

Phenotypic and physiological characterization of two *Jatropha curcas* accessions in drought stress conditions

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Abstract

Drought tolerance of *Jatropha curcas* accessions from different geographical and climatic origins has been poorly investigated.

In order to enlighten putative impact of plant origin on plant behavior towards stress, we have characterized the drought and recovery responses of two accessions of *Jatropha curcas* with different geographical and climate provenances (arid climate - Cape Verde islands, and wet tropical climate - Indonesia).

Light response photosynthetic curves were performed for the two accessions. Our results suggest that the relationships between light use efficiency, CO₂ fixation and photoinhibition are similar for both *J. curcas* accessions tested.

Preliminary drought assays were performed to optimize stress imposition, duration and intensity. Stress was imposed on 71 days-old plants either by water withhold for 36 days, or by gradual irrigation reduction until 15% of field capacity was reached (for 28 days), in both cases followed by one week of recovery under normal irrigation. Water withhold resulted in a gradual stress imposition, which can be useful when looking for putative discriminating behaviors between accessions in response to stress. Also, to promote this gradual reduction plants should be used in the 3 to 5 leaves stage.

With this knowledge, an optimized drought assay was performed. Plants (36 days-old, 3-5 leaves stage) were subjected to drought by water withhold until soil water content reached a value of 10% of initial field capacity. Leaf gas exchange, chlorophyll *a* fluorescence, chlorophyll content, leaf water status and morphological parameters were evaluated along the drought and recovery period. No significant morpho-physiological differences were detected between the two accessions, either in control conditions or in response to drought stress and after recovery period. Both accessions showed a similar response. Nevertheless, some differences were observed for biomass allocation (especially in root percentage of dry matter) and in leaf shedding. Furthermore, both accessions maintained a high leaf water status due to a strict stomatal control and reduction of leaf total area (decreasing leaf expansion, leaf production and, under severe stress, shedding of older leaves).

Keywords: *Jatropha curcas*; water stress; accessions; morphology; leaf gas exchange and chlorophyll *a* fluorescence; water relationships.

Sumário

A tolerância à seca de ecotipos de diferentes origens geográficas e climáticas de *Jatropha curcas* tem sido pouco estudada.

Para compreender o possível impacto da origem dos ecotipos sobre o seu comportamento durante o déficit hídrico, foi efectuada uma caracterização das respostas à seca e recuperação de dois ecotipos de *Jatropha curcas* com diferentes proveniências geográficas e climáticas (clima árido - ilhas de Cabo Verde, e clima tropical húmido - Indonésia).

Foram realizadas curvas de resposta fotossintética à luz para os dois ecotipos. Os resultados das mesmas sugerem que as relações entre eficiência do uso de luz, fixação de CO₂ e fotoinibição são semelhantes para ambos os ecotipos de *J. curcas* testados.

Foram realizados ensaios de seca preliminares para otimizar a imposição, duração e intensidade do déficit hídrico. Para tal, o déficit hídrico foi imposto em plantas com 71 dias, através de supressão de rega por 36 dias, ou redução gradual da rega até um valor mínimo de 15% da capacidade de campo (por 28 dias), ambos seguidos por uma semana de recuperação com rega. A imposição por supressão de rega, resultou numa imposição de déficit hídrico mais gradual, que é mais indicada quando se pretende comparar possíveis comportamentos discriminantes entre ecotipos em resposta a déficit hídrico.

Após optimização das condições, um ensaio de seca foi realizado. Plantas com 36 dias de idade (3-5 folhas) foram submetidas a déficit hídrico por supressão de rega até 10% da capacidade de campo ser atingido. Trocas gasosas, fluorescência da clorofila *a*, conteúdo de clorofila, conteúdo hídrico foliar e parâmetros morfológicos foram avaliados ao longo do período de déficit hídrico e recuperação. Não foram observadas diferenças morfo-fisiológicas significativas entre os dois ecotipos. Ambos os ecotipos apresentaram uma resposta idêntica. Embora, algumas diferenças tenham sido observadas na alocação de biomassa (especialmente na percentagem de matéria seca das raízes) e na queda de folhas. Porém, ambos os ecotipos mantiveram um elevado conteúdo hídrico nas folhas, originado por um controle estomático rigoroso, bem como uma redução da área foliar total (diminuindo a expansão foliar, produção de novas folhas e, em condições extremas de déficit hídrico, queda de folhas mais velhas).

Palavras-chave: *Jatropha curcas*; stress hídrico; ecotipos; morfologia; trocas gasosas e fluorescência clorofilina; relações hídricas.

List of abbreviations

μmol	Micro mole
%	Percentage
°C	Degree centigrade
A _n	Net photosynthesis
ATP	Adenosine triphosphate
Chl <i>a/b</i>	Chlorophyll <i>a</i> to <i>b</i> ratio
Chl _{<i>a</i>}	Chlorophyll <i>a</i>
Chl _{<i>b</i>}	Chlorophyll <i>b</i>
C _i	Intercellular CO ₂ concentration
cm	Centimeter
CO ₂	Carbon dioxide
DW	Dry weight
E	Transpiration rate
ER	End of recovery
ETR	Electron transport rate
FC	Field capacity
FW	Fresh weight
g	Gram
g _s	Stomatal conductance
h	hours
H ₂ O	Water
L	Litre
LCP	Light compensation point
LED	Light-emitting diode
LRC	Light response curves
LSP	Light saturation point
M	Molar
m	Meter
Max	Maximum
mg	Milligram
min	Minutes

Min	Minimum
ml	Milliliter
mm	Millimeter
mol	Mole
MS	Maximum stress
NADPH	Nicotinamide adenine dinucleotide phosphate
nm	Nanometer
ng	Nanogram
O ₂	Oxygen
PPFD	Photosynthetic photon flux density
ppm	Parts <i>per</i> million
RH	Relative humidity
rpm	Rotations <i>per</i> minute
RNA	Ribonucleic acid
DNA	Desoxyribonucleic acid
RT	Room temperature
RuBisCO	Ribulose-1.5-bisphosphate carboxylase oxygenase
RWC	Relative water content;
s	second
S:R	Shoot to root dry mass ratio
se	Standart error
SLA	Specific leaf area;
SWA	Soil water availability;
TC1	Tested conditions 1
TC2	Tested conditions 2
TLA	Total leaf area
TW	Turgid weight
v	Volume
WS	Water stress
WUE	Water use efficiency
WW	Well watered
Φ _{PSII}	Photosystem II efficiency

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1 Introduction

1.1. *Jatropha curcas*

Jatropha curcas or physic nut (common name) was first described by Linnaeus (Divakara *et al.*, 2010). The genus name *Jatropha* derives from the Greek word *jatros* (doctor) and *trophe* (food) and it is associated with its ancient medicinal use as a purgative. Moreover, it is a stem-succulent tree or shrub that has been described as drought tolerant and capable of growing in marginal and poor soils (Heller, 1996; Divakara *et al.*, 2010). Furthermore it is emerging as a potential source of biodiesel (Fairless, 2007). Other characteristics like easy propagation, rapid growth, short gestation period, high oil content and low seed cost, makes it a promising crop to grow in semi-arid and poor soil conditions without competing with food production for land use (Divakara *et al.*, 2010; Fairless, 2007). In addition, the genome size of *Jatropha* is fairly small (~410 million base pairs) and was been recently sequenced (Sato *et al.*, 2010).

Besides its undeniable potential, *Jatropha curcas* is still considered an undomesticated plant showing considerable performance variability (Fairless, 2007; Achten *et al.*, 2008; Achten *et al.*, 2010a), and major knowledge gaps still exist with regards to eco-physiology traits which limit our understanding and the capacity to predict this species agronomic performance (*e.g.* biomass and seed yield in response to the environment). To reduce the risk of future unsustainable practices and to improve future crop performance, further selection, breeding and domestication of *Jatropha* is critical (Achten *et al.*, 2010a).

1.1.1 Biology and ecology

Jatropha curcas is a small stem-succulent tree or large shrub (Fig. 1 A) belonging to the Euphorbiaceae family, which can reach a height of three to 5 m, but can attain a height of 8 or 10 m under favorable conditions. The plant shows articulated growth straight trunk, thick branches with a soft wood and a life expectancy of up to 50 years (Heller, 1996). Leaves are deciduous and are shed during the winter months, although *J. curcas* can withstand lower temperatures and even a light frost. Leaves can also be shed during long periods of drought due to reduce transpiration loss (Kumar and Sharma, 2008). Leaves are alternate but apically crowded (Heller, 1996). Usually, five roots are formed on seedlings, one being central and four peripheral (Heller, 1996).

Flowering normally occurs during the wet season, however in humid regions, flowering can occur throughout the year (Heller, 1996). The inflorescence is axillary

paniculate polychasial cymes formed terminally on branches and are complex, possessing main and co-florescences with paracladia (Fig. 1 B). The plant is monoecious with male and female flowers on the same plant and in the same inflorescence (Fig. 1 C), occasionally hermaphroditic flowers can be presented (Heller, 1996; Achten *et al.*, 2010a). According to Little *et al.* (1974), *Jatropha curcas* flowers are greenish yellow colored and occur in terminal long and peduncled paniculate cymes. The same authors describe the inflorescences as a bunch of green trilocular ellipsoidal fruits yielding approximately 10 or more ovoid fruits, with the exocarp remaining fleshy until the seeds are mature (Fig. 1 D). After drying, capsules split into 3 valves, with at least two having an oblong black seed (Fig. 1 E) (Little *et al.*, 1974). *Jatropha* can set seed after either insect or self-pollination, although self-pollination is less frequent and, in 25% of the cases, leading to abortion before maturation (Achten *et al.*, 2010a).

A recent review by Maes *et al.* (2009a) has compiled extensively the species climate growing requirements. These authors have defined the climatic conditions by combining the locations of herbarium specimens with corresponding climatic information, moreover they describe that 90% of the specimens grew in areas with a mean annual rainfall above 944 mm *per year*, an average minimum temperature above 10.5 °C and a mean annual temperature range between 19.3–27.2 °C.

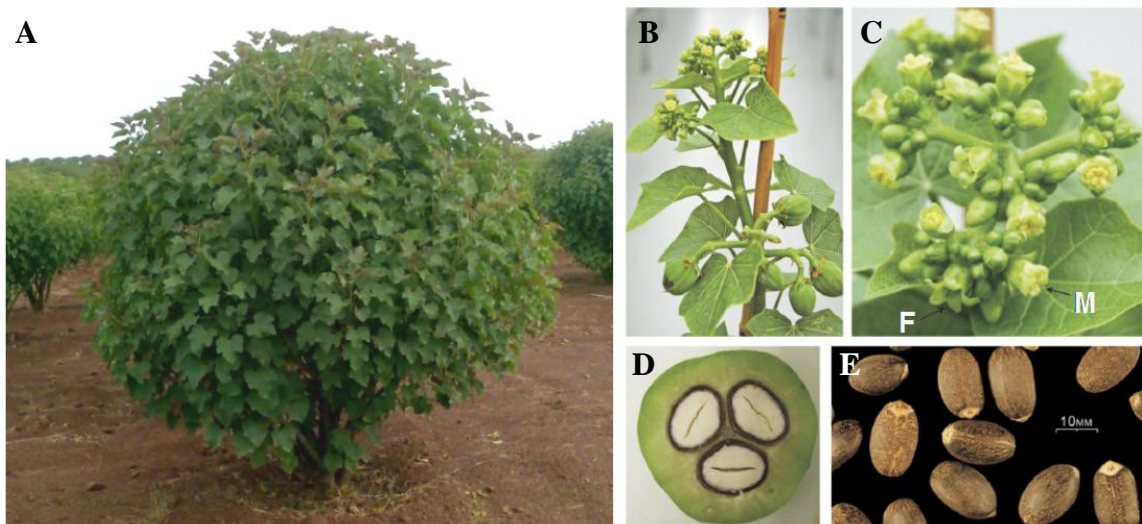


Figure 1 - *Jatropha curcas*: A) adult plant; B) flowers and developing seed pods; C) inflorescence containing both male staminate flowers (M) and female pistillate flowers (F); D) seed pod containing three developing seeds in a fruit cross-section; E) mature seeds (King *et al.*, 2009; van der Putten *et al.*, 2010).

1.1.2 Species distribution

Jatropha is original from Mexico and continental Central America (Heller, 1996). The Portuguese in the 16th Century, have spread *J. curcas* out of its centre of origin to Africa where they have established commercial plantations for soap and lamp oil production in Cape Verde Islands and Guinea Bissau (Heller, 1996). Later, *Jatropha* genotypes adopted in Western Africa were spread across other Portuguese colonies in Africa (Mozambique, Angola) and into Asia (India, China and Indonesia). Currently *Jatropha curcas* grows in tropical areas worldwide (Sub-Saharan African countries, Southeast Asia, India) (Fig. 2).

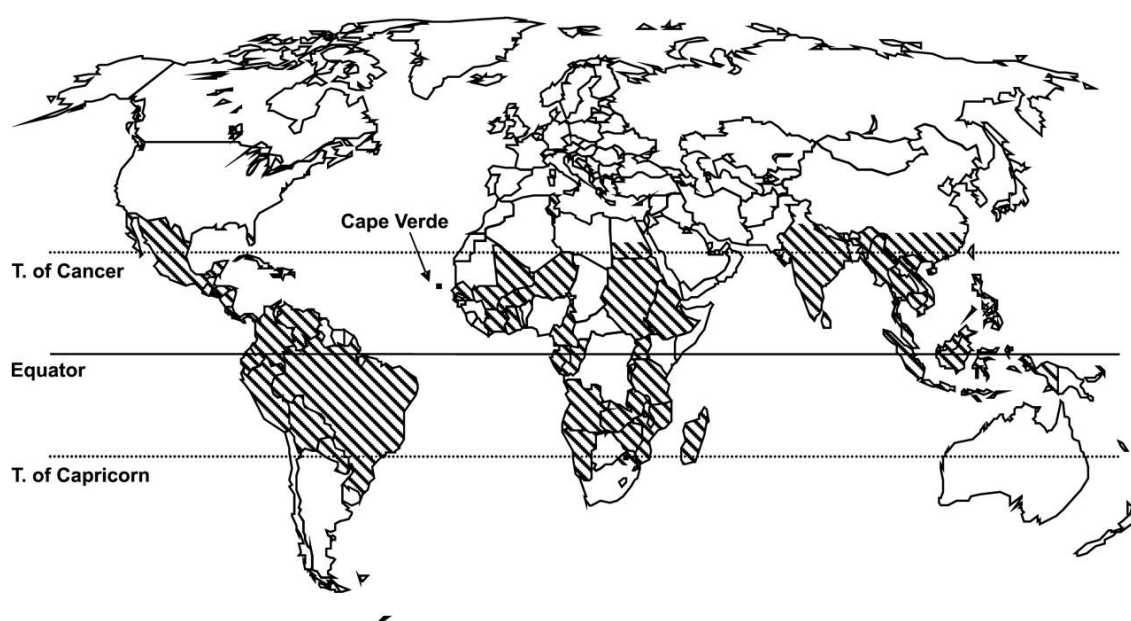


Figure 2 - Global distribution of *J. curcas*. Shaded regions indicate areas in which *J. curcas* is presently growing (King *et al.*, 2009).

1.1.3 Genetic diversity

Studies based on genetic markers revealed low levels of genetic diversity in *Jatropha* landraces from China (Sun *et al.* 2008) and only modest levels of diversity in India (Basha and Sujatha, 2007; Ranade *et al.*, 2008). Low levels of genetic diversity in landraces from Mali, Kenya and Tanzania were also reported by Nielsen *et al.* (in Achten *et al.*, 2010a). Moreover, the use of microsatellite markers (simple sequence repeats) has revealed that, even in its centre of origin, *Jatropha curcas* shows a low genetic variation (Sun *et al.*, 2008). When characterizing two Mexican accessions of *Jatropha* (one toxic and one nontoxic), with various markers (microsatellites, amplified fragment length polymorphism and random amplification of polymorphic DNA) Pamidimarri *et al.* (2009) verified that even though they could

discriminate between the accessions, no variation was found between individuals within each accession. Achten *et al.* (2010a) have presenting a good revision on the subject and according to these authors this data could be an indication of a population structure with a high level of homozygosity.

