Its absence in *Arabidopsis thaliana* causes metabolic changes that are sensed and trigger appropriate compensatory responses (Rouhier *et al.*, 2004; Lamkemeyer *et al.*, 2006).

The peroxiredoxin Q (PrxQ) in *Jatropha curcas* was not regulated during mild stress in E5 but was significantly upregulated in E2 (Fig.5.61). During maximum drought stress, it was observed an increasing trend in transcript levels for the two experiments with high intensity, but in slower droughting process. E2 showed an increasing trend (n.s.), that was statistically higher in E3; in E5 there might also be an upregulation if compared to previous values, but there was

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no control value for this point. At maximum stress, a second regulation pattern was observed for the experiments with the most unfavorable conditions: E1 (n.s.) and E4 (p < 0.01) had reduced transcript levels when compared to controls. After re-watering, PrxQ transcript upregulation was then observed in experiments with progressive water reduction after one month, that included high illumination levels, and the downregulation was spotted for the experiments with a stronger handicap, either by droughting speed (E1) or reduced light intensity (E4).

PrxQ		Mild	Severe	RW
Jatropha	E1	n/a	=C (↓)	↓
	E2	↑	↑	↑
	E3	n.m.	↑	↑
	E4	n.m.	↓	= C
	E5	=	n/a	n/a

Figure 6.6 Regulation of Jc *PrxQ* during drought. Arrows indicate if the trend indicated upregulation or downregulation. (=C) was the annotation when stressed and control plants had similar transcript levels. (n.m.) indicate not measured for this experiment and (n/a) indicates that control values to compare were not available.

So far, there has not been reported PrxQ enzyme activity. Therefore, no further analysis was performed.

Conclusion

Reactive oxygen species (ROS) are regularly produced in all sites with electron transport chains like the chloroplast; to prevent this danger, the plant uses an antioxidant system that scavenges those radicals, to maintain balance. Drought is known to induce ROS production and to reduce photosynthesis activity, but in natural conditions, especially the tropical ones, plants must adapt to this constant stressor.

This work presented one of the first compilations for the study of the transcriptional regulation for plastidic protection in tropical trees during drought stress, including the description of partial sequences (for *Swietenia*) and a detailed characterization (for *Jatropha*) of the most abundant and responsive genes in the plastidic antioxidant system. The observed gene expression was compared with the enzymatic activity of those genes, previously reported for *J. curcas*, and for other species, as a reference frame. Based on the physiological parameters observed it was confirmed a different strategy of growth and stress avoidance in the two species. *Jatropha curcas*, considered a drought tolerant species, proved to have a more sensitive antioxidant system, that responded differentially, depending on the environmental conditions. On the contrary, *Swietenia macrophylla*, considered a drought-sensitive plant species, presented one type of regulation in all evaluated conditions. Though the assumptions of regulation trends were based on slight variations, this flexible gene expression was also correlated with the growth strategy observed for each case. This work will serve as a starting point for further studies on gene regulation and in the metabolism of drought tolerance in naturally adapted plants.

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