Establishment of a regeneration and transformation system of *Balanites aegyptiaca* L. and investigation on the physiological responses to abiotic stress of different genotypes

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A: (3)
…[2]Es ist eine ausführliche Darstellung voranzustellen, die eine kritische Einordnung der Forschungsthemen und wichtigsten Erkenntnisse aus den Publikationen in den Kontext der wissenschaftlichen Literatur zum Thema vornimmt …

Die voranzustellende ausführliche Darstellung ist in dieser Arbeit aufgeteilt in die Kapitel 1 und 7.

B: (3)
…vornimmt sowie die individuellen eigenen Beiträge und ggf. die Beiträge weiterer Autoren an den jeweiligen Publikationen darlegt.

Chapter 2:

Khamis G & Papenbrock J (2014): Newly established drought-tolerant plants as renewable primary products as source of bioenergy, Emirates Journal of Food and Agriculture, 26, doi: 10.9755/ejfa.v26i12.19108

• The structure of the review was designed by JP.

• GK wrote the chapter on "Morphological, physiological and metabolic adaptations of xerophytes to drought".

Chapter 3:

Khamis G & Papenbrock J. Investigation on the genetic diversity among *Balanites aegyptiaca* (Desert date) provenances by amplified fragment length polymorphism analysis.
• Collected *Balanites aegyptiaca* seeds from different regions.
• GK established the AFLP system in *B. aegyptiaca* and generated the data.
• The data were analyzed by GK.

• The manuscript was written by GK and JP.

Chapter 4:

Khamis G, Schaarschmidt F and Papenbrock J. Effect of water deficiency on different genotypes of *Balanites aegyptiaca.*

• GK collected the materials and conducted the drought experiment.

• GK carried out the methods to analyse the *B. aegyptiaca* response to water stress and collected the data.

• GK and JP analyzed the data.

• The statistical analyses were mainly performed by FS.

• The manuscript was written by GK, FS and JP.

Chapter 5:

Khamis G, Schaarschmidt F and Papenbrock J. Effect of water deficiency and recovery time on different genotypes of *Balanites aegyptiaca.*

• GK collected the materials and conducted the drought and recovery experiment.

• GK carried out the methods to analyse the *B. aegyptiaca* response to water stress and collected the data.

• GK and JP analyzed the data.

• The statistical analyses were mainly performed by FS.

• The manuscript was written by GK, FS and JP.

Chapter 6

Khamis G, Winkelmann T Papenbrock J. Establishment of a regeneration and transformation system of *Balanites aegyptiaca*

• GK carried out the laboratory experiments and generated the data.

• GK, TW and JP analyzed the data.

• The manuscript was written by GK, TW and JP.

Summary

Balanites aegyptiaca L. (Del.) is a multi-purpose tree belonging to the family of Balanitaceae. The species is considered as drought-tolerant. Distributed in arid lands in the tropics and subtropics it serves as a source of many secondary metabolites and has a potential for biodiesel production. To study the relation between the genetic diversity and geographical distribution amplified fragment length polymorphism (AFLP) analysis was performed to evaluate the genetic diversity of twelve provenances of *B. aegyptiaca* collected from different geographical regions. The results of cluster analysis and the principal components analysis indicated that *B. aegyptiaca* individual samples are distributed in three main clades. There is no clear relation between the genetic variation and the geographic distribution while this variation might be obtained through the capacity of outcrossing pollination in *B. aegyptiaca*. A study was conducted to compare and examine the morpho-physiological responses to water stress of six different *B. aegyptiaca* genotypes collected from different regions. Different regimes of soil volumetric water content (VWC 35% as a control, VWC 20% as moderate, and VWC 5% as a severe drought stress) were chosen to finally select the most drought-tolerant genotype under greenhouse conditions. Several growth parameters, stomata conductance, photochemical efficiency and metabolite contents were analyzed to investigate and compare the drought impact among *B. aegyptiaca* genotypes. At severe drought stress each genotype showed an independent strategy to cope with the water shortage through a significant reduction in biomass parameters, early stomata closure combined with small changes in photochemical efficiency and producing high concentrations of ascorbic acid. A regeneration and transformation system was established in *B. aegyptiaca* through nodal explants with axillary buds and cotyledon explants excised from seeds collected from three different provenances. There were no significant differences between provenances with respect to their response to plant growth regulator concentrations and provenance-plant growth regulator concentrations interaction. 6-Benzyladenine (BA) was significantly more effective in shoot induction through nodal explants whereas thidiazuron (TDZ) was more efficient in the number of produced shoots per cotyledon explant. Three different strains of *Agrobacterium tumefaciens* were examined to establish first steps towards a transformation system in *B. aegyptiaca* and based on the highest survival rate and highest number of explants gave positive GUS assay and PCR *A. tumefaciens* GV3101 strain was used for further transformation experiments of *B. aegyptiaca* to produce salt-tolerant *B. aegyptiaca.*

Keywords. *Balanites aegyptiaca*, drought stress, genetic variation, recovery, regeneration, transformation.

Zusammenfassung

Balanites aegyptiaca L. (Del.) ist eine Baumart aus der Familie der Balanitaceae, die vielfältig genutzt werden kann. Die Art gilt als trockentolerant und kommt weit verbreitet in ariden und semiariden Gebiete in den Tropen und Subtropen vor. Verschiedene Pflanzenorgane dienen als Quelle sekundärer Metabolite, und die Früchte können möglicherweise für die Biodieselproduktion genutzt werden. Um die Beziehung zwischen genetischer Vielfalt und geografischer Verteilung zu untersuchen, wurden AFLP (**a**mplified **f**ragment-**l**ength **p**olymorphism)-Analysen von DNA aus Früchten von zwölf *B. aegyptiaca*-Provenienzen aus Afrika und Asien durchgeführt. Die Ergebnisse der Clusteranalyse und der Hauptkomponentenanalyse zeigen, dass die einzelnen *B. aegyptiaca*-Proben in drei Hauptkladen eingeordnet werden können. Es gibt keine klare Beziehung zwischen genetischer Variabilität und der geographischen Verteilung. Sechs verschiedene *B. aegyptiaca*-Genotypen aus verschiedenen Regionen wurden hinsichtich ihrer morphologischen und physiologischen Reaktionen auf Wasserstress verglichen. Unterschiedliche volumetrische Wassergehalte (VWC 35% als Kontrolle, VWC 20% moderater und VWC 5% starker Trockenstress) wurden ausgewählt, um die Genotypen mit der größten Trockentoleranz unter Gewächshausbedingungen zu identifizieren. Es wurden verschiedene Wachstumsparameter, die stomatäre Leitfähigkeit, die photochemische Effizienz und Metabolitgehalte analysiert, um die Auswirkungen von Trockenstress auf *B. aegyptiaca*-Genotypen zu untersuchen und zu vergleichen. Bei starkem Trockenstress reagierte jeder Genotyp mit einer etwas unterschiedlichen Strategie, um die Wasserknappheit zu bewältigen, so durch eine deutliche Reduzierung der Biomasse, durch Verschließen der Stomata kombiniert mit kleinen Änderungen in der photochemischen Effizienz und durch Produktion großer Mengen an Ascorbinsäure. Außerdem wurde ein Regenerations- und Transformationsystem von *B. aegyptiaca* durch Achselknospen- und Keimblattexplantate von Samen drei verschiedener Herkünfte etabliert. Es gab keine signifikanten Unterschiede zwischen den Herkünften in Bezug auf ihre Reaktion auf die eingesetzen Pflanzenwachstumsregulatoren. 6-Benzyladenin (BA) war signifikant wirksamer in der Sprossinduktion von Achselknospenexplantaten, während Thidiazuron (TDZ) effizienter war in der Zahl der produzierten Sprosse pro Keimblattexplantat. Drei verschiedene Stämme von *Agrobacterium tumefaciens* wurden untersucht, um eine erste Schritte zu einem Transformationssystem in *B. aegyptiaca* aufzubauen. Auf der Grundlage der höchsten Überlebensrate und die höchste Zahl der Explantate gaben positive GUS-Assay und PCR *A.*

tumefaciens GV3101-Stamm wurde für die weitere Transformationsexperimente verwendet von *B. aegyptiaca* zu salztoleranten *B. aegyptiaca* produzieren

Schlüsselwörter: *Balanites aegyptiaca,* genetische Variation, Regeneration, Transformation, Trockenstress.

Contents

Abbreviations:

ABA	Abscisic acid
AFLP	Amplification Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BA	6-benzyladenine
BTL	Biomass to liquid
CAM	Crassulacean acid metabolism
cDNA	Complementary Deoxyribonucleic Acid
CTAB	Cetyl trimethylammonium Bromide
CWSI	Crop water stress index
DHNs	Dehydrins
DM	Dry matter
DNA	Deoxyribonucleic Acid
dNTP	Desoxyribonucleic triphosphate
dS	Deci Siemens
DTT	Dithiotreitol
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
ERD10	Early responsive to dehydration 10
ETR	Electron transport rate

FAO	Food and Agriculture Organization of the United Nations
FAME	Fatty acid methyl esters
F_v/F_m	Maximal PS II quantum yield
FW	Fresh weight
ΔF/Fm’	Photochemical efficiency
GA3	Gibberellic acid
GUS	Beta-glucuronidase
Ha	Hectare
IBA	Indole-3-butyric acid
IRD	Infrared dye
LEA	Late Embryogenesis Abundant
LWC	Leaf water content
MS	Murashige and Skoog
NAA	α-naphthalene acetic acid
NaOH	Sodium hydroxide
NEM	*N*-ethylmaleimide
NH_3	Ammonia
NRC	National Research Council
PAM	Pulse-amplitude-modulation
PAR	Photosynthetically active radiation
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction

PGR	Plant growth regulator
pH	Power of hydrogen
QTL	Quantitative trait loci
RAPD	Random Amplified Polymorphic DNA
RL-Buffer	Restriction ligation buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Rounds per minute
RWC	Relative water content
SD	Standard deviation
SEM	standard error of mean
TAA	Total ascorbic acid
Taq	*Thermus aquaticus* polymerase
TCA	Trichloro acetic acid
TDR	Time-domain reflectometry
TDZ	Thidiazuron
µM	Micro molar
UPGMA	Unweighted pair group method with arithmetic mean
USDA	United States Department of Agriculture
VWC	Volumetric water content

Chapter 1

General introduction

Global warming and food production

During the last few decades the rhythm of climate change increased and the level of ozone (O_3) and atmospheric carbon dioxide (CO_2) accelerated in several agricultural areas in the world (Lobell and Gourdji 2012). Climate changes enhance the increase in temperature and evaporation with a reduction in precipitation and rainfall causing a limitation in water resources. These factors increase the drought periods in semi-arid areas resulting in a reduction in food crops and the yield of domestic animals (FAO 2012; Nelson et al. 2014). On the one hand, the plant growth and development are speeded up in the warmer climate conditions. On the other hand, increasing the temperature above the optimal level caused high reduction in yield. It was reported that in maize the increase in daily temperature to more than 30°C decreases the yield by 1.7% and the increase in night temperature from 27°C to 32°C reduce the yield in rice by 90% (Thornton et al. 2014).

Drought stress and the impact on food production

Drought is a climatic condition considered as one of the main limitations in food production and subsistence for more than two billion people located in the dry regions which represent 41% of the global land area (Solh and Ginkel 2014). Drought occurred when land was affected with periodic extreme climate condition with rates of precipitation below normal for a certain period of time weeks, months or even years (Dai 2011).

The limitation of water for agriculture use affects the food production in different regions in the world (FAO 2012). Climate change influences the food security and human mobility through its negative impact on food production, the capability of countries to import food, and the capacity of people to buy food, where people migrate to areas which have more food supplies and livestock (Celia 2013). In 2010 several factors affected the wheat production in the leading exporter's countries where the wheat harvest was reduced, in Canada by approximately 25% in response to the rainfall in spring of 2010, in Russia, Ukraine and Kazakhstan the crop was decreased because of drought and forest fires. The winter drought in China enforces the government to buy wheat from the international market to recover the draw caused through drought. Also storms affected the wheat production in USA during the end of January 2011. All of these factors increased the wheat trading from US $4 in July 2010 into 8.50 to US $9 a bushel in February 2011. The price of bread was surged in Egypt

which is considered as one of the largest wheat importers. So during the Arab spring in 2011 the people highlighted their dissatisfaction about the political corruption and the increasing food prices and living costs (Celia 2013; Johnston and Mazo 2013).

So drought can be classified into three types (Fig. 1): 1. Meteorological drought, when the precipitation occurred below the normal level for a time of weeks, months or years, this can be combined with increasing of temperature up the normal. 2. Agricultural drought occurred with dry soils for a period of time as a result of decrease in the precipitation beneath the normal average or low rainfall and up-normal evaporation. 3. Hydrological drought, which takes place when river stream flow, lakes, reservoirs and water reserves in aquifers decreased under the mean level for long time (Dai 2011).

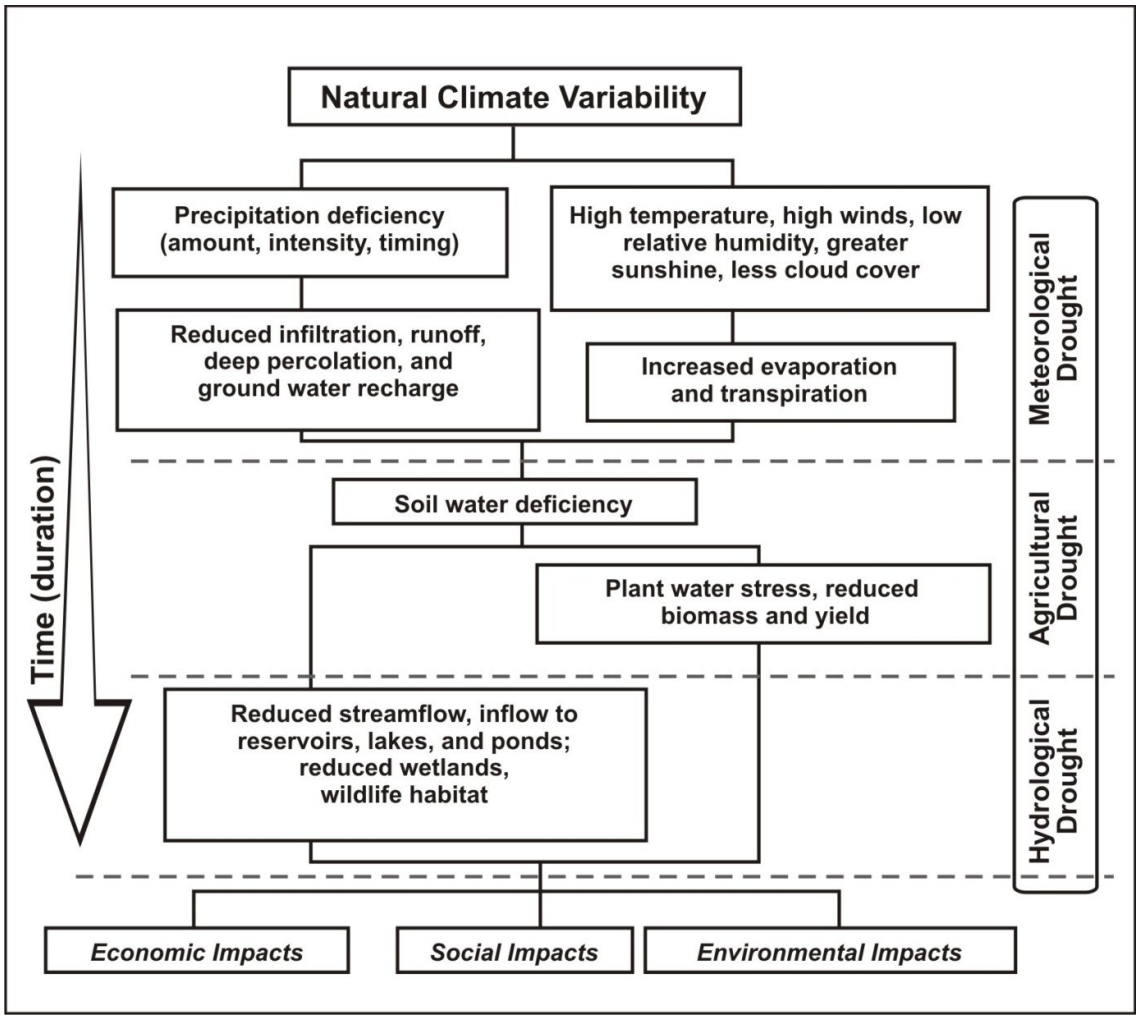

Figure 1. Climate change and the impacts of drought occurrence through several types of drought effects (Source: National Drought Mitigation Centre, University of Nebraska-Lincoln, U.S.A.).

Plant response to drought stress

Drought stress caused more than 40% reduction in the mean yields of crop plants in USA during the time of 1939 and 1978, while abiotic stress has a negative effect on plant growth and development through several physiological, morphological, molecular and biochemical alterations (Boyer 1982; Wang et al. 2003). Plant resistance to water shortage was divided into three levels, escape, avoidance and tolerance. To escape the drought impact, plants showed a high extent of developmental flexibility to finish their life time before the occurrence of physiological water stress. The avoidance strategy to cope with drought effects is through prevention the tissue desiccation by decreasing the water loss and increases the water absorption. This avoidance strategy occurred through several traits, such as stomata closure, dense trichomes, deep root system, and shedding the older leaves. Plants tolerate the drought stress through osmotic adjustment, increase the rigidity of cell walls and produce smaller cells, while the shrubs and trees in the arid regions show high levels of solute concentrations in living cells combined with reduction in the photosynthetic efficiency and stomata conductance (Chaves et al. 2003).

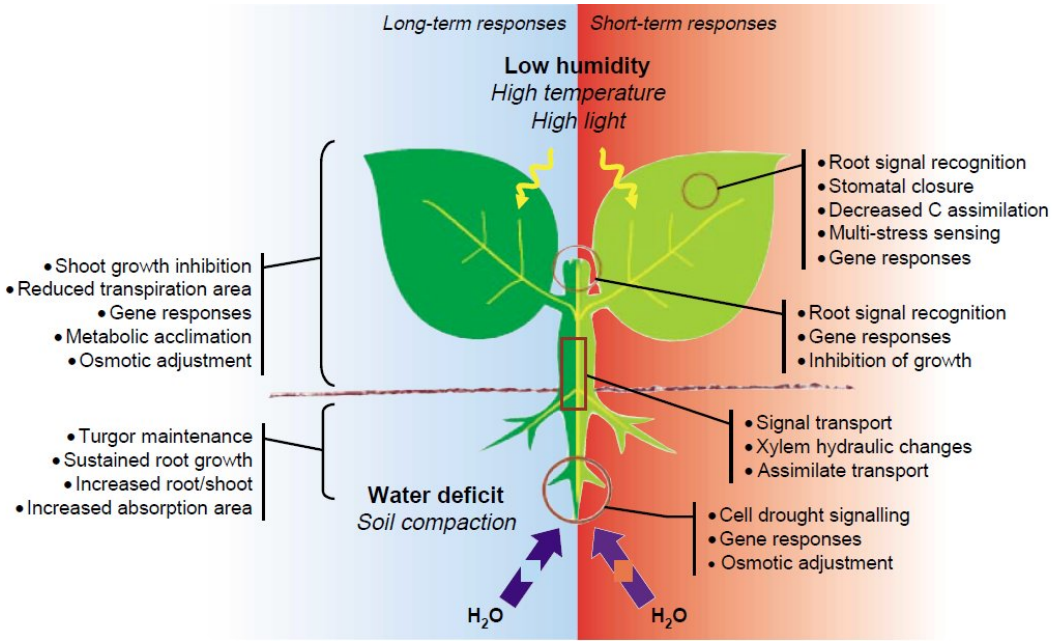

Figure 2. Long and short term response of the plant to drought stress conditions (Chaves et al 2003).

Plants respond to water stress through several strategies to overcome the stress. These strategies can be divided into short- and long-term responses (Fig. 2). In the long-term

response plants adapt to survive with small water content through biochemical changes to decrease the osmotic potential inside the cells by the biosynthesis of compatible solutes. They produce a high amount of absicic acid (ABA) and late embryogenesis abundant proteins (LEA), beside that there are growth changes (Fig. 3). Under water stress conditions the plant water content decreases resulting in shrinking of the cells and affect on the cell expansion. To enhance the water absorption and keeping the osmotic pressure under water stress, the plants increase their root length and growth to improve the water uptake from the soil. In the short-term response, stomata closure is considered as first response to water stress. Stomata closure occurres when the turgur pressure on the guard cells decreases also the ABA signal which influences the stomata closure, resulting in reduction in CO_2 uptake, transpiration and photosynthesis and also in reduction of the absorption of nutrients and water (Arve et al. 2009).

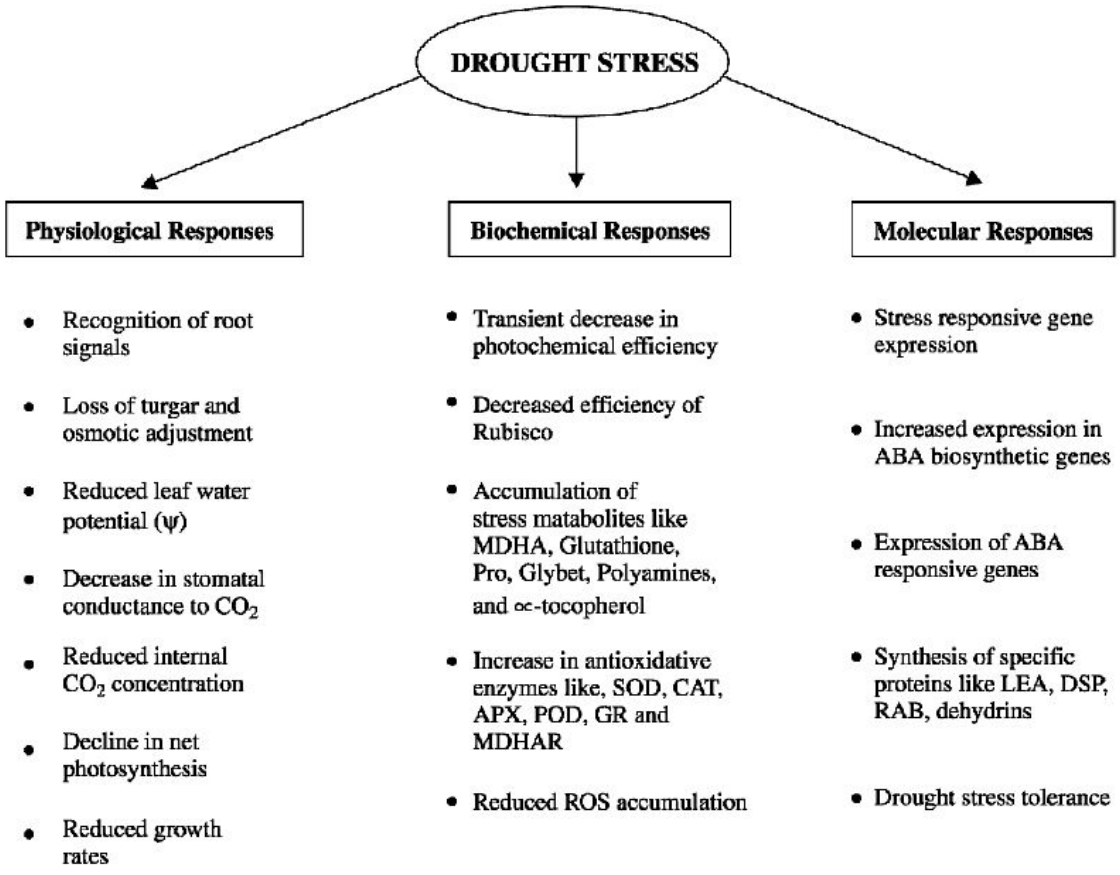

Figure 3. Classification of plant responses to drought stress (Oliveira et al. 2013).

At the molecular level there are many genes that are induced in plants to overcome abiotic stress. Understanding this strategy is a prerequisite for the genetic modification to produce abiotic stress-tolerant plants.1. Genes participating in transcription control and signalling cascade (MAP kinases). 2. Genes responsible for protein and membrane protections (heat shock proteins and LEA proteins). 3. Genes that contribute to water and ion absorption and transport (ion transporter and aquaporins) (Wang et al. 2003).

New drought-tolerant crops

The limitation of water resources in the planet makes it necessary to search for crops better adapted for the cultivation on dry lands (Borrell et al. 2014). The increase in the world human population is combined with a decrease in the water resources. Therefore improvement and production of drought-tolerant crops to prevent the reduction in crop yield under water stress will be even more important (Arve et al. 2009). Several plant species were improved through classical breeding methods or genetic modification to produce plants more tolerant to abiotic stress that could be cultivated in the arable and non-arable land with minimum water requirements for bioenergy and secondary metabolites. One example is "stay green sorghum" that is adapted to cope with water stress conditions and shows greener leaves and stems through the grain filling time and finally increases the tolerance to drought stress and increases the grain mass and yield and was used for biofuel production (Borrell et al. 2014). *Euphorbia tirucalli* L. is widely distributed in various regions under different climatic conditions and exhibits a high capacity as drought-tolerant crop plant serving as source of biofuel, rubber and phytochemicals (Hastilestari et al. 2013). *Jatropha curcas* L. is a drought-tolerant plant species, cultivated in tropical and subtropical regions while the seeds are used for biodiesel production (Fini et al. 2013).

In the following parts, the potential of *Balanites aegyptiaca* as drought-tolerant plant that could be cultivated in arid and semi-arid regions mainly for secondary metabolites and bioenergy is described.