On the other hand, in a study with 13 provenances and landraces tested in two places in Senegal and another two in Cape Verde conducted by Heller (1996), significant differences were found at all sites in terms of vegetative growth. At one of the test sites in Cape Verde, provenances were significantly different concerning the number and weight of capsules and the number and weight of seeds *per* shrub, as analyzed 25.3 months after planting (Heller, 1996). Genotype×Environment interactions between sites were significant in Senegal, but not in Cape Verde (Heller, 1996).

Ginwal *et al.* (2004) compared plants from 10 Indian landraces after 6–24 months, and found large significant variations, attributing more than 80% of the total phenotypic variance to the seed source (Ginwal *et al.*, 2004). Phenotypic studies for *J. curcas* seed properties reveal a high phenotypic range for oil content ranging from 28 to 39% and 100 seed weight from 49.2 to 64.9 g in accessions from Indian landraces (Kaushik *et al.*, 2007) and similar results were found in another study with Indian landraces, where the oil content ranged from 29.9 to 37.1% and the 100 seed weight from 57 to 79 g (Rao *et al.*, 2008). Moreover, Popluechai *et al.* (2009), reported appreciable variability exists in physiological and biochemical traits (*e.g.* seed size, water use efficiency and seed oil content).

Recent studies suggested that this phenotypic variation can be epigenetically controlled (Popluechai *et al.*, 2009; Yi *et al.*, 2010).

1.1.4 Economical aspects

Historical records show that *J. curcas* was used traditionally by native Indians of Central America and perhaps South America, as herbal medicine (van der Putten *et al.*, 2010). Moreover, all parts of *Jatropha* (seeds, leaves and bark) have been used in traditional medicine and for veterinary purposes (Kumar and Sharma, 2008). The oil has a strong purgative action and is also widely used for skin diseases and to soothe pain such as that caused by rheumatism (Heller, 1996; Kumar and Sharma, 2008). The leaves and latex are used in healing of wounds, refractory ulcers, and septic gums and as a styptic in cuts and bruises (Heller, 1996; Kumar and Sharma, 2008).

Due to the toxicity of the leaves and its fast growth and resilience, *J. curcas* is often used as hedge or living fence since it is not browsed by cattle (Heller, 1996; Kumar and Sharma, 2008; van der Putten *et al.*, 2010). Additionally, Kumar and Sharma (2008), reported that oil and aqueous extracts from oil have been used as insecticide to control insect pests in cotton, potato and corn.

Furthermore, *J. curcas* plantations can be used in soil conservation by growing in wastelands avoiding wind erosion, increasing soil moisture retention and carbon sequestration. Moreover, it has been pointed as a strong candidate for phytoremediation due to its fast growing habit, tolerance to adverse environment conditions, production of high biomass with little maintenance, profuse root system, and ability to accumulate heavy metals many folds from fly ash without attenuating plant growth (Jamil *et al.*, 2009). The fact that *J. curcas* is not edible is also a major advantage.

As briefly explained, *Jatropha curcas* has many uses (Fig. 3) but its major interest arose in recent years due to the high quality of its oil for biodiesel production, and the high oil content in seeds, reaching 40% of the seed weight, according to Achten *et al.* (2008). Of course, there are several other candidates for bio-diesel production, such as soybean, rapeseed, groundnut, sunflower, which, however, have the disadvantage of being edible. Additionally, *J. curcas* is able to grow in wasteland which is of major importance for developing countries like India, that have a dearth of huge quantity of edible oil (6.31 million tonnes) for internal use, and cannot afford using edible oils for bio-diesel production (Divakara *et al.*, 2010). Thus, *J. curcas* not only meets the American and European standards, but it also gained importance in tropical and sub-tropical countries (Tiwari, 2007).

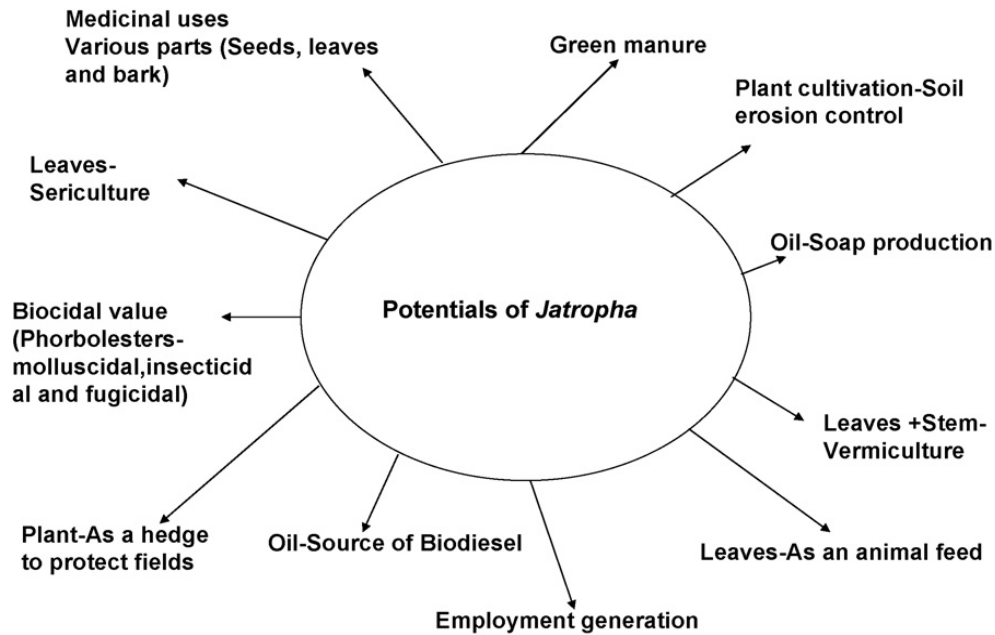


Figure 3 - Potential economical uses for *Jatropha curcas* (Kumar and Sharma, 2008).

Besides the interest in the oil itself, the by-products of oil extraction (frequently called as seed cake or press cake) can be further used as green manure (Achten *et al.*, 2008; Kumar and Sharma, 2008), biogas production, and if available in large quantities it can be used as a fuel for steam turbines to generate electricity (Kumar and Sharma, 2008).

1.2 Abiotic stresses

Plants are sessile organisms with growth and development extremely influenced by environmental conditions (Szabados *et al.*, 2011). To cope with extreme conditions plants have evolved mechanisms in response to these environmental factors. In fact, plants can perceive abiotic stresses and select the appropriate responses altering metabolism, growth and development (Bartels and Sunkar, 2005). Water scarcity, extreme temperatures or high salinity will result in a reduction of cellular water content, enhancing the cellular osmotic potential, and generating osmotic stress in plants. However, plants have antioxidant and oxygen reactive species scavenging compounds capable of responding and maintain a cellular redox homeostasis (Szabados *et al.*, 2011). Some of the physiological responses to drought, cold and salt stress may include reduction of growth (shoot and root) and photosynthetic activity, accumulation of reactive oxygen species, changes in metabolite profiles, alteration in ion transport and compartmentalization (Szabados *et al.*, 2011).

1.2.1 Water stress

Drought is a major environmental factor determining plant productivity and distribution (Bartels and Sunkar, 2005) since drought reduces the soil water potential and the ability of plants to take up water, and this quickly reduces the rate of cell expansion in growing tissues (Chaves *et al.*, 2011). Moreover, drought is one of the major abiotic stresses in areas of *J. curcas* cultivation (Divakara *et al.*, 2010). Understanding plant tolerance to drought is therefore of fundamental importance.

1.2.1.1 Morpho-physiological adaptations to water limiting conditions

Plants have developed a wide diversity of morphological and physiological mechanisms to tolerate drought (Blum, 1996). To limit water loss, leaf area expansion is often lowered, either by growth reduction and/or leaf shedding (Boyer, 1970), resulting in a reduction of total leaf area and of transpiration. However the reduced photosynthetic leaf area, will lead to a reduction of assimilates flux to the meristematic and growing tissues of the plant, in both leaves and roots, although leaves are often more affected (Szabados *et al.*, 2011). At the same time, water stress will reduce stomatal conductance in the older leaves, limiting their photosynthetic rate. Parameters of leaf gas exchange, such as measurements of stomatal conductance and photosynthesis, as well as chlorophyll fluorescence (a measure of Photosystem II efficiency) are strongly affected by water deficiency due to diffusion limitations across stomata and mesophyll (Chaves *et al.*, 2009). Stomatal closure will thus lead not only to oxidative stress, but also to increased heat because of the reduced transpiration, thus generating a superimposed heat stress with leaf temperatures often rising up to 5 or 6 °C above air temperature (Chaves *et al.*, 2009, 2011). Therefore, the plant ability to dissipate excess radiation when subjected to drought is an indicator of its tolerance to water stress conditions (Chaves *et al.*, 2003).

It is therefore of the major importance to characterize leaf gas exchanges and chlorophyll fluorescence responses to water stress and recovery (Chaves and Oliveira, 2004; Chaves *et al.*, 2011). Moreover, leaf gas exchange characteristics not only influence plant adaptation to drought but they can also vary with genotypes (Chaves *et al.*, 2010). The carbon balance of a plant enduring a water-stress period may depend on the rate and degree of photosynthetic recovery, and on the rate and degree of photosynthetic mechanisms decline during water depletion (Flexas *et al.*, 2007).

1.3. Objectives

Jatropha curcas ability to growth in marginal and dry soils has been poorly explored because the research in this species focuses mainly on the chemical and physical properties of seed oil. To our knowledge, only two studies have compared the responses to drought in accessions of different provenances, namely of Ethiopia, India and Thailand (Maes *et al.*, 2009b and Achten *et al.*, 2010b). Considering that certain provenances may differ relative to others in their behavior towards water scarcity, in this study we aimed to characterize the morpho-physiology of two *Jatropha curcas* accessions originating from contrasting environments (arid and wet tropical climates).

2 Materials and methods

2.1 Plant material

Two accessions of *Jatropha curcas* originating from distinct climate regions were used. One accession was obtained from the wet tropical climate of Indonesia (GPS coord: S3° 14' 28.82", E102° 57' 44.95") and another from the arid climate of Cape Verde islands (GPS coord: N14° 57' 16.11", W23° 36' 19.68"). The two accessions were cultivated in Cape Verde islands and seeds produced there were kindly provided by Quinvita.

The seeds were germinated at 28 °C, in a wet environment and clean sand. Homogeneous 10 day old seedlings were transplanted to 7.5 L pots with a soil mixture of sand, peat and soil (3:1:1) supplemented with a commercial fertilizer (Osmocote slow release, 16N+9P₂O₅+12K₂O+2,5MgO, Scotts, Netherlands) (10.5 g/pot). Plants were maintained well watered (at field capacity) until the beginning of the treatments.

2.2 Plant analysis

2.2.1 Morphological parameters

Plant growth was monitored weekly for all tested conditions (see sections 4 and 5). Measurements of stem grow were made by analyzing stem length (measured vertically from the substrate surface until the apical meristem in cm) and diameter (measured at the basis in cm), as well as number of leaves (>2 cm in length) *per* plant. At harvest collection points, fresh weight (g) was determined for leaves, roots and shoots. Dry weight (g) was determined by drying at 70°C until constant weight was achieved. The percentage of dry matter [(fresh weight/dry weight) * 100] and shoot to root dry mass ratio (S:R) were calculated. Specific leaf area (SLA) was calculated as leaf area *per* unit dry mass (cm² g⁻¹ DW). Leaf area was determined after the harvesting of the plants, by isolation of all leaves and measuring total leaf area (TLA) *per* plant using a color image analysis system (WinDIAS 2, Delta-T Devices, UK).

2.2.2 Water relations parameters

2.2.2.1 Water availability in the substrate

Soil water availability (SWA) was calculated as: $SWA = [(Pot\ weight - Minimum\ Pot\ Weight) - (Maximum\ Pot\ Weight - Minimum\ Pot\ Weight)] * 100$. Minimum pot weight was

considered the pot weight without any water. For assess minimum pot weight, the soil was spread in a fine layer and air dried in the glasshouse until a constant weight was achieved, normally three weeks. Maximum pot weight was considered the pot weight at field capacity.

2.2.2.2 Leaf relative water content determination

Six leaf discs (19mm in diameter) were collected from the three youngest fully expanded leaves and weighed (fresh weight - FW). These leaf discs were allowed to float in the dark at room temperature with the abaxial leaf surface facing distilled water. After 24 hours absorbing water the discs were gently cleaned with absorbent paper to remove any water excess and weighed (turgid weight - TW). Finally, after drying at 70°C for 48 hours, the dry weight (DW) was recorded. RWC was calculated according to Barrs and Weatherly (1962) using the formula: $RWC (\%) = [(FW-DW)/(TW-DW)]*100$.

2.2.3 Leaf physiology

2.2.3.1 Determination of chlorophyll content in leaves

Chlorophyll was determined according to the method of Lichtenthaler (1987). Three circles (19 mm in diameter) were collected from the three youngest fully expanded leaves and immediately immersed in liquid nitrogen and stored at -80 °C until use. Frozen leaf tissue was ground in liquid nitrogen, weighted and transferred to a sample flask covered with aluminium foil containing 20 ml of 80% acetone and transferred to -20°C for 24 h. Measurements, were performed after 4 h at room temperature. Absorbance was measured with a spectrophotometer (DU-70 Spectrophotometer, Beckman, USA) at 663.2 and 646.8 nm in a quartz cuvette. The chlorophyll *a* (Chl_a) and chlorophyll *b* (Chl_b) content (mg g⁻¹ FW) were calculated as follows: $Chl_a = (12.25 \cdot A_{663.2} - 2.79 \cdot A_{646.8})/(v/FW)$; $Chl_b = (21.5 \cdot A_{646.8} - 5.1 \cdot A_{663.2})/(v/FW)$; where $A_{646.8}$ is the absorbance at 646.8 nm and $A_{663.2}$ is the absorbance at 663.2 nm, *v* is volume (ml). Chlorophyll *a* to *b* ratio (Chl *a/b*) was also calculated.

2.2.3.2 Leaf gas exchange and chlorophyll *a* fluorescence measurements

Leaf gas exchange was monitored with a portable infrared gas-exchange meter (Li-6400; Li-Cor Inc., USA) equipped with an artificial red-blue light-emitting diode (LED) source, and with an integrated fluorescence chamber head. Measurements of net photosynthesis (A_n , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), transpiration rate (E , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) were made periodically (see 4.2.3 and 5.2.3) between 10 a.m. and 2 p.m. with a block

temperature set at 28°C, light intensity set at 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a CO_2 concentration of 400 ppm and air flow rate of 500 $\mu\text{mol s}^{-1}$ in the youngest fully expanded leaf. Simultaneous measurements of chlorophyll *a* fluorescence allowed determination of the photosystem II efficiency (Φ_{PSII}).

2.3 RNA

2.3.1 RNA collection from leaves and roots

Young leaves (~2cm in length, Fig. 4 A) were collected for RNA extraction at early moderate, moderate, late moderate and maximum drought, as well as in the recovery week (1st, 3rd and last day of recovery). Root material (Fig. 4 B) was collected at moderate and maximum drought, as well as in the recovery week (1st, 3rd and last day of recovery). For root tips samples, soil was carefully removed to avoid contamination.

Collection points correspond to a pool of 3-6 plants (more details regarding collection points and pools can be found in *addendum I*). Collected plant samples (leaves and roots) were immediately frozen in liquid nitrogen and stored at -80 °C until further use.

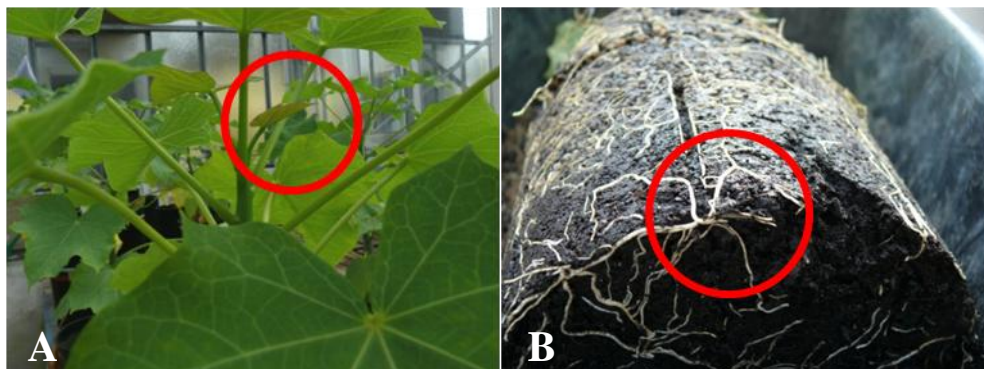


Figure 4.- Example of material collected for RNA extractions for: A) leaf and B) root.

2.3.2 RNA extraction

The frozen material was carefully ground in liquid nitrogen to a fine powder. Eighty milligrams of material were used for total RNA extraction using the RNeasy plant mini kit (RNeasy plant mini kit, Qiagen, Germany). RNA from leaf material was extracted using the RLC lysis buffer supplemented with 0.2% of a PEG 20000, as suggested by Gehrig *et al.* (2000). On the other hand, RNA was extracted from root material using the RLT lysis buffer (RNeasy plant mini kit). All the other extraction protocol steps were performed according to the manufacturer's instructions (detailed protocol in *addendum II*). After the extraction,

samples were treated with DNase (Turbo DNA-free Kit, AM1907, Ambion, USA) according to the manufacturer's instructions (see *addendum II*) to remove any DNA contamination.

2.3.3 RNA quantification and quality

RNA was quantified by spectrophotometer (NanoDrop 3300, Thermo Scientific, USA), and quality/purity was evaluated by the ratio $A_{260/280}$ and the ratio $A_{260/230}$. Moreover, RNA integrity and quantification was checked by electrophoresis. Total RNA (300 or 500 ng) was fractionated in a 1% agarose gel stained with ethidium bromide. The RNA gel was visualized using the Image Analyzer Gel DocTM XR+ (Biorad, USA).

2.4 Statistical analysis

Analysis of variance (ANOVA) was performed (SigmaPlot 11, USA). When significant differences occurred, means were separated by the Tukey's studentized range test at $P < 0.05$.

3. Photosynthetic response to light

3.1 Introduction

The level of irradiance is an important environmental factor on which plants depend to grow. Photosynthesis can present light-dependent and -independent reactions. In the light-dependent reaction, light energy is used to produce adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH). Subsequently, in the light-independent reaction, carbon is fixed into carbohydrates (Taiz and Zeiger, 2006).

However, under very high irradiance, the photosynthetic apparatus may absorb excessive light energy, resulting in the inactivation of the chlorophyll containing reaction centers of the chloroplasts (Bertamina *et al.*, 2006). As a consequence, photosynthetic activity is depressed by photoinhibition (Osmond, 1994). In contrast, under low irradiances, insufficient ATP is produced to allow further carbon fixation and carbohydrate biosynthesis, which leads to reduced plant growth (Baltzer and Thomas, 2007). Leaf gas exchange and chlorophyll *a* fluorescence measurements are a rapid and non invasive measurement that provides information regarding photosynthetic performance of plants (Maxwell and Johnson, 2000; Long and Bernacchi, 2003). In eco-physiological studies, light response curves (LRC) can provide information about the efficiency at which light is used by photosynthesis. Both, the light compensation and light saturation points and photosynthetic efficiency can be estimated through photosynthetic light response curves (Taiz and Zeiger, 2006). The point on the light curve where photosynthesis no longer increases with increasing light intensities is called light saturation point, and is where the light-dependent reactions are producing more ATP and NADPH than can be used by the light-independent reactions (Smith, 1936). Light compensation point (LCP) is the point where the rate of photosynthesis exactly matches the rate of respiration, at this point, the uptake of CO₂ through photosynthetic pathways is exactly matched to the respiratory release of CO₂, and the uptake of O₂ by respiration is exactly matched to the photosynthetic release of O₂ (Taiz and Zeiger, 2006). Moreover, differences in the shape of LRC may unveil inter- and intra-specific differences (Singsaas *et al.*, 2001). Furthermore, the light response curve of *J. curcas* remains not well described in literature.

In order to better understand the photosynthetic performance of the two *Jatropha curcas* accessions, the response of leaf gas exchange and chlorophyll fluorescence to different irradiance intensities was tested in well watered plants.

3.2. Method to analyse leaf gas exchange and chlorophyll *a* fluorescence

Response curves were performed with a portable infrared gas-exchange meter (Li-6400; Li-Cor Inc., USA) equipped with an artificial red-blue LED source and an integrated fluorescence chamber. Net photosynthesis (A_n , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), transpiration rate (E , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and internal CO_2 concentration (C_i , ppm) were measured. Simultaneous measurements of chlorophyll *a* fluorescence allowed determination of photosystem II efficiency (Φ_{PSII}) which translate the efficiency of photochemistry (Maxwell and Johnson, 2000). Electron transport rate (ETR, $\mu\text{mol electron m}^{-2} \text{ s}^{-1}$) was calculated as $\text{ETR} = \Phi_{\text{PSII}} \times \text{PPFD} \times 0.5 \times 0.84$; 0.5 was used as the fraction of excitation energy to photosystem II and 0.84 as the fraction of light absorption (Schreiber *et al.*, 1998). Instantaneous water use efficiency (WUE, $\mu\text{mol CO}_2 \mu\text{mol}^{-1} \text{ H}_2\text{O}$) was calculated as A_n/E . Block temperature was set at 28°C, with a CO_2 concentration of 400 ppm and an air flow rate of 500 $\mu\text{mol s}^{-1}$. Photosynthetic photon flux density (PPFD) was gradually decreased from 2000 to 0 (2000; 1750; 1500; 1200; 900; 700; 500; 250; 100; 70; 50 and 0 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$) in order to avoid limitation of photosynthesis at high light due to insufficient stomatal opening caused by the initial lower light intensities (Singsaas *et al.*, 2001). A 2-3 min acclimation period was performed between the measurements of the 12 light levels. Determinations were performed in well lit, fully expanded leaves, between 11 a.m. and 4 p.m., in 71 days old plants. Five replications were used *per* accession (one leaf *per* plant).

Light response curves were interpreted according to Taiz and Zeiger (2006), where the linear phase corresponds to the fast increase of photosynthesis in response to increased irradiation. Light compensation point (LCP) was extrapolated from the linear phase equation and corresponds to the PPFD value when A_n is zero. The light saturation point (LSP) corresponds to the value of PPFD from which further increase of PPFD is not followed by an increase in A_n .

3.3 Results

3.3.1 Leaf gas exchange

Net photosynthesis responded positively to the light increase. In both accessions A_n increased rapidly as PPFD increased to $250 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (this corresponding to the linear phase). Then there was a slow increase to a maximum ($1200 \mu\text{mol photon m}^{-2} \text{s}^{-1}$, corresponding this to LSP), remaining constant as light intensity was increased to $2000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (Fig. 5 A and Table 1). The LCP was estimated to be approximately 1.6 and $1.2 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (for the wet tropical and the arid accessions, respectively) (Table 1).

Regarding g_s (Fig. 5 B), the wet tropical accession increased g_s until $700 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (rapid until $250 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ and then gradual), followed by a soft decrease until $1750 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ followed by a slight increase until $2000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$. On the other hand, the arid accession showed a rapid increase of g_s values until a light intensity of $250 \mu\text{mol photon m}^{-2} \text{s}^{-1}$, remaining constant until maximum light intensity ($2000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$).

The transpiration light response curve appeared to be a marked single-peak curve (Fig. 5 C), with E values rapidly increasing from 0 to $250 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ followed by a gradual increase until $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$. At high irradiance levels (from 1200 to $2000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) the arid accession presented slightly higher E values compared with the wet tropical accession.

The internal CO_2 light response curve is presented in Fig. 5 D. C_i values rapidly decrease until an irradiance level of $250 \mu\text{mol m}^{-2} \text{s}^{-1}$, slowly increasing afterwards until the maximum light level is reached ($2000 \mu\text{mol m}^{-2} \text{s}^{-1}$). The C_i response pattern is identical for both accessions, although from $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ the wet tropical accession presents slightly lower values of C_i .

The two accessions had identical water use efficiency in response to light (Fig. 6). WUE rapidly increase until $250 \mu\text{mol photon m}^{-2} \text{s}^{-1}$, reaching a maximum of $\sim 4 \mu\text{mol CO}_2 \text{mol}^{-1} \text{H}_2\text{O}$ and stabilizing thereafter.

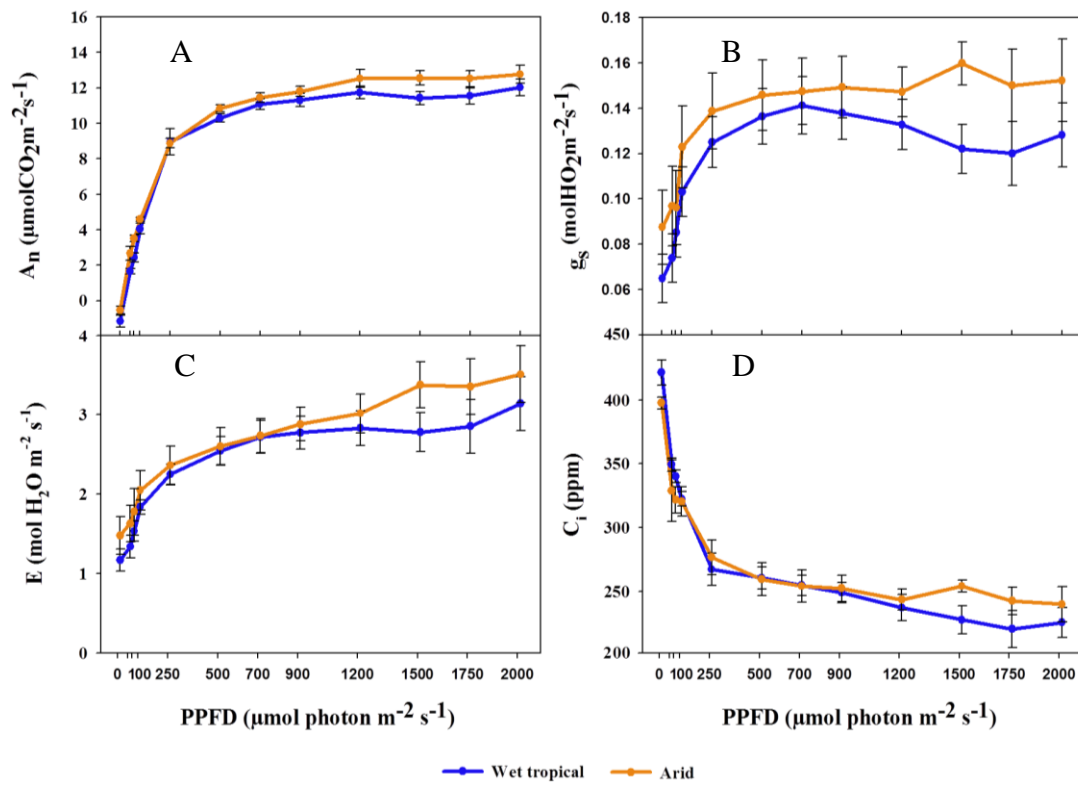


Figure 5 - Light response curves for two *Jatropha curcas* accessions (original from a wet tropical or arid climates): A) Net photosynthesis (A_n); B) stomatal conductance (g_s); C) transpiration rate (E); and D) intercellular CO_2 concentration (C_i). Values are means \pm se ($n=5$).

Table 1: Photosynthetic parameters for the two *Jatropha curcas* accessions (original from a wet tropical or arid climates). Light saturation and light compensation points (LSP and LCP, respectively) as well as maximum net photosynthesis (A_n maximum) were estimated from the light response curves.

	LSP ($\mu\text{mol photon m}^{-2} \text{s}^{-1}$)	LCP ($\mu\text{mol photon m}^{-2} \text{s}^{-1}$)	A_n maximum ($\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$)
Wet tropical	1200	1.6	12.02
Arid	1200	1.2	12.76

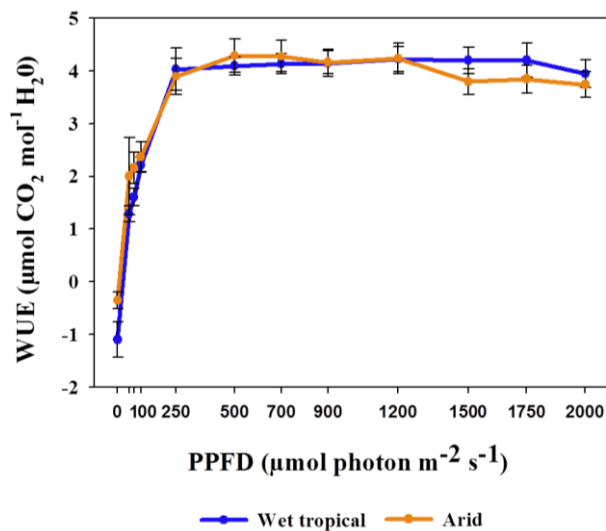


Figure 6 - Instantaneous water use efficiency (WUE) light response curve of two *Jatropha curcas* accessions. Values are means \pm se ($n=5$).

3.3.2 Chlorophyll *a* fluorescence

Leaf chlorophyll *a* fluorescence was measured under light conditions. Photosystem II efficiency (Φ_{PSII}) and electron transport rate (ETR) responses to light are presented in Fig. 7. Φ_{PSII} showed a steady decline, for both accessions (Fig. 7 A). Additionally, ETR increased rapidly as light increased up to 500 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ but decreased above 700 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$, in both accessions (Fig. 7 B).

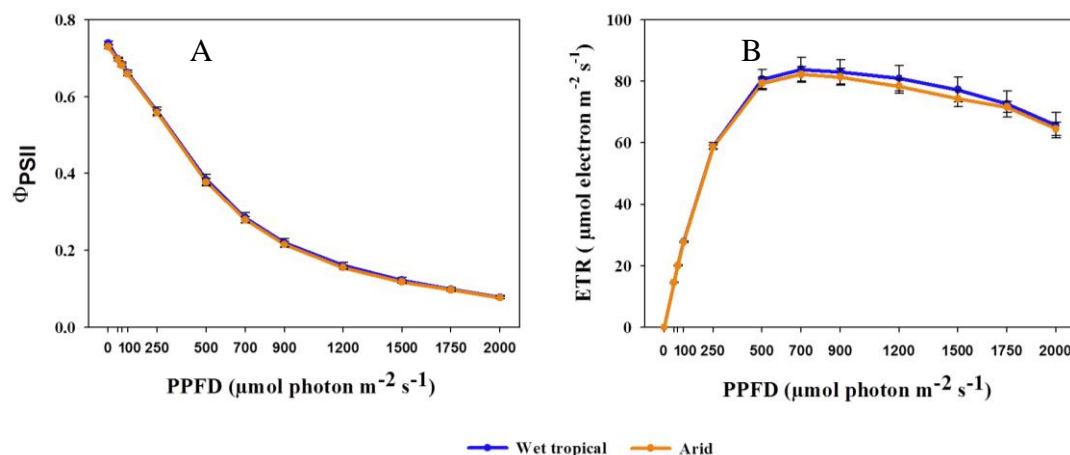


Figure 7 – Light response curves of two *Jatropha curcas* accessions: A) Photosystem II efficiency (Φ_{PSII}); B) electron transport rate (ETR). Values are means \pm se (n=5).

3.4 Discussion and conclusions

Light response curves can help to characterize the photosynthetic performance characteristics and the photosystem performance of different accessions of *Jatropha*, for which information is limited.

However, in this study, no significant differences were observed between accessions for the responses to light of leaf gas exchange (Fig. 5), water use efficiency (Fig. 6) and chlorophyll *a* fluorescence (Fig. 7). Yong *et al.* (2010), measured photosynthetic light response curves to characterize three different *Jatropha curcas* accessions (from China, Cambodia and India). These authors found no differences between accessions.