Taxonomic classification of *Balanites aegyptiaca*

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida

Order: Sapindales

Family: Zygophyllaceae/Balanitacea

Genus: *Balanites*

Species: *Balanites aegyptiaca* (NRCS, USDA).

Plant description

Balanites aegyptiaca is considered as one of the most widely distributed woody plant species in Africa. It is a spiny, evergreen and deciduous shrub or tree up to l0 m tall and exhibits different characters. With a spherical crown the short trunk usually branches close to the base. It has yellow or green thick thorns (8-10 cm long); the bark is dark and ranges from brown to grey. Bright green and leathery leaves with two separate obovate leaflets range between 2.5 to 6 cm, the young leaves show fine hairs. Flowers are aromatic with yellowish-green colours found in the leaf axils. A long fruit with narrow drupe approximately 2.5 to 7 cm long and with a diameter of 1.5 to 4 cm is formed. The fruits turn from green to yellow at the maturation stage (Fig. 4). The pulp is edible with bitter sweet. Seeds are light brown, very hard, approximately 1.5 to 3 cm long, and compose 50 to 60% of the fruit. One kg contains approximately 500 dry seeds. The tree starts to flower and produce fruit at an age of 5 to 7 years and the maximum production time is at 15 to 25 years (Sands 2001; Chothani et al. 2011). *Balanites aegyptiaca* is considered partially autocompatible, exhibits a large fruit abortion based on a small fruit/flower ratio and an allopollination ratio of approximately 37% that might be related to wind and insects like Halictidae and Dipterae (Ndoye et al. 2004; Dubey et al. 2011).

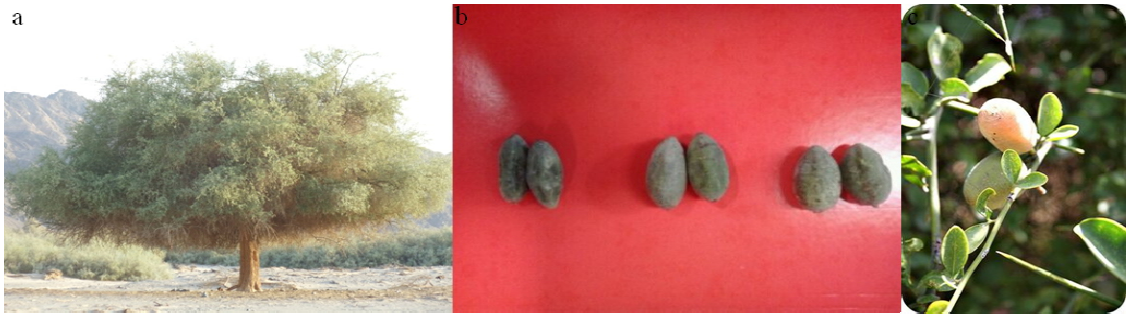

Figure 4. *Balanites aegyptiaca* tree and fruits (a and b) from Wadi El-Gemal National Park, Egypt. (G. Khamis), (c) Mature *B. aegyptiaca* fruits on the tree (SENENEWS.com).

Habitat and distributions

Historically, the plant was found in Egypt since 4,000 years while the kernels of *B. aegyptiaca* fruits were reported in the graves of the twelfth dynasty. It is distributed in several African countries (Sudan, Senegal, Somalia, Egypt, Zambia, Zimbabwe and in Asia through Yemen and Jordan valley) and in the Middle East, Arabian countries and India. The species is distributed in the regions with annually 400-1000 mm rainfall, grown in a wide range of soil varied between deep sands to sandy loam or clays (Sands 2001).

Active compounds and uses

Saponins were used in the folk medicine in various countries in Asia and Africa and are used in food, medical, and veterinary industries. Saponins were extracted from several parts of *B. aegyptiaca* such as roots, stem bark, leaves, fruit, pulp, seed kernel, and mesocarp (Fig. 5). Saponins are glycosides composed of sugar residues connected by oxygen with multiring complex compounds. *Balanites aegyptiaca* organs are very rich in saponins and contains different types of saponins; called balanitin 1 to 7 (Varshncy and Janin 1979; Varshncy and Vyas 1982; Yadav and Panghal 2010) extracted from the kernel and the pulp. The balanitin 1, 2 and 3, diosgenin and alkaloids were found and extracted from the root wood and stem bark, also balanitin 1 was extracted from the stem wood where diosgenin was used as a natural source of steroidal hormones in pharmaceutical industries (Liu et al. 2005). The branches and leaves of *B. aegyptiaca* from Egypt were used to extract six glycosidic flavonoides characterized as quercetin-3-rufinoside, 3-rutinoside, quercetin 3-glucoside, 3-glucoside, 3-7-diglucoside and 3-rhamnogalactoside of isorhamnetin (Maksoud and El-Hadidi 1988). *Balanites aegyptiaca* fruits are very rich in saponins which are distributed with 7.2% in the mesocarp and 6.7% in the kernel (Watt and Brandwijk 1962). The kernel contains 44-51% w/w oil and mainly consists of triglycerides with low amounts of diglycerides, sterol esters, tocopherols, and phytosterols (Abu-El-Futuh 1983). The transesterfication of *B. aegyptiaca* oil provides the possibility to ester the oil of *B. aegyptiaca* as fuel for biodiesel production; the properties of the biodiesel in engine performance were recorded (Deshmukh and Bhuyar 2009). The in vitro antioxidant activity of *B. aegyptiaca* and *Artocarpous heterophyllus* was evaluated using 2, 2- diphenyl-1-picryl hydrazyl (DPPH) free radical method, the aqueous extract of *Balanites* leaves exhibited highest antioxidant activity (42.14%) than the acetone extract from the same plant, also the acetone extract of *Artocarpus* seed showed the lowest antioxidant activity (12.34%), the presence of saponins in the aqueous extract of *Balanites*

leaves and their absence in the acetone extract indicate a possible role for the saponins in antioxidant activity. (Meshram and Umbarkar 2011).

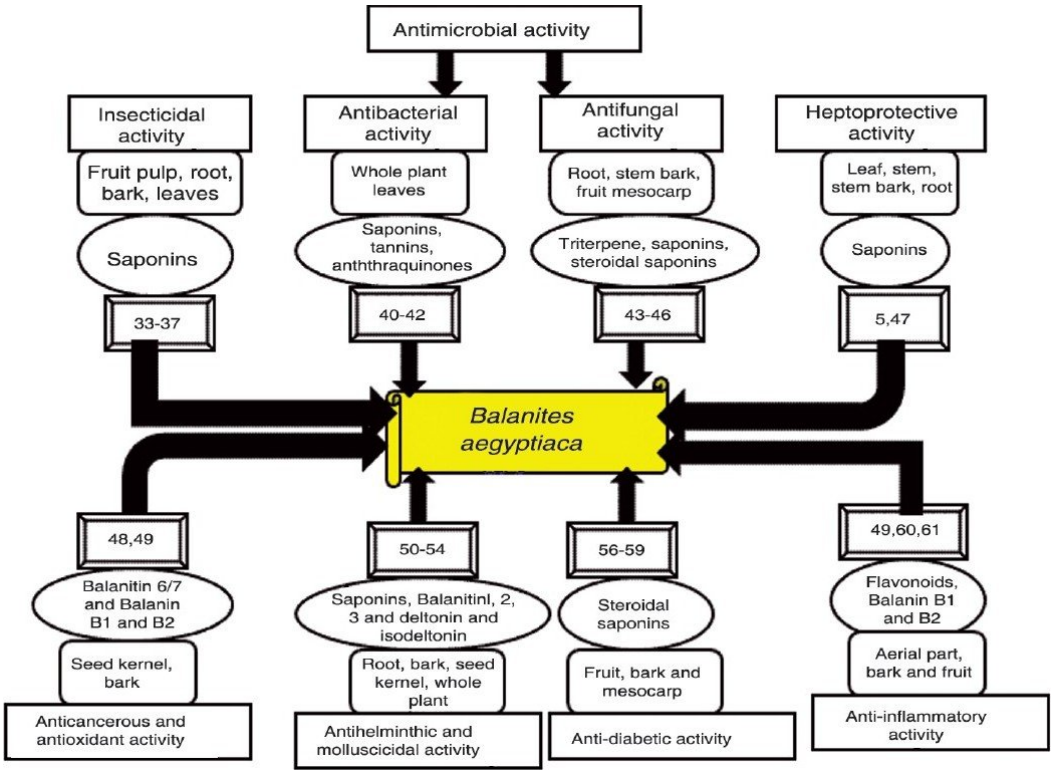

Figure 5. The pharmacological activities for components extracted from *B. aegyptiaca* parts and the numbers revealed to the references and details of the authors according to Yadav and Panghal (2010).

Pharmacological activities

Balanites aegyptiaca extracts exhibit activity as natural larvicidal versus the mosquito larvae (Wiesman and Chapagain 2003; Chapagain and Wiesman 2005). Saponins are distributed in several parts of the plants and are able to interact with the cuticle membrane of the mosquito larvae (Morrissey and Osbourn 1999). Several reports showed the potential activity of the plant species as antimicrobial, antibacterial (Doughari et al. 2007), and antifungal because of the presence of saponins (Farid et al. 2002). Also hepatoprotective activity was reported from the extracts of several *B. aegyptiaca* parts (leaf, stem and roots) in the treatments of Wistar albino rats (Ojo et al. 2006). The anti-cancer and antioxidant activities were recorded when a combination of 72% balanitin-7 and 28% balanitin-6 was used *in vitro* for the treatments of

six different human cancer cell lines (Noula et al. 2008). The plant species also contains compounds acting anti-inflammatory and analgesic activity (Gaur et al. 2008).

Several parts of *B. aegyptiaca* that exhibit medicinal properties were used in the folk medicine by the local people in Africa. The bark was used to heal the mental diseases, yellow fever, and epilepsy. Boiled root was used for stomach pain (Ojo et al. 2006). Plant leaves showed anti-helminthic activities and were used for curing anthrax, and to clean malignant wounds (Hamid et al. 2001). The fruit could treat whooping cough, skin diseases and sleeping sickness (Ojo et al. 2006).

The genetic diversity of *B. aegyptiaca*

The *Balanites* genus includes nine species and eleven infra-specific taxa (Sands 2001). The *B. aegyptiaca* tree is widely distributed through a broad range of environments and soils providing large phenotypic variation in crown shape, seed, fruit, leaves, and the fruiting and flowering time (Hall and Walker 1991; Hall 1992; Sands 2001; Elfeel et al. 2009). It exhibits a large variation in fruits and seeds among and within geographical sources (Zobel and Talbert 2003). So the capability of the tree to overcome drought conditions is different among the provenances and geographical sources (Elfeel et al. 2007). There is a limitation in the information about genetic variation of this species among the geographic habitats. A genetic variability in *B. aegyptiaca* has only been recorded through peroxidase isozyme analysis of different germplasm pools (Chamberlain 1992).

The potential of *B. aegyptiaca* as a drought-tolerant plant

Balanites aegyptiaca showed drought-tolerant characters through distinct anatomical leaf features, such as abundant sunken stomata on both leaf surfaces and thicker leaf laminae. Thick waxy cuticles and a thick layer of trichomes on the leaves of *B. aegyptiaca* reflects excess of light and might form a protective shield against high irradiance in the arid habitat (Radwan 2007). These leaf characters seem to contribute one of the major adaptive features for drought tolerance on this species and also a deep tap root system (Elfeel et al. 2007). The capability of the plant to produce fruits under arid conditions was recorded and the tree is not damaged through forest fire and flooding, except the young trees. Therefore it can be cultivated in various arid desert environments in Africa and South Asia (Hall and Walker 1991).

Micropropagation of *B. aegyptiaca*

The classical propagation in *B. aegyptiaca* was done through vegetative cutting, seeds and root suckers but these methods are inadequate for mass multiplications because of the limitation in low survival and germinations rates for seeds and vegetative cutting, also the low rate of root induction through vegetative cutting (Anis et al. 2010).

Various efforts were reported for *in vitro* propagation of *B. aegyptiaca* through nodal explants with axillary buds (Ndoye et al. 2003; Anis et al. 2010), direct shoot morphogenesis from root segments (Gour et al. 2005; Varshney and Anis 2013) and *in vitro* shoot induction and plant regeneration from nodal explants (Siddique and Anis 2008). Several explants such as young thorns, apical buds, root segments and cotyledon explants were used for callus induction (Gour et al. 2007). Also root explants were used to produce somatic embryos (Saharan et al. 2011). Kinetin at 1 mg l^{-1} combined with NAA at 0.2 mg l^{-1} mixed with 4 g l^{-1} casein hydrolysate was more efficient for shoot number obtained from nodal explants. On the other hand increase the sucrose concentration to 70 g l^{-1} separately or mixed with kinetin and NAA caused suppression of the shoot number per explants (Dawah et al. 2013). Kinetin at 1 mg l^{-1} combined with NAA 0.2 mg/l and 40 mg l^{-1} adenine sulphate gave the highest number of shoots from shoot tip explants, also kinetin at 1 mg l^{-1} combined with 0.2 mg l^{-1} NAA plus 0.25, 0.50 or 1 mg l^{-1} diphenyl urea gave the highest number of shoot per explants for nodal and shoot tip explants, finally, NAA at 0.2 mg l^{-1} combined with 8 mg l^{-1} phloroglucinol enhance the vegetative growth for nodal and shoot explants (El-Mekawy et al. 2012). Gour and Kant (2011) investigated the effect of low cost gelling agent and carbon source on the *in vitro* rooting of *B. aegyptiaca* and *Phyllanthus emblica* microshoots, the results showed that, the presence of isabgol as gelling agent in the rooting media for *B. aegyptiaca* was equivalent to that from agar and isabgol could reduce the cost of gelling agents by 44%, also sugar table could be used for the in vitro rotting of *B. aegyptiaca* and *Phyllanthus emblica* microshoots. Hwida and El-Kader (2012) delivered an efficient method for in vitro preservation of shoot culture on *Balanites aegyptiaca*, the preservation conducted through encapsulation of shoot explants at 1,2 and 3 g l^{-1} of sodium alginate in presence and absence of MS media supplemented with 0.5 mg l^{-1} BA besides the control treatment, the cultures were stored for 12 months and MS medium supplemented with 0.5 mg l^{-1} BA with or without ABA at 10 and 15 mg l^{-1} gave the highest survival rate (100%). The genetic stability of the plantlets was confirmed through RAPD analysis. A micropropagation system was established in *B. aegyptiaca* from axillary shoot bud multiplication, the genetic uniformity among the

regenerated plantlet was confirmed through Inter-simple sequence repeat (ISSR) markers, 117 bands were obtained where 115 bands were monomorphic and 2 bands were polymorphic so 98.2% genetic uniformity was confirmed and the plantlets were true-to-type to the mother plant (Varshney and Anis 2013). For the short term conservation of germplasms Varshney and Anis (2014) developed a method for the encapsulation of nodal explants obtained from *in vitro* proliferated shoot. The encapsulation conducted through 3% sodium alginate and 100 mM $CaCl_2$ x 2 H_2O, which gave 80% response to convert the encapsulated nodal explants into plantlets through the *in vitro* propagation protocol and the survival rate after acclimatization was 90% and the genetic stability of this synseed was confirmed through ISSR.

Effect of salt stress on *B. aegyptiaca*

The US National Research Council (NRC) reported that the species is naturally grown and distributed in one of the most drought-affected regions in the world. Considered among the 24 high priority neglected crops in Africa providing needs of different food supplies, medicinal compounds and fuel wood for the people living in these regions. Nevertheless the plant species was neglected in the scientific communities (NRC 2008). Radwan et al. (2000) reported that *B. aegyptiaca* seedlings are sensitive at low levels of salinity (12 dS m^{-1}) which is considered as saline irrigation water, the salinity level at 24 dS m^{-1} was harmful for the growth of the seedlings by reducing the photosynthesis and transpiration rates. Also the effect of salt stress on three varied sources of *B. aegyptiaca* (SD1 and SD2 from Sudan and KSA from Saudi Arabia) was investigated through varied levels of salt concentrations (control, 5, 8 and 10 dS m^{-1}), the results showed that under salt stress, seedlings exhibited lower biomass and growth. The level of sodium was increased in the leaves by increasing the salt concentration in the soil, also tannin was increased at increased salinity and was significantly different among the sources. Also the three sources showed significant differences in the growth parameters and according to the plants response to salt stress there was variability in the salt tolerance among different intra-specific sources of *Balanites* species (Elfeel et al. 2013). In this way it is advantageous to improve the ability of this plant species to overcome salt stress in order to cultivate *B. aegyptiaca* in arid and non-arid land in various soil types.

Aims of this study

1. Collection of *B. aegyptiaca* seeds from different areas, and study the genetic diversity among *B. aegyptiaca* provenances from different geographical places by *amplified fragment length polymorphism* techniques (AFLP).
2. Investigation of the morpho-physiological responses of *B. aegyptiaca* sources to water stress to select the most drought-tolerant source that could be recommended for the cultivation in arid and semi-arid environments.
3. Establishment of an efficient *in vitro* propagation method implying shoot proliferation from nodal and adventitious shoot regeneration from cotyledon explants.
4. Preparation of plasmid constructs containing gene responsible for some abiotic stress tolerance for later transfer via *Agrobacterium tumefaciens*, to produce salt-tolerant *B. aegyptiaca* plants that can be cultivated in the non-arable, saline areas.

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Chapter 2

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REVIEW ARTICLE

Newly established drought-tolerant plants as renewable primary products as source of bioenergy

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Abstract

Drought-tolerant plants, also called xerophytes, have developed during evolution a huge spectrum of morphological, physiological and metabolic adaptations to a shortage of water. Due to global climate change large areas of land are threatened by increasing water limitation and therefore drought stress. In addition, energy becomes limiting for an increasing world population and renewable local energy sources are needed. The major food and fodder plants such as wheat, rice, corn and soybean do not show high drought tolerance. There are a number of genetic approaches to increase drought tolerance of these species; some of the genetically modified plants show good results in the greenhouse but when the plants are challenged by field conditions the promising results cannot be reproduced. One option to overcome the problem is to develop new crop plants from already highly drought-tolerant plants. We would like to focus on drought-tolerant plants grown on non-arable land as primary source of bioenergy. Some plants species are already locally used. Promising ecotypes could be used in breeding programs to improve the agricultural and economic values of these plants. For the selection and the development of new crops plants as sources for bioenergy the description of an optimal plant could be the starting point. It will be reported about promising preliminary results and economic uses of xerophytes from the genera Jatropha, Balanites and Euphorbia as new crop plants for the production of ethanol, biodiesel, biofuel, biogas and biomass-to-liquid. Finally, the pros and cons of plants as source of bioenergy in water-limited areas will be discussed.

Key words: Drought-tolerance, New crop species, Non-arable land

1. Introduction

The supply of fossil fuel will gradually decrease (Longwell, 2002; Tsoskounoglou et al., 2008). Therefore, efforts are made to substitute fossil energy sources. One of these sources is solar energy that is unlimited and plants have the capability to capture this energy through photosynthesis. The yield of photosynthesis is stored as biomass and has the potential to contribute to the energy needs. Selecting inexpensive and abundant biomass feedstock is important to obtain biofuel production with minimal cost. This has lead to the large scale conversion of arable land for cultivating energy plants (Klein-Goldewijk et al., 2004). However, global warming makes agriculture facing a range of serious environmental problems such as depletion of water resources and the increase of dry areas (Dai, 2012).

Therefore it is important to find plants for growing on marginal and non-arable land in which food crops cannot be cultivated. Drought stress is one of the major environmental limitations with tremendous effects on the plant growth and development (Harb et al., 2010; Song et al. 2012). Drought stress causes a decrease in the crop productivity and nearly 28% of the world's soil surface is too dry for regular crop yields (Bray et al., 2002; Ambrosone et al., 2013). In addition, the area of agricultural land decreased, for example between 2004 and 2011 from 37.9% to 37.6% (The World Bank Annual Report, 2013). One important factor for this decrease is the climate change. According to US National Oceanic and Atmospheric Administration (2008) combined global land and ocean surface temperature from January to December 2008 was 0.49°C above the 20th century average of 13.9°C. Since 1880, the annual combined global land and ocean surface temperature has increased at a rate of 0.05°C per decade. This rate has increased to 0.16°C per

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decade over the past 30 years (US National Oceanic and Atmospheric Administration, 2008). The trend in rainfall variability has created a greater frequency of dryer years, roughly doubling the number of dry areas in the world from about 15% in 1950 to 30% in 2005 (The World Bank Annual Report, 2013) and finally resulting in huge degraded land areas (Table 1). In addition there is an increase in the world population with expected 9 billion in the year 2050 increasing the need for food and energy. On the one hand the remaining land has to be used for the production of food, on the other hand fast decrease in mineral oil resources make a big demand to search for renewable resources in fuel and there is an increasing demand for energy locally produced with low technically input. One solution might be the cultivation of stress-tolerant plants as renewable primary products as source of bioenergy. Biodiesel has been already produced from several edible plant oils such as soybean, sunflower and coconut, but with the reduction in the agricultural land in the world, this could make a competition between the food crops and oil crops for biodiesel (Hill et al., 2006; Chapagain et al., 2009). In this review, different aspects of the cultivation of new drought-tolerant crop plants as source of energy will be elucidated from different point of views.

Table 1. Globally available land (data are taken from Metzger and Hüttermann 2009).

Use	Area (in billion ha)
Arable land	1.54
Pasture	3.39
Forest	3.95
Deserts	2.56
Steepland	1.47
Degraded areas (former fields, forests, grazing land)	3.50
Total land area	12.91

2. Morphological, physiological and metabolic adaptations of xerophytes to drought

Plants respond to drought stress by reprogramming a number of metabolic and physiological mechanisms (Morison et al., 2008; Ahuja et al., 2010; Park et al., 2012) to overcome the water deficit. The reduction in plant water content induces stomatal closure resulting in a reduced transpiration rate, a reduced rate of photosynthesis and finally in a decrease of growth and gain of biomass. In parallel, an accumulation of reactive oxygen species (ROS) and of compounds such as proline and mannitol is observed. Higher concentration of abscisic acid and the synthesis of new proteins in response to water deficit can be measured (Lichtenthaler et al., 1981; Yordanov et al., 2003).

Among xerophytes, a term referred to plants that have adapted to live in dry and arid habitats, are approximately 20,000 useful plant species (Wickens, 1998; Akashi et al., 2011). Xerophytes have distinct morphological characters helping the plant to survive in arid and dry ecosystems. The root systems are well developed and richly branched with a long tap root, the stem covered with wax and hairs often stores water and becomes succulent, and the overall surface area, especially of the leaves, is reduced. Sometimes the leaves fall down soon after they have been created (*Euphorbia* species). In *Opuntia* species the leaves have a spine-like structure whereas *Bromelia* species show a rosette arrangement which helps the leaves in severe light to minimize the transpiration. Some desert grasses (*Ammophila* and *Stipa* species) present unifacial leaves, where the stomata exist on the upper epidermis to stop the transpiration (Bendre and Kumar, 2010).

About 85% of the plant species are so called C_3-plants and fix carbon dioxide (CO_2) directly to the C_5 body 1,5-ribulose-bis-phosphate by the enzyme 1,5-ribulose-bis-phosphate-carboxylase-oxygenase (RubisCO). The first measurable organic carbon molecule containing the fixed CO_2 consists of three carbon atoms and it is either used for the production of C_6 sugars or for the regeneration of the C_5 body in the Calvin Cycle. At higher temperature and under dry condition when the CO_2 concentration in the chloroplasts decreased below 50 ppm, RubisCO binds and fixes increasing amounts of oxygen instead of CO_2 leading to the process of photorespiration which consumes energy and makes photosynthesis less efficient for the plant (Moore et al. 1995). In the early response of water stress when the relative water content in the leaves decreases to less than 70%, there is a negative effect on the C_3 photosynthesis by decreasing the photosynthetic activity and the stomatal conductance, and by increasing the CO_2 assimilation to recover and balance the effect of water stress (Cornic et al., 2000; Ghannoum, 2009). When the leaf water stress decreased to 30% in C_3 plants even with high CO_2 concentration and saturating light, the efficiency of photosynthesis is affected and it shows a reduction in CO_2-uptake under drought stress due to stomatal closure (Kaiser, 1987; Cornic et al., 1991).

Many grasses are drought-tolerant due to their C_4 metabolism when a pre-fixation of CO_2 to a C_3

body occurs spatially separated from the normal Calvin cycle because of their higher water use efficiency. About 10% of the plant species adapted to high temperature and water deficiency use alternative CO_2 fixation ways such as the Crassulacean Acid Metabolism (CAM). Cacti, orchids and species from the Asclepiadoideae subfamily, such as the wax plant, *Hoya carnosa* (L.f.) R.Br. (1810) which has distinct waxy foliage for example, take CO_2 up at night and fix it into malic acid which is stored in vacuoles for photosynthesis. Under dry conditions and to reserve water, the stomata are closed during the day and CO_2 is released from malate and fixed through the Calvin cycle (Moore et al. 1995). So the stomatal closure during the day reserves the water from evaporation under heat condition or water stress. Some plants change from C_3 to CAM under water stress or other environmental conditions (Bastide et al., 1993). In summary, CO_2 can be bound also under low water conditions with closed stomata during the day, however, the plant has to invest about three times more energy to produce the same amount of biomass than a C_3 plant.