Maximum values of net assimilation (A_n) have been reported to vary between 12 and 19 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for mature leaves of *J. curcas* plants growing under a light intensity of about 900 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Yong *et al.*, 2010; Juan-Yu *et al.*, 2011). The large range of values presented in the literature for maximum A_n are probably due to differences in leaf developmental stage and fertilizer application. In this study A_n maximum was of about 12 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for both accessions. This value may be related to different conditions used, especially the ones related to growing light conditions. Our plants were grown in low light conditions ($141 \pm 16 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and it is well established that plants grown under low light tend to have lower photosynthetic capacity reaching lower A_n maximum and lower light saturation points compared to plants grown in high light (Bjorkman, 1981; Taiz and Zeiger, 2006; Dai *et al.*, 2009).

Moreover, in the tested conditions, the LCPs were about 1.6 and 1.2 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for wet tropical and arid accession, respectively. These are low light compensation points and are typical of shade plants which have LCP between 1 to 5 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (Taiz and Zeiger, 2006). Shade plants also tend to present lower respiration rates, so in these growing conditions lower photosynthesis brings rates of CO_2 exchange to zero (Taiz and Zeiger, 2006), as we observed.

Senevirathna *et al.* (2003) reported for rubber tree plants grown in low light, that A_n tended to saturate at low PPFD. However, this was not observed for our both accessions as they presented light-saturated photosynthetic capacity at 1200 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$. Yong *et al.* (2010) have reported light saturation points between 750 and 1500 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (for *Jatropha* plants grown under high light). This suggests that even growing in low light, both *J. curcas* accessions presented a high light saturation point. This is in accordance with

the findings of Matos *et al.* (2009), who found that *Jatropha* plants growing under low light reduced LCP and A_n maximum compared with plants grown in high light conditions, but the LSP was not changed.

Regarding stomatal conductance, the results of g_s in response to light for the wet tropical accession suggest that this accession has a higher stomatal sensitiveness to high irradiance, since a slightly decline was observed in g_s for irradiances higher than 700 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ (the same was not observed for the arid accession). Moreover this reduction did not affect A_n values. Additionally, C_i reduction at increasing irradiation was not caused by stomatal limitations, because g_s values increased with increasing light (Fig. 5 B and D), but rather due to higher CO_2 consumption in photosynthesis. Moreover this decrease in C_i is limiting A_n above the light saturation point by the pools of Calvin cycle intermediates, which can affect the carboxylation activity of RuBisCO or the metabolism of the triose phosphates (Long and Bernacchi, 2003; Taiz and Zeiger, 2006).

No significant differences were observed for WUE between accessions (Fig. 6), suggesting that the different origins of both accessions does not affect water use efficiency in the present conditions.

Chlorophyll *a* fluorescence measurements of Φ_{PSII} , showed a constant decrease of Φ_{PSII} with increasing light (Fig. 7 A). At light saturation levels (1750 – 2000 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$) Φ_{PSII} was almost null. This indicates that the reaction centers of photosystem II are been progressively closed as light increases (Maxwell and Johnson, 2000). While photosynthesis is increasing, the majority of the light received by the leaf is being used for photochemistry (A_n) resulting in decreased light energy dissipation by fluorescence, since these processes occur in competition (Maxwell and Johnson, 2000). ETR values are proportionally increasing as the Φ_{PSII} decreases, which is in accordance with the increasing closure of photosystem II reaction centers, since the ETR value indicates the relative quantity of electrons passing through photosystem II (Tezara *et al.*, 2003).

From this study we could observe that the photosynthetic response to light of both *J. curcas* accessions is similar. However, in further studies it would be important to analyze plants growing under different light regimes. Further characterization of the photosynthetic efficiency in response to different temperatures and CO_2 concentrations, would also complement this photosynthetic characterization.

4 Establishing conditions for drought assays in *Jatropha*

4.1 Introduction

Jatropha curcas ability to growth in marginal and dry soils has been poorly explored, opposite to the numerous studies analyzing the chemical and physical properties of seed oil. A few studies were recently published reporting the plant performance (biomass production and allocation) and plant-water relationships, leaf gas exchange and osmotic adjustment in conditions of limited water availability (e.g. Maes *et al.*, 2009b; Achten *et al.*, 2010b; Pompelli *et al.*, 2010; Silva *et al.*, 2010a,b). However the plant age, the water stress induction conditions, and the duration and intensity of the stress are highly variable, which makes it impossible to integrate or compare results. Stress imposition (intensity and duration) will determine the nature and extent of the effects of water deficit (Chaves *et al.* 2002). To optimize duration and intensity of the water stress treatment, two preliminary drought assays were conducted using both *J. curcas* accessions, in order to determine the appropriate conditions to better characterize them. The objective of these experiments was to determine the best water stress imposition, measurements and sampling periodicity. Also these experiments were crucial to establish the RNA collection points.

4.2 Methods for drought assays

4.2.1 Environmental conditions

Two independent experiments, identified as TC1 and TC2, were carried out in a growth chamber (3x6x2.8 m) with partially controlled conditions of temperature with average values of $28\pm 2^{\circ}\text{C}/20\pm 4^{\circ}\text{C}$ (day/night), and relative humidity with average values of $35\pm 5\%/75\pm 5\%$ (day/night). The photoperiod was of 12 h and average light intensity at the plant level was of $172\pm 66 \mu\text{mol photons m}^{-2}\text{s}^{-1}$, provided by two types of lamps [mercury (MASTER HPI-T Plus 400W, Philips, Belgium) and sodium (son-T-agro 400W, Philips, Belgium)].

4.2.2 Treatments

Plants of the two accessions were subjected to drought (water stress - WS) or grown under well watered conditions – WW (control). The drought treatments consisted in subjecting 71 days old plants to either water withhold for 36 days (TC1) or to a gradual decrease of soil water availability (10% reduction of soil water availability every 2 days on a weight base) for

28 days (TC2), followed by a re-hydration period of 7 days. Control treatment consisted in daily irrigation. A schematic representation of the experimental design can be found in Fig.8.

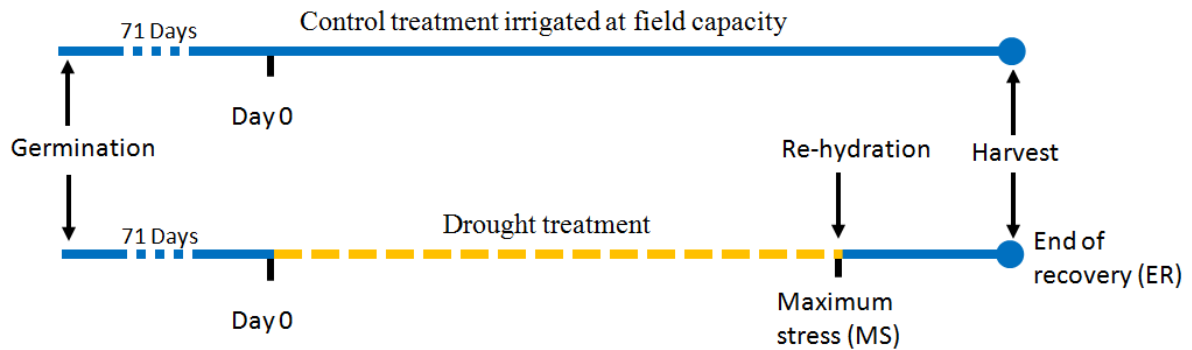


Figure 8 - Experimental design used to establish drought conditions. Water stress was achieved by water withhold for 36 days (TC1) or by gradually decreasing irrigation, until reaching 15% field capacity (after 28 days) (TC2), followed by one week of recovery (daily irrigation). Control plants were maintain at well watered conditions and used as reference. Harvest of plants was performed at the end of the recovery (ER).

4.2.3 Measurements and sampling time

All measures and sample collection were performed in 5 plants *per* treatment between 10 a.m. and 2 p.m.. Samplings for relative water content and chlorophyll content were performed at maximum stress (MS) and end of recovery (ER). Destructive measurements were performed at harvest in ER (Fig. 8) for both tested conditions.

Leaf gas exchange measurements were performed frequently along the two tested conditions. Namely, for TC1 at 0; 13; 19; 27; 31 and 36 (MS) days of water withhold and at the 1st, 2nd, 5th and last day of the recovery week, and for TC2 measurements were performed at day 0; 4; 7; 11; 15; 20; 26 and 28 (corresponding to ~100; 80; 70; 60; 50; 40; 30; 20 and 15% SWA) and at the 1st; 2nd; 3rd; 4th; 5th and last day of the recovery week.

4.3 Results

4.3.1 Water relations parameters

4.3.1.1 Soil water availability variation

During water stress by water withholds (TC1) the soil water availability was gradually decreased for both accessions decreasing in average ~2% *per* day and reaching a minimum of 30% (Fig. 9 A). Regarding TC2, soil water availability was decreased by 10% every 2 days and reached a minimum of 15% (Fig. 9 B).

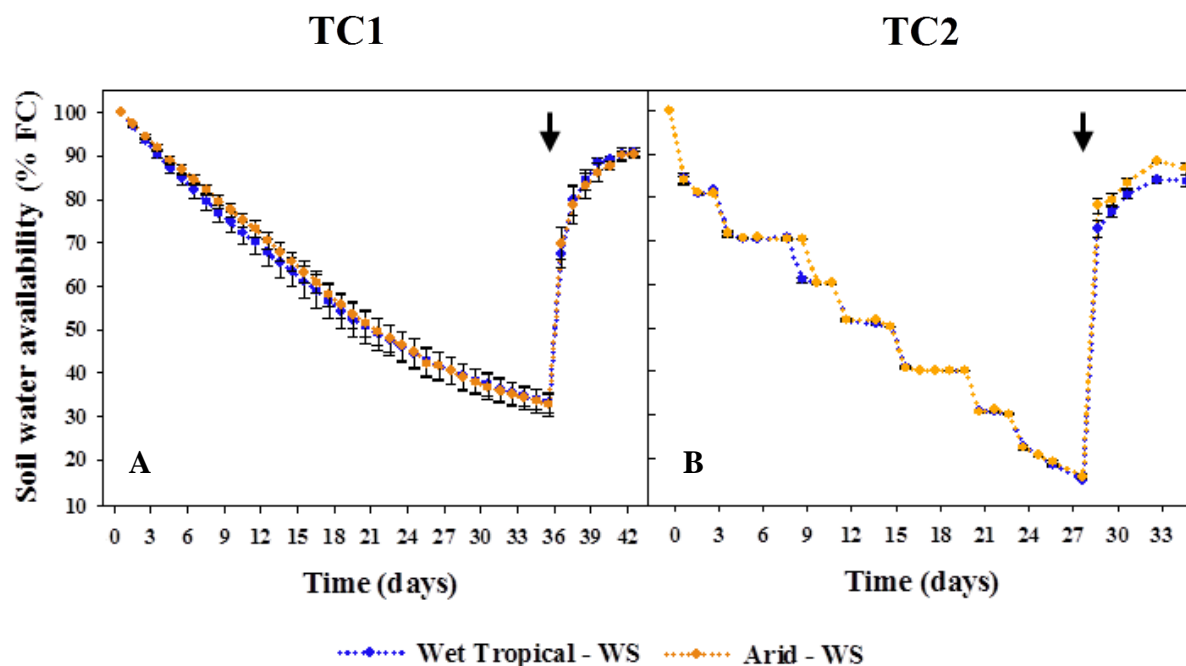


Figure 9 - Soil water availability along the drought treatments. Soil water availability was monitored and recorded during water stress imposition by: A) water withhold for 36 days (TC1) or B) gradually decreasing irrigation, until reaching 15% field capacity (after 28 days) (TC2). Both followed by seven days of recovery. FC stands for soil field capacity. Arrows stand for re-hydration day. Points represent means \pm se (n=5).

4.3.1.2 Leaf relative water content

Leaf RWC was not altered by the water stress treatment in both tested conditions (Table 2), suggesting that stomatal control (observed in the gradual reduction of g_s during WS) is efficient in reducing water loss and maintaining a good leaf water status. No significant differences were found between accessions subjected to WS or WW.

Table 2: Effect of drought stress in leaf relative water content (RWC) of two *Jatropha curcas* accessions. Sampling points were performed at maximum stress (MS) and end of recovery (ER). Values are means \pm se (n=5).

		TC1		TC2	
		MS	ER	MS	ER
Wet tropical	WW	67.10 \pm 1.59	70.00 \pm 2.75ab	66.43 \pm 0.62b	72.43 \pm 1.81
	WS	73.16 \pm 5.70	79.56 \pm 3.77a	73.45 \pm 2.63ab	71.90 \pm 3.62
Arid	WW	69.34 \pm 0.62	67.61 \pm 2.05b	75.63 \pm 2.51ab	75.66 \pm 1.25
	WS	75.25 \pm 1.56	75.02 \pm 2.58ab	82.87 \pm 3.32a	69.92 \pm 1.13

Treatment means with a different letter differ significantly according to Tukey's test ($p < 0.05$).

4.3.2 Morphology

Growth inhibition and resume after re-hydration was observed in the two tested conditions for both *J. curcas* accessions (Fig.10 and Table 3). Growth arrest was more pronounced and occurred earlier in TC1, stem diameter (Fig. 10 A) arresting growth from day 21 until day 36 (50-30% SWA). Furthermore, some growth inhibition was observed for wet tropical accession from day 14 to 21 (70-50% SWA) (the same was not observed for arid accession). Stem length (Fig. 10 B) showed growth arrest for both accession from day 7 until day 36 (80-30% SWA). At day 29 (40% SWA) a clear difference was observed between stress and control groups.

Regarding the number of apical leaves (Fig. 10 E), in TC1 emergence of new leaves was arrested from day 14 to day 36 (70-30% SWA), and leaf expansion was also affected. In fact, by harvest time, the total leaf area in both drought stressed accessions showed a 9 x reduction compared with controls (p-value<0.001). Senescence of older leaves was observed only for the wet tropical accession after prolonged stress (from day 29 to 36 of water withhold, 40-30% SWA). In TC2 conditions no difference was observed between stress and control groups (Fig.10 B, D, F), growth inhibition occurred at severe stress from day 21 to day 28 (30-15% SWA).

Although plants used for TC1 and 2 were of the same age (71 days), clear differences were observed for stem length and leaf number. At day 0 plants of TC1 presented ~3.5 leaves and ~15.2 cm long stems, against the ~12.9 and ~35.5 cm long stems of TC2 plants. Stem diameter at the base was similar in plants of both tested conditions (1.1 cm TC1 and 1.2 cm TC2).

In TC1 plants, we found a significant increase in the percentage of dry matter (p-values<0.001) in water stress conditions, with no significant differences between accessions. In TC1 dry matter percentage in leaves, stem and roots was always higher in drought stressed plants, independently of the provenance, while this not always occurred in TC2 (Table 3). Regarding shoot to root ratio a significant reduction was observed for plants subjected to TC1 water stress (p-value<0.001), suggesting that *Jatropha* severely reduced aerial part growth during water limiting conditions. In TC2 however, no major differences were found at harvest between accessions and treatments (Table 3), except for root percentage of dry matter. Root percentage of dry matter was significantly higher in the arid accession subjected to drought.

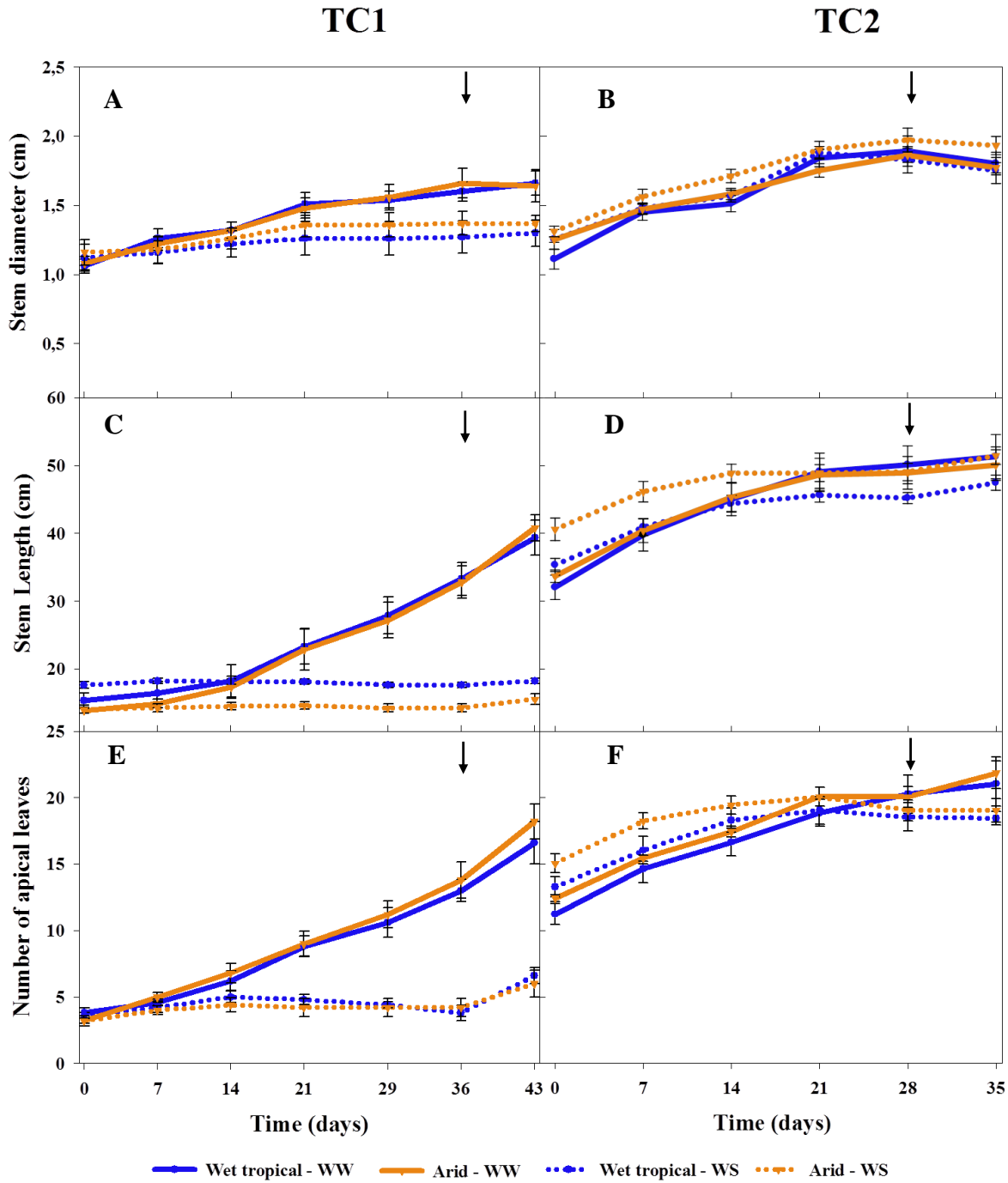


Figure 10 - Effect of drought stress and recovery on morphology of two *Jatropha curcas* accessions: A-B) Stem diameter; C-D) stem length; E-F) number of apical leaves. Arrows indicate re-watering day. Values are means \pm se (n=5).

Specific leaf area (SLA) (Table 3), is an indicator of leaf thickness, and has often been observed to reduce under drought conditions. This was observed at harvest time for TC1 in both accessions subjected to stress (WW versus WS, $p=0.007$) with a reduction of 19 and 12% SLA compared to control plants in wet tropical and arid accessions, respectively.

Table 3: Effect of drought stress on percentage of dry matter, total leaf area (TLA), specific leaf area (SLA) and shoot to root dry mass ratio (S:R) of two *Jatropha curcas* accessions. Sampling was performed at end of recovery (ER). Values are means \pm se (n=5).

			Dry matter (%)			TLA (cm ²)	SLA (cm ² g ⁻¹)	S:R
			Leaf	Stem	Root			
TC1	Wet tropical	WW	11.7 \pm 0.5b	12.9 \pm 0.8c	12.0 \pm 0.5bc	2144 \pm 330a	315.6 \pm 19.2	4.2 \pm 0.3ab
		WS	17.4 \pm 0.6a	16.8 \pm 0.8a	15.2 \pm 0.7ab	234 \pm 43b	256.0 \pm 7.3	2.8 \pm 0.2b
	Arid	WW	11.7 \pm 0.7b	12.8 \pm 0.7bc	9.6 \pm 1.2c	2140 \pm 215a	302.2 \pm 21.9	4.8 \pm 0.5a
		WS	19.2 \pm 2.7a	16.9 \pm 0.3ab	15.8 \pm 1.0a	267 \pm 55b	267.1 \pm 4.2	3.1 \pm 0.4b
TC2	Wet tropical	WW	16.1 \pm 1.7	5.6 \pm 0.1	14.9 \pm 0.3ab	3510 \pm 349	241.2 \pm 5.4	2.7 \pm 0.1
		WS	14.1 \pm 0.7	6.1 \pm 0.9	13.8 \pm 1.1b	2808 \pm 99	254.1 \pm 8.3	3.0 \pm 0.2
	Arid	WW	13.9 \pm 0.5	5.8 \pm 0.5	14.2 \pm 0.3ab	3645 \pm 202	257.3 \pm 14.0	2.6 \pm 0.1
		WS	14.7 \pm 0.3	7.1 \pm 0.5	16.9 \pm 0.6a	3129 \pm 101	247.2 \pm 3.4	2.9 \pm 0.1

Treatment means with a different letter differ significantly according to Tukey's test ($p<0.05$).

4.3.3 Chlorophyll content

Some fluctuations were observed in the chlorophyll content of *Jatropha curcas* plants (Fig.11). At the end of the stress imposition in TC1, the Chl_b percentage in total chlorophyll content of the wet tropical accession increase from 25% (control) to 35% in water stress plants, this resulted in a decrease of Chl *a/b* (Fig. 11). In contrast, at the end of the stress period in TC1, the percentage of Chl_b in total chlorophyll content of the arid accession decreased from 33% (control) to 27% in water stress plants, which resulted in a slight increased of Chl *a/b* (Fig. 11). After the recovery week, the percentage of Chl_b was similar for all treatments (25% wet tropical-WW; 28% wet tropical-WS; 26% arid-WW; 27% arid-WS), and no variations in Chl *a/b* were observed.

In TC2 minor variations were observed in chlorophyll content. Moreover, a change in the percentage of Chl_b from 29 (control) to 32% (stress) was observed resulting in a decrease in Chl *a/b* (Fig.11), under drought, for both accessions. By the end of the recovery week values of Chl *a/b* were very uniform for all treatments.

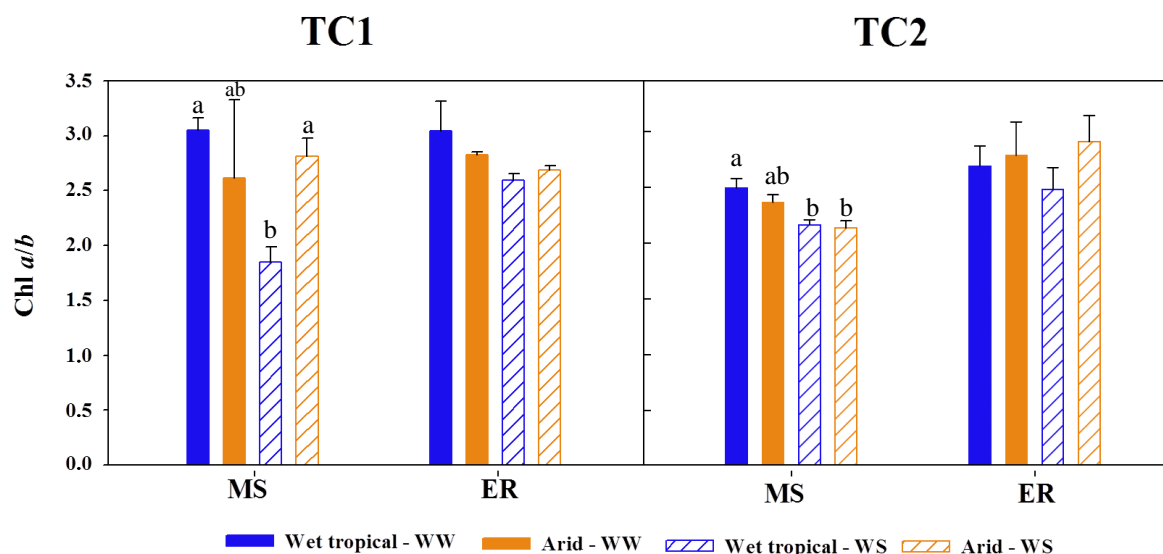


Figure 11 - Effect of drought stress and recovery on chlorophyll content. Chlorophyll *a* to *b* ratio of two *Jatropha curcas* accessions. Sampling points were performed at maximum stress (MS) and end of recovery (ER). Values are means \pm se (n=5). Treatment means with a different letter differ significantly according to Tukey's test ($p < 0.05$).

4.3.4 Leaf gas exchange and chlorophyll *a* fluorescence

The water stress treatment by water withhold (TC1) induced a progressive decrease in net photosynthesis (Fig. 12 A), stomatal conductance (Fig. 12 C) and photosystem II efficiency (Fig. 12 E). The reduction started earlier for the wet tropical accession, although no significant differences were found between accessions. Small restrictions in A_n appeared by day 7 (85% SWA) to wet tropical accession and day 19 (60% SWA) to arid accession, with a reduction of 31% and 7% respectively. The decrease continued until it reached a reduction of 51% for both accessions at day 36 (MS, SWA of 30%). For g_s , the decrease was gradual from day 13 (70% SWA) until day 36 (MS, 30% SWA), moreover by day 36 the decrease on g_s was of 75% for wet tropical and 86% for arid accessions (as compared to control). The reduction pattern of Φ_{PSII} was very similar to the one observed for A_n , with an earlier reduction for the wet tropical accession (at day 19, 60% SWA) and a late reduction for the arid accession (at day 31) (40% SWA). However, under drought conditions, the arid accession presented higher Φ_{PSII} values during stress initiation. Furthermore, by day 36, both ecotypes presented a similar reduction in Φ_{PSII} as compared to the respective controls (39% and 31% of reduction, for the wet tropical and the arid, respectively).

Regarding TC2, a gradual decrease of g_s (Fig.12 D) was observed for both accessions, suggesting an acclimatory response to the increase of water scarcity in the soil (as observed for g_s in TC1). A reduction of 42% and 33% (wet tropical and arid accessions) was observed at day 11 (60% of SWA), and it continued to decrease until it reached a reduction of 52% and 54% (wet tropical and arid accessions) at day 20 (40% SWA), reaching a final reduction of 96% and 99% at day 28 (MS, 15% SWA). Surprisingly, A_n restriction was only observed at day 23 (30% of SWA) with a decrease of 49% and 34% for wet tropical and arid accessions, reaching a reduction of 77% and 78% at day 28 (MS, SWA of 15%) (Fig. 12 B). For Φ_{PSII} , an abrupt reduction was observed at day 26 (20% of SWA) reaching 58% and 73% (wet tropical and arid accessions, respectively) and stabilizing until day 28 (MS, 15% SWA).

At the end of the recovery week the normal values of leaf gas exchange and chlorophyll fluorescence were recovered for both accessions (wet tropical and arid) and tested conditions (TC1, TC2), moreover, in TC2 by day 29 (1st day of recovery week) the values obtained for all parameters tested (A_n , g_s , Φ_{PSII}) were identical to the control. It is important to emphasize that TC2 experiment resulted in a more severe reduction of leaf gas exchange and chlorophyll fluorescence reaching levels at maximum stress of 77% reduction for A_n , 98% reduction for g_s and 65% reduction for Φ_{PSII} , while in TC1 the corresponding reduction values were 51% for A_n , of 86% for g_s and 35% for Φ_{PSII} (both accessions analyzed together).

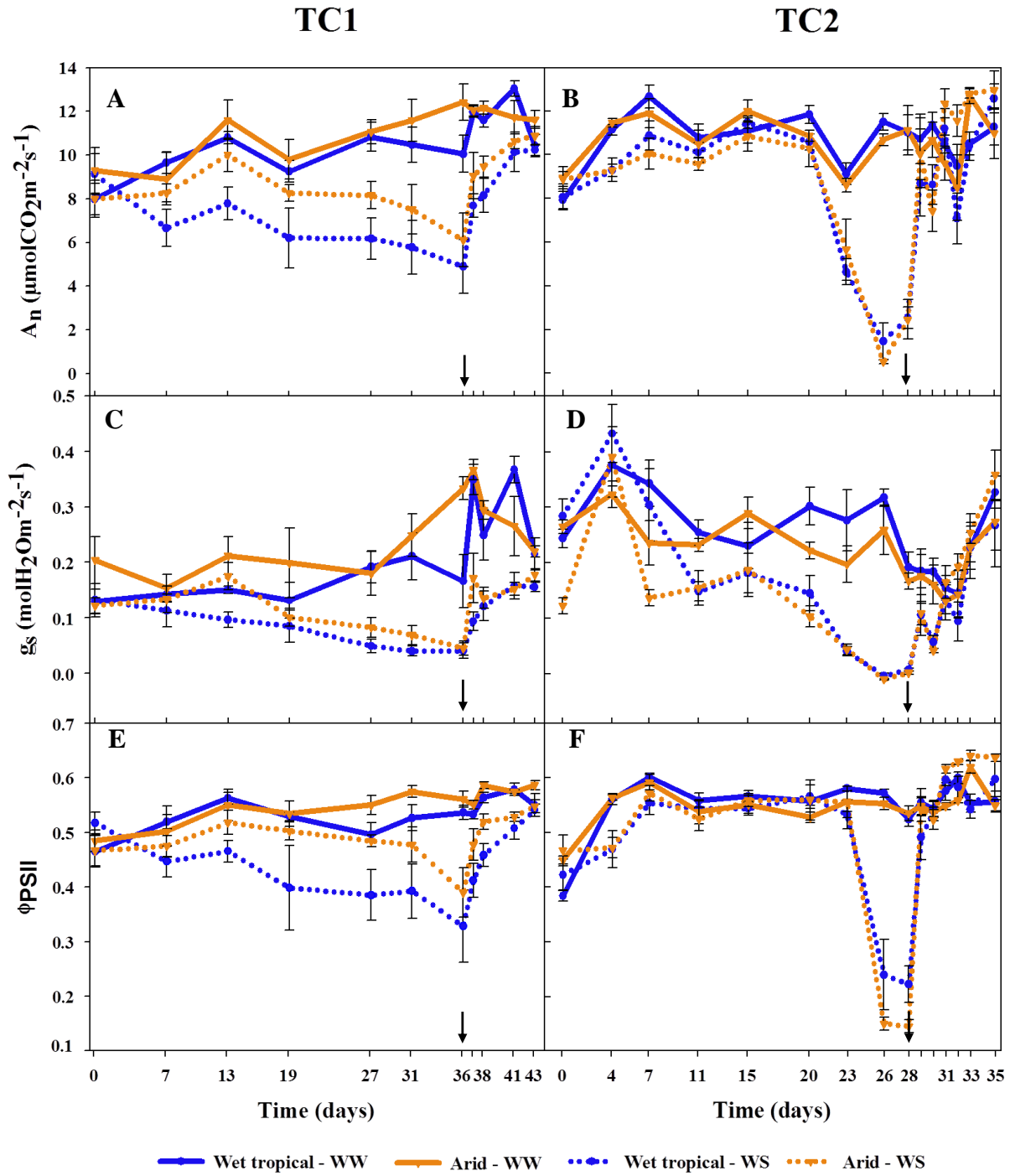


Figure 12 - Effect of drought stress and recovery on leaf gas exchange and chlorophyll *a* fluorescence of two *Jatropha curcas* accessions: A-B) net photosynthesis (A_n); C-D) stomatal conductance (g_s); E-F) photosystem II efficiency (Φ_{PSII}). Arrows indicate re-watering day. Values are means \pm se ($n=5$).

4.4 Discussion and conclusions

In both tested conditions relative water content was not affected by drought (Table 2), which agrees with reported data (Silva et al, 2010a,b; Díaz-López *et al.*, 2012) and will be further discussed in section 5.

Growth was significantly affected by water withhold (Fig. 10 TC1) but no significant reduction was observed for TC2 (Fig. 10 TC2). This suggests that even with a very low percentage of water available, *J. curcas* plants were able to maintain a growth rate. Moreover, this is in accordance with the findings of Achten *et al.* (2010b) who observed that water withhold would cause growth arrest (as observed in TC1), but maintaining plants at low SWA (40%) would allow plants to continue growing, although at a slower rate than controls. In fact in TC2, plants could continue growing even under reduced SWA and no difference was observed between stress and control plants.