3. Methods for quantifying drought tolerance

Stomatal conductance and infrared thermography are techniques that can be useful in the analysis of drought tolerances (Jones, 2007). Under drought stress conditions stomatal closure leads to a reduction of water loss for the plants. Conductivity and leaf temperature measurements allow the observation of stomata behavior. Both are non-destructive methods. Stomata conductivity measurements with a porometer have the disadvantage that the measurement is done only punctually on a leaf. This results in a large mean variance of the data. It is possible to investigate the stomatal behavior and thereby temperature changes of a leaf or even of complete plants with infrared thermography. The differences in stomatal conductance and the leaf temperatures between drought and control groups are significant for many plant species and the determination of these parameters can be used for the identification of drought tolerant species and genotypes (Jones et al., 2002; Guretzki and Papenbrock, 2013). The suitability of chlorophyll fluorescence measurements as an early valuable indicator of stress impact on plants was assumed by many researchers based on its high sensitivity. Therefore it was used in many screenings for drought tolerant genotypes. However, for several species analysis of chlorophyll fluorescence does not appear to be sensitive enough to detect early symptoms of drought stress. Under mild to moderate drought stress, the closing of the stomata is the main reason for changes in photosynthesis as summarized by Medrano et al. (2002). The factor "variable fluorescence" divided by the "maximal fluorescence" (F_v/F_m) is considered as a meaningful, fast-measured indicator for stress on plants. For non-stressed C_3 plants values of about 0.83 are expected, a decline in F_v/F_m indicates a lower potential tolerance against oxidative stress (Björkman and Demmig, 1987). In several studies only severe water limitation for a longer period of time lead to a significant reduction of F_v/F_m in comparison to the control group (Woo et al., 2008). The response of these plants matches with the "Threshold for Tolerance Model" (Sperdouli and Moustakas, 2012). According to this model, tolerance mechanisms are started with a lag time or are induced by threshold concentrations (Barcelo and Poschenrieder, 2002). Moderate stress causes less damage to the plant, because stress adaptation processes and repair mechanisms start in the plant whereas during mild drought conditions the stress threshold was not reached. Therefore, the plants are more affected under mild drought stress reflected by stronger altered chlorophyll fluorescence values in comparison to the moderate group (Lichtenthaler, 1998; Sperdouli and Moustakas, 2012). Important for the characterization of drought tolerance in different species and genotypes is therefore the application of the appropriate strength of drought stress, then chlorophyll fluorescence measurements might be used efficiently (Guretzki and Papenbrock, 2013). Overall, a combination of infrared thermography and porometer measurements in conjunction with traditional growth parameter like biomass investigations is advisable for analyzing plants grown under greenhouse conditions. These methods are also suitable for many species because measurements are easy and quick to handle. Thereby the different effects of drought stress on the plant can be analyzed in order to filter out drought-tolerant genotypes from a selection of genotypes (Guretzki and Papenbrock, 2013).

Currently, many approaches are used to improve the drought tolerance of well-established crop plants. In conventional breeding drought tolerance is selected together with plant productivity. On commercial varieties displaying drought tolerance are crossed with susceptible, higher-yield plants. Marker-assisted breeding assists and speeds up conventional breeding. In addition, the genetic factors responsible for part of

the phenotypic variation observed for a quantitative characteristic, named quantitative trait loci (QTL), are being identified opening a great potential to accelerate the whole process (Xoconostle-Cazares et al., 2010). Plant breeding through genetic engineering opened a wide range of possibilities to improve the ability of the plants to grow in drought stress. However, one has to keep in mind that drought tolerance is a multigenic trait. Several genes were used to produce transgenic plants; these genes were noted in other plants or microorganisms to be induced under abiotic stress, so far with limited success (Xoconostle-Cazares et al., 2010). However, instead of producing transgenic plants, exploitation the potential of already stress-tolerant plants as new crop plants might be more promising.

4. From promising preliminary results to economic use of xerophytes as new crop plants

4.1. Overview on the production of bioenergy from plant biomass

There are different ways how crop plants can be used for the production of bioenergy. Currently, a lot research is going on to find the optimal way for certain plant species with respect to yield, cost, practicability and sustainability. Plants can be used to produce biogas, ethanol, petrol, biodiesel or biomass can be converted by the Fischer-Tropsch process to liquid synthetic fuels (BTL).

4.1.1. Biogas

Biogas is a product of the anaerobic digestion of solid organic biomaterials resulting gaseous mixture mostly methane, carbon dioxide and some impurities such as H_2S, NH_3, water vapor, N_2, dust and siloxanes (Deublein and Steinhauser, 2011). The weak points of this method are odors and producing corrosive volatile sulfur compound (Ward et al., 2008). This process is theoretically an energy balance. The energy of organic material built up by photosynthesis is balanced with the degradation of organic material plus methane combustion (Deublein and Steinhauser, 2011). The result of combustion (CO_2 and H_2O) can be reused for photosynthesis. Thus, there are no substances lost and the energy circle is closed. To increase the yield of biogas, careful selection of the input material is necessary. The highest methane yield from degradation of the same amount of biomaterial are lipids, then followed by protein and carbohydrates (Deublein and Steinhauser, 2011). Proteins might yield impurities such as NH_3 and H_2S that decreased the efficiency of biogas formation. Biogas can contain 40-70% methane which has a heating value of 5 to 7.7 kWh m^{-3}. Several simple fermentation systems have been developed which can be operated locally also by non-experts. However, it is essential to keep them tight. If only 5% of the produced methane is emitted from the storage tank, the positive climate effect of the energetic use of biogas is completely cancelled because the global warming effect of methane is at least a factor of 23 higher than CO_2 (Weiland, 2003).

4.1.2. Bioethanol

Bioethanol can be produced from very common sugar cane, potato, manioc and corn. Currently, the raw product always contains starch or sugar as fermentable carbohydrates and all plant species used are also valuable food and fodder plants. The basic steps for large scale production of ethanol are: microbial (yeast) fermentation of sugars, distillation, dehydration and denaturing (optional). Prior to fermentation, some crops require the hydrolysis of soluble carbohydrates such as cellulose and starch into simple sugars (Weiland, 2003). Enzymes are used to convert starch into sugar. Because the use of starch and sugar-containing crop plants a starting product the production of bioethanol remains ethically difficult and new ways for bioethanol production are being investigated. The use of ligno-cellulose containing biomass is more sustainable because cellulose, hemicelluloses and lignin are not digestible for humans and feedstock; therefore the tank or plate conflict can be avoided. Recently, a pulping process for lignocellulosis biomass conversion was developed (Alkaline Polyol Pulping, AlkaPolP). Lignocellulosis-containing biomass is dissolved in alkaline glycerol for several minutes at normal pressure. The cellulose-rich pulp is filtered, lignin- and hemicelluloses are separated from the solute which is regenerated for further use. Cellulose, lignin and hemicelluloses can be used after further characterization as raw material for the different industrial uses (Hundt et al., 2013). However, just for the production of bioenergy simpler cellulosic technologies are being developed.

4.1.3. Petrol

There are some species of certain families which accumulate hydrocarbons of high molecular weight (up to 10,000 Da). These petroplants have lactiferous canals in their stem and secrete a milky latex. The latex can be either continuously tapped like *Hevea brasiliensis* (Willd. Ex. Juss) Muell. Arg latex and stored or extracted from the biomass by using the organic solvents. The product rich in long chain hydrocrackable hydrocarbons is called as 'biocrude'. After conversion into short chain

hydrocarbons biocrude yields about 70.6% energy, out of which 22% as kerosene and 44.6% as gasoline. It was hoped that petroplants can yield petroleum more than 40 to 45 barrel acre^{-1} (Calvin, 1978).

4.1.4 Biodiesel

For the production of biodiesel fatty acid methyl esters (FAME) have to be synthesized from vegetable oil because their physical characteristics are closer to those of fossil diesel fuels than pure vegetable oils (Chapagain et al., 2009). Biodiesel and fossil diesel have different properties. Therefore different additives are needed to improve the characteristics of biodiesel at low temperature and to reduce the oxidation processes. To produce FAME oils and fats from different sources (vegetable, oil, animal fats and waste cooking oils) are transformed in a process called transesterification. In the presence of a catalyst a glyceride reacts with an alcohol and a mixture of fatty acids esters plus alcohol is synthesized. The products of the transesterification reactions are raw biodiesel and raw glycerol. The raw biodiesel needs to be cleaned to obtain the usuable biodiesel whereas the glycerol produced can be used for various industrial purposes (food, cosmetics, oleochemistry.) The main feedstocks are oil seeds (rape, sunflower, soybean, oil palm), used cooking oil, waste animal fat (http://www.biofuelstp.eu/ accessed 15 June 2013).

4.1.5. Biomass to liquid (BTL)

There are several technical processes and devices in the development how biomass such as straw or wood can be converted to liquid synthetic fuel. So far there only pilot systems have been established but the results are promising with respect to yield and CO_2 balance. There are four phases: 1. Gasification, the original energy carrier will be converted into a usable synthetic gas. 2. Gas purification and conditioning for further synthesis. 3. Hydrocarbon synthesis in a Fischer-Tropsch process to more complex hydrocarbons as hydrocarbon raw products (paraffins, oleofines and oxygen-containing compounds) with different chain length. 4. Processing and conditioning. The hydrocarbons are processed to the final fuel. However, the process is not yet technically optimized and needs high level technical equipment (Hundt et al., 2013).

4.2. Optimal, hypothetical plant for the production of bioenergy in a sustainable way

One could speculate about the perfect plant for the sustainable production of renewable energy under water-limiting conditions. Following aspects might be important: A low need for water, rapid attainment of maximum growth rate (drought avoidance), the capacity to outlive water shortage for a period of time, undemanding for fertilization, easy to propagate promising lines, robust with respect to pathogens, no allelopathy effects for the same species and also for other species for repeated growth on the same field or re-cultivation by other species, perennial due to a higher efficiency, sufficiently high yields also under limiting conditions to make the cultivation economically feasible, suitable for the production of bioenergy by at least one process, and native to the respective flora to avoid escape as an invasive plant.

For many regions in the world the non-xerophyte *Zea mays* L. seems to be an almost perfect plant because it can be easily grown and offers high yields. In the fermentation process the methane percentage is high. In the first generation of energy plants only the starch-containing seeds have been taken based on the existing conversion technology. In the second generation the corn stover, the "leftover" portion of the corn plant after harvest, including corn cobs, stalks and leaves, has been used a lignocellulosic feedstock in pilot devices still in the development of new conversion technologies. In Europe, the biogas from corn is considered to have the highest yield with 5,780 m^3 ha^{-1} year^{-1} (Weiland, 2003). In temperate regions *Brassica napus* L. is a very attractive plant for the production of ethanol and biodiesel. However, both plants are also an important source of food and both plant species need to be grown on fertile soil. Both plant species are annual plants. It is reported that perennial plants are more sustainable and have a better balance with respect to greenhouse-gas production than annual crop plants on the field. Actually, energy savings and greenhouse gas reductions reached by the use of annual crops are negative or at least lower than those for perennial crops as was shown by life-cycle (Karp and Shield, 2008). For the tropical and subtropical regions *Jatropha curcas* L. was highlighted as a perfect plant for the production of biofuel (see chapter 4.3.4).

4.3. Drought-tolerant plants for the production of renewable primary products as source of bioenergy

Sometimes different market catagories for the use of bioenergy crops are defined. They can be used for power generation such as electricity, heat, and a combination of heat and power or they can be used for the production of liquid transport fuels.

Many energy plants or plant organs can be used for both market catagories (Karp and Shield, 2008). The bioenergy yield can be either quantified as the amount of dry matter (DM) or biomass (C) per area of land used for the conversion or as the bioenergy obtained from this biomass for the production of bioethanol, biodiesel or heat and electricity. The composition of the plant DM varies considerably among bioenergy crops (for consensus values for quality attributes see (Karp and Shield, 2008) and this has significance for conversion to energy and thus for bioenergy yields.

4.3.1. Plant sources of biogas

Many rhizomatous grasses are used for the production of biogas. Some experiments with drought-tolerant grasses have been conducted. Greenhouse experiments with *Eragrostis tef* (Zucc.) Trotter from Ethiopia has shown that this plant has a methane potential in the same range as corn (about 300 ml g^{-1}). Teff´s high methane potential together with its possibility to grow on wastelands makes it an interesting potential substrate for biogas production. Teff can be grown and perform well on abandoned lands e.g. waterlogged or wastelands where other crops such as corn cannot be successfully cultivated. This implies that there could be a window of opportunity to cultivate the crop for biogas production without interfering with food production (Nigatu et al. 2012). Sudan grass (*Sorghum sudanense* = *Sorghum* × *drummondii* (= *S. bicolor* × *S. arundinaceum*) (Steud.) Millsp. & Chase has high yields and only modest requirements on the soil. The species can adapt to dry periods quickly. A disadvantage might be slow growth. Plantations need a labor-intensive start. More breeding progress is needed to optimize genotypes for dry, non-arable land (Bibi et al., 2010).

4.3.2. Production of ethanol

World ethanol production from plants for transport fuel tripled between 2000 and 2007 from 17 billion to more than 52 billion liters, widely used in the USA and Brazil. The main sources are corn in the USA and sugar cane in Brazil (El Bassam, 2010). Advantages are high yields and several harvest per year. On the other hand, their tolerance to abiotic stress, such as drought is low. Currently, there is some progress to identify and breed more drought tolerant sugar cane genotypes (de Almeida Silva et al., 2008). But they also act as food and fodder plants. A more promising plant might be the perennial grass *Miscanthus x giganteus* Greef et Deu, Poaceae, from Southeast Asia, a hybrid of *M. sinensis* and *M. sacchariflorus*. *Miscanthus x giganteus* is characterized by a very high gain in biomass due to the fact that it is a hybrid and that is C_4 photosynthetic pathway as many other grasses. It has a low demand for fertilization and plant protection, however, it needs watering for optimal growth. Recently, different genotypes of *Miscanthus x giganteus* have been investigated for its drought and salinity tolerance and some QTLs have been identified which could be used for further breeding (Grare, 2010). In Germany it produces up to 30 t ha^{-1} $year^{-1}$ dry mass whereas even fast growing tress such as poplar only produce about 16 ha^{-1} $year^{-1}$ (Lieberei and Reisdorff, 2012). Another advantage is the simple further post-harvest processing because the above ground shouts die already on the field and are dry at harvest. The technical aspects of using *Miscanthus x giganteus* as a source of biomass, for example in the form of pellets, biofuel and even as construction material are already well investigated because research is massively supported. Therefore if the cultivation of drought and salt tolerant *Miscanthus x giganteus* genotypes on non-arable land will be successful the transfer of knowledge on further processing can be easily done.

Recently, an Agave-to-ethanol project was set up. Two native species from the Agavaceae *Agave tequilana Weber* and *Agave angustifolia Haw.* and varieties thereof were cultivated in semi-arid areas of Mexico. Some varieties possess a three times higher sugar content than sugarcane. These high quality agaves are very good feedstock material for bioethanol due to their high total sugar density and content, their high weight of the fruit and stems and their high density of plants per hectare. The two agave species have low water requirements and need low maintenance during the cultivation and harvest cycles of six years The researchers estimate that varieties of *A. tequilana* can yield up to 7,000 l ha^{-1} $year^{-1}$ of distilled ethanol (Burger, 2008). These data sound very promising and species from this family might be also attractive for cultivation in other semi-arid regions on marginal land. However, still some technical problems of controlled crushing and extracting the sugar need to be optimized.

4.3.3. Plant sources of petrol

Many *Eucalyptus* species contain high contents of essential oils which could be used for the petrol production. *Eucalyptus globulus* Labill. contains about 3.5% essential oils in the leaves. Selection programs for drought tolerant hybrids *E. globulus x nitens* obtained positive results (Navarrete-Campos et al., 2013). Therefore there are a number of examples of cultivating *Eucalyptus* species in the

tropics and subtropics and use them as cash crop, for construction and as a source of energy (Guinand and Lemessa, 2001; Lieberei and Reissdorff, 2012). But *Eucalyptus* species cannot provide the same wide variety of different products as indigenous species and in addition has negative effects on soil fertility, creates soil erosion and dries up the land by influencing the ground water system (Guinand and Lemessa, 2001).

Species from the genus *Euphorbia* in the family Euphorbiaceae have a high milk sap or latex content consisting of diterpene and triterpene esters (with a very high energy content of 46.9 kJ g^{-1} even in comparison to lipids with 38.9 kJ g^{-1}), resins and proteins. Therefore they are candidates for the production of petrol. *Euphorbia tirucalli* L. has a high salinity and drought tolerance (Janssens et al., 2009; Hastilestari et al., 2013) and it survives in a wide range of habitats even under conditions in which most crops and other trees cannot grow. The coverage of *E. tirucalli* includes tropical arid areas with low rainfall, poor soil condition but it is not frost tolerant (Van Damme, 2001). The same author mentions that *Euphorbia* subg. *tirucalli* consists of ca. 30 species and its distribution is through the Paleotropical region in Madagascar, the Cape region, East Africa, and Indochina. It is also utilizable for other applications such as a source of pharmacological activities as described in Hastilestari et al. (2013). According to Calvin (1978) the hydrocarbon of the latex is able to produce the equivalent of 10 to 50 barrels of oil per acre. However, more recent results revealed that extraction of hydrocarbon to yield methane is less efficient than using the biomass in biogas (Loke et al., 2011).

Sow et al. (1989) reported based on research carried out in Kenya that 80,000 *E. tirucalli* plants per hectare yield 20 t $year^{-1}$, and it`s potential annual methane production is around 3,000 m^3 per year. Loke et al. (2011) showed based on field experience in Colombia that 30 t ha^{-1} $year^{-1}$ yielded 8,250 m^3 ha^{-1} biogas. One m^3 of biogas can generate 1.44 kWh m^{-3}, so totally the output is 11,880 kWh $ha^{-1}year^{-1}$. The authors also reviewed a comparison of different conversion techniques to get electricity and mentioned that biogas production has the highest yield (11,800 kWh) compared to oil extraction (6,600 kWh) and gasifier (3,700 kWh). This biogas yield was calculated from estimated yield of 273 m^3 biogas (60% methane) per ton dry matter of *E. tirucalli*. The biogas technique results in 50.7% higher yield than that of oil palm only (5,858 kWh). However, no details on the scientific data are publicly available. In Europe, the biogas from corn is considered to have the highest yield. Compared to maize (5,780 m^3 ha^{-1} $year^{-1}$) and forage beet and leaf (5,800 m^3 $ha^{-1}year^{-1}$), the biogas yield of *E. tirucalli* is smaller; however, it is higher than wheat (2,960 m^3 ha^{-1} $year^{-1}$) and rape (1,190 m^3 ha^{-1} $year^{-1}$) (Weiland, 2003). Crops proposed for biogas production should be selected and contain low amount of lignin because lignin structures are normally resistant to degradation under anaerobic conditions (Weiland, 2003). The content of lignin of *E. tirucalli* might be quite low as the stem is highly succulent and non woody.

Euphorbia lathyris L. is an annual native herb to Southern Europe, northwest Africa, and eastward through southwest Asia to western China but was also introduced to the USA. Research on this species was originally initiated by Calvin (1978) in the USA. The biennial plant can well be cultivated on marginal land and produces 20 t ha^{-1} $year^{-1}$ dry matter. The hydrocarbons which contain around 30% triterpenoids can be converted in to petrol. By optimizing the cultivation and extraction procedures by organic solvents such as hexane the yield in petrol was between 3,000 and 4,000 L ha^{-1} (Lieberei and Reissdorff, 2012). The experiments were taken up by researchers in India and *E. lathyris* was successfully cultivated on semi-arid and arid regions of Rajasthan, India. Gain in biomass, hydrocarbon yield and productivity of *E. lathyris* could be improved during subsequent experiments (Garg and Kumar, 1989).

There are a number of other plants containing hydrocarbons, mainly in the form of rubber (Sanderson, 2006). However, some of them are not drought-tolerant, such as *Hevea brasiliensis* (Euphorbiaceae), *Copaifera langsdorfii* and *C. mutijuga* (both Fabaceae), or they are too difficult to cultivate and thus the yield is too low for the production of bioenergy, such as *Euphorbia abyssinica* and *E. resinifera*. (Euphorbiaceae). They still might be a promising source for special kinds of rubber such as *Taraxacum koksaghyz* (Compositae) and *Calotropis procera* (Asclepiadaceae). Some might be interesting drought-tolerant candidates for future research as source of bioenergy, such as *Hardwickia pinnata* (Asclepiadaceae), *Parthenium argentatum* (Asteraceae) and *Dipterocarpus turbinatus* (Dipterocarpaceae).

4.3.4. Plant sources of biodiesel

Many plant species which are adapted to grow in non-arable land and dry ecosystems produce considerable amounts of triglycerides which could

be used in the biofuel production through esterification processes (Chapagain et al., 2009). *Elaeis guineensis* Jacq., the African oil palm has many uses and offers many coproducts. The cultivation on degraded land is well possible. However, the production of palm oil has detrimental effects on the environment and is not considered to be a sustainable biofuel. The deforestation occurring as a result of the growing demand for this plant (15 Mio. ha for biofuel), has made scarce natural habitats for orangutans and other rainforest dwellers. More carbon is released during the life cycle of a palm oil plant for its use as a biofuel than is emitted by the same volume of fossil fuels, also due to a high input of fertilizer and pesticides (Carlson et al., 2012).

Jatropha curcas L., family Euphorbiaceae, is a drought tolerant shrub distributed in central and South America, South East Asia, India and Africa with modest demands for nutrients and capital inputs (Schmook and Serralta-Peraza, 1997; Costa et al., 2010). It was shown that it protects against soil erosion. Cultivation is labor-intensive, at least when starting a plantation, and so far the yields are relatively small maybe due to little cultivation experience worldwide. The plants are propagated through cuttings or by seeds. As a result of the high demand of this plant as a source of biodiesel, biotechnological approaches were used to increase the production through mass micro-propagation and regeneration. Thus a method for in-vitro propagation of *J. curcas* was established through nodal explants and green cotyledon explants for somatic embryogenesis (Kalimuthu et al., 2007). There were some attempts to improve the content of unsaturated fatty acids and to enhance the chilling resistance in *J. curcas* through genetic transformation (Luo et al., 2007). In *J. curcas* seeds the oil content ranges between 30-50% by weight, while in the kernel the range is between 45-60%. From the properties and engine test results it has been established that 40–50% of *J. curcas* oil can be substituted for diesel without any engine modification and preheating of the blends (Pramanik, 2003) or even up to 100% according to Hoa et al. (2012). The properties of diesel and *J. curcas* oil are shown in Table 2 (Hoa et al., 2012).

Table 2. The properties of diesel and *J. curcas* oil from Viet Nam.

Properties	Diesel	*J. curcas* oil
Density (gm/cc), 300°C	0.836-0.850	0.918
Cetane No.	46	51
Flash point, 0°C	45-60	101
Calorific value (Kcal/kg)	10478	9432
Sulfur (%)	0.43	0.33

Table 2 shows that the cetane value of *J. curcas* oil is higher than diesel, thus it is suitable for diesel engines. The sulfur content in *J. curcas* (0.33) is lower in comparison to diesel (0.43). and therefore, the use of *J. curcas* oil for engines is environmentally better. With respects to safety and storage, *J. curcas* is safer in storage and transport because the flash point of diesel is a half of *J. curcas*. Overall, the calorific value by volume is the same thus it is unnecessary to change the amount of fuel supply. The cetane number and emission parameter are 80% lower from biodiesel when compared by mineral diesel, particularly the emission of hydrocarbons and particulate matter (Makkar et al., 2009). Comparing with oil from *J. curcas* grown in India (Pramanik, 2003) (Table 3) is becomes obvious that cultivation of plants, soil type, use of different genotypes and processing influences the properties of the final oil quality drastically.

Table 3. Comparison with *J. curcas* oil produced in Viet Nam and in India.

Properties	Viet Nam	India
Density (gm/cc), 300°C	0.918	0.93292
Cetane No.	51	38
Flash point, 0°C	101	210
Calorific value (Kcal/kg)	9432	9123.913
Sulfur (%)	0.33	n.d.

n.d. not determined.

As shown in Table 4 the quality of the *J. curcas* oil meets for example the European quality standard for biodiesel. Therefore the biodiesel can be used in today's vehicle fleets already on the market worldwide and may also offer a viable path to sustainable transportation, i.e. lower greenhouse gas emissions and enhanced mobility, even in remote areas.

Table 4. Properties of *J. curcas* biodiesel compared to European standard (Francis et al., 2005).

Properties	*Jatropha* biodiesel	European standard	Remarks [a]
Density (g cm^{-3} at 20°C)	0.87	0.86-0.900	+
Flash point (°C)	191	>101	+
Cetane no. (ISO 5165)	57-62	>51	+ + +
Viscosity mm^2/s at 40°C	4.20	3.5-5.0	+
Net cal. val. (MJ/L)	34.4	-	-
Iodine No.	95-106	<120	+
Sulphated ash	0.014	<0.02	+
Carbon residue	0.025	<0.3	++

[a] + indicates that *J. curcas* performs better than the European standard.

Although *J. curcas* possesses a high drought tolerance the original theoretical calculations for the yield of *J. curcas* were based on growth under optimal agricultural conditions and therefore there was a big discrepancy among theoretical yield and actual yields when grown on non-arable yields (Karp and Shield, 2008). Although it was meant to be grown on non-arable land, plantations were then set up on arable land instead of cultivating plants for food production and *J. curcas* plants were cultivated like food plants by watering, fertilizing and pesticide treatments to obtain higher yields. The yield was of course much higher on fertile land but the original idea was foiled (Francis et al., 2005). In addition, it was shown that both seeds and leaves show some toxicity against human beings and animals because of several secondary components such as phorbol esters, curcains, trypsin inhibitors and others. These compounds decrease the benefits to use the seed cake as animal feed because the detoxification process for this compounds is tedious and expensive (Abdulla et al., 2011). Therefore the Indian government stopped the cultivation of *J. curcas* to avoid a competition of food and fodder plants on the fields in a country where enough food is still not available for all human beings.