Both tested conditions revealed different patterns of leaf gas exchange and chlorophyll *a* fluorescence response towards drought (Fig. 12). Moreover, although the stomatal conductance presented the same pattern in both tested conditions (gradual decrease) net photosynthesis and photosystem II efficiency presented completely different responses. In TC1 both A_n and Φ_{PSII} were gradually decrease (Fig. 12 TC1), on the other hand in TC2, A_n and Φ_{PSII} rapidly decreased only when soil water availability was very low (<30%) (Fig. 12, TC2). Furthermore, although plants had the same age in both tested conditions, differences in the developmental stages of both groups of plants were observed, and the plants of tested conditions 2 had a significantly higher leaf area (9 fold) and consequently higher transpiration surface, thus requiring more water. These differences in growth and in leaf gas exchange response were probably due to the fact that plants used in TC1 suffered a spider mite infestation at a young stage (15 days old seedling). Although the infestation was controlled and healthy plants were available by the beginning of the experiment, this could have caused unpredictable changes in development. Since the biotic stress caused the plants in TC1 to have a lower leaf area, this could justify the differences observed in leaf gas exchange response to water stress.

Although some difference in growth and leaf gas exchange patterns were observed between accessions in TC1, for most cases these were not significant. This was probably because the decrease in soil water content was slower in TC1, allowing the accessions to

gradually acclimatize to the reducing water in the soil. The reduced leaf area of plants in TC1 was probably another important factor contributing to the patterns observed.

From these experiments, we may suggest that in the future experiments aiming to compare ecotypes healthy plants with 3-5 leaves should be used and water stress treatment applied by water withhold until a 15% (or less) water availability is achieved in soil. Based on the results of TC1, this will promote a slower decline in the tested parameters, mimicking more natural conditions, and allowing to better explore putative differences in behavior of the plants under study.

5 Comparing two *Jatropha curcas* accessions during water stress

5.1 Introduction

Jatropha curcas has been described as drought tolerant and capable of growing in marginal and poor soils (Heller, 1996; Fairless, 2007; Divakara *et al.*, 2010). However *J. curcas* ability to growth in marginal and dry soils has been poorly explored.

Recently, several studies have been conducted regarding *J. curcas* drought tolerance (plant-water relationships, leaf gas exchange and osmotic adjustment) and performance (biomass production and allocation) (Maes *et al.*, 2009b; Achten *et al.*, 2010b; Pompelli *et al.*, 2010; Silva *et al.*, 2010a,b; Díaz-López *et al.*, 2012). However, studies comparing the responses of different accessions to water stress are scarce, and only two studies targeted water stress response in accessions from different provenances, namely Ethiopia, India and Thailand (Maes *et al.* 2009b; Achten *et al.* 2010b). These authors reported no effect of accession provenance on growth rate, plant-water relationships or drought tolerance. Moreover, it seemed that the growth conditions used had a higher effect on plant performance during stress imposition, than the genetic differences encountered between the accessions (Achten *et al.* 2010b). Nevertheless, further studies are needed to compare the performance of different accessions to water limiting conditions and to other stresses.

In this context, a drought assay was performed, according to the previously optimized conditions (see section 4) to characterize morpho-physiologically two accessions of *J. curcas* from two different provenances (wet tropical and arid climate) subjected to water stress as compared to control conditions.

5.2 Specific methods for water stress assay

5.2.1 Plant growth conditions

Drought assay was carried out in a greenhouse with a natural photoperiod (29 June to 24 August 2011, Oeiras, Portugal) and an average light intensity at the plant level of $411 \pm 226 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (Fig. 13). Temperature (°C) and relative humidity (%) were recorded during August (from day 36 to day 56 of the experiment) with a thermo-hydrograph (8147, Lufft, Germany) and the specific variation can be found in Fig. 14. Temperatures reached average values of 29 ± 3 to $20 \pm 2^\circ\text{C}$ (day/night), and relative humidity of 39 ± 8 to $69 \pm 4\%$ (day/night).

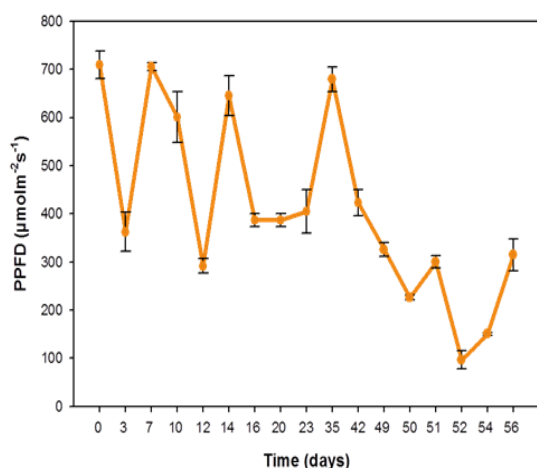


Figure 13 - Light variation along the drought assay. Light was recorded at measurement time (11 a.m. to 1 p.m.) at plant level with a PPFD sensor coupled in the chamber head of the infrared gas analyzer. Mean \pm se (n=20-25).

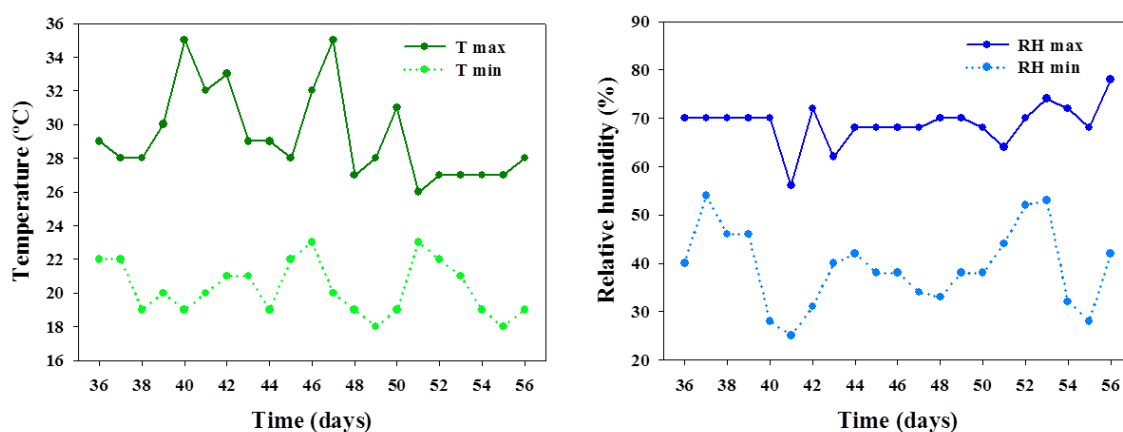


Figure 14 - Relative humidity and temperature variation (maximum and minimum) in the greenhouse during August (2011). Values refer to day 36 to day 56 of drought assay.

5.2.2 Treatments

Plants with 36 days, were subjected to 49 days of water withhold followed by one week of recovery (water stress treatment - WS) or kept at field capacity (well watered - WW). Pots positions were randomly changed weekly along the experiment to avoid light/shade influences.

5.2.3 Sampling

Destructive measurements were performed at harvest time, at moderate stress (day 13); maximum stress (day 49); 1st (day 50), 3rd (day 52) and end of recovery (day 56) (Fig. 15). Additionally leaf discs were collected for RWC and chlorophyll content determination at the beginning of the assay (day 0). Plants harvested at days 50 and 52 (1st and 3rd day of re-

watering) were not sampled for chlorophyll content. Leaf gas exchange and chlorophyll *a* fluorescence measurements were performed between 11 a.m. and 1 p.m..

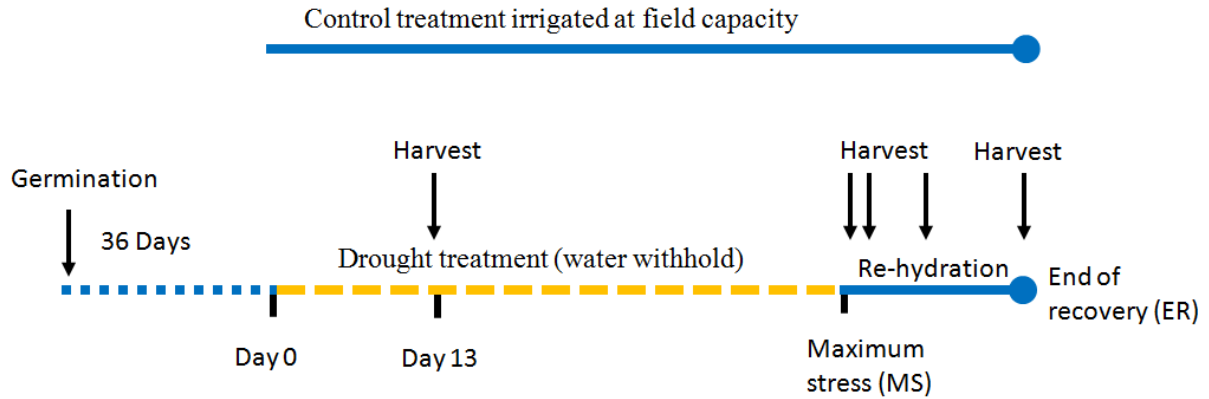


Figure 15 - Experimental design used to determine the effect of progressive drought. Water stress was imposed in 36 days-old *J. curcas* plants of two accessions by water withhold for 49 days followed by 7 days of recovery (well watered conditions). Control plants were maintain at well watered conditions and used as reference. Harvest of plants were performed at moderate stress (day 13); maximum stress (day 49); 1st (day 50), 3rd (day 52) and end of recovery (day 56) for destructive morphological measurements and tissue collection.

5.3 Results

5.3.1 Water relations parameters

5.3.1.1 Soil water availability (SWA) variation

The variation of SWA along the drought experiments can be found in Fig. 16. Along the water stress treatment, SWA decreased rapidly until day 15 (reaching 30%) and had a slower decline until it reached 10% at day 49 (MS) for both accessions.

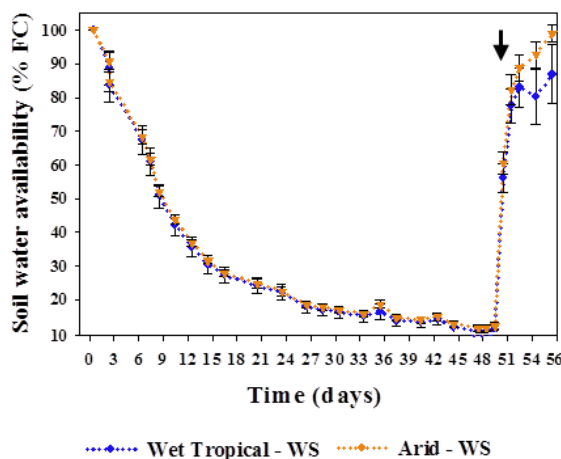


Figure 16 - Soil water availability along the drought treatments. Soil water availability was monitorized and recorded during water stress imposition and re-hydration. FC stands for soil field capacity. Arrow stands for re-watering day. Points represent means \pm se (n=5-6).

5.3.1.2 Leaf water status

RWC was maintained at control level throughout the water stress period for both accessions (Table 4). Moreover, even at maximum stress (10% SWA) no significant differences were found between treatments or accessions. Although no differences were also found at the first and third day of recovery, at the end of the recovery week the wet tropical accession showed a significantly lower RWC (p -value=0.031) as compared with the control (wet tropical WW).

Table 4: Effect of drought stress in leaf relative water content (RWC) of two *Jatropha curcas* accessions. Sampling points were performed at the beginning of the stress period (day 0); moderate drought (day 13); maximum stress (day 49) and during the recovery (day 50, 52 and 56). Values are means \pm se [n=6 (day 0); 3 (day 13) and 5-6 (day 49 and 56)].

		Time (days)					
		0	13	49	50	52	56
Wet tropical	WW	66.1 \pm 2.1	65.8 \pm 1.3	70.0 \pm 1.0	69.8 \pm 1.9	71.92 \pm 1.2	69.5 \pm 1.2a
	WS	68.4 \pm 2.8	61.1 \pm 3.3	67.5 \pm 3.2	71.2 \pm 4.4	74.69 \pm 4.1	64.1 \pm 1.9b
Arid	WW	63.1 \pm 1.5	66.5 \pm 1.9	71.0 \pm 2.6	69.1 \pm 2.4	72.18 \pm 0.7	68.0 \pm 0.6ab
	WS	70.3 \pm 3.1	59.3 \pm 4.6	71.9 \pm 1.2	68.4 \pm 0.8	73.80 \pm 0.6	67.0 \pm 1.1ab

Treatment means with a different letter differ significantly according to Tukey's test ($p < 0.05$).

5.3.2 Growth performance of *J. curcas* accessions

Plant growth was arrested at day 14 (30% SWA) and resumed after re-hydration for all non-destructive morphological parameters measured (Fig. 17).

Water stress induced a significant reduction of all growth parameters (number of leaves, stem length and diameter) ($p < 0.001$). Moreover, stem length (Fig. 17 B) growth was the first parameter affected, showing a reduction from day 7 to 14 (65–30% SWA). By the end of the stress period (MS 10% SWA), stem diameter presented a reduction of 41 and 38%, stem length reduced 23 and 21% and number of leaves reduced 50 and 42% as compared with control plants of wet tropical and arid accession, respectively. Indeed the number of leaves was the morphological parameter most affected by water stress in both accessions. This was not only due to absence of formation of new leaves but also to leaf shed. Furthermore, leaf shed was observed by day 28 (20% SWA) for the wet tropical accession and by day 42 (15% SWA) for the arid accession (Fig. 17 C).

Total percentage of dry matter was not altered during drought for both accessions, moreover, during recovery a significant decrease occurred, especially after 7 days of recovery, suggesting that after re-hydration plants are quickly increasing their water content, thus reducing their percentage of dry matter (Table 5). The same pattern was observed for the percentage of dry matter of leaves and stem. Interestingly, in the arid accession, plants leaf dry matter during recovery (days 52 and 56) was significantly lower.

On the other hand, when analyzing the root percentage of dry matter some interesting results were observed (Table 5). At moderate stress (day 13, 30% SWA), differences could be observed in root dry matter. Independently of the accession, drought increased root percentage of dry matter from 9.8 to 18.8 for wet tropical and 12.7 to 15.9 for arid accession, suggesting that roots are the first tissue to suffer dehydration. In the following collection point (day 49, maximum stress) the same pattern was observed, although with no significant differences. Though, by the 1st day of re-hydration differences are still found between the root percentage of dry matter between treatments (higher in WS compared with WW), after 3 days of re-hydration (day 52) roots are again fully hydrated and the values of dry matter percentage are at control levels, suggesting that the root system was not damaged and roots could quickly rehydrate.

Total leaf area *per* plant was significantly reduced by drought stress (Table 7). Specific leaf area (Table 7) was also higher in stressed plants although this difference was only significant at moderate stress (day 13) and end of recovery (day 52 and 56). Moreover, no significant differences were observed for shoot to root ratio during drought, although in day 13 plants subjected to stress presented lower values. By the end of the recovery period both accessions subjected to drought showed significant higher shoot to root ratio suggesting that both are investing in the aerial part biomass production.

The morphological aspect of plants at maximum stress is shown in Fig. 18.