Another very promising candidate for growth in desert areas might be *Balanites aegyptiaca* L. (Del.). It belongs to the family of Balanitaceae, is a multipurpose xerophytic tree distributed in the tropical and arid lands in Northern Africa, West Asia and Arabia. Its common name is desert date (Hall and Walker, 1991; Bhandari et al., 1995; Chapagain et al., 2009; Anis et al., 2010). *Balanites aegyptiaca* trees grow up to heights of 6 to 8 m and live until 100 years with annually crop production of 125 kg of fruit and the first fruit after 5 to 6 years and the oil could be extracted from the kernel and used through ester preparation to produce biodiesel (Deshmukh et al., 2009). The composition of the fatty acids at different pressing temperatures is shown (Table 5).

Table 5. Fatty acid composition of *Balanites aegyptiaca* kernel oil as a function of pressing temperature (Mohamed et al., 2002).

Fatty acid	50°C mg/g oil	115°C mg/g oil
C16:0	95.6	89.7
C18:0	94.5	89
C18:1	287.6	270.5
C18:2	263.9	259.9
C18:3	12.6	9.7
UFA (%) [a]	74.8	75.14
Total	754.2	718.8

[a] Unsaturated fatty acids = (g of unsaturated fatty acids / g of total fatty acids) × 100

Chapagain et al. (2009) reported the production of biodiesel through six different genotypes of desert date. The greatest oil content was 46.7% and the total unsaturated fatty acid content was in *B. aegyptiaca* less than in the soybean (Table 6) and rapeseed and very similar to *J. curcas* and *Argania spinosa* (L.) Skeels. With the decreasing contents of unsaturated fatty acids the viscosity is increased and this is a considerable factor in the quality of biodiesel. The oil quality parameters have been investigated (Table 6) and the produced biodiesel has been tested for engine analysis which gave a good engine performance.

Table 6. Analysis of the quality of Desert date oil extracted from extruder machine and compared with soy oils (Chapagain et al., 2009).

Parameters	Desert date	Soybean
Iodine value	97.7	74.2
Melting point (°C)	3 to 10	8 to 20
Refractive index	1.5142	1.505
Saponification value (mg) NaOH/(g)	175.91	187.4
Unsaponifiable (%)	0.68	1.58
Specific gravity	0.9013	0.9140
Moisture (%)	0.5	0.1
Viscosity (cp)	49	42.4

Another tree plays an already an important role as source of biofuel. *Millettia pinnata* (L.) Panigrahi (formlerly known as *Pongamia pinnata*), Fabaceae, is native in tropical and temperate Asia. *Millettia pinnata* is well-adapted to arid zones due to its dense network of lateral roots and its thick, long taproot. The non-edible seed oil has been found to be useful in diesel generators and it is being explored as feedstock for biodiesel produced by transesterification of the crude oil with methanol in the presence of KOH as catalyst. Important fuel properties of methyl esters of *M. pinnata* biodiesel compare well (viscosity mm²/s at 40ºC, 4.8; flash point, 150°C) with German biodiesel standards (Karmee and Chadha, 2005). It grows naturally in many arid parts of India and is one of the few crops well-suited to commercialization by India's large rural population. Several unelectrified villages use the *M. pinnata* oil in simple processing techniques, to create their own grid systems to run water pumps and electric lighting. To be able to better exploit the advantages of M. pinnata several aspects of its biology are currently under investigation, such as nitrogen fixation (root biology, nodulation), stress tolerance, especially salinity tolerance, ways of propagation, breeding potential, and the regulation of genes involved in fatty acid biosynthesis. Another important aspect in the carbon sequestration in relation to carbon credits. After extraction of oil from M. pinnata the leftovers could be used as protein-rich feed supplement for animals (Scott et al., 2008). However, in other non-Asian countries *M. pinnata* distributed quickly and was listed as invasive species. The same property makes the native Chinese tree *Sapium sabiferum* (L.) Roxb., Euphorbiaceae, which is moderately drought tolerant, less usable. Because of its invasive nature it is not recommendable as a new crop plant, except in China.

There are several drought-tolerant C_4-grasses such as *Andropogon gerardii* Vitman (big bluestem), native to the Great Plains of North America and due to its stunted growth even more drought-tolerant *Panicum virgatum* L. (switchgrass) native to North America including Canada and Mexico. Both species are being tested as sources of biomass for the biofuel production, either for bioethanol or biogas. An interesting option for their growth might be a polyculture to exploit their different growth optima in different regions and climatic conditions. Recently, the C_3-grass *Arundo donax* L. (giant reed) native to East and South Asia but now found in many countries with Mediterranean climate. It is promoted in the USA as new source of renewable biomass source but many environmentally concerned people warn about the invasive nature of this grass (Lieberei and Reissdorff, 2012).

4.3.5. Plant sources of biomass to liquid (BTL)

Virtually all drought-tolerant plants can be used as raw material, however, the processes are technically elaborate, energy-consuming and still in a pilot state.

5. Plants as source of bioenergy: Pros and Cons

There are a number of arguments used by promoters on the one hand and by opponents on the other hand. All arguments, cons and pros, should be carefully proven to select the best plants and follow the best strategy for energy production.

Contra: The cultivation of energy plants reduces the land area for the cultivation of food plants. *Pro*: Energy plants can be cultivated without replacing food and fodder plants. For example, the area in Germany for agricultural use sums up to 16.7 Mio ha, 57% of this area is used for the growth of fodder, 28% for food, and 12% for the growth of energy plants (Metzger and Hüttermann, 2009). If one thinks about the re-cultivation of degraded land worldwide by stress-tolerant energy plants there is a huge area which could be used because about one third of land which could be used for agriculture is degraded (Table 1).

Contra: Energy plants generate monoculture and destroy diversity. *Pro:* Energy plants could also enrich agriculture by cross-cultivation, intercropping, crop rotation and agroforestry of several species.

Contra: Industrialized countries destroy the rain forest. *Pro*: Currently, 2% of the fields worldwide are used for the cultivation of energy plants and about 5% of the palm oil produced is used for energy production (Metzger and Hüttermann, 2009).

Contra: Energy plants need even more fertilizer and pesticides. *Pro*: Energy plants save fertilizer and pesticides because there are less monocultures and there is more crop rotation. Smaller amounts of herbicides are applied because strict monocultural cultivation is not as important for the cultivation of food.

Contra: Energy plants are door openers for genetic engineering. *Pro*: As the example Germany shows, no genetically engineered corn is grown at all.

Contra: Cultivation of plants for the energy production cause worldwide price explosion and hunger crisis. *Pro*: The costs for raw products are controlled and influenced by the global market.

Prices for wheat, corn and soybean increased drastically, almost three times for one ton of wheat since about 2000 according to the FAO (http://www.fao.org/economic/ess/ess-economic/en/accessed 30th of June 2013). The rise of people into the middle class leads to a high demand for meat, because the increased consumption of meat is one sign of having risen to the middle class. Fodder consists mainly of wheat and corn. Therefore the growth of plants for energy production contributed only little to the increase in prices for basic foodstuff. Energy plants cannot be used as punching ball.

6. Future perspectives

We have to face the growing world population and their energy demand. Therefore providing renewable resources are absolutely essential. It was shown in this review that there are a number of plants which can be cultivated in a sustainable way on non-arable land. The yields will be not as high as when grown on arable land with irrigation, fertilizer and plant protection agents but the arable land should be reserved for the cultivation of food and to a lower part for fodder plants. Some of the promising plants presented are not native to the respective region of country. However, this is an important aspect because from the plantation of plants, especially of grasses, individuals can escape and invade the native ecosystem. The areas for the cultivation of energy plants are restricted. The utilization of perennial or at least biannual plants is more sustainable and exhibits a better greenhouse gas balance than cultivation of annual plants. There are huge areas of degraded land (about 3.5. billion ha). If only half of this land (1.75 billion ha) could be used for the cultivation of stress-tolerant, mainly drought tolerant, plants a big contribution to the worldwide energy supply could be offered. One quarter (0.9 billion ha) could produce about half of the yearly fuel consumption with an average production of 1.2 t ha^{-1} of plant oil or ethanol yield. On the other quarter fast growing trees with a yearly average dry biomass gain of 10 t about one third of primary energy consumption could be covered as recommended (Pieprzyk, 2009).

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Chapter 3

Investigation on the genetic diversity among *Balanites aegyptiaca* (desert date) provenances by amplified fragment length polymorphism analysis

Abstract

Balanites aegyptiaca is a drought-tolerant plant species distributed in tropical and sub-tropical regions in Africa and Asia. In this study, amplified fragment length polymorphism (AFLP) analysis was used to evaluate the genetic diversity of twelve provenances of *B. aegyptiaca* collected from different geographical regions. Through four primer combinations a total number of 510 bands were produced and 477 (93.5%) of which were polymorphic. The results of cluster analysis and principal component analysis indicated that *B. aegyptiaca* individual samples were distributed in three main clades. This study suggested that there is no clear relation between the high genetic variation among *Balanites* sources and the geographic distribution while this variation might be obtained through the percentage of outcrossing pollination in *B. aegyptiaca* and also the movement of animal-intake seeds or winds influence the spreading of the seeds through several geographic regions. *Balanites* sources showed a large number of polymorphic AFLP marker bands.

Keywords: Allopollination, genetic distance, molecular marker, woody plant species.

Introduction

Balanites aegyptiaca (desert date) belongs to the family of Balanitaceae (Hall and Walker 1991). The *Balanites* genus comprises nine species and eleven infra-specific taxa (Sands 2001). It is a spiny, evergreen and multi-purpose tree distributed in different African and Asian countries across the arid and semi-arid areas to the sub-humid savanna delivering many necessities of life, like food supplies in the hard times (edible fruits), medicinal compounds, fuel wood, and pesticides (NRC 2008; Clement et al. 2011). According to a report published by the US National Research Council (NRC) the plant is neglected in the scientific communities despite it is considered among the 24 high priority lost crops in Africa and Asia (NRC 2008).

The distribution of the tree species across a broad range of environments and soils provides large phenotypic variation in crown shape, seed, fruit, leaves, and the fruiting and flowering time (Hall and Walker 1991; Hall 1992; Sands 2001; Elfeel et al. 2009). *Balanites aegyptiaca* showed high variance in fruits and seeds among and within geographical sources (Zobel and Talbert 2003). Weber and Sotelo Montes (2009) have investigated the phenotypic connection between the density of the wood and tree growth through eleven provenances sampled in one dry site in Niger, and they found that wood density and tree growth are positively correlated. However, the correlation varied among provenances and there was increasing in the mean stem diameter and tree height of provenances in the sample area from the high humid to drier parts. Elfeel et al. (2007) examined the drought effect on *B. aegyptiaca* seedlings collected from eight geographical regions and found that seedlings from provenances grown on clay showed the highest mortality, more than those from sandy clay loam suggesting a correlation between the drought-tolerance and the soil type of sources. So the ability of the tree to adapt to drought conditions is significantly different among the provenances and geographical sources.

There is a scarcity in the knowledge about genetic variation of this species among the geographic habitats. So far, genetic variability in *B. aegyptiaca* has only been investigated through peroxidase isozyme analysis of different germplasm pools (Chamberlain 1992). Amplified fragment length polymorphism (AFLP) is a DNA fingerprint method where PCR amplification is used to detect genomic restriction fragments without previous knowledge of the DNA sequences (Vos et al. 1995). Recently, the patterns of genetic variation in a tree species was investigated through biochemical and molecular marker analysis, such as isozyme and random amplified polymorphic DNA (RAPD) markers in teak (*Tectona grandis*

L. f.) (Balasundaran 2010). Genetic variation and differentiation of five Oak species through isozymes and microsatellites (Curtu et al. 2007). Also AFLP and microsatellites were used to study the genetic variation of Ackee (*Blighia sapida*) through 279 individuals from 6 wild and 8 cultivated populations (Ekué et al. 2011). Investigate of the genetic differentiation among 54 Indonesian species of Dipterocarpaceae and study the genetic diversity and the structure of nine *Shorea* species collected from two different locations was performer through AFLP (Cao et al. 2006, 2009). This study aimed to use AFLP techniques to investigate the genetic diversity among the *B. aegyptiaca* sources collected from different geographical regions and determine the relationship between geographical distribution and genetic diversity.

Material and methods

Plant material

From twelve provenances of *B. aegyptiaca* fruits have been collected from different geographical locations as one plant per provenance in Sudan, Egypt, Togo, Ethiopia, Ghana, Saudi Arabia and Yemen (Table 1). The seeds were germinated for 1 month and leaves from one plant were randomly selected and harvested for DNA extraction. The seeds collected from Wadi El-Gemal were divided into two groups: group a1 was collected from three trees (a1, a2 and a3) in the entrance of the valley close to the coast of Red Sea. The second group b1 was collected from another three trees (b1, b2 and b3) 5 to 10 km away from the entrance.

Table 1. Provenances, origin and coordinates of locations of collections used in this study.

Provenances	**Country**	**Coordinates**
Sudan	Sudan	Not known
Cairo	Giza, Zoo, Egypt	30°1'27.158"N; 31°12'49.731"E
Wadi El-Alaqi (1)	Park of the Faculty of Sciences, University of South Valley, Aswan, Egypt	24°5'20.177"N; 32°53'59.385"E
Wadi El-Gemal (a1)	Park of the Faculty of Sciences, University of South	24°41'16.573"N; 35°5'1.248"E

Wadi El-Gemal (b2)	Valley, Aswan, Egypt	24°41'16.573"N; 35°5'1.248"E
El-Kharga Oasis (a3)	Park of the Faculty of Sciences, University of South Valley, Aswan, Egypt	24°5'20.177"N; 32°53'59.385"E
Togo	Togo	Not known
Ethiopia	Ethiopia	Not known
Ghana	Ghana	00° 00' 59.1"N; 05° 56' 50.7"E
Medina	Saudi Arabia	Not known
Jazan	Saudi Arabia	Not known
Yemen	Yemen (Tohama)	Not known

DNA extraction and AFLP technique

DNA extraction was performed using the Plant Nucleospin II Kit (Macherey & Nagel, Düren, Germany) as described in the manufacturer's manual. The quality of DNA was tested on agarose gels stained with ethidium bromide. A microplate reader with micro-volume plates (Synergy Mx Multi-Mode, BioTek, Germany) was used to measure the DNA concentration. The four step AFLP method was conducted according to Vos et al. (1995) with minor modifications. (1) *Restriction.* The enzyme combination of 0.5 µl *Eco*RI (10 U μl^{-1}) and 0.3 µl *Mse*I (10 U μl^{-1}) was used to digest 250 ng of genomic DNA in a total volume of 25 µl including 1 × RL buffer (10 mM Tris/HCl, 10 mM MgAc, 50 mM KAc, 5 mM DTT, pH 7.5). This mixture was incubated overnight at 37°C. (2) *Ligation of adaptors*. The produced restriction fragments were ligated to specific *Mse*I (50 pmol) and *Eco*RI (5 pmol) adaptors (MWG Biotech Eurofins, Ebersberg, Germany) in a total volume of 5 µl reaction mix containing 0.5 µl of *Eco*RI adaptor, 0.5 µl of *Mse*I adaptor, 0.5 µl 10 x RL-buffer, 0.6 µl of 10 mM ATP, 0.05 µl of T4-DNA-ligase (1 U μl^{-1}) and 2.85 µl H_2O. The reaction mix was incubated for 3.5 h at 37°C. (3) *Pre-amplification*. The pre-amplification was performed in a total volume of 50 µl including 5 µl template of digested and ligated DNA, 1.5 µl (50 ng) of *Eco*RI+A and *Mse*I+A primers (Table 2), 1 µl of *Taq* polymerase (MBI Fermentas, St. Leon-Rot, Germany), 1 × Williams buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$,

0.001% gelatine), 5 µl dNTPs (0.25 mM) and 31 µl H_2O. The PCR program was conducted through 94°C for 5 min, then 20 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by 10 min at 72°C. (4) *Amplification.* The pre-amplified PCR product was diluted 1:20 with 1 TE Buffer (10 mM Tris/HCl pH 7.5, 1 mM EDTA) and 2.5 µl of the diluted pre-amplification product was used for the selective PCR amplification which was performed in a total volume of 10 µl containing of 2.5 µl *Eco*RI-IRD primer (2 ng μl^{-1}) and 0.3 µl *Mse*I primer (50 ng μl^{-1}) through four primer combinations *Eco*RI+ACA/*Mse*I+ATC, *Eco*RI+ACC/*Mse*I+ATC, *Eco*RI+ACA/*Mse*I+ACA and *Eco*RI+ACC/*Mse*I+ACA (Eurofins MWG Operon) (Table 2), 1 µl dNTPs (2 mM), 0.05 µl *Taq* polymerase (5 U μl^{-1}), 1 µl 10 x William buffer and finally 2.65 µl H_2O. The PCR program started with 94°C for 5 min, one cycle was performed for 94°C for 30 s, 65°C for 30 s and 72°C for 1 min. After that the annealing temperature was decreased by 0.7°C per cycle for the next 11 cycles, then 24 cycles starting with 94°C for 30 s, annealing temperature 56°C for 30 s and final extension at 72°C for 10 min. After final amplification 20 µl of AFLP dye (98% formamide, 10 mM EDTA, and 0.05% pararosaniline) was added to each sample. Mixtures were warmed up to 72°C for 5 min. By following the manufacturer's instructions the samples were loaded onto 6% AFLP gels on the 4300 DNA Analyzer (LI-COR, Biosciences, Germany).

Table 2. Sequences of adaptors and primers used in this study.

Adaptor/Primer		**Sequences**
*Eco*RI adaptors		5'-CTCGTAGACTGCGTACC-3
		5'AATTGGTACGCAGTCTAC3'
*Mse*I adaptors		5'-GACGATGAGTCCTGAG-3'
		5'-TACTCAGGACTCAT-3'
Pre-selective primers (*Eco*RI+A)		5'-GACTGCGTACCAATTCA-3'
Pre-selective primers (*Mse*I+A)		5'-GATGAGTCCTGAGTAAA-3'
Selective primer (*Eco*RI + ACA)		5'-GACTGCGTACCAATTCACA3'
Selective primer (*Mse*I + ACA)	Set 1	5'GATGAGTCCTGAGTAAACA-3'
Selective primer (*Eco*RI + ACC)		5'GACTGCGTACCAATTCACC3'
Selective primer (*Mse*I + ACA)	Set 2	5'GATGAGTCCTGAGTAAACA3'
Selective primer (*Eco*RI + ACA)		5'-GACTGCGTACCAATTCACA-3'
Selective primer (*Mse*I + ATC)	Set 3	5'-GATGAGTCCTGAGTAAATC3'
Selective primer (*Eco*RI + ACC)	Set 4	5'-GACTGCGTACCAATTCACC-3'

Selective primer (*Mse*I + ATC)	5'-GATGAGTCCTGAGTAAATC-3'

Data analysis

The data was manually scored comparing with the pictures where the binary data was organized and only the polymorphic fragments were scored as band present (1) and band absent (0). Absent and present binary data of 12 individuals and 477 polymorphic loci was used as the basis for the analysis. In the present study, the band-based approach based on Bonin et al. (2007) was used for the analysis in the individual. Dice coefficient (Dice 1945) was used to calculate the similarity among 12 individuals. The cluster analysis was prepared through unweighted pair group method with arithmetic mean (UPGMA) based on Dice index (Nei and Li 1979). Bootstrap values (based on 1000 re-sampling) was used to estimate the reliability of the clustering pattern. The NTSYSpc version 2.20 was applied to prepare Principle components analysis (PCoA) of the correlation matrix to test the relationship among the individuals.

Results

A total number of 510 bands were generated with the four AFLP primer combinations, where each primer combination gave a different number of polymorphic fragments (Table 3).

Table 3. Selected primer combinations and polymorphism rates for AFLP (Amplified fragment-length polymorphism) analysis of provenances.

Pre amplification	**Final amplification**	**Total bands**	**Polymorphic bands**	**Polymorphism %**
*Eco*RI+A/*Mse*I+A	*Mse*I+ACA and *Eco*RI 616	168	148	88.09
*Eco*RI+A/*Mse*I+A	*Mse*I+ACA and *Eco*RI 617	88	85	96.59
*Eco*RI+A/*Mse*I+A	*Mse*I+ATC and *Eco*RI 616	173	169	97.68
*Eco*RI+A/*Mse*I+A	*Mse*I+ATC and *Eco*RI 617	81	75	92.59
Total		510	477	93.52

The highest number of polymorphic fragments was obtained from the combination *Eco*RI 616 + ATC/*Mse*I and the average number of polymorphic loci detected was about 119 per primer combination. Genetic similarity values among the 12 individual samples of *B.*

aegyptiaca ranged from 0.246 to 0.591 based on the Dice index. The data obtained from cluster analysis indicated that *B. aegyptiaca* individual samples were distributed in three main clades; El-Kharga (a3) in one clade, the second clade was Wadi El-Gemal (a1 and b2), and the rest of the individuals were collected in the third clade (Alaqi1, Ghana3, Cairo, Ethiopia, Medina, Yemen, Jazan, Sudan and Togo) (Fig. 1). This result was consistent with the data obtained by the Eigen vectors analysis of PCoA (Fig. 2) where the *B. aegyptiaca* individuals were distributed in the same three clades.

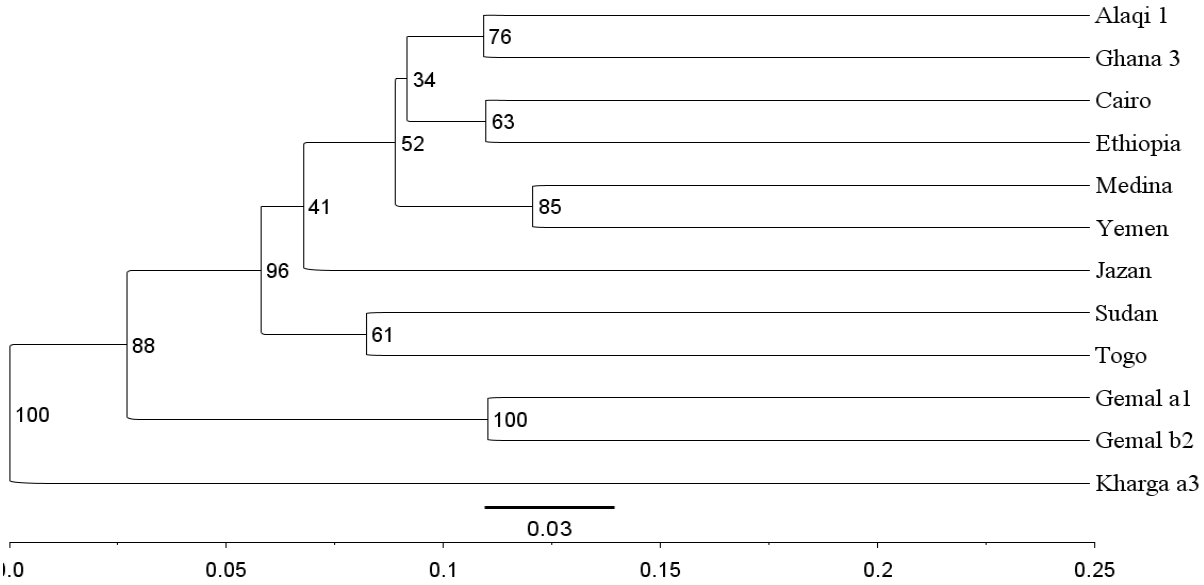

Figure 1. UPGMA-based dendrogram of *B. aegyptiaca*. Performed from 477 AFLP markers. Confidence limits for the dendrogram are based 1000 bootstrap replications. The dendrogram was generated by Free Tree (Hampl et al. 2001).

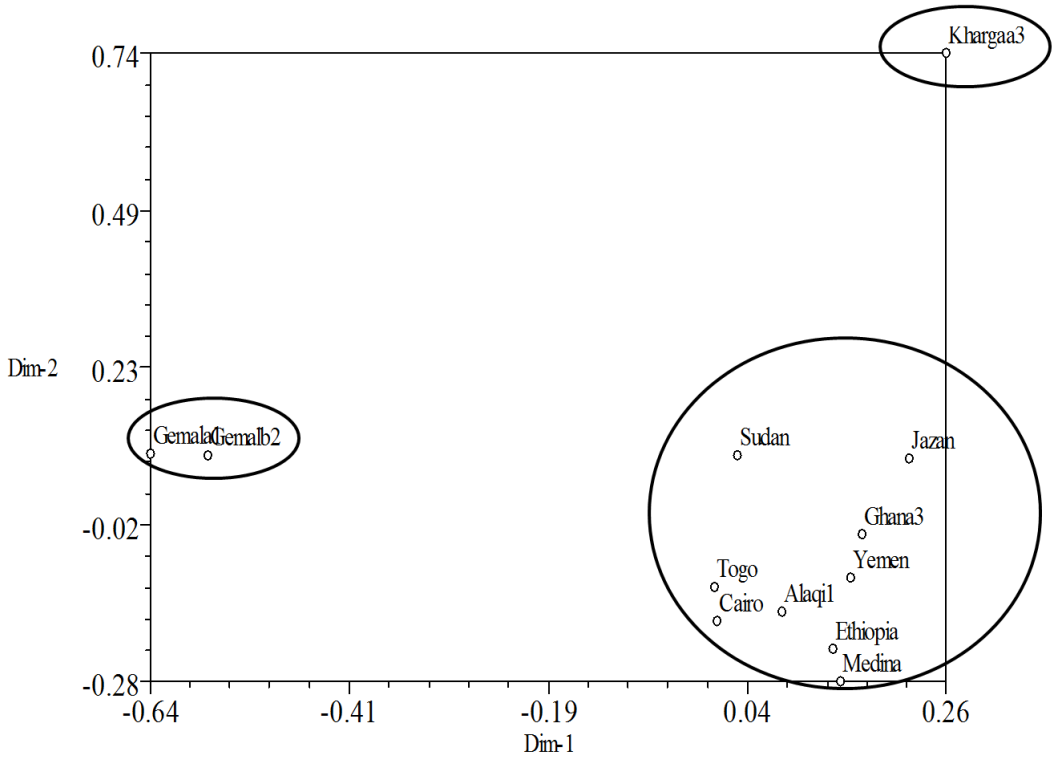

Figure 2. Principle components analysis (PCoA) based on 477 AFLP markers. The matrix plot was performed by NTSYSpc, 2.20 (Rohlf 2005). The *B. aegyptiaca* individuals are distributed in three clades.