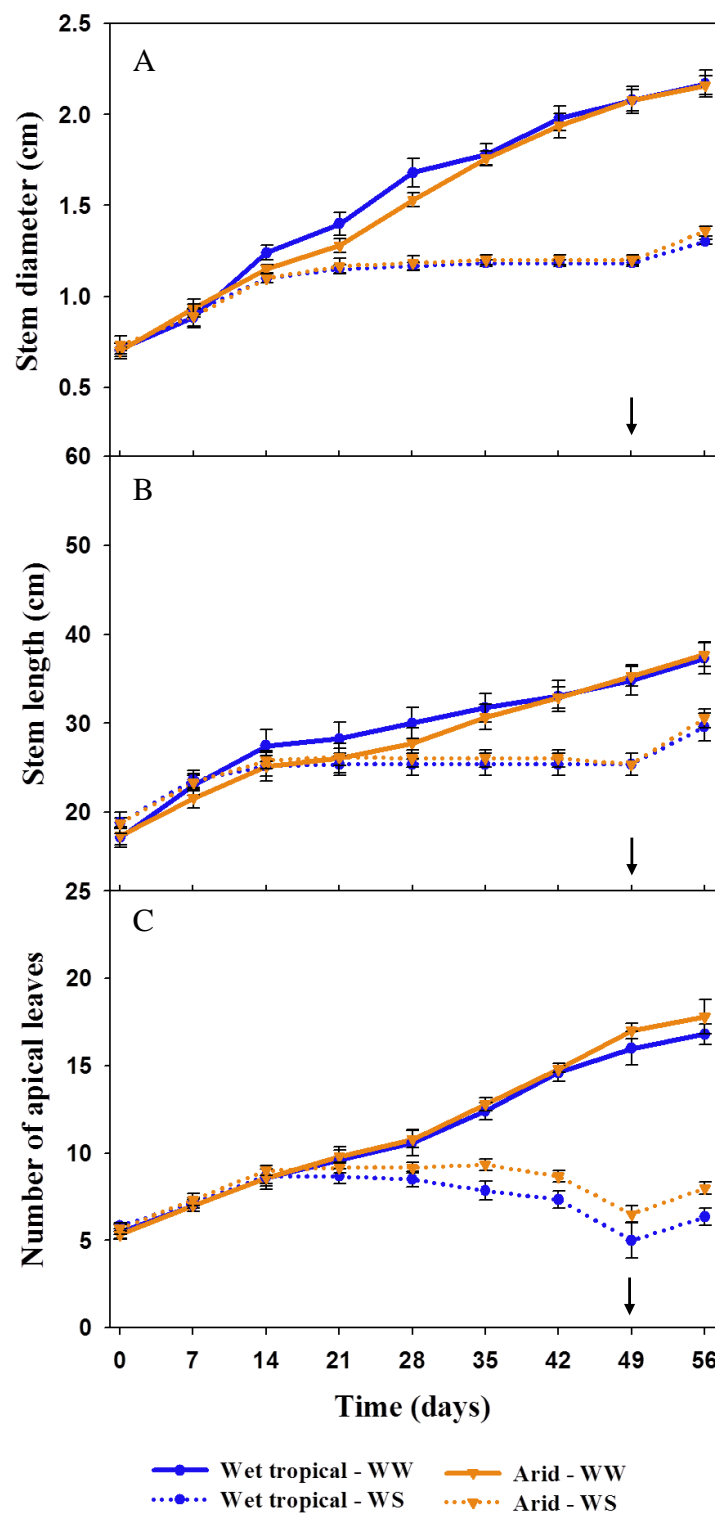


Figure 17 - Effect of drought stress and recovery on morphology. A) stem diameter; B) stem length and C) number of apical leaves. Arrows indicate re-watering day. Values are means \pm se (n=5-6).

Table 5: Effect of moderate drought (day 13) and maximum stress (day 49) and recovery (day 50, 52 and 56) on dry matter partition (leaf, stem, root). Values are means \pm se (n=3-6).

		Time (days)				
		13	49	50	52	56
Leaf dry matter (% relative to fresh weight)						
Wet tropical	WW	16.1 \pm 0.1	16.7 \pm 0.5	16.7 \pm 0.5	14.5 \pm 0.4a	16.2 \pm 0.4a
	WS	14.2 \pm 0.2	17.1 \pm 0.5	16.1 \pm 0.1	12.9 \pm 0.2bc	14.8 \pm 0.2 b
Arid	WW	16.2 \pm 0.8	16.8 \pm 0.6	16.8 \pm 0.6	14.2 \pm 0.2ab	16.1 \pm 0.3a
	WS	14.0 \pm 0.5	16.2 \pm 0.1	16.3 \pm 0.6	12.3 \pm 0.4c	13.5 \pm 0.2c
Stem dry matter (% relative to fresh weight)						
Wet tropical	WW	11.1 \pm 0.5	19.0 \pm 0.9	19.0 \pm 0.9a	17.7 \pm 0.5a	18.9 \pm 0.6a
	WS	10.9 \pm 0.6	17.1 \pm 0.5	15.2 \pm 0.7b	12.0 \pm 0.4b	12.8 \pm 0.5b
Arid	WW	11.4 \pm 0.3	18.7 \pm 0.7	18.7 \pm 0.7a	18.9 \pm 0.6a	18.2 \pm 0.6a
	WS	10.4 \pm 0.6	15.6 \pm 0.9	14.4 \pm 0.6b	11.8 \pm 1.3b	11.8 \pm 0.3b
Root dry matter (% relative to fresh weight)						
Wet tropical	WW	9.8 \pm 0.1c	16.9 \pm 0.8	16.9 \pm 0.8	15.3 \pm 0.02	17.6 \pm 0.4a
	WS	18.8 \pm 1.2a	28.5 \pm 1.5	25.5 \pm 0.3	15.8 \pm 0.2	15.7 \pm 0.4b
Arid	WW	12.7 \pm 1.4 bc	17.0 \pm 0.2	17.0 \pm 0.2	15.9 \pm 0.6	16.5 \pm 0.3ab
	WS	15.9 \pm 0.6 ab	27.2 \pm 0.7	25.9 \pm 0.6	16.9 \pm 3.9	14.2 \pm 0.3c
Total dry matter (% relative to fresh weight)						
Wet tropical	WW	13.3 \pm 0.3	17.7 \pm 0.7	17.7 \pm 0.7	16.0 \pm 0.4ab	17.7 \pm 0.4a
	WS	13.5 \pm 0.3	18.5 \pm 0.4	16.9 \pm 0.4	12.9 \pm 0.1b	13.9 \pm 0.3b
Arid	WW	13.9 \pm 0.3	17.6 \pm 0.5	17.6 \pm 0.5	16.4 \pm 0.4a	17.1 \pm 0.3a
	WS	13.1 \pm 0.4	17.4 \pm 0.7	16.6 \pm 0.5	12.6 \pm 1.3b	12.8 \pm 0.2b

Treatment means with a different letter differ significantly according to Tukey's test ($p < 0.05$).

Table 6: Effect of moderate drought (day 13) and maximum stress (day 49) and recovery (day 50, 52 and 56) on total leaf area, specific leaf area and shoot to root dry mass ratio. Values are means \pm se (n=3-6).

		Time (days)				
		13	49	50	52	56
Total leaf area (cm ²)						
Wet tropical	WW	1054 \pm 129	2223 \pm 301a	2223 \pm 301a	2045 \pm 130a	2031 \pm 117a
	WS	712 \pm 1260	529 \pm 97b	481 \pm 74b	671 \pm 39b	811 \pm 91b
Arid	WW	947 \pm 188	2474 \pm 348a	2474 \pm 348a	2123 \pm 129a	2007 \pm 141a
	WS	523 \pm 133	516 \pm 190b	464 \pm 45b	410 \pm 67b	858 \pm 45b
Specific leaf area (cm ² g ⁻¹)						
Wet tropical	WW	196 \pm 2b	174 \pm 9	174 \pm 9	182 \pm 12b	181 \pm 6b
	WS	225 \pm 4a	213 \pm 5	203 \pm 13	259 \pm 7a	234 \pm 4a
Arid	WW	200 \pm 3b	167 \pm 4	167 \pm 4	184 \pm 12b	181 \pm 5b
	WS	235 \pm 5a	165 \pm 19	198 \pm 9	243 \pm 9a	244 \pm 4a
Shoot to root ratio						
Wet tropical	WW	7.0 \pm 0.4	3.9 \pm 0.2	3.9 \pm 0.2	3.8 \pm 0.3ab	4.0 \pm 0.1ab
	WS	5.8 \pm 1.2	4.6 \pm 0.7	3.9 \pm 0.1	5.0 \pm 0.1a	5.6 \pm 0.6a
Arid	WW	6.4 \pm 0.6	3.9 \pm 0.3	3.9 \pm 0.3	3.4 \pm 0.4b	3.4 \pm 0.2b
	WS	4.4 \pm 1.0	4.1 \pm 0.6	3.6 \pm 0.1	3.8 \pm 0.4ab	5.0 \pm 0.3a

Treatment means with a different letter differ significantly according to Tukey's test ($p < 0.05$).

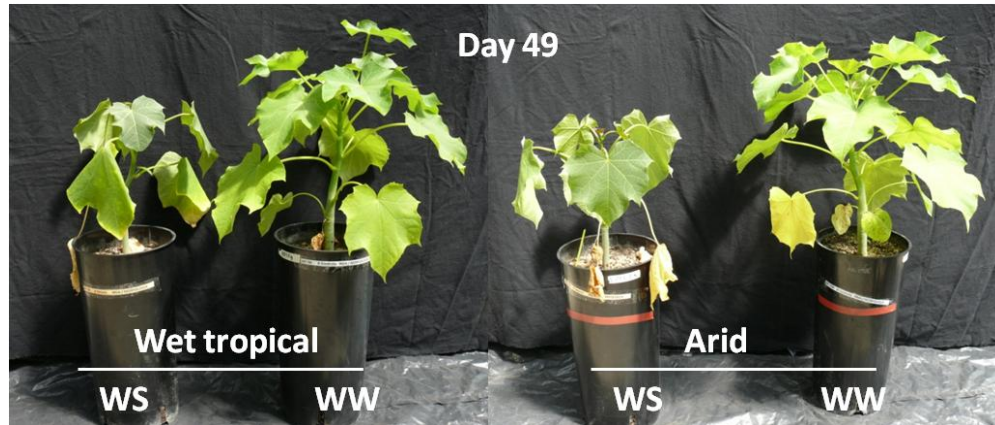


Figure 18 - Morphological aspect of plants at maximum stress (day 49). Plants from wet tropical or arid accessions subjected to 49 days of water withhold (WS) or well watered conditions (WW).

5.3.3 Leaf physiology (gas exchange, chlorophyll *a* fluorescence and chlorophyll content)

Concerning leaf gas exchange and chlorophyll *a* fluorescence measurements (net photosynthesis, stomatal conductance and photosystem II efficiency), no significant differences were found between accessions (Fig. 19). Moreover, water stress significantly influenced (independently of the accession) all measured parameters ($p < 0.001$).

After 12 days of water withhold (40% SWA), net photosynthesis was reduced in both accessions by 41 and 32% for wet tropical and arid climates, respectively compared to the control (WW). While water withhold period increases, net photosynthesis continues to be reduced for stressed plants. In fact, at maximum stress (10% SWA), it could be observed a reduction of 94 and 96% for wet tropical and arid accessions as compared to the control (WW).

A strict stomatal control was observed for both accessions. In relation to the control treatment (well-watered plants), stomatal conductance (Fig. 19 B) was reduced by 20% for both accessions after one week of water withhold (60% SWA). After 12 days of water withhold (SWA of 40%), stomatal conductance showed a reduction of 89 and 87% for wet tropical and arid accession, respectively. When minimum soil water availability was achieved (10%), stomatal conductance reached values close to zero. Interestingly, the reduction on stomatal conductance was previous to the reduction of net photosynthesis.

Regarding chlorophyll *a* fluorescence measurements, Φ_{PSII} of stressed plants remained at control levels until day 16 of water withhold (25% SWA). At day 20, Φ_{PSII} started to decline for both accessions, while on day 23 (20% SWA) the reduction observed was of 77 and 72% for wet tropical and arid accessions as compared with the control. These values remained throughout the drought stress period until maximum stress.

After re-hydration a complete recovery was observed for all measured parameters (values at control levels) within 5 days of recovery (day 54).

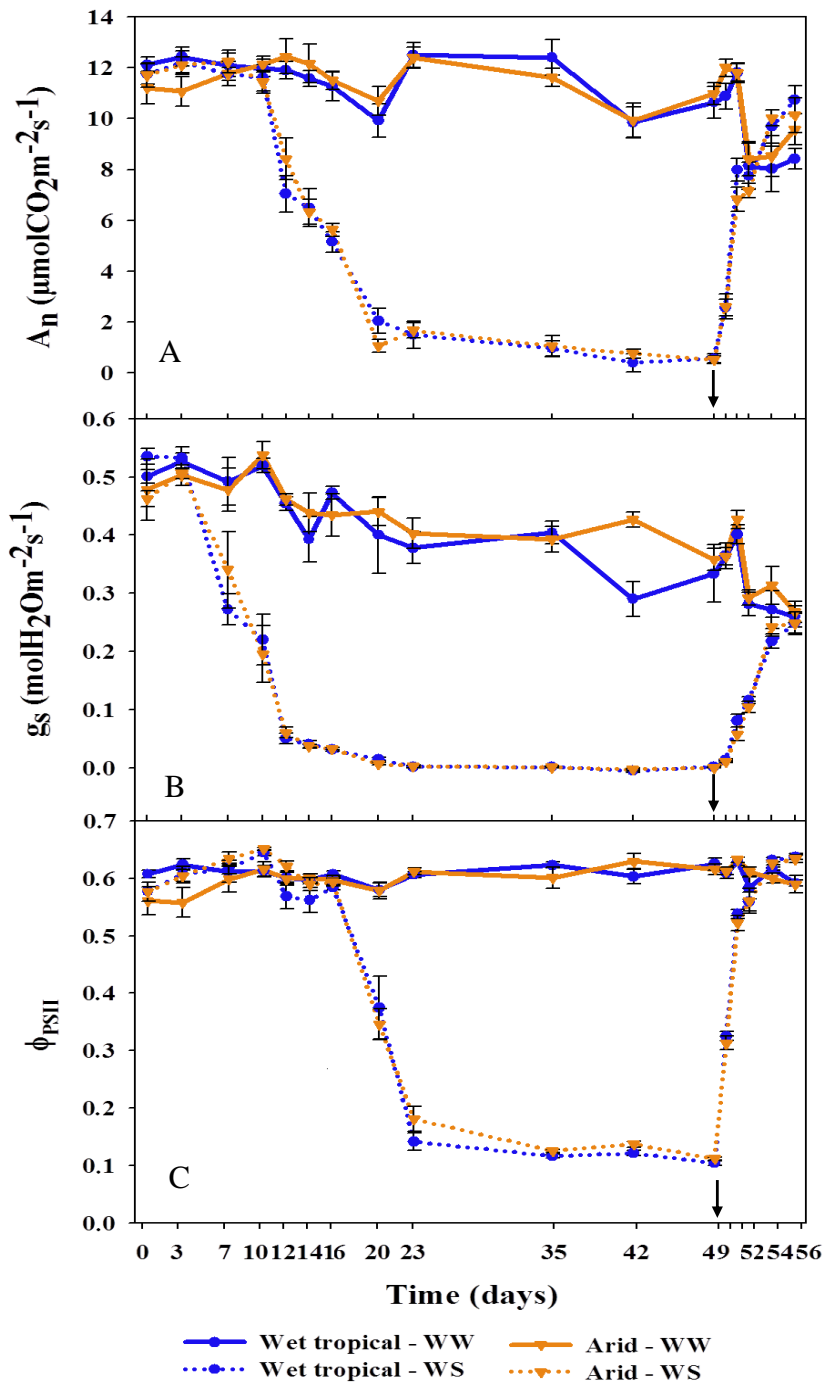


Figure 19 - Effect of drought stress and recovery on leaf gas exchange and chlorophyll *a* fluorescence. A) net photosynthesis (A_n); B) stomatal conductance (g_s) and C) photosystem II efficiency (Φ_{PSII}). Arrows indicate re-watering day. Values are means \pm se (n=5-6).

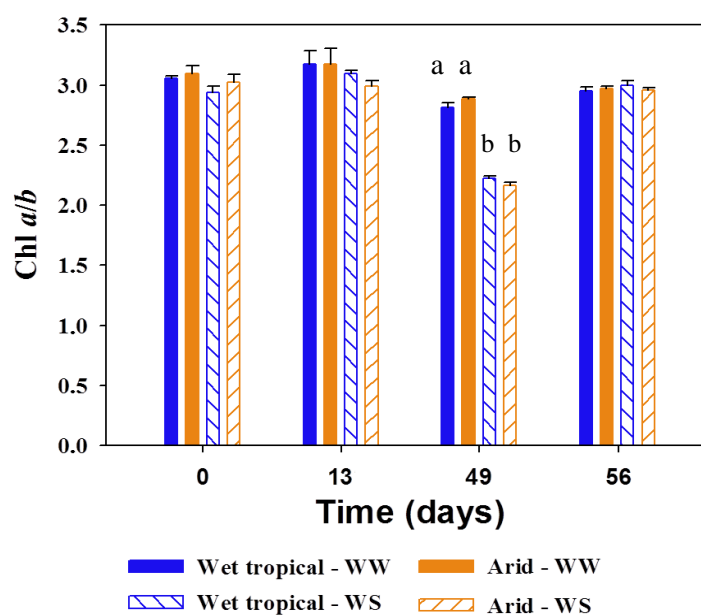


Figure 20 - Effect of drought stress and recovery on chlorophyll *a* to *b* ratio. Arrows indicate re-watering day. Values are means \pm se ($n=5-6$). Treatment means with a different letter differ significantly according to Tukey's test ($p<0.05$).

No differences in the chlorophyll *a* and *b* distribution were found in the beginning of the assay (day 0) or at moderate drought (day 13, 30% SWA). At maximum stress (10% SWA), however, the chlorophyll *b* content increased in response to drought switching from 25% in control to 32% in stress plants, which resulted in a decrease of the chlorophyll *a/b* ratio (Fig. 20) for both accessions.

5.3.4 RNA extraction

During the drought assay RNA material was collected for leaf and root samples at different time points along drought and recovery (*Addendum II*, Table 7). We were able to extract RNA from all collection points. RNA content was around 0.21-0.30 mg g⁻¹ FW for leaf material and the $A_{260/280}$ ratio ranged from 1.98 to 2.04 independently of the treatment or accession, indicating high purity of the samples (*Addendum III*, Table 8). Samples were also fractionated in 1% agarose gel and no degradation was detected (*Addendum III* Fig. 21). For root material, lower RNA content (compared with leaf samples) was observed (0.06-0.09 mg g⁻¹ FW) with higher heterogeneity in $A_{260/280}$ values, which ranged from 1.82 and 1.90, suggesting that root is a difficult material for RNA extractions, nevertheless samples were not degraded (*Addendum III*, Fig. 22).

5.4 Discussion and conclusions

The analyzed parameters showed no significant different trends during water deficit and recovery when comparing accessions in the tested conditions.

Both *Jatropha curcas* accessions presented a high leaf water status (same as control plants) even at severe stress (MS, 10% SWA) (Table 4). This is in accordance with the literature, independently of the stress imposition *Jatropha* plants maintain a high relative leaf water content (Silva *et al.*, 2010a,b; Díaz-López *et al.*, 2012) and with our previous findings while optimizing the drought conditions assay (see section 4). This is probably achieved by the strict stomatal control observed from day 7 to the end of the stress period (60 – 10% of SWA, Fig. 19 B) and reduction on transpiration area.

In fact, total leaf area was drastically reduced for both accessions (Table 6). This response was achieved by arresting new leaf production (Fig. 17 C), combined with the reduction of leaf expansion (resulting in a higher SLA in stressed plants, Table 6) and leaf shed at severe stress (20-10% SWA). This strategy allowed the plants to reduce transpiration area and therefore avoid water loss. Moreover, younger leaves tend to be more resistant to drought than older leaves. This increased tolerance may be particularly relevant in plants where a severe reduction in the size of the leaf canopy occurs as a result of shedding of older leaves, allowing a faster recovery after re-hydration (Pereira and Chaves, 1993). Although leaf loss occurred earlier and was more pronounced in the accession from the wet tropical climate, no significant differences were found between the numbers of leaves for the two accessions. These results are in accordance with the findings of Maes *et al.* , (2009b) and Achten *et al.* (2010b), that have submitted 62 days-old *J curcas* plants of three accessions (from Ethiopia, India and Thailand) to water withhold and 40% of SWA. They reported that after 30 days of water withhold plants from all accessions started to shed older leaves. Moreover, the same was observed, though in a less pronounced way, for those accessions subjected to mild stress (40% SWA). These authors have also reported stem growth arrest (length and diameter) 14 days after water withhold for the three accessions. The same was observed in our results (Fig. 17 A and B), with both accessions arresting growth 2 weeks after water withhold. However, no stem shrinking was observed in our results contrary to the findings of these authors (Maes *et al.*, 2009b; Achten *et al.*, 2010b).

No differences were found in shoot to root ratio during the drought period (Table 6). However, the lower values of shoot to root ratios of WS plants at moderate drought (day 13, 30% SWA) seems to be caused by increase in root biomass production and/or reduction of

leaf expansion, since WS plants showed lower leaf areas (although not significant) and significant higher specific leaf area (thicker leaves). The wet tropical accession showed, at the 3rd day of recovery, an earlier increase in the shoot to root ratio that was maintained onwards. On the other hand, an increase in the shoot to root ratio was only observed at the last day of recovery for the arid accession. This increase in the recovery period suggests a rapid investment in the aerial (shoot + stem) biomass production. Although no significant differences were found for total percentage of dry matter during drought (Table 5), at moderate stress (day 13) root percentage of dry matter was altered in the WS plants, with a significant increase for the wet tropical accession. All together, this suggests that the wet tropical accession can adjust biomass partition faster than the arid accession in response to water stress, investing in root biomass at moderate stress, and rapidly investing in the aerial growth after re-hydration.

Photosynthesis and growth are the primary processes affected by drought (Chaves, 1991). The reduction of growth was concomitant with the decrease of photosynthesis. Our results showed a reduction of photosynthesis after 12 days of water withhold (40% SWA). Pompelli *et al.* (2010), have shown that 11 month-old *J. curcas* plants subjected to water stress showed a reduction on net photosynthesis after two days of water withhold. Moreover, after four days of drought stress, plants showed net photosynthesis values close to zero, with the same pattern as stomatal conductance. On our experiment values of net photosynthesis (Fig. 19 A) ranged values close to zero after 20 days of water withhold, this slower decrease may be related with the developmental stage of the plants, since 11 moth-old plants (used by Pompelli *et al.*, 2010) probably presented a higher leaf area and therefore transpiration rate. What is interesting is that these authors reported the exact same pattern of net photosynthesis and stomatal conductance. The same synchronized reduction of stomatal conductance and net photosynthesis was reported by Silva *et al.* (2010a,b) and Díaz-López *et al.* (2012). However, we have observed that for the studied *Jatropha curcas* accessions, stomatal conductance starts to decrease prior to net photosynthesis (Fig. 19 A and B). This pattern was also observed in our previous experiment (TC2). This suggests that reduction on photosynthesis is limited by non-stomatal characteristics. Probably this reduction in photosynthesis was caused by an increase in leaf temperature and/or inability of the plant to dissipate excess energy inducing photoinhibition. This is corroborated by the photosystem efficiency II (Fig. 19 C) rapid decrease after arrest of photosynthesis, suggesting an increase in thermal dissipation. Moreover at maximum stress (day 49, 10% SWA) a reduction was observed in the Chl *a* to *b*

ratio (Fig. 20), due to an increase of Chl_b synthesis. Chl_b synthesis is required for realizing the functions of light harvesting and also for excess energy dissipation (Tanaka *et al.* 2001). The observed increase of the Chl_b in the ratio is most likely related to the increasing need of stressed plants to dissipate excessive energy and avoid photodamage.

Concluding, drought significantly reduced stem growth, leaf area, biomass, leaf gas exchange, chlorophyll *a* fluorescence and the chlorophyll *a* to *b* ratio for both *J. curcas* accessions. On the other hand, an increase for specific leaf area was observed. No differences were observed in the relative water content of the plants suggesting that *Jatropha* strict control of stomata and reduction of transpiration area efficiently reduces water loss.

6 General conclusions and future perspectives

A morpho-physiological characterization of two *Jatropha curcas* ecotypes from different origins was performed.

Our results suggest that the relationships between light use efficiency, CO₂ fixation and photoinhibition are similar for both *J. curcas* accessions tested. Moreover, this was, to the best of our knowledge, the first time that these accessions were characterized providing insights into the photosynthetic response of these accessions to light. Also, this data can be used for further characterization studies and to be compared with other accessions, allowing the possible use of these values to predict biomass production for agronomical uses.

Drought assay conditions were established in order to allow stress to be imposed gradually. This gradual imposition will be useful when looking for putative discriminating behaviors between accessions in response to stress. Our results showed that plants should be used in the 3 to 5 leaves stage and should be subjected to water withhold to promote a gradual water stress response.

With the optimized conditions plants were subjected to drought by water withhold until soil water content reached a value of 10% of initial field capacity. In the tested conditions and evaluated parameters, we were not able to detect significant morpho-physiological differences between the two accessions in control conditions or in response to drought stress and recovery. Both accessions showed an almost identical response. Although, some differences were observed for biomass allocation (especially in root percentage of dry matter) and in leaves shedding. Furthermore, both accessions are capable of maintaining a good leaf water status due to a strict stomatal control and reduction of leaf total area (decreasing leaf expansion, leaf production and, under severe stress, shedding of older leaves). Indeed, stomata play an important role in regulation of leaf gas exchange between the interior of the leaf and the atmosphere, preventing water loss. Moreover, both accessions showed the same strategy to cope with water stress.

Recent studies reveal a narrow genetic diversity between *Jatropha curcas* ecotypes spread around the world beyond its origin center (Sun *et al.* 2008; Basha and Sujatha, 2007; Ranade *et al.*, 2008; Pamidimarri *et al.*; 2009). This assumption could, in part, explain the absence of phenotypic differences between accessions under drought. However, despite the limited genetic diversity within *J. curcas* accessions, appreciable variability has been reported for phenotypic traits (Heller, 1996; Rao *et al.*, 2008; Popluechai *et al.*, 2009). Thus, we would

expect phenotypic plasticity between accessions from different provenances. The seeds used in these experiments have been collected from trees of both accessions growing in Cape Verde islands. We could speculate that some adaptation may have occurred to the Cape Verde islands climate in the wet tropical accession, thus leading to similar responses between accessions. For future trials it would be interesting to perform a similar assay with seeds collected from the original climates and compare the results.

During the optimized assay RNA was collected from roots and leaves. Meanwhile, in the end of 2010, *Jatropha curcas* genome was sequenced (Sato *et al.*, 2010) and publicly available at <http://www.kazusa.or.jp/Jatropha/> (*Jatropha* genome database). Having in mind the fast evolution of next generation sequencing technologies, isolated RNA under drought versus control conditions can be used for future works, like to perform a RNA-seq analysis. A whole transcriptomic analysis by RNA-seq will provide an insight regarding the transcriptomic network of this drought tolerant plant. This type of analysis may also aid in the search for good candidate genes targeting drought tolerance. Such strategy may be useful for the improvement of drought sensitive species.

Addenda

Addendum I: RNA collection time points

Table 7: Leaf and root collection time points for RNA extraction of the two *Jatropha curcas* accessions. Plants of 36 days from two different provenances (accessions from wet tropical and arid climates) were subjected to control (daily watered) or stress conditions (water withhold for 49 days, followed by one week of recovery). The plus sign (+) stands for tissue collection.

Time of drought stress	Wet tropical		Arid		Number of plants used <i>per</i> RNA pool
	Leaf	Root	Leaf	Root	
Day 0	+	-	+	-	6
Day 7 (Early moderate drought)	+	-	+	-	6
Day 13 (Moderate drought)	+	+	+	+	3
Day 35 (Late moderate drought)	+	-	+	-	5-6
Day 49 (Maximum drought)	+	+	+	+	3
Day 50 (First day of recovery)	+	+	+	+	3
Day 53 (Third day of recovery)	+	+	+	+	3
Day 56 (End of recovery period)	+	+	+	+	5-6

Addendum II: RNA extraction protocol

RNeasy plant mini kit (Qiagen, Germany) extraction protocol:

1. Fine ground plant material was weighted (about 80 mg).
2. Lysis buffer (450 µl) was added to the sample and vigorously vortexed.
3. The lysate was transferred to the QIAshredder spin column, placed in a 2 ml collection tube and centrifuged for 2 min at full speed. The supernatant was transferred to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube.
4. Half volume of ethanol (96–100%) was added to the cleared lysate, and mixed immediately by pipetting.
5. The sample was transferred to an RNeasy spin column.
6. Buffer RW1 (700 µl) was added to the RNeasy spin column.
7. Buffer RPE (500 µl) was added to the RNeasy spin column and centrifuged for 15 s at 10000 rpm. The flow-through was discarded.
8. Buffer RPE (500 µl) was added to the RNeasy spin column and centrifuged for 2 min at 10000 rpm. The flow-through was discarded.
9. The RNeasy spin column was placed in a new 1.5 ml collection tube. RNase-free water (30 µl) was added directly to the spin column membrane and centrifuged for 1 min at 10000 rpm.
10. RNA was stored at -20°C until further use.

Turbo DNA-free Kit (Ambion, USA) protocol:

1. One time TURBO DNase Buffer and 1 µL TURBO DNase were added to the RNA, and gently mixed.
2. Incubated at 37°C for 30 min.
3. One time DNase Inactivation Reagent was added and gently mixed.
4. Incubated for 5 min at room temperature.
5. Centrifuged at 10000 rpm for 1.5 min.
6. RNA was transferred to a fresh tube and stored at -20 °C until further use.

Addendum III: RNA quantification

Table 8: RNA quantification by spectrophotometer (NanoDrop 3300, Thermo Scientific, USA). The ratios $A_{260/280}$ and $A_{260/230}$ are quality/purity indicators (good quality for $A_{260/280}$ is about 2.00 and for $A_{260/230}$ is about 1.80). RNA content is presented as mg of RNA *per* gram of fresh weight. Values are means \pm se (n=4-8).

		$A_{260/280}$	$A_{260/230}$	RNA (mg g ⁻¹ FW)
Leaf samples				
Wet tropical	WW	2.04 \pm 0.02	1.84 \pm 0.24	0.24 \pm 0.03
	WS	2.00 \pm 0.02	1.70 \pm 0.11	0.27 \pm 0.04
Arid	WW	1.98 \pm 0.02	1.74 \pm 0.09	0.30 \pm 0.06
	WS	2.01 \pm 0.01	1.86 \pm 0.07	0.21 \pm 0.03
Root samples				
Wet tropical	WW	1.82 \pm 0.07	0.93 \pm 0.18	0.07 \pm 0.01
	WS	1.90 \pm 0.07	1.10 \pm 0.13	0.09 \pm 0.03
Arid	WW	1.83 \pm 0.09	1.06 \pm 0.16	0.08 \pm 0.04
	WS	1.90 \pm 0.10	0.94 \pm 0.16	0.06 \pm 0.01

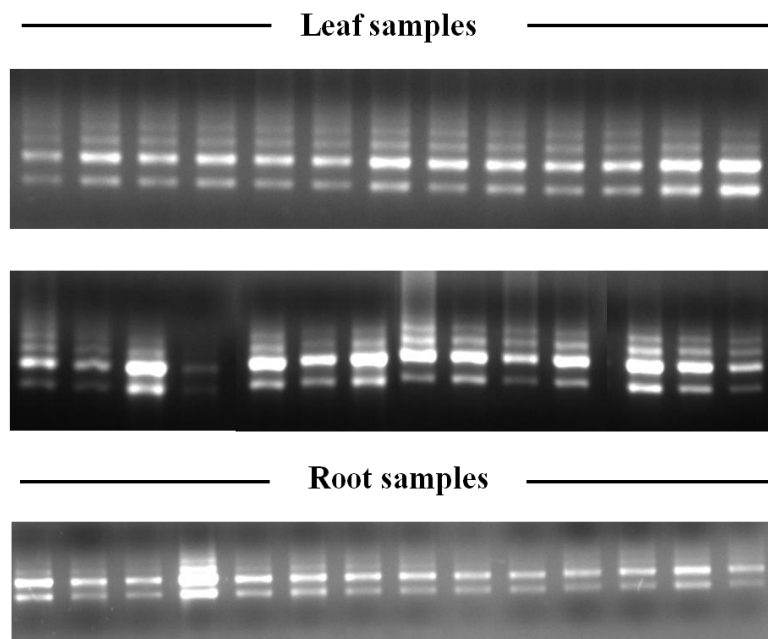


Figure 21 - RNA integrity checked by electrophoresis. 500 ng (leaf samples) or 300 ng (root samples) of total RNA were fractionated in a 1% agarose gel stained with ethidium bromide.

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