Discussion

This study aimed to analyze the genetic diversity among the *B. aegyptiaca* provenances collected from different geographical places by using the AFLP technique, and to determine the relationship between geographical distribution and genetic diversity. The *B. aegyptiaca* species is highly variable in many of its characters. Also the variety *aegyptiaca* was defined as largely variable among the other four varieties (ferox, pallida, quarrei, and tomentosa) within the species that were classified according to several features such as distribution, flowers, fruits and spines, and that are distributed in different soils ranging from deep sand to sandy loams and clays (Sands 2001). Unfortunately, there is no clear information available about the coordinate locations of samples collected from Sudan, Ethiopia, Togo, Jazan, Medina, and Yemen. Through a total number of 510 bands there were 477 (93.5%) polymorphic bands. Based on the results obtained from this study the provenances can be designated as genotypes. The high percentage of polymorphism was observed in several woody plants such as 98.2% polymorphism obtained from the AFLP analysis of 39 genotypes of Mexican pineapple germplasm collections (Paz et al. 2005) and 87.4% polymorphism were

obtained from 56 genotypes of *A. comosus* and one of *Bromelia pinguin* were investigated with 191 AFLP markers to study the genetic diversity of Cuban pineapple germplasm (Paz et al. 2012) and 88.5% polymorphism obtained from AFLP analysis of nine natural population of *Hibiscus tileaceus* from 145 individuals (Tang et al. 2003).

Clustering and principal components analysis discriminated the 12 genotypes of *B. aegyptiaca* collected from different regions into three clades. This discrimination related to the AFLP fingerprint of each sample and was not closely related to the geographic distribution. The relation between genetic diversity and geographic places in *B. aegyptiaca* has been investigated by Chamberlain (1992). The author used isoelectric focusing of peroxidase isozymes to characterize different germplasm pools of *B. aegyptiaca*. The results revealed that the banding pattern of a single genotype was consistent and the plants from the same mother tree showed minimal banding patterns variation compared to the plants from the same provenance but collected from different parentage. There was also a higher variability in peroxidase isozyme expression of the plants from different provenances than those from the same provenances. The plants obtained from different countries showed some remarkable bands and variability in peroxidase isozyme expression (Chamberlain 1992). The varieties of *B. aegyptiaca* species showed also variations in some morphological characters where the phenotypic variations in fruit and seed morphology were investigated in four natural populations of *B. aegyptiaca* in eastern Niger. The results indicated that there were significant geographic variations in some parameters within populations while the sample from drier parts showed heavier fruits and kernels with longer and narrower seeds and exhibit low variability within tree in fruit and seed width (Abasse et al. 2010).

Elfeel et al. (2009) investigated the relation between seed germination and their provenances distributions through seeds of *B. aegyptiaca* collected from eleven provenances and the seeds were cultivated in two different soil type (silt and sand). The results showed that a highly significant effect of soil on the germination and growth parameters, and the cluster analysis displayed grouping of provenances depending on the overall growth parameters, nevertheless the grouping was not related to soil type or the diffused rainfall in the provenances.

Balanites aegyptiaca is a woody plant species and is considered partially autocompatible and exhibit a large fruit abortion based on a small fruit/flower ratio. The allopollination ratio of approximately 37% might be related to wind and insects like Halictidae and Dipterae (Ndoye et al. 2004; Dubey et al. 2011). Hamrick et al. (1992) reported that the woody species that are distributed in a wide geographic range and have outcrossing breeding systems through wind

and insects or spreading seeds by animal feeding showed a high genetic diversity within species and populations with low variation among populations.

So this study suggests that there is no clear relation between the high genetic variation within *Balanites* sources under investigation and the geographic distribution of these genetic variations among this sources might be obtained through the capacity of outcrossing pollination in *B. aegyptiaca* through Halictidae and Dipterae also the spreading of the seeds through various geographic areas besides the high number of polymorphic AFLP marker bands produced from each individual. The high genetic diversity within populations with low diversity among countries was reported in many outcrossing woody plants species such as *Uapaca kirkiana* (Mwase et al. 2006), *Swietenia macrophylla* (Gillies et al. 1999), and *Populus tremuloides* (Yeh et al. 1995).

This is a first report based on AFLP fingerprint to investigate the genetic diversity among different genotypes of *B. aegyptiaca* collected from different countries. For the future perspective there is a demand to increase the size of populations and collected samples. This should be combined with morphological and cytological analysis for samples under investigation to make a clear conclusion about the genetic diversity among the *B. aegyptiaca* populations and the relation between geographical distance and genetic diversity.

Conclusion

The data obtained from the band-based approach indicate that 12 *B. aegyptiaca* genotypes collected from different geographical locations were separated into three main clades. This data was confirmed through cluster analysis and the Principal Co-ordinates Analysis. The high genetic variation among the individuals was observed in many of outcrossing woody plant species so here the genetic diversity among *B. aegyptiaca* sources could be more related to the AFLP fingerprint of each source. *Balanites aegyptiaca* as a woody plant species with a percentage of outcrossing that might responsible about the variation among *Balanites* sources.

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Chapter 4

Effect of water deficiency on different genotypes of *Balanites aegyptiaca*

Abstract

Water deficit is strongly affecting plant development and production. With the decrease in rainfall in many areas and the shortage of arable land for growing food crops, there is a demand to find alternative plant species that could be cultivated in non-arable land for food supplies and bioenergy. *Balanites aegyptiaca* is a multi-purpose tree belonging to the family of Balanitaceae distributed in North and West Africa, and West Asia. The species is considered as drought-tolerant serving as a source of many secondary metabolites and having a potential for biofuel production. This study aimed to examine and compare the morpho-physiological responses to water stress of six different *B. aegyptiaca* genotypes collected from different regions. Different regimes of soil volumetric water content (VWC 35% as a control, VWC 20% as moderate and VWC 5% as a severe drought stress) were chosen to finally select the most drought-tolerant genotype under greenhouse conditions. Several growth parameters, stomata conductance, photochemical efficiency and metabolites contents were analyzed to investigate and compare the drought impact among *B. aegyptiaca* genotypes. The results indicate that at severe drought stress each genotype has an independent strategy to cope with the water shortage through a significant reduction in biomass parameters, early stomata closure combined with small changes in photochemical efficiency and producing a high concentration of ascorbic acid. Finally, we found that i) *Balanites* genotypes showed different morphological and physiological responses to cope with the water shortage, ii) collections of two or three parameters could distinguish the effect of water deficit among the genotypes, and iii) genotypes El-Kharga and Cairo showed higher drought tolerance compared to the other genotypes. They showed the lowest magnitude of biomass reduction and early stomata closure as strategy of saving leaf water content under severe drought stress.

Keywords: *Balanites aegyptiaca* L., biometrical growth parameters, stomata conductance, volumetric water content, water deficit.

Introduction

Water shortage is one of the major environmental limitations and has a tremendous effect on plant growth and development (Harb et al. 2010; Song et al. 2012). Drought stress causes a decrease in crop productivity and nearly 28% of the world's soil surface is too dry to produce regular sufficient crop yields (Bray et al. 2002; Ambrosone et al. 2013). Plants respond to water shortage through several different mechanisms to overcome their water deficit. Therefore, the response of a plant to water stress can be considered a quantitative trait that is controlled through several regulatory pathways by reprogramming a number of metabolic and physiological mechanisms (Morison et al. 2008; Ahuja et al. 2010; Park et al. 2012; Khamis and Papenbrock 2014). The reduction in plant water content induces stomata closure resulting in a reduced transpiration rate, a reduced rate of photosynthesis and finally in a decrease of growth and gain of biomass. Stomata pore size controlled through guard cells is considered as the main switching machinery that ultimately determines the water use efficiency and the exchange rates of CO_2 for photosynthesis as a process of plant adaptation to water stress (Song et al. 2014). Plants respond to variation in soil water content through root signal(s) originated from the root tips transferred via the xylem to the leaf (Cornic and Massacci 1996) by increasing the concentration of abscisic acid (ABA) up to 30- fold in the guard cell apoplast (William and Outlaw 2003). The increased concentration of ABA in the cytosol of guard cell enhances the ions flow through the cell membrane and deporting the K^+ and Cl^- via outflow channels. This reduction in solute concentration agitates the water out from the cell and when the guard cell looses water, the cell volume decreases with loss of turgor and finally stomata closure (Joshi-Saha et al. 2011; Watkins et al. 2014). As a result of stomata closure, the uptake of net CO_2 by leaves decreases resulting in a reduction of CO_2 concentration in the chloroplast (Cornic and Massacci 1996). Water stress also affects the plant growth through inhibition of the cell elongation and of the leaf expansion resulting in reduction of net photosynthesis. And water stress induces changes in the photosynthetic apparatus and its pigments, such as accumulation of de-epoxidised xanthophyll cycle pigments, antheraxanthin and zeaxanthin (Farooq et al. 2009). The reduction in water availability combined linearly with the increase in reactive oxygen species causing lipid peroxidation, membrane injuries, protein oxidation and enzyme inactivation (Berlett and Stadtman 1997; Blokhina et al 2003; Sairam et al. 2005; Farooq et al. 2009).

Balanites aegyptiaca (desert date) is a xerophytic tree belonging to the family of Balanitaceae and is distributed in tropical and non-tropical areas in North and West Africa and West Asia

(Bhandari 1995; Siddique and Anis 2008). It is considered as a multi-purpose tree because desert date kernels are rich in oil content, up to 46.7%, that was successfully tested for biodiesel production (Chapagain et al. 2009). The kernels and various parts of the plants also contain several types of saponins that show activity in anti-cancer therapies and as foaming agents in the detergent production (Mohamed et al. 2002; Chapagain et al. 2008). Besides, the roots and fruits contain beneficial secondary metabolites that were suggested to be used for medical treatment, such as antibacterial and anti-leishmanial, and the production of oral contraceptives (El-Tahir et al. 1998). *Balanites aegyptiaca* has drought-adapted anatomical leaf characteristics such as thick waxy cuticles, numerous sunken stomata on both leaf surfaces and thicker leaf laminae. A thick layer of trichomes on the leaves of *B. aegyptiaca* reflects excess of light and might form a protective shield against high irradiance in the arid habitat (Radwan 2007). These leaf traits seem to contribute one of the major adaptive features for drought tolerance of this species (Elfeel et al. 2007). The plant is also considered as drought-tolerant because of its deep tap root. Besides, it has the ability to produce fruits under arid conditions and the tree is not damaged through grass fire and flooding, except the young trees. Therefore, it can be cultivated in several arid desert environments in Africa and south Asia (Hall and Walker 1991). Dependent on these distinguished benefits as drought-tolerant plant, *B. aegyptiaca* could be recommended for cultivation programs in arid and semi-arid habitats in Africa.

The main objectives of this study are i) to investigate the morpho-physiological responses of *B. aegyptiaca* to water stress through different regimes of soil water content, ii) to compare the plant´s responses to water stress among different genotypes of *B. aegyptiaca* that were collected from different regions.

Material and methods

Plant material and experimental conditions

Plant propagation for the water stress experiments was conducted through 5-7 cm cuttings that were taken from the mother plants and planted in pots of six *B. aegyptiaca* populations were collected from different countries (Table 1). The cuttings propagated in pots filled with CL T soil type (Einheitserde, Sinntal-Altengronau, Germany) a soil with 30% clay content. The plants were covered by plastic boxes for 4 weeks and then plants with a good root formation were transferred to 9 cm pots length filled with 700 cm^3 soil. Plants were irrigated every two days with normal water and once per week with water containing 0.25% Wuxal Top N fertilizer consist of 120 g kg^{-1} nitrogen, 40 g kg^{-1} phosphorus pentoxide, 60 g kg^{-1} potassium oxide, 0.1 g kg^{-1} boron, 0.04 g kg^{-1} copper, 0.2 g kg^{-1} iron, 0.01 g/kg molybdenum, 0.04 g kg^{-1} zink and 0.12 g kg^{-1} manganese (Aglukon, Düsseldorf, Germany). Propagation of these cuttings was done in the greenhouse for 8 weeks at a temperature of 22°C and 12 h light/dark condition. If the outside light conditions did not enclose appropriate light intensity inside the greenhouse, extra light was supplied to obtain a constant quantum fluence rate of approximately 350 µmol $m^{-2}s^{-1}$ (sodium vapor lamps, SON-T Agro 400, Philips, Amsterdam, Netherlands).

After 8 weeks of propagation under green house condition the drought stress experiment was conducted for four weeks and the plants were divided into three different groups dependent on soil volumetric water content (VWC) using Fieldscout device based on time domain reflectometry (TDR) (Spectrum Technologies, Plainfield, USA). Each treatment comprises five plants per genotype. Balanites sources in their habitat grown in broad range of soil from deep sand to clay loam and heavy clay, according to preliminary experiments *Balanites* sources gave more biomass and growth when propagated on clay loam substrate compared to sand, so here in this experiment clay loam was used as soil substrate. According to the manual of the Fieldscout instrument, clay loam substrate has a maximum water holding capacity at 35% which was considered as a control and a permanent wilting point at 20% VWC where water content less than this value will lead to permanent damage, so it considered here as a moderate stress. To investigate the effect of severe drought stress on the plants 5% VWC has been used. During the drought experiment plants were irrigated every 2 days, the water amount was calculated based on the water deficit calculation (D) of the Fieldscout; one mm equals 8 ml water for each pot based on the truncated cones formula.

Table 1. Genotypes, origin and coordinates of the seed collection used in this study.

Genotypes	**Locations**	**Coordinates**	**Annual average temperature (˚C)**	**Annual average precipitation (mm)**
El-Kharga	Park of Faculty of Sciences, University of South Valley, Aswan, Egypt	24°5'20.177"N; 32°53'59.385"E	25	1
Wadi El-Alaqi	Park of Faculty of Sciences, University of South Valley, Aswan, Egypt	24°5'20.177"N; 32°53'59.385"E	26	1
Wadi EL-Gemal	National park of Wadi EL-Gemal, Red Sea, Egypt	24°41'16.573"N; 35°5'1.248"E	24	3
Cairo	Giza, Zoo, Egypt	30°1'27.158"N; 31°12'49.731"E	21	26
Sudan	Sudan	not known	28	361
Togo	Togo	not known	27	1156

Plant growth analysis

Shoot length, fresh and dry weight, and leaf water content were examined after four weeks drought experiment. In addition the changes in plant morphology under severe drought stress were monitored. The plant height was determined by measuring the longest shoot. The above ground plant material was harvested and weighed for fresh weight examinations (FW), and then samples were oven-dried at 80˚C for 48 h and weighed for dry weight determination (DW). The numbers of plants tested per treatment were 5 from each genotype, only genotype El-Kharga was 3 plants for each treatment. Leaf water content was calculated according to this equation LWC= (FW-DW)/FW * 100.

Stomata conductance and chlorophyll fluorescence measurements

Young but fully expanded leaves were used for chlorophyll fluorescence measurements (Junior-PAM, Heinz Walz GmbH, Effeltrich, Germany) and determination of stomata conductance using a porometer (AP4 Delta-T Devices, Burwell, UK) as non-destructive methods. During the four week experiment, every Tuesday at 9 at the morning five leaves per treatment from each genotype were dark-adapted for 30 min by specific clips (Heinz Walz GmbH) and light curves in ten-second frequency were used for chlorophyll fluorescence measurements. From these measurements photochemical efficiency (ΔF⁄Fm') = (Fm'- Fs) Fm') was recorded. At the fourth week the same leaves were used to examine the water vapour stomatal conductance (mmol m^{-2} s^{-1}) using a porometer AP4 (Delta-T Devices).

Measurements of ascorbic acid

The determination of ascorbic acid was modified based on published protocols (Kampfenkel and Montagu 1995; Stevens et al. 2006; Gillespie and Ainsworth 2007). Fifty mg fresh mass from bulk samples of leaf were grinded in liquid nitrogen and weighed into two reaction tubes, 500 µl (6%) ice cold trichloro acetic acid (TCA) was added and vortexed incubated on ice for 15 min and then centrifuged at 18,400 x g for 20 min. The supernatant was taken and kept on ice, 10 µL of cold 75 mM phosphate buffer pH 7.0 and 10 µL of blank, standard, or sample were added to a 96-well microplate (Sarstedt AG and Co., Nümbrecht, Germany) (standards ranging from 1 mM to 0.0625 mM). Ten µl of 10 mM DTT were added to every second sample for the TAA measurements. After 10 min, 10 µl of *N*-ethylmaleimide (NEM) (0.5%) were added to the same second sample. To every other well, 20 µl of water were added. After 2 min incubation 100 µl of reaction mix was added. The reaction mix consisted of two parts 10% TCA, one part $FeCl_3$, two and a half part 43% H_3PO_4, two parts 4% α-α'-bipyridyl solved in 70% ethanol. After incubation for 30 min at 37°C the absorbance was read by the mircoplate reader (Synergy Mx, BioTek Germany, Bad Friedrichshall, Germany) at 523 nm.

Statistical analysis

Before statistical analysis, fresh weights, dry weights and conductance were log-transformed to achieve normal distributed residuals and variance homogeneity. To visualize potential redundancy of those physiological variables that were obtained for each individual plant,

principal component analysis (PCA) was performed after standardization (Venables and Ripley 2002).

To analyze significance of effects for single variables, two-factorial analysis of variances (ANOVA) was performed, with main effects for genotype, VWC, and genotype-VWC interaction. Because severe heterogeneity of variances could not be removed by transformations, the leaf water content was analyzed in a generalized least square model (GLS), instead of ANOVA, to allow for different residual variances in different VWC levels (Pinheiro and Bates 2000). For detailed investigation of the genotype-VWC interactions, data were split according to genotypes, and all pair-wise comparisons (Tukey test) of the VWC levels were performed for each genotype separately. The results of this test are visualized in the bar charts. Likewise, data were split according to the levels of VWC and all pair-wise comparisons among genotypes were tested (Tukey test) on each VWC level separately (details in supplementary material). Further, interaction contrasts (Gabriel et al. 1973) were used to compare the magnitude of differences between VWC levels among the genotypes (supplementary material). Finally, variance components of two-factorial random effect models (genotype, VWC and genotype-VWC interaction) were estimated to visualize the proportion of the total variance due the different factors and the residual error on a comparable scale. All computations were performed in R-3.1.0 (R Core Team 2014), using the package multcomp (Hothorn et al. 2008) for the Tukey test and compact letter display, the packages nlme (Pinheiro and Bates 2000) and lme4 (Bates et al. 2014) for GLS and random effect models, as well as the packages ggplot2 (Wickham 2009) and ade4 (Dray and Dufour 2007) for the figures.

Results

Effect of water stress on growth parameters

Plant height was significantly reduced with decreasing the soil water contents (P=0.0001; Supplementary Table 1) in genotypes Wadi Al-Alaqi, Wadi El-Gemal, Sudan and Cairo (Fig. 1a). There was no significant difference related to the genotypes-VWC interaction (P=0.845; Supplementary Table 1). The impact of water limitation observed in the increasing of shoot length between the first day and the last day of the experiment, whereas the total increase in stem length between the first day and last day of the experiment at each treatment has been

calculated as a percentage describing the gain in stem length. At VWC 5% the plants grew very slowly and there was almost no increase in stem length (0, 0.32, 1.93, and 3.76%) in genotypes Wadi El-Gemal, Wadi El-Alaqi, Sudan and Togo, respectively. Genotypes Cairo and El-Kharga represent the highest increase in stem length at the same soil water content (57.5 and 11.9%), respectively. Genotype Cairo and El-Kharga showed the highest gain in stem length at VWC 35% (59.0 and 56.1% respectively), and at VWC 20% genotypes Cairo and Wadi El-Alaqi (69.0 and 44.4%, respectively) had the highest increase in stem length.

The fresh weight of leaves (FW) was reduced with decreasing VWC (Fig. 1b) through all the genotypes (P=0.0001; Supplementary Table 1). Leaf FW showed significant differences between El-Kharga and Sudan at VWC 35 and 20%, there was no significant difference among the genotypes at VWC 5% (Supplementary Table 2) although plants in each genotype separately represent significant decreases at different soil water contents (P=0.0008; Supplementary Table 1). Overall at VWC 35% all genotypes showed the highest biomass fresh and dry weight, only genotype El-Kharga showed highest leaf FW (2.38 ± 0.26 g) at VWC 20%. Genotype Sudan at VWC 35% had the highest leaf FW (9.86 ± 0.34 g) in comparison to genotypes Cairo, El-Kharga and Togo (P=0.0206, 0.0005 and 0.0022; Supplementary Table 3), respectively. Also Wadi El-Alaqi has significantly more leaf FW than El-Kharga (P=0.0498; Supplementary Table 3). And the lowest leaf FW was obtained with genotypes Togo (0.32 ±0.13 g) at VWC 5%. The reduction in leaf FW between both of control, moderate stress and severe drought stress is significant larger in Sudan than in El-Kharga (P=0.0203; Supplementary Table 4), also the reduction in leaf FW between the control treatment (VWC 35%) and severe drought stress (VWC 5%) was significantly higher in genotypes Sudan, Wadi El-Gemal and Togo than in genotype El-Kharga (P=0.0028, 0.121 and 0.0803; Supplementary Table 4).

Under water deficit the decrease in stem fresh weight (Fig. 1c) was not significant among the genotypes (Supplementary Table 2) but there was a significant difference among the plants within the same genotype at different soil water contents (P=0.0001; Supplementary Table 1). Beside the reduction in biomass at different soil water contents, plant water content among genotypes was decreased with increasing water limitations. There were significant differences among genotypes at VWC 35% and no significant differences were observed under moderate and severe drought stress (Fig. 1d). Significant difference was observed among genotypes Cairo, El-Kharga, Wadi El-Gemal and Wadi El-Alaqi and no significant difference between genotypes Sudan and Togo at the same soil water content (P=0.0001; Supplementary Table 1). Genotype Cairo had the highest value (75.98%), where there was no

significant difference at VWC 20 and 5% between the treatments and among the genotypes. Genotype Wadi El-Alaqi represents the lowest value at VWC 5% (14.86%). Beside that other morphological changes have been monitored under severe drought stress: leaves shedding in Wadi El-Gemal genotype, leaf rolling and necrosis in genotype Togo, and chlorosis of many leaves in genotype Wadi El-Alaqi. Also under severe stress most of the plants from Wadi El-Alaqi were severely wilted or died, two plants were survived from five in genotypes Wadi El-Gemal, Sudan and Togo, and one plant only died from genotypes Cairo and El-Kharga.

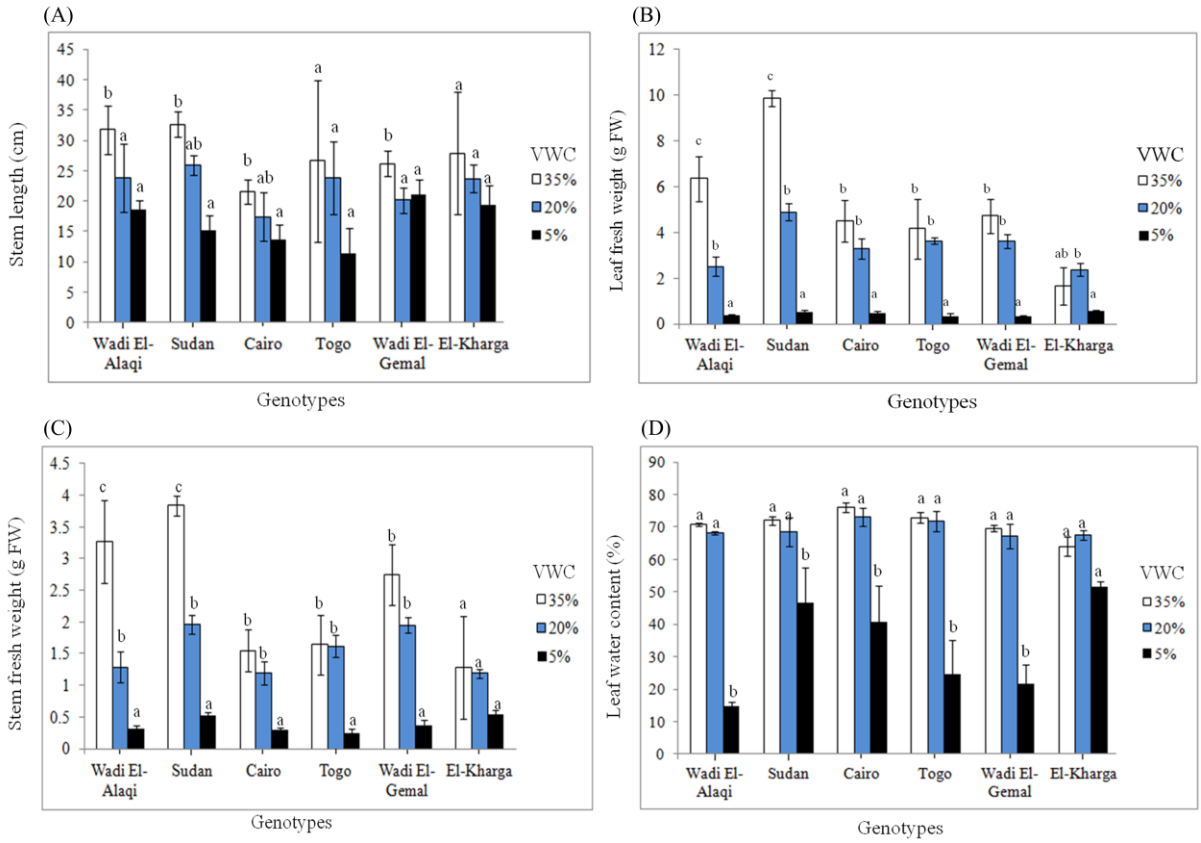

Figure 1. Effect of water stress on growth parameters. Effect of water stress on (A) Stem length, (B) Leaf fresh weight, (C) Stem fresh weight, (D) Leaf water content of *B. aegyptiaca* genotypes after 4 weeks of different water stress treatments, VWC 35%, VWC 20% and VWC5%. Values are means (± S.E.), n = 3 to 5. Within each genotype water stress treatments followed by the same letter do not show significant differences, following Tukey procedure (p<0.05).

Determination of stomata conductance

The decrease in stomata conductance was highly significant under the three level of soil water contents in each genotype (Fig. 2), also there are clear significant differences among genotypes dependent on the genotype-VWC interaction at control and moderate drought stress treatments (P=0.0025; Supplementary Table 1). These significant differences were smaller under severe drought stress where El-Kharga and Sudan significantly differed from Togo and Wadi El-Gemal. The reduction in stomata conductance between control and

moderate stress was significantly larger in Wadi El-Alaqi than in El-Kharga (*P*=0.0160; Supplementary Table 4). The difference in stomata conductance values between control and severe stress in Wadi El-Gemal genotype was significant, on the other hand, there was a significant reduction between control and severe stress in El-Kharga and Cairo genotypes (*P*=0.0382 and 0.0025; Supplementary Table 4). The increase between moderate and severe stress was highly significant in Wadi El-Gemal compared to the reduction between moderate and severe stress in El-Kharga and Cairo genotypes (*P*=0.0001 and 0.0002; Supplementary Table 4). Genotype El-Kharga showed higher stomata conductance with VWC 20% (290 mmol m^{-2} s^{-1}) and Cairo with VWC 35% (265 mmol m^{-2} s^{-1}), whereas lowest conductance was obtained through genotypes El-Kharga and Sudan at VWC 5% (21.32 and 21.81 mmol m^{-2} s^{-1}), respectively. There was a reduction in stomata conductance observed in genotype Sudan at the three levels of soil water contents. The plants from Wadi El-Alaqi were severely wilted and died by the third week at VWC 5%.

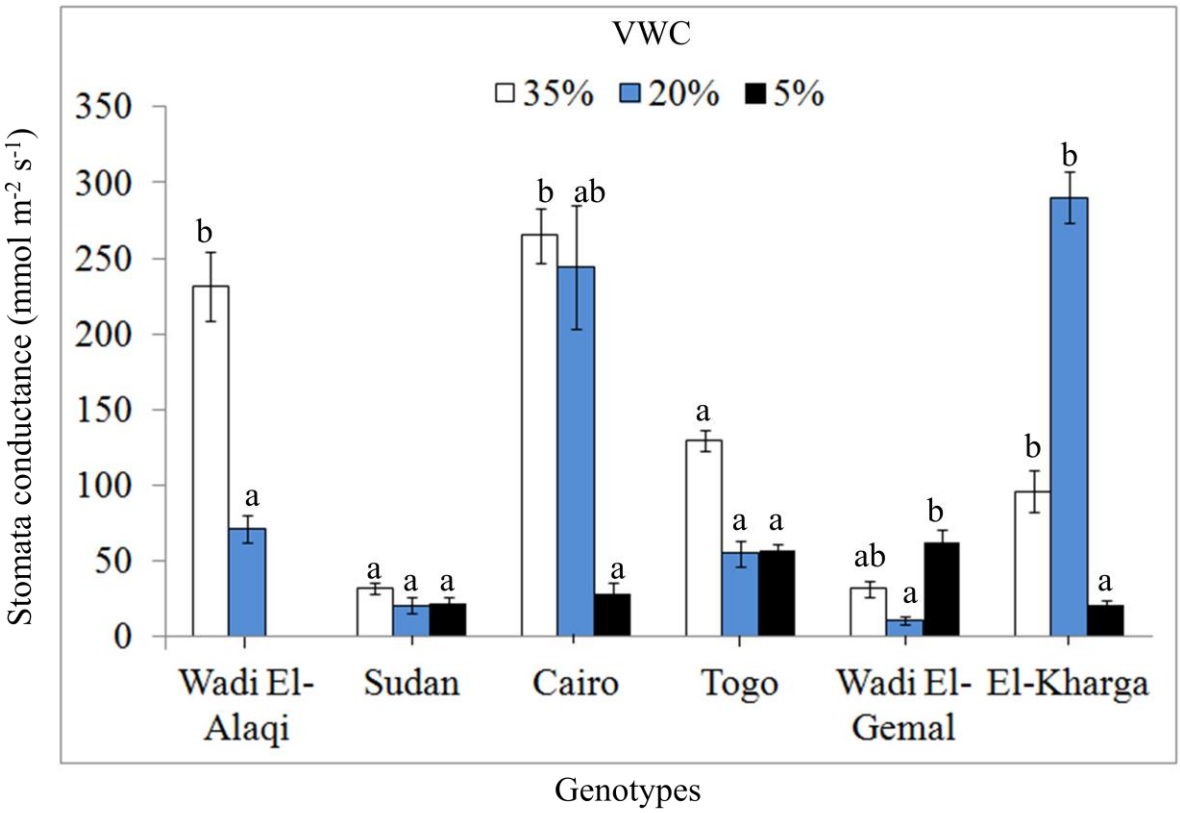

Figure 2. Effect of water limitation on stomata conductance. Effect of water shortage on leaf conductance (mmol m^{-2} s^{-1}) of *B. aegyptiaca* genotypes after 4 weeks of different water stress treatments, VWC 35%, VWC 20% and VWC 5%. Values are means (± S.E.), n = 3 to 5. Within each genotype water stress treatments followed by the same letter do not show significant differences, following Tukey procedure (p<0.05). The plants from Wadi El-Alaqi were severely wilted and died by the third week at VWC 5%.

Principal component analysis for biomass and leaf conductance variables

Fresh and dry mass of stem and leaves are summarized with about equal weights as the first component (Fig. 3 left), explaining roughly 70% percent of variability in the data (Supplementary Table 5). Observations of fresh and dry weights were highly correlated among each other, suggesting that these variables provide mutually exchangeable information on the biomass effects of drought stress in this highly controlled experiment. The second component mainly consists of conductance and explains roughly 18% of the variation (Supplementary Table 5). In this overall view, the variability of conductance is uncorrelated to the four biomass variables; overall changes in conductance do not go along with the overall changes in the biomass variables. The third component contrasts the leaf water content with the dry mass and stomata conductance, and accounts for 10% of the variance. That is, roughly 98% of the variance can be accounted through three components, major parts of the joint variation in these six physiological parameters, may be explained by only two or three variables (Supplementary Table 5). The location of genotypes and treatment groups with respect to the first and second component is depicted. The first component (biomass) mainly distinguishes the severe drought stress VWC 5% observations from the moderate drought stress VWC 20% and control VWC 35% observations. The second component (stomata conductance) could distinguish the control treatment VWC 35% (high conductance) from the moderate drought stress VWC 20% (low conductance) observations, but to distinguish the severe drought observations from the moderate and control treatments this was not clear through some genotypes compared to the first component (Fig. 3 right).

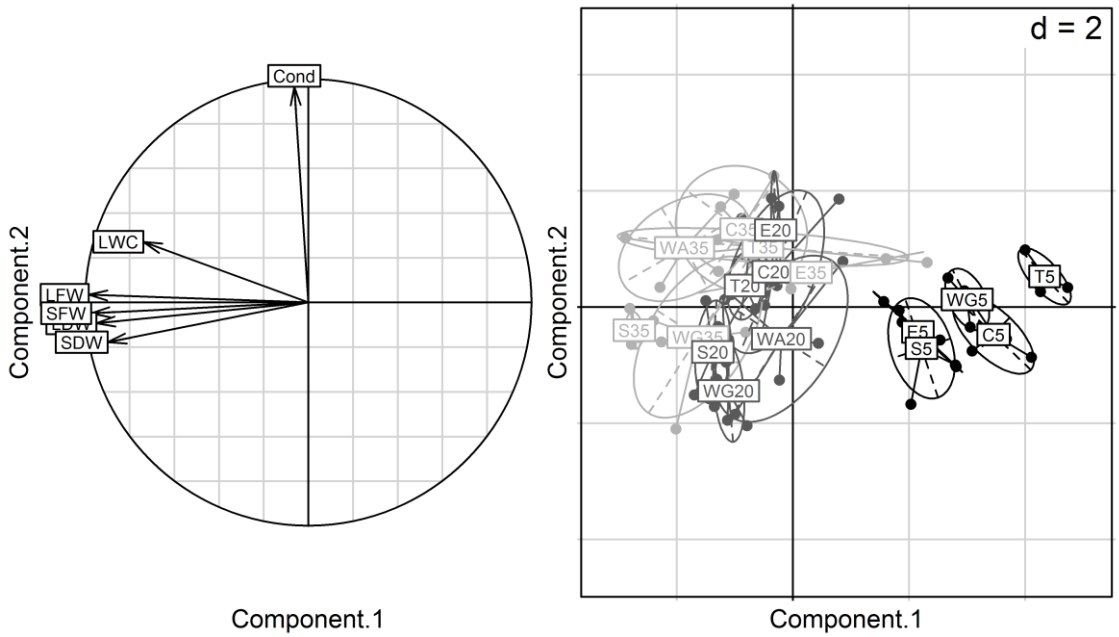

Figure 3. Principal component analysis for biomass and stomata conductance variables. Biplots with biomass as first component (abscissa) and stomata conductance as second component (ordinate) of the principal component analysis. Left: arrows depict how original variables contribute to the 1st and 2nd component; nearly parallel arrows with the same direction indicate correlation close to 1 among the original variables. (LFW, SFW: leaf and stem fresh mass; LDW, SDW, leaf and stem dry mass; LWC, leaf water content; Cond: Conductance). Right: grouping (genotype and VWC) of single plant observations according to the 1st and 2nd component; light gray, dark gray and black indicate VWC levels 35, 20 and 5%, respectively; genotype labels: C: Cairo, E: El-Kharga, S: Sudan, T: Togo, WG: Wadi El-Gemal, WA: Wadi El-Alaqi.

Comparing variance components

The results of the variance components analysis revealed that the first component of the PCA analysis and the biomass variables showed a large proportion of volumetric water content (VWC) variance, whereas the second components from PCA (stomata conductance) showed large proportion to the residual error and a high proportion of genotypes interaction with VWC (Fig. 4). The leaf water content showed high a proportion of VWC variance where the variance of genotypes and genotypes interaction with VWC is estimated zero. Shoot length showed high proportion of VWC and residual error with small proportion of genotypes variance effect.

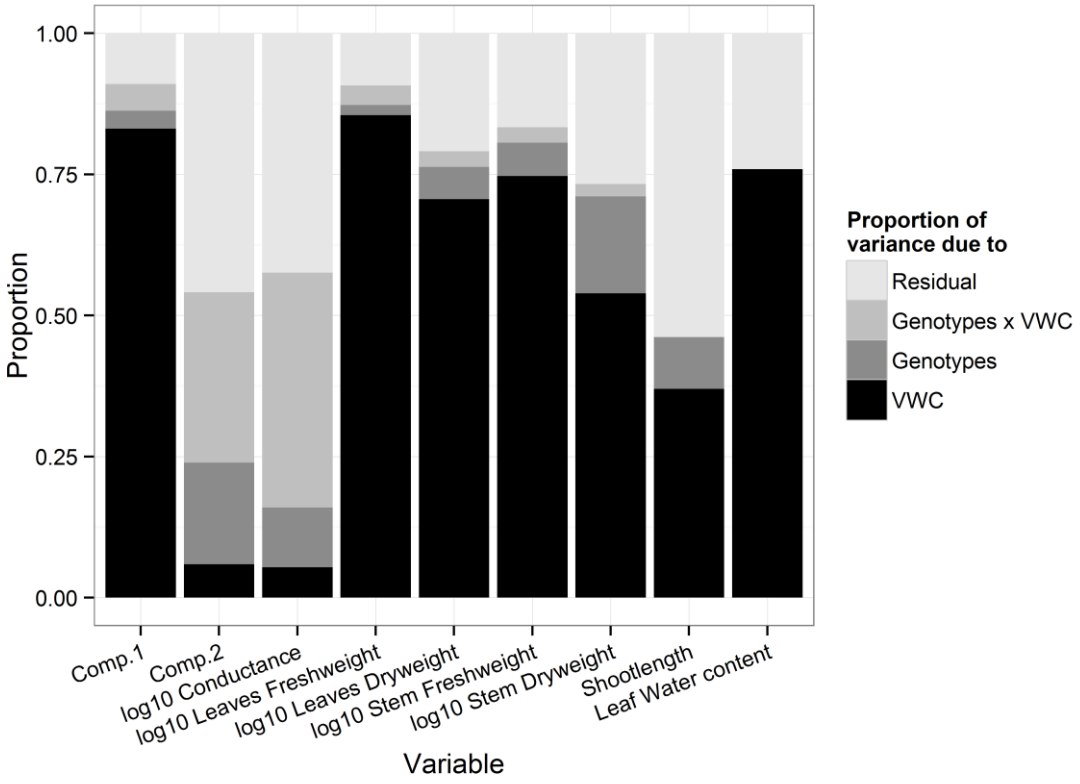

Figure 4. Comparing variance components. Estimated variance components, expressed as proportions of the total variance, for seven variables measured for single plants and the 1st and 2nd component derived from PCA. Variance due to VWC represents mean differences among VWC levels, variance due to genotypes represents overall differences among genotypes, genotype-VWC-interaction represents different reaction on VWC levels between genotypes, and residual (error) variance represents variability of single observations due to differences among single plants or measurement error.

Chlorophyll fluorescence measurements

The impact of water limitation on chlorophyll fluorescence was examined through photochemical efficiency (ΔF⁄Fm'). The measurements showed that there were no significant differences among the genotypes per treatment. Only at the second week there was a significant decrease in photochemical efficiency (Fig. 5) observed with genotype Wadi El-Alaqi at VWC 5%. The values were close at the 4th week and the impact of water stress was not clear among the genotypes and treatments. At the 4th week the mean average of photochemical efficiency for all treatments was between (0.439:0.603).

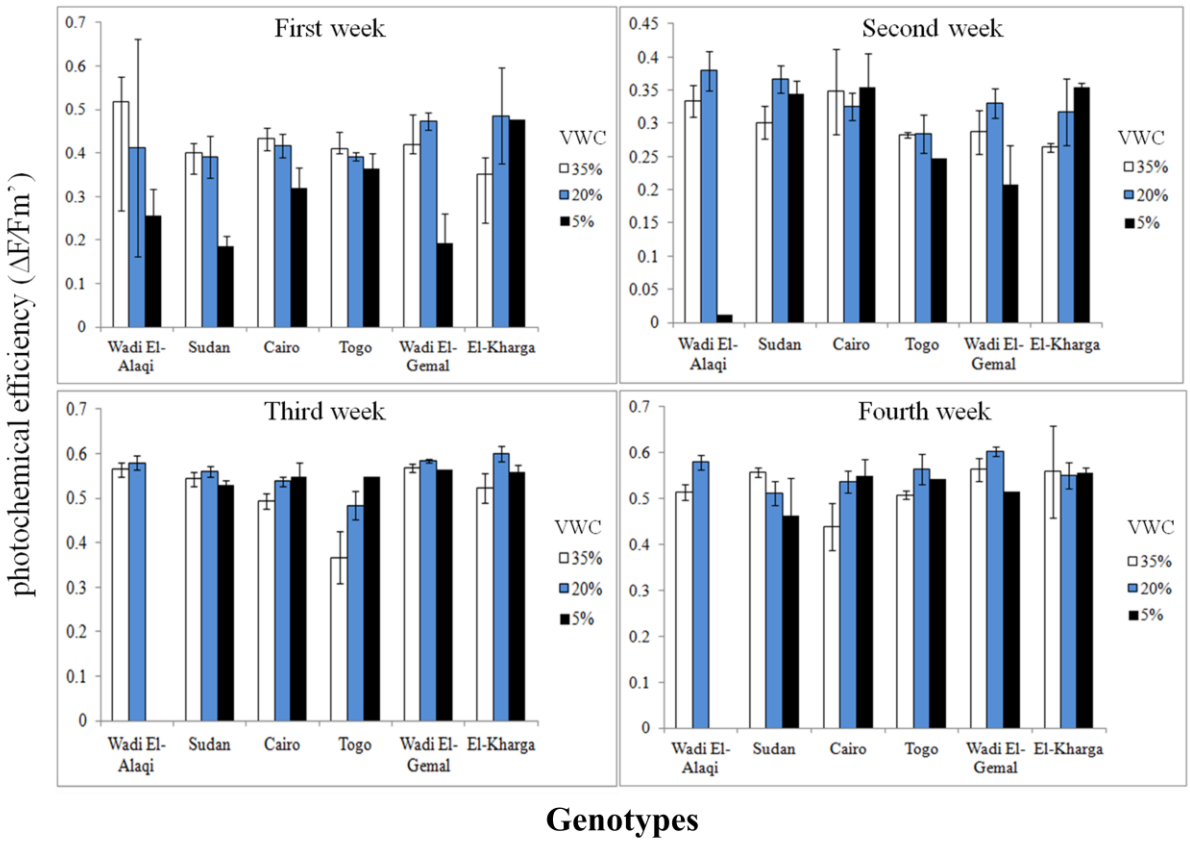

Figure 5. Effect of water stress on chlorophyll fluorescence (photochemical efficiency). Effect of water deficit on photochemical efficiency (ΔF/Fm') of *B. aegyptiaca* genotypes at different water stress treatments, VWC 35%, VWC 20% and VWC 5%. (1) Drought effect after week one, (2) week two, (3) week three, (4) week four. Values are means (± S.E.), n = 3 to 5. The plants from Wadi El-Alaqi were severely wilted and died by the third week at VWC 5%.

Total ascorbic acid

The results of the determination of total ascorbic acid (TAA) showed that there was a difference in TAA concentration among the genotypes and the plants produced the highest concentrations of TAA at well watered condition with VWC 35%. Under this treatment the highest TAA concentrations were obtained from genotypes El-Kharga, Togo and Sudan (608.9, 527.9 and 263.0 mg 100 g^{-1} FW, respectively), and decreased under water deficit (Fig 6). At VWC 20% only genotypes Wadi El-Alaqi and Cairo had the highest TAA concentration (285.2 and 165.4 mg 100 g^{-1} FW, respectively). Only genotype Wadi El-Gemal produced the highest concentration of TAA under severe drought condition VWC 5% (602.6 mg 100 g^{-1} FW). The plants from Wadi El-Alaqi were severely wilted and died by the third week at VWC 5%, the plants from Wadi El-Gemal were severely wilted under VWC 5% so only one sample was used from this genotype.

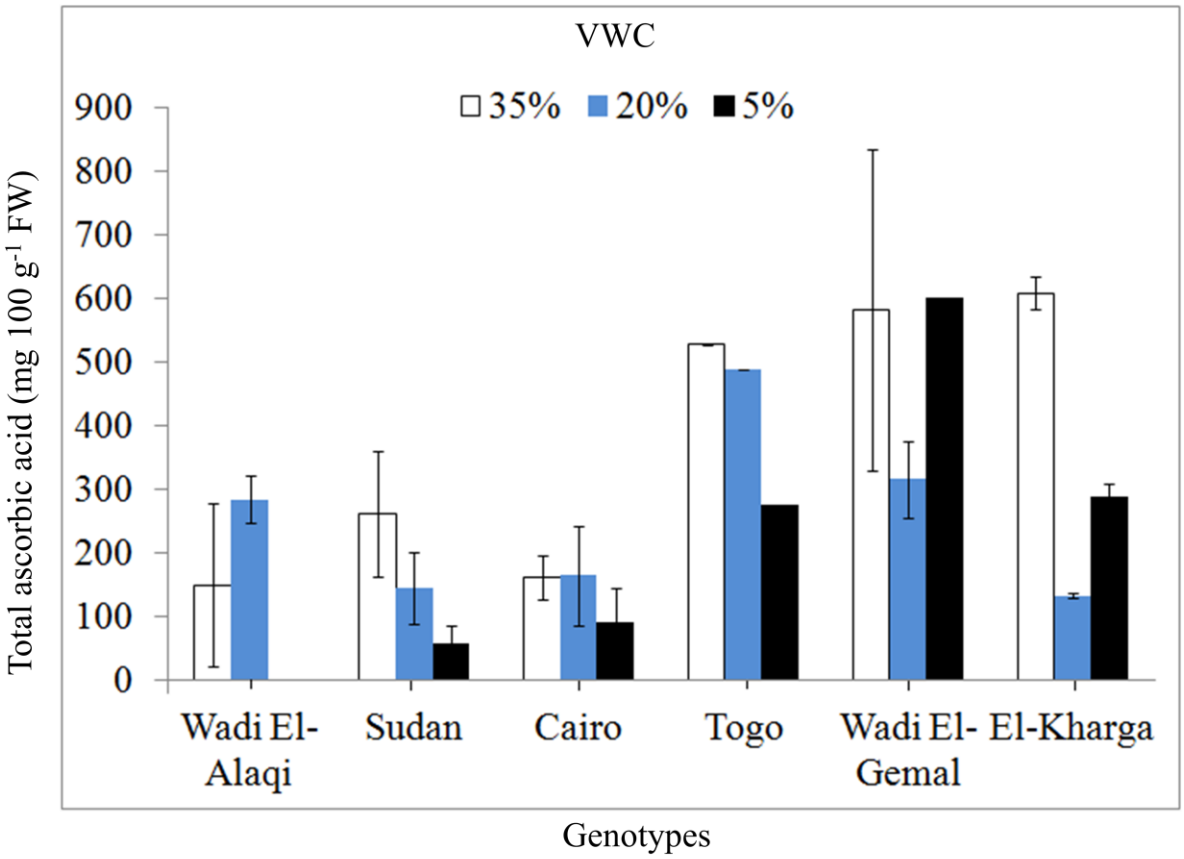

Figure 6. Effect of water stress on total ascorbic acid (TAA) (mg 100 g^{-1} FW), of *B. aegyptiaca* genotypes after 4 weeks of different water stress treatments, VWC 35%, VWC 20% and VWC 5%. Values are means (± S.D.), n = 1 to 3. The plants from Wadi El-Alaqi were severely wilted and died by the third week at VWC 5%.

Discussion

Major results are that *B. aegyptiaca* genotypes differed in their morphological and physiological responses to cope with the water shortage. Only two to three parameters could distinguish the water stress levels among the genotypes. The objective of this study was to examine and compare the effects of water deficit among different genotypes of *B. aegyptiaca* to recommend the best genotype for cultivation in arid and semi-arid areas.

We consider VWC 35% as control treatment; the plants produced the highest above ground biomass compared to moderate and severe stress and no sign of wilting symptoms or stress. Moderate drought with VWC 20%; there was slight decrease in above ground biomass compared to the control with decrease in stomata conductance but no wilting or stress signs. Severe drought stress with VWC 5%; drastic reduction in growth and the stomata conductance with different signs of wilting and stress, with decrease in survival rate in some genotypes. The increase in plant height was almost stopped under severe drought with a

reduction in leaf and stem water content in genotypes Togo, Wadi El-Gemal and Wadi El-Alaqi. This reduction in growth parameters under water stress conditions could be explained in the following way: the plant avoids the stress via decreasing the biomass and shoot extension, resulting in reduction of growth parameters. According to Farooq et al. (2009) water deficit decreases the rate of the cell division, cell elongation and expansion, resulting in reduction of growth. This is consistent with a previous study on seedlings of *B. aegyptiaca* collected from eight geographical sources and the results indicated that, the seedlings mortality was significantly reduced under water deficit also a reduction in shoot and leaf weight were recorded with an increase in the root weight and root to shoot ratio (Elfeel et al. 2007).

Under severe drought stress *B. aegyptiaca* genotypes showed different morphological responses to cope with stress: Wadi El-Gemal genotype showed leaves shedding, leaf rolling and necrosis was observed in genotype Togo. In genotype Wadi El-Alaqi most of the leaves showed chlorosis. This is consistent with the observation of Engelbrecht and Kursar (2003) investigating seedlings of 28 woody plant species. They defined the severely wilted as "very strong change of leaf angle or change of leaf surface structure with beginning leaf necrosis". This was also noticed in *Jatropha curcas* L.: the plants adapted to water limitation through selective leaf shedding as a drought tolerance strategy (Fini et al. 2013) and with Mediterranean and tropical tree species under arid or semi-arid conditions (Sanchez-Blanco et al. 2002). The effect of water stress on growth parameters was examined in *Euphorbia tirucalli* L. (pencil tree) genotypes that were collected from different countries to study the effect of water limitation on *E. tirucalli* through different VWC. After eight weeks experimental treatment, all the genotypes under well water treatment showed an increase in shoot fresh and dry weight, plant height and plant water content, while under severe drought stress there were significant decreases among the genotypes in plant height, dry biomass and chlorophyll fluorescence (Hastilestari et al. 2013).

Finally, *B. aegyptiaca* genotypes at severe drought stress (VWC 5%) did not show significant differences in growth parameters among the genotypes compared to the clear significant differences at control and moderate drought treatments among the genotypes (Supplementary Table A2). Otherwise, the reduction in biomass of plants grown in control and moderate stress conditions in comparison to the severe water stress was highly significant in some genotypes (Sudan, Wadi El-Alaqi, Wadi El-Gemal and Togo) compared to the others (El-Kharga and Cairo) which might be a good indicator for the water deficit effect in these genotypes.

Under water limiting conditions a reduction in stomata closure was observed in all *B. aegyptiaca* genotypes where stomata closure was considered as early plant response to water stress (Cornic and Massacci 1996). Thus under severe drought stress *B. aegyptiaca* showed this water saving mechanism in genotypes Cairo and El-Kharga which decrease the losses in plant water content in the range between 40.7 and 51.59% in leaves and between 72.4 and 53.7% in stems. This result from genotypes Cairo and El-Kharga could explain the gain in plant height at severe drought stress where Cairo represents high stem water content under severe stress treatment. According to Fini et al. (2013) the responses of different sources of the oil-producing, drought-tolerant *J. curcas* to water stress were investigated. The authors found that this species has several mechanisms to avoid water stress, such as early stomata closure. Under growth chamber conditions four week old seedlings of *J. curcas* were grown and subjected to five different water stress treatments for four weeks experiment (100% field capacity) as a control treatment and 5, 50, 25 and 0% FC as water stress conditions. At severe (0% FC) and moderate drought stress (25% FC), the plant showed a strategy to withdraw the water stress through a decreasing in plant height and stomata conductance. These reactions resulted in a decrease of the transpiration rate and reduced the losses in leaf water content (Díaz-López et al. 2012).

In barley cultivars under soil moisture limitation a decrease in stomata conductance combined with a reduction in the yield and leaf water potential was observed. Otherwise with soil moisture availability there were increasing stomata conductance values combined with an increase of leaf water status and the yield observed (Siddique et al. 1990). In genotypes Sudan, Wadi El-Gemal and Togo at moderate drought stress conditions, there is a reduction in stomata conductance with high plant water content. This reduction in stomata conductance could be more related to soil moisture, where several studies suggested that the stomata conductance is more related to soil moisture than to leaf water content, also genotype Sudan showed no significant differences in stomata conductance with early stomata closure at the three level of soil water content where this reduction in stomata conductance at control treatments might be related to the water logging at this level which might affect the root system in this genotype. Also under water limitation, the dehydrated roots send a chemical signal (ABA) to the leaves which affected stomata closure with stable plant water content at the high levels (Farooq et al. 2009).

In the principal component analysis, the first component (biomass) clearly distinguishes the observations of VWC 5% from the VWC 35% and 20% treatments where the decrease in biomass (represented by fresh and dry weights) is a clear indicator of severe stress induced by

the VWC 5% treatment to all genotypes. With respect to the first component, there is no clear distinction between the VWC 20% and 35% treatments, suggesting that the VWC 20% treatment induced mostly a moderate stress which did not lead to remarkable decrease in biomass. Differences between treatments and genotypes are less clear in the second and third component. The second component (mainly dependent on stomata conductance) shows some differences between genotypes in the VWC 35 and 20% treatments, however, between group differences are less clear relative to the variation within groups of the same genotypes and VWC treatment. The high correlation of leaves and stems fresh- and dry mass indicates that measurement of only some of these variables might be sufficient to assess the effects of severe drought stress on biomass, at least in highly controlled experimental setups.

Variance components analysis indicates that the biomass variables (first component in PCA) show a large proportion of environmental variance (VWC effects) such that these variables might be used as indicators for severe drought stress over all the genotypes. Also leaf water content might be used as an indicator for drought stress due to a high proportion of variance due to VWC, however, the relatively large proportion of residuals error (large differences between single plants within a treatment group) suggests that it may be less useful than the biomass variables. Shoot length showed less proportion of VWC related variance along with a high proportion of residual error. This high residual variability should be mostly due to variations in the stem length between the plants in the same genotypes sometimes and among the genotypes although the experiment was starting with approximately the same length of vegetative cuttings. The stomata conductance (second component of the PCA) showed no clear and consistent response to drought stress treatments across genotypes, as indicated by the low proportion of VWC variance and high proportion of genotype x VWC interaction. However, the use of stomata conductance to distinguish drought-tolerant from drought stress-susceptible genotypes appears to be restricted because the proportion of residual variance for stomata conductance is high. For future analyses it would thus be helpful to include more single plants if this high variation is a property of single plants, or to perform repeated measurements of conductance on single plants if this residual error is mainly due to the measurement method.

Chlorophyll fluorescence determinations in summary indicate that there were no remarkable changes in photochemical efficiency (ΔF⁄Fm') at the three levels of soil water content in each genotype. Only genotype Wadi El-Alaqi showed a significant decrease in the second week (Fig. 5). This might be consistent with Fini et al. (2013), through examining the drought avoidance strategy in three different accessions of *J. curcas*, the authors found that the

Suriname genotype showed a decrease in plant dry weight and total leaf area with a reduction in Fv/Fm and stomata conductance whilst the values of Fv/Fm were not affected in the genotype from Brazil that was considered as the best drought-tolerant source under those experimental conditions also after leaf shedding, only a few leaves were still attached to the stem, where the biomass aggregation in stem under water limitation could help in carbon assimilation to the whole plant. In our experiment, at the fourth week of treatment genotype Sudan showed the highest photochemical efficiency values at control treatments (Fig. 5). In genotype El-Kharga the values were very close at the three levels of soil water content, where genotype El- Kharga showed no significant differences in the shoot length, leaf water content and stem fresh weight at the three levels of soil water content. Genotype Cairo had higher values at severe drought treatments; this could be explained from the high stem water content measured in these treatments. In conclusion, the measurements of photochemical efficiency (ΔF⁄Fm') do not represent a remarkable change even under severe drought conditions. This was previously reported in four genotypes of *Lablab purpureus* L. investigated by Guretzki and Papenbrock (2013) to compare the response mechanism of water stress among genotypes. Under drought conditions there was a significant decrease in growth parameters and stomata conductance among the genotypes. However, the data from chlorophyll fluorescence measurements were not strong tools to examine the plant response to mild stress.

Summarizing the data of TAA measurements indicate that under control condition *B. aegyptiaca* produced a high concentration of TAA compared to other plant species (445, 228.3, and 53 mg 100 g^{-1} FW) in Indian gooseberry, Guava and Orange respectively (USDA 2007). Under abiotic stress conditions AA has an important role as electron donor in reactive oxygen species scavenging (ROS) (Zhang 2013). In *Zea mays* L. under water stress treatments, TAA was decreased where AA has increased the ability of the plant to overcome water stress through scavenging of ROS and reduce its damage effect under stress conditions (Dolatabadian et al. 2009). The role of TAA under water stress conditions was investigated in several plant species. Under drought stress, transformed *Arabidopsi*s with EsWAX1 (isolated from *Eutrema salsugineum* with genes involved in wax biosynthesis) showed increase in the level of TAA and this reduced the negative effect of drought stress compared to the control (Zhu et al. 2014). In canola plants drought stress of 60% field capacity reduced the growth parameters, leaf chlorophyll contents and enzyme activity where AA foliar applied with different levels (100 and 150 mg L^{-1}) increased the shoot and root fresh and dry weight,

enzyme activity and non photochemical quenching (NPQ) under drought stress conditions (Shafiq et al. 2014).

Conclusion

Balanites aegyptiaca is considered as a drought-tolerant plant. In this study, several genotypes were used to investigate the mechanisms these genotypes use to cope with low, moderate and severe water limitation at the three level of soil water content. The genotypes differed in their range of reactions to water limitation. Dependent on the plant response to water stress and survival rate, the genotypes under investigation could be divided into four groups; i) Sensitive to severe drought: this group including genotype Wadi El-Alaqi, under severe water stress four plants from five were dead at the end of the experiment with steep reductions in all growth and physiological parameters. ii) Medium drought-tolerant: this group including genotypes Wadi El-Gemal and Togo, both genotypes showed steep reductions in growth parameters and only two plants from five survived. In genotypes Wadi El-Gemal most of the leaves were fallen down but the stem was still green, in genotype Togo, leaf necrosis and rolling was observed under severe water stress. iii) Mild-drought stress-tolerant: this group including genotype Sudan, where two plants from five were dead. Genotype Sudan showed high reduction in growth parameters under severe drought stress compared to the control and revealed early stomata closure which kept the leaf and stem water content in save level. iv) Highly drought-tolerant: this group including genotypes Cairo and El-Kharga only one plant died from Cairo and El-Kharga and no remarkable change in leaf structure was recorded under severe stress conditions. So under severe stress genotypes Cairo and El-Kharga showed moderate reduction in growth parameters and high level of leaf water content compared to the other genotypes under the same conditions. Also genotype Sudan showed early stomata closure and high leaf water content under severe stress.

Finally, based on the data provided through this experiment, genotypes El-Kharga, Cairo and Sudan could be recommended for further drought stress experiment to examine the potential of these genotypes to recover after severe stress and finally select the most drought tolerant genotype.

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Supplementary Tables

Supplementary Table 1. Two-factorial ANOVA. Shown are the significant effects of the interaction between factors (volumetric water content, genotypes, VWC and genotypes interaction and residual error) and different variables (the log of leaf fresh weight, stem fresh weight, leaf conductance), the shoot length, leaf water content and first, second component from principal coordinate.

Variable	**Effect**	**Df**	**SS**	**MS**	**F**	***P* value**	**Sign.**
LFW (log)	VWC	2	17.86	8.93	254.39	0.0000	***
	Genotypes	5	0.86	0.17	4.87	0.0008	***
	VWC:Genotypes	10	0.93	0.09	2.65	0.0098	**
	Residuals	59	2.07	0.04			
SFW (log)	VWC	2	9.30	4.65	121.50	0.0000	***
	Genotypes	5	1.26	0.25	6.57	0.0001	***
	VWC:Genotypes	10	0.69	0.07	1.79	0.0822	.
	Residuals	59	2.26	0.04			
Cond (log)	VWC	2	2.37	1.18	12.16	0.0000	***
	Genotypes	5	6.51	1.30	13.37	0.0000	***
	VWC:Genotypes	9	5.09	0.57	5.80	0.0000	***
	Residuals	81	7.89	0.10			
Shoot length	VWC	2	1732	866	18.22	0.0000	***
	Genotypes	5	727	145	3.06	0.0157	*
	VWC:Genotypes	10	263	26	0.55	0.8455	
	Residuals	62	2947	48			
Comp. 1 (PCA)							
	Genotypes	5	31.47	6.29	10.99	0.0000	***
	VWC	2	210.87	105.43	184.09	0.0000	***
	Genotypes:VWC	9	15.64	1.74	3.03	0.0055	**
	Residuals	52	29.78	0.57			
Comp. 2 (PCA)							
	Genotypes	5	22.77	4.55	8.90	0.0000	***
	VWC	2	7.54	3.77	7.37	0.0015	**
	Genotypes:VWC	9	15.55	1.73	3.38	0.0025	**
	Residuals	52	26.61	0.51			
Wald-test for effects in GLS model					Statistic	*P* value	
LWC	VWC	2			46.40	0.0000	***
	Genotypes	5			6.63	0.0001	***
	VWC:Genotypes	10			0.90	0.5425	

Supplementary Table 2. Shown are the significant differences among the genotypes regarding to different variables (the log of Leaf fresh weight, stem fresh weight, leaf conductance), the shoot length and leaf water content at different level of volumetric water content.

Variable	VWC	Cairo	El-Kharga	Sudan	Togo	Wadi El-Gemal	Wadi El-Alaqi
LFW (log)	35	ab	a	b	ab	ab	ab
	20	ab	a	b	ab	ab	a
	5	a	a	a	a	a	a
SFW (log)	35	a	a	a	a	a	a
	20	a	a	a	a	a	a
	5	a	a	a	a	a	a
Conductance (log)	35	c	c	ab	bc	a	c
	20	bc	c	a	ac	a	ab
	5	ab	a	a	b	b	
Shoot length	35	a	a	a	a	a	a
	20	a	a	a	a	a	a
	5	a	a	a	a	a	a
LWC	35	c	a	bc	bc	ab	b
	20	a	a	a	a	a	a
	5	a	a	a	a	a	a

Supplementary Table 3. The effect of water stress at the level of genotypes-VWC interaction through different variables (the log of leaf fresh weight, stem fresh weight, stomata conductance, the shoot length, leaf water content, first and second component from principal coordinate according).

Variable	Comparison	Estimate	SD error	Test stat	*P* value	Sign.
LFW (log)	((35 - 5):El-Kh) - ((35 - 5):Cairo)	-0.556	0.216	-2.572	0.1710	
	((35 - 5):Sudan) - ((35 - 5):Cairo)	0.298	0.178	1.675	0.6687	
	((35 - 5):Togo) - ((35 - 5):Cairo)	0.024	0.183	0.134	1.0000	
	((35 - 5):WEl-G) - ((35 - 5):Cairo)	0.069	0.178	0.390	1.0000	
	((35 - 5):WEl-A) - ((35 - 5):Cairo)	0.083	0.190	0.436	0.9999	
	((35 - 5):Sudan) - ((35 - 5):El-Kh)	0.854	0.208	4.105	0.0028	**
	((35 - 5):Togo) - ((35 - 5):El-Kh)	0.581	0.212	2.736	0.1189	
	((35 - 5):WEl-G) - ((35 - 5):El-Kh)	0.626	0.208	3.007	0.0637	
	((35 - 5):WEl-A) - ((35 - 5):El-Kh)	0.639	0.219	2.919	0.0800	
	((35 - 5):Togo) - ((35 - 5):Sudan)	-0.273	0.173	-1.582	0.7265	
	((35 - 5):WEl-G) - ((35 - 5):Sudan)	-0.228	0.168	-1.363	0.8466	
	((35 - 5):WEl-A) - ((35 - 5):Sudan)	-0.215	0.181	-1.186	0.9183	
	((35 - 5):WEl-G) - ((35 - 5):Togo)	0.045	0.173	0.260	1.0000	
	((35 - 5):WEl-A) - ((35 - 5):Togo)	0.059	0.186	0.316	1.0000	
	((35 - 5):WEl-A) - ((35 - 5):WEl-G)	0.014	0.181	0.075	1.0000	
	((35.20 - 5):El-Kh) - ((35.20 - 5):Cairo)	-0.376	0.178	-2.112	0.3877	
	((35.20 - 5):Sudan) - ((35.20 - 5):Cairo)	0.214	0.153	1.403	0.8266	
	((35.20 - 5):Togo) - ((35.20 - 5):Cairo)	0.131	0.155	0.844	0.9875	
	((35.20 - 5):WEl-G) - ((35.20 - 5):Cairo)	0.093	0.153	0.611	0.9986	
	((35.20 - 5):WEl-A) - ((35.20 - 5):Cairo)	-0.051	0.167	-0.303	1.0000	
	((35.20 - 5):Sudan) - ((35.20 - 5):El-Kh)	0.590	0.172	3.434	0.0203	*
	((35.20 - 5):Togo) - ((35.20 - 5):El-Kh)	0.507	0.174	2.909	0.0803	.
	((35.20 - 5):WEl-G) - ((35.20 - 5):El-Kh)	0.470	0.172	2.731	0.1214	
	((35.20 - 5):WEl-A) - ((35.20 - 5):El-Kh)	0.326	0.185	1.760	0.6131	
	((35.20 - 5):Togo) - ((35.20 - 5):Sudan)	-0.083	0.148	-0.559	0.9993	
	((35.20 - 5):WEl-G) - ((35.20 - 5):Sudan)	-0.121	0.145	-0.832	0.9885	
	((35.20 - 5):WEl-A) - ((35.20 - 5):Sudan)	-0.265	0.160	-1.650	0.6844	
	((35.20 - 5):WEl-G) - ((35.20 - 5):Togo)	-0.038	0.148	-0.256	1.0000	
	((35.20 - 5):WEl-A) - ((35.20 - 5):Togo)	-0.182	0.163	-1.115	0.9400	
	((35.20 - 5):WEl-A) - ((35.20 - 5):WEl-G)	-0.144	0.160	-0.897	0.9819	
SFW (log)	((35 - 5):El-Kh) - ((35 - 5):Cairo)	-0.531	0.226	-2.350	0.2618	
	((35 - 5):Sudan) - ((35 - 5):Cairo)	-0.017	0.186	-0.094	1.0000	
	((35 - 5):Togo) - ((35 - 5):Cairo)	-0.045	0.191	-0.238	1.0000	
	((35 - 5):WEl-G) - ((35 - 5):Cairo)	0.032	0.186	0.172	1.0000	

	((35 - 5):WEl-A) - ((35 - 5):Cairo)	0.042	0.199	0.210	1.0000	
	((35 - 5):Sudan) - ((35 - 5):El-Kh)	0.513	0.217	2.363	0.2542	
	((35 - 5):Togo) - ((35 - 5):El-Kh)	0.486	0.222	2.191	0.3435	
	((35 - 5):WEl-G) - ((35 - 5):El-Kh)	0.563	0.217	2.590	0.1633	
	((35 - 5):WEl-A) - ((35 - 5):El-Kh)	0.573	0.229	2.503	0.1938	
	((35 - 5):Togo) - ((35 - 5):Sudan)	-0.028	0.180	-0.155	1.0000	
	((35 - 5):WEl-G) - ((35 - 5):Sudan)	0.049	0.175	0.282	1.0000	
	((35 - 5):WEl-A) - ((35 - 5):Sudan)	0.059	0.189	0.313	1.0000	
	((35 - 5):WEl-G) - ((35 - 5):Togo)	0.077	0.180	0.428	0.9999	
	((35 - 5):WEl-A) - ((35 - 5):Togo)	0.087	0.194	0.449	0.9999	
	((35 - 5):WEl-A) - ((35 - 5):WEl-G)	0.010	0.189	0.052	1.0000	
	((35.20 - 5):El-Kh) - ((35.20 - 5):Cairo)	-0.438	0.186	-2.355	0.2587	
	((35.20 - 5):Sudan) - ((35.20 - 5):Cairo)	-0.110	0.159	-0.693	0.9965	
	((35.20 - 5):Togo) - ((35.20 - 5):Cairo)	0.047	0.162	0.288	1.0000	
	((35.20 - 5):WEl-G) - ((35.20 - 5):Cairo)	0.025	0.159	0.156	1.0000	
	((35.20 - 5):WEl-A) - ((35.20 - 5):Cairo)	-0.101	0.175	-0.576	0.9991	
	((35.20 - 5):Sudan) - ((35.20 - 5):El-Kh)	0.328	0.179	1.825	0.5700	
	((35.20 - 5):Togo) - ((35.20 - 5):El-Kh)	0.485	0.182	2.662	0.1404	
	((35.20 - 5):WEl-G) - ((35.20 - 5):El-Kh)	0.463	0.179	2.579	0.1674	
	((35.20 - 5):WEl-A) - ((35.20 - 5):El-Kh)	0.337	0.193	1.747	0.6215	
	((35.20 - 5):Togo) - ((35.20 - 5):Sudan)	0.157	0.155	1.016	0.9629	
	((35.20 - 5):WEl-G) - ((35.20 - 5):Sudan)	0.135	0.152	0.893	0.9824	
	((35.20 - 5):WEl-A) - ((35.20 - 5):Sudan)	0.010	0.168	0.059	1.0000	
	((35.20 - 5):WEl-G) - ((35.20 - 5):Togo)	-0.022	0.155	-0.142	1.0000	
	((35.20 - 5):WEl-A) - ((35.20 - 5):Togo)	-0.147	0.170	-0.865	0.9854	
	((35.20 - 5):WEl-A) - ((35.20 - 5):WEl-G)	-0.125	0.168	-0.749	0.9942	
Cond (log)	((20 - 35):El-Kh) - ((20 - 35):Cairo)	0.481	0.240	2.003	0.5716	
	((20 - 35):Sudan) - ((20 - 35):Cairo)	-0.019	0.233	-0.084	1.0000	
	((20 - 35):Togo) - ((20 - 35):Cairo)	-0.067	0.273	-0.244	1.0000	
	((20 - 35):WEl-G) - ((20 - 35):Cairo)	-0.246	0.255	-0.965	0.9924	
	((20 - 35):WEl-A) - ((20 - 35):Cairo)	-0.357	0.222	-1.610	0.8233	
	((20 - 35):Sudan) - ((20 - 35):El-Kh)	-0.501	0.245	-2.044	0.5445	
	((20 - 35):Togo) - ((20 - 35):El-Kh)	-0.548	0.283	-1.933	0.6222	
	((20 - 35):WEl-G)-((20 - 35):El-Kh)	-0.727	0.266	-2.738	0.1563	
	((20 - 35):WEl-A)-((20 - 35):El-Kh)	-0.838	0.234	-3.585	0.0160	*
	((20 - 35):Togo) - ((20 - 35):Sudan)	-0.047	0.278	-0.170	1.0000	
	((20 - 35):WEl-G) -((20 - 35):Sudan)	-0.227	0.260	-0.873	0.9964	

	((20 - 35):WEl-A) -((20 - 35):Sudan)	-0.337	0.227	-1.488	0.8819	
	((20 - 35):WEl-G) - ((20 - 35):Togo)	-0.180	0.296	-0.607	0.9998	
	((20 - 35):WEl-A) - ((20 - 35):Togo)	-0.290	0.268	-1.084	0.9828	
	((20 - 35):WEl-A)-((20 - 35):WEl-G)	-0.111	0.249	-0.444	1.0000	
	((5 - 35):El-Kh) - ((5 - 35):Cairo)	0.172	0.275	0.624	0.9997	
	((5 - 35):Sudan) - ((5 - 35):Cairo)	0.630	0.245	2.573	0.2249	
	((5 - 35):Togo) - ((5 - 35):Cairo)	0.584	0.297	1.962	0.6010	
	((5 - 35):WEl-G) - ((5 - 35):Cairo)	1.190	0.289	4.118	0.0025	**
	((5 - 35):Sudan) - ((5 - 35):El-Kh)	0.459	0.269	1.708	0.7679	
	((5 - 35):Togo) - ((5 - 35):El-Kh)	0.412	0.317	1.299	0.9451	
	((5 - 35):WEl-G) - ((5 - 35):El-Kh)	1.019	0.309	3.293	0.0382	*
	((5 - 35):Togo) - ((5 - 35):Sudan)	-0.047	0.292	-0.160	1.0000	
	((5 - 35):WEl-G) - ((5 - 35):Sudan)	0.560	0.283	1.976	0.5917	
	((5 - 35):WEl-G) - ((5 - 35):Togo)	0.607	0.330	1.840	0.6833	
	((5 - 20):El-Kh) - ((5 - 20):Cairo)	-0.310	0.264	-1.172	0.9710	
	((5 - 20):Sudan) - ((5 - 20):Cairo)	0.650	0.257	2.533	0.2433	
	((5 - 20):Togo) - ((5 - 20):Cairo)	0.650	0.300	2.165	0.4594	
	((5 - 20):WEl-G) - ((5 - 20):Cairo)	1.437	0.292	4.918	0.0002	***
	((5 - 20):Sudan) - ((5 - 20):El-Kh)	0.960	0.263	3.654	0.0132	*
	((5 - 20):Togo) - ((5 - 20):El-Kh)	0.960	0.305	3.142	0.0587	.
	((5 - 20):WEl-G) - ((5 - 20):El-Kh)	1.746	0.297	5.872	0.0000	***
	((5 - 20):Togo) - ((5 - 20):Sudan)	0.000	0.299	0.001	1.0000	
	((5 - 20):WEl-G) - ((5 - 20):Sudan)	0.787	0.290	2.708	0.1688	
	((5 - 20):WEl-G) - ((5 - 20):Togo)	0.786	0.330	2.385	0.3218	
Comp. 1	((35 - 5):El-Kh) - ((35 - 5):Cairo)	-2.502	0.874	-2.863	0.0670	.
	((35 - 5):Sudan) - ((35 - 5):Cairo)	0.606	0.718	0.843	0.9662	
	((35 - 5):Togo) - ((35 - 5):Cairo)	0.506	0.788	0.643	0.9918	
	((35 - 5):WEl-G) - ((35 - 5):Cairo)	0.295	0.769	0.384	0.9997	
	((35 - 5):Sudan) - ((35 - 5):El-Kh)	3.107	0.840	3.697	0.0073	**
	((35 - 5):Togo) - ((35 - 5):El-Kh)	3.008	0.901	3.339	0.0200	*
	((35 - 5):WEl-G) - ((35 - 5):El-Kh)	2.797	0.885	3.161	0.0319	*
	((35 - 5):Togo) - ((35 - 5):Sudan)	-0.099	0.750	-0.132	1.0000	
	((35 - 5):WEl-G) - ((35 - 5):Sudan)	-0.310	0.731	-0.424	0.9994	
	((35 - 5):WEl-G) - ((35 - 5):Togo)	-0.211	0.800	-0.264	1.0000	
	((35.20 - 5):El-Kh)-((35.20-5):Cairo)	-1.921	0.720	-2.669	0.1048	
	((35.20 - 5):Sudan) - ((35.20 - 5):Cairo)	0.206	0.616	0.334	0.9999	
	((35.20 - 5):Togo) - ((35.20 - 5):Cairo)	0.937	0.686	1.367	0.7587	
	((35.20 - 5):WEl-G) - ((35.20 - 5):Cairo)	0.308	0.675	0.456	0.9990	
	((35.20 - 5):Sudan) - ((35.20 - 5):El-Kh)	2.127	0.694	3.063	0.0417	*
	((35.20 - 5):Togo) - ((35.20 - 5):El-Kh)	2.858	0.757	3.777	0.0056	**
	((35.20 - 5):WEl-G) - ((35.20 - 5):El-Kh)	2.229	0.747	2.982	0.0502	.
	((35.20 - 5):Togo) - ((35.20 - 5):Sudan)	0.732	0.659	1.110	0.8866	
	((35.20 - 5):WEl-G) - ((35.20 - 5):Sudan)	0.102	0.648	0.158	1.0000	
	((35.20 - 5):WEl-G) - ((35.20 -	-0.629	0.715	-0.881	0.9585	

	5):Togo)					
Comp. 2	((20 - 35):El-Kh) - ((20 - 35):Cairo)	1.462	0.810	1.804	0.7087	
	((20 - 35):Sudan) - ((20 - 35):Cairo)	0.361	0.660	0.547	0.9999	
	((20 - 35):Togo) - ((20 - 35):Cairo)	0.070	0.697	0.101	1.0000	
	((20 - 35):WEl-G) - ((20 - 35):Cairo)	-0.191	0.660	-0.290	1.0000	
	((20 - 35):WEl-A) - ((20 - 35):Cairo)	-0.834	0.679	-1.228	0.9600	
	((20 - 35):Sudan) - ((20 - 35):El-Kh)	-1.101	0.794	-1.386	0.9179	
	((20 - 35):Togo) - ((20 - 35):El-Kh)	-1.392	0.826	-1.685	0.7814	
	((20 - 35):WEl-G) - ((20 - 35):El-Kh)	-1.653	0.794	-2.081	0.5233	
	((20 - 35):WEl-A) - ((20 - 35):El-Kh)	-2.296	0.810	-2.833	0.1365	
	((20 - 35):Togo) - ((20 - 35):Sudan)	-0.291	0.679	-0.428	1.0000	
	((20 - 35):WEl-G) - ((20 - 35):Sudan)	-0.552	0.640	-0.862	0.9965	
	((20 - 35):WEl-A) - ((20 - 35):Sudan)	-1.194	0.660	-1.811	0.7029	
	((20 - 35):WEl-G) - ((20 - 35):Togo)	-0.261	0.679	-0.385	1.0000	
	((20 - 35):WEl-A) - ((20 - 35):Togo)	-0.904	0.697	-1.296	0.9444	
	((20 - 35):WEl-A) - ((20 - 35):WEl-G)	-0.643	0.660	-0.975	0.9914	
	((5 - 35):El-Kh) - ((5 - 35):Cairo)	0.791	0.826	0.957	0.9926	
	((5 - 35):Sudan) - ((5 - 35):Cairo)	1.503	0.679	2.214	0.4335	
	((5 - 35):Togo) - ((5 - 35):Cairo)	1.307	0.745	1.755	0.7384	
	((5 - 35):WEl-G) - ((5 - 35):Cairo)	2.462	0.727	3.385	0.0348	*
	((5 - 35):Sudan) - ((5 - 35):El-Kh)	0.712	0.794	0.896	0.9954	
	((5 - 35):Togo) - ((5 - 35):El-Kh)	0.516	0.851	0.606	0.9998	
	((5 - 35):WEl-G) - ((5 - 35):El-Kh)	1.671	0.836	1.998	0.5796	
	((5 - 35):Togo) - ((5 - 35):Sudan)	-0.196	0.709	-0.276	1.0000	
	((5 - 35):WEl-G) - ((5 - 35):Sudan)	0.959	0.691	1.388	0.9173	
	((5 - 35):WEl-G) - ((5 - 35):Togo)	1.155	0.756	1.528	0.8620	
	((5 - 20):El-Kh) - ((5 - 20):Cairo)	-0.671	0.756	-0.888	0.9957	
	((5 - 20):Sudan) - ((5 - 20):Cairo)	1.142	0.660	1.731	0.7540	
	((5 - 20):Togo) - ((5 - 20):Cairo)	1.237	0.727	1.700	0.7716	
	((5 - 20):WEl-G) - ((5 - 20):Cairo)	2.653	0.709	3.739	0.0124	*
	((5 - 20):Sudan) - ((5 - 20):El-Kh)	1.813	0.739	2.454	0.2918	
	((5 - 20):Togo) - ((5 - 20):El-Kh)	1.908	0.800	2.385	0.3288	
	((5 - 20):WEl-G) - ((5 - 20):El-Kh)	3.324	0.784	4.242	0.0026	**
	((5 - 20):Togo) - ((5 - 20):Sudan)	0.095	0.709	0.133	1.0000	
	((5 - 20):WEl-G) - ((5 - 20):Sudan)	1.511	0.691	2.186	0.4516	
	((5 - 20):WEl-G) - ((5 - 20):Togo)	1.416	0.756	1.873	0.6633	

Supplementary Table 4. The variance proportion of each component used in the principal coordinate analysis.

Variable	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5	Comp. 6
Cond (log)	-0.03	0.94	0.33	-0.03	0.01	0.00
LFW (log)	-0.48	0.03	-0.08	0.42	-0.11	-0.75
LDW (log)	-0.46	-0.09	0.27	0.57	-0.10	0.60
SFW (log)	-0.48	-0.05	0.04	-0.28	0.83	0.02
SDW (log)	-0.44	-0.17	0.40	-0.61	-0.49	-0.05
LWC	-0.36	0.26	-0.80	-0.18	-0.23	0.26
Prop. Var	0.70	0.18	0.10	0.02	0.01	0.00

Supplementary Table 5. Indicate the genotypes, VWC interaction and its effect on the significant production of leaf fresh weight among the genotypes according to the VWC in the soil.

Genot. VWC interaction	Estimate	*p*.value	Signif.
(El-Kharga - Cairo) :35	-1.197	0.7924	
(Sudan - Cairo) :35	1.847	0.0206	*
(Togo - Cairo) :35	-0.370	1.0000	
(Wadi El-Gemal - Cairo):35	0.749	0.9481	
(Wadi El-Alaqi - Cairo):35	0.981	0.7885	
(Sudan - El-Kharga):35	3.044	0.0005	***
(Togo - El-Kharga):35	0.827	0.9856	
(Wadi El-Gemal - El-Kharga):35	1.946	0.0945	.
(Wadi El-Alaqi - El-Kharga):35	2.179	0.0498	*
(Togo - Sudan):35	-2.217	0.0022	**
(Wadi El-Gemal - Sudan):35	-1.098	0.4630	
(Wadi El-Alaqi - Sudan):35	-0.865	0.8606	
(Wadi El-Gemal - Togo):35	1.119	0.5276	
(Wadi El-Alaqi - Togo):35	1.352	0.3125	
(Wadi El-Alaqi - Wadi El-Gemal):35	0.233	1.0000	
(El-Kharga - Cairo):20	-0.036	1.0000	
(Sudan - Cairo):20	1.047	0.5404	
(Togo - Cairo):20	0.491	0.9989	
(Wadi El-Gemal - Cairo):20	0.774	0.9007	
(Wadi El-Alaqi - Cairo):20	-0.133	1.0000	
(Sudan - El-Kharga):20	1.083	0.7062	
(Togo - El-Kharga):20	0.527	0.9994	
(Wadi El-Gemal - El-Kharga):20	0.809	0.9508	
(Wadi El-Alaqi - Togo):20	-0.624	0.9886	
(Wadi El-Alaqi - Wadi El-Gemal):20	-0.906	0.7502	
(El-Kharga - Cairo):5	1.304	0.4897	
(Sudan - Cairo):5	1.241	0.3615	

(Togo - Cairo):5	-0.877	0.9361	
(Wadi El-Gemal - Cairo):5	0.453	0.9999	
(Sudan - El-Kharga):5	-0.063	1.0000	
(Togo - El-Kharga):5	-2.181	0.0280	*
(Wadi El-Gemal - El-Kharga):5	-0.851	0.9696	
(Togo - Sudan):5	-2.118	0.0114	*
(Wadi El-Gemal - Sudan):5	-0.788	0.9600	
(Wadi El-Gemal - Togo):5	1.330	0.5658	

Chapter 5

Effect of water deficiency and recovery time on different genotypes of *Balanites aegyptiaca*

Abstract

Drought stress has a tremendous effect on plant development and production. With the decrease in water resources and the reduction of arable land in many areas around the world, there is a demand to find alternative plant species that could be cultivated in non-arable land for food supplies and bioenergy. *Balanites aegyptiaca* L. is considered as drought-tolerant tree belonging to the family of Balanitaceae, naturally distributed in Africa and Middle East. The species having a potential for biofuel production. This study aimed to examine and compare the morpho-physiological responses to water stress and the potential for recovery after withholding water stress among five different *B. aegyptiaca* genotypes collected from different regions. Different regimes of soil volumetric water content (VWC 25% as a control, VWC 15% as moderate and VWC 5% as a severe drought stress) were chosen to finally select the most drought-tolerant genotype under greenhouse conditions. Several growth parameters, stomata conductance, quantum efficiency and thermal imaging were analyzed to investigate and compare the drought impact and the capability for recovery among *B. aegyptiaca* genotypes. The results indicate that, at severe drought stress each genotype showed significant reduction in biomass parameters with increasing in root length and biomass, reduction in stomata conductance combined with a decrease in quantum efficiency and in the other side there was increasing in the leaf temperature under severe stress. Finally, we suggest that i) *Balanites* genotypes showed different morphological and physiological responses to cope with the water shortage and all genotypes were recovered after withholding the effect of water stress, ii) the reduction in growth parameters and the increasing in root biomass are remarkable factors explained the effect of severe water stress among *Balanites* sources, and iii) genotype El-Kharga showed higher drought tolerance compared to the other genotypes and showed the lowest magnitude of biomass reduction and early stomata closure as strategy of saving leaf water content under severe drought stress with high potential to recover after withholding the severe water stress.

Keywords: *Balanites aegyptiaca* L., growth parameters, stomata conductance, thermal imaging, water deficit, recovery.

Introduction

The reduction in water resources for agricultural use influences the food production in several regions around the world (FAO 2012). Agricultural drought is one of the main climatic conditions that takes place when the precipitation rate decreased under the normal level during a period of time that affect the availability of food supplies in dry regions (Dai 2011; Solh and Ginkel 2014) resulting in a decrease in crop productivity, approximately 28% of the world's soil surface is too dry for sufficient crop production (Bray et al. 2002; Ambrosone et al. 2013). Chaves et al. (2003) indicated that the plant response to drought stress through avoidance and tolerance mechanism enabled a high developmental flexibility when the drought occurred to overcome the stress and decrease the water loss by stomata closure, while under severe stress the plant cope the stress through changes in growth parameters, produce and accumulate high concentration of compatible solutes in living cells. The reduction in plant water content decrease the turgor pressure in guard cells that control the pore size in stomata and resulting in stomata closure followed by decrease in transpiration rate, net CO_2 assimilations, reduction in photosynthesis efficiency and finally in a decrease of growth and gain of biomass (Song et al. 2014).

Balanites aegyptiaca (Desert date) belonging to the family of Balanitaceae is a multi-purpose xerophytic tree widely distributed across several regions in Africa. It is a spiny, evergreen and deciduous shrub or tree up to l0 m tall and exhibits different characters to adapt water stress (Sands 2001). Due to the high oil content, up to 46.7%, in the kernels the tree was successfully used for biodiesel production (Chapagain et al. 2009). The plant parts very rich in several types of saponins that were used as foaming agents in the detergent production besides the production of oral contraceptives also the plant showed pharmacological activity as antibacterial, anti-leishmanial and anti-cancer therapies (El-Tahir et al. 1998; Mohamed et al. 2000; Chapagain et al. 2008). The plant is highly tolerant to drought stress where its deep tap root system enabled the plant to survive and distribute across different arid and non arid regions in Africa (Hall et al. 1991). Their leaf features consider one of the major adaptive characters for drought tolerance of this species (Elfeel et al. 2007). Also the thick layers of trichomes on the leaves of *B. aegyptiaca* protect the plant from the high irradiance through reflecting excess of light in the arid habitat (Radwan 2007). For their distinct features to adapt water shortage *B. aegyptiaca* could be recommended for cultivation programs in arid and semi-arid habitats in Africa.

Based on the reduction in growth parameters, early stomata closure and consuming high concentration of total ascorbic acid to cope severe water stress, the highest three drought tolerance genotypes (Chapter 4) El-Kharga, Sudan and Cairo were used in this study with another two genotypes from Medina and Yemen for the following objectives i) to investigate the morpho-physiological responses of *B. aegyptiaca* to water stress through different regimes of soil water content and another soil type, ii) to compare the plant´s responses to water stress and recovery time among different genotypes of *B. aegyptiaca* that were collected from different region and finally, iii) select the most drought tolerant genotypes that have the capability to adapt the water stress and fast recovers after stress to finally give a recommendation about the most drought-tolerant genotype under greenhouse conditions that could be used for further cultivation and selection in arid and semi-arid environments.

Material and methods

Plant material and experimental conditions

The most drought tolerant genotypes (El-Kharga, Cairo and Sudan) from the last drought experiment (Chapter 4) besides two other genotypes from Medina and Yemen (Table 1) were used to study the plant response to water stress and recovery. So cuttings with 5-7 cm highest were taken from each mother plants and propagated in pots filled with CL T soil type (Einheitserde, Sinntal-Altengronau, Germany) a soil with 30% clay content. The plants were covered by plastic boxes for 4 weeks and then plants with a good root formation were transferred to 9 cm pots length filled with 700 cm^3 soil. Plants were irrigated every two days with normal water and once per week with water containing 0.25% Wuxal Top N fertilizer consist of 120 g kg^{-1} nitrogen, 40 g kg^{-1} phosphorus pentoxide, 60 g kg^{-1} potassium oxide, 0.1 g kg^{-1} boron, 0.04 g kg^{-1} copper, 0.2 g kg^{-1} iron, 0.01 g kg^{-1} molybdenum, 0.04 g kg^{-1} zink and 0.12 g kg^{-1} manganese (Aglukon, Düsseldorf, Germany). Propagation of these cuttings was done in the greenhouse for 8 weeks at a temperature of 22°C and 12 h light/dark condition. If the outside light conditions did not enclose appropriate light intensity inside the greenhouse, extra light was supplied to obtain a constant quantum fluence rate of approximately 350 µmol $m^{-2}s^{-1}$ (sodium vapor lamps, SON-T Agro 400, Philips, Amsterdam, Netherlands).

After 8 weeks of propagation under green house condition the drought stress experiment was conducted for four weeks followed by two weeks recovery and the plants were divided into three different groups dependent on soil volumetric water content (VWC) using Fieldscout device based on time domain reflectometry (TDR) (Spectrum Technologies, Plainfield, USA). Each treatment comprises ten plants per genotype. *Balanites* sources in their habitat grow in a broad range of soil from deep sand to clay loam and heavy clay. For the experiment described in Chapter 4 *Balanites* sources were grown in clay loam substrate. To check the effect of soil substrate on growth and drought tolerant in this experiment another soil substrate was used from clay-loam: sand (2:1) substrate as soil substrate. According to the manual of the Fieldscout instrument, clay-loam: sand (2:1) has a maximum water holding capacity at 25% which was considered as a control and a permanent wilting point at 15% VWC where water content less than this value will lead to permanent damage, so it considered here as a moderate stress. To investigate the effect of severe drought stress on the plants 5% VWC has been used. During the drought experiment plants were irrigated every 2

days, the water amount was calculated based on the water deficit calculation (D) of the Fieldscout; one mm equals 8 ml water for each pot based on the truncated cones formula.

Table 1. Genotypes, origin and coordinates of the seed collection used in this study.

Genotypes	Locations	Coordinates
El-Kharga	Park of Faculty of Sciences, University of South Valley, Aswan, Egypt	24°5'20.177"N; 32°53'59.385"E
Cairo	Giza, Zoo, Egypt	30°1'27.158"N; 31°12'49.731"E
Sudan	Sudan	not known
Medina	Saudi Arabia	not known
Yemen	Tohama, Yemen	not known

Plant growth analysis

Leaf, stem and root dry weight, and leaf, stems and root water content and also leaf area, root length and shoot to root ratio were examined after 4 weeks drought experiment and after 2 weeks recovery period. The leaf number was counted manually every week during 4 weeks drought stress and 2 weeks recovery. In addition the changes in plant morphology under severe drought stress were monitored. The plant height was determined by measuring the longest shoot every week during 4 weeks drought stress and 2 weeks recovery also the leaf length multiplied with leaf width was calculated every week. Each treatment started with 10 plants from each genotypes, where five plants were harvested after drought experiment and five plants were fixed and used to measure leaf number, plant height and leaf length*width that were harvested after two weeks recovery. The above ground plant material was harvested and weighed for fresh weight examinations (FW), and then samples were oven-dried at 80°C for 48 h and weighed for dry weight determination (DW). Leaf, stem and root water content was calculated according to this equation LWC= (FW-DW)/FW * 100. The number of plants per each treatment was 5.

Stomata conductance, chlorophyll fluorescence and thermal imaging measurements

Young but fully expanded leaves were used for chlorophyll fluorescence measurements (Junior-PAM, Heinz Walz GmbH, Effeltrich, Germany) and determination of stomata conductance using a porometer (AP4 Delta-T Devices, Burwell, UK) as non-destructive methods. After the 4 weeks drought experiment and 2 weeks recovery, at 9:00 in the morning five leaves per treatment from each genotype were dark-adapted for 30 min by specific clips (Heinz Walz GmbH) and light curves in ten-second frequency were used for chlorophyll fluorescence measurements. From these measurements quantum efficiency (Fv/Fm) was recorded (as a non-destructive method depends on the plant interaction with light). Then the same leaves were used to examine the stomata conductance (mmol m^{-2} s^{-1}) using a porometer AP4 (Delta-T Devices). The number of plants per each treatment was 5.

Further analysis were performed on the same leaves through thermal imaging investigation (as a non-destructive method depends on the plant interaction with light) was conducted in the afternoon with the camera T360 (FLIR Systems, Wilsonville, USA) according to Grant et al. (2006) and Guretzki and Papenbrock (2013). The camera was switched on at least 30 min before taking the first picture. Fully expanded and youngest leaves were used. The dry (T_{dry}) was performed in one leaflet of the paired leaflets of a leaf. The other one was considered as wet (T_{wet}) reference. The wet reference leaflets were wetted with water on both sides while dry reference leaflets were covered with petroleum jelly. The terminal leaflet of the same leaf was used as sample (T_{leaf}). T_{dry} was examined at least 5 min after the covering with petroleum jelly, T_{wet} immediately after using the water. The analysis of the thermal imaging pictures were conducted using the software FLIR Quick Report 1.2 SP2 (FLIR Systems). For each image, the object parameters were set to emissivity 0.95, atmospheric temperature 23°C, reflected apparent temperature 23°C, relative humidity 45%/50% and distance 0.2 m. Based on the results the index I_G was calculated through the formula $I_G = (T_{dry} - T_{leaf})/ (T_{leaf} - T_{wet})$.

Statistical analysis

To analyze significance of effects for single variables, 3-factorial ANOVA were used for the 18 single variables, to sort out variables that show a similar response in week 4 and 6 from others that show an interaction effect of VWC every week (overall reaction to drought stress is different after recovery). Another effect through the interaction of genotype with VWC and week (the differences in the drought stress effect among genotypes showed change in plant

response between drought experiment (week 4) and recovery (week 6). For detailed investigation of the genotype-VWC interactions, data were split according to genotypes. All pair-wise comparisons (Tukey test) of the VWC levels were performed for each genotype separately. Data were split according to the levels of VWC and all pair-wise comparisons among genotypes were tested (Tukey test) on each VWC level separately (details in supplementary material). Further, interaction contrasts (Gabriel et al. 1973) were used to compare the magnitude of differences between VWC levels among the genotypes (supplementary material). All computations were performed in R-3.1.0 (R Core Team 2014), using the package multcomp (Hothorn et al. 2008) for the Tukey test and compact letter display, the packages nlme (Pinheiro and Bates 2000) and lme4 (Bates et al. 2014) for GLS and random effect models, as well as the packages ggplot2 (Wickham 2009) and ade4 (Dray and Dufour 2007) for the figures.

Results

Effects of water stress on growth parameters

Stem length; Under control condition VWC 25% and moderate stress VWC 15% there was continually increasing stem lengths from the first day and during 4 weeks drought experiment and after recovery time while genotype El-Kharga showed the highest stem length in the first day (18.1 cm) at 25% VWC which increase during 4 weeks drought stress (31.4 cm) and (33.4 cm) after 2 weeks recovery. Under severe stress VWC 5% the increasing in stem length almost stopped between the second and third week (Fig. 1).

Genotype Cairo showed a steep decrease in stem length between the second and third week until the fourth week while three plants died under severe drought stress so another three plants from the same treatment were used instead of the died plants. For Cairo, at the third week there was a significantly lower increasing in stem length under severe stress VWC 5% when compared to the increasing in stem length through control treatment VWC 25% this significance was persisted and slightly increased until week 6 after recovery. Also genotype Cairo at week 4 and thereafter, there was a significantly lower increasing in stem length through VWC 15% treatments as compared to control treatment VWC 25%. Genotype El-Kharga showed a significant decrease in stem length under severe stress VWC 5% compared to well-watered condition VWC 25% this stress was starting the second week and persisting

until week 6 after recovery. The slightly reduced and consistent increasing in stem length in VWC 15% compared to VWC 25% is not significant. For Medina genotype there was no significant decrease in stem length at any week neither in 5% compared to VWC 25% nor in VWC 15% compared to VWC 25% while there was steep reduction occurred during weeks (3,4 , 5 and 6) in VWC 5% compared to VWC 25%. For Sudan and Yemen there was a significant decrease in stem length under severe stress at VWC 5% compared to control treatments VWC 25% during weeks (4, 5 and 6). Also in genotype Sudan the stem length at moderate stress was higher than the control treatment during the four weeks drought experiment and after two weeks recovery.

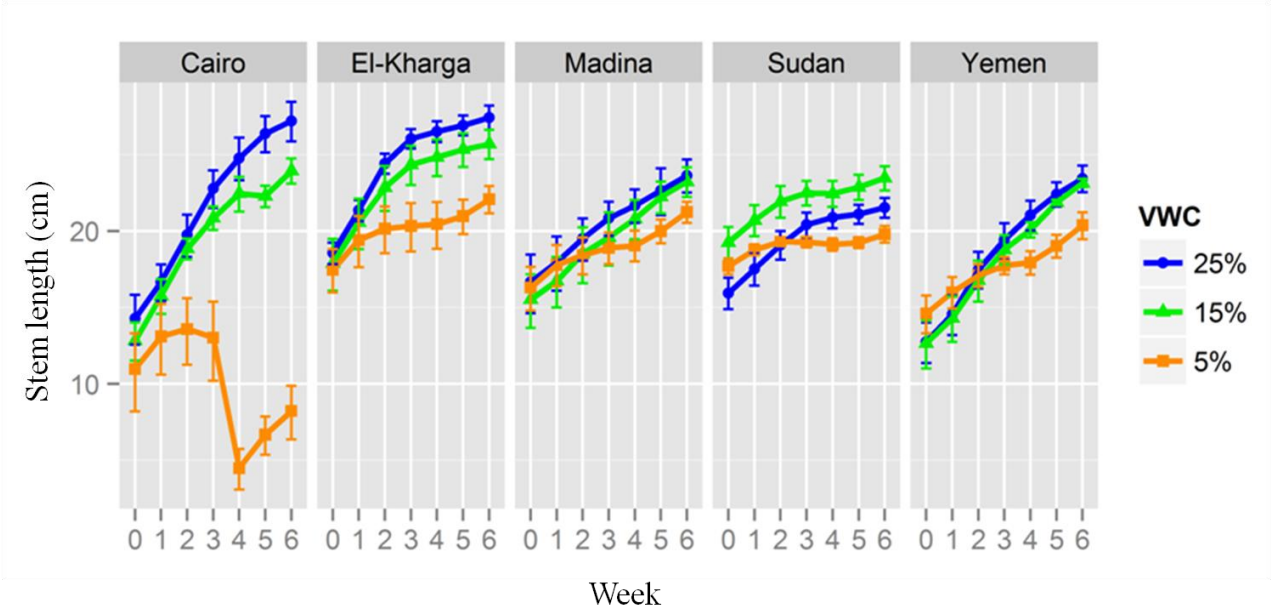

Figure 1. Effect of water stress on stem length during 4 weeks drought stress (VWC 25%, VWC 15% and VWC5%), followed by 2 weeks recovery under well-watered conditions (VWC 25%) of five *B. aegyptiaca* genotypes. Values are means (± S.E.), n= 5.

Leaf number; under control treatment and moderate stress the leaf number was significantly increased (Fig. 2) among the genotypes during four weeks drought experiment and after two weeks recovery. Under severe stress at VWC 5% all the genotypes showed a reduction in leaf number starting from the second week and after withholding the water stress effect the leaf number at VWC 5% treatment was slightly increase from the fifth week. Genotype Yemen showed the highest number of leaves (178 leaves) after 4 weeks drought stress under control treatment. The lowest reduction in leaf number obtained from genotype Cairo (7 leaves) after four weeks severe stress at VWC 5% and slowly increased to be (18 leaves) after two weeks recovery. There were differences of increments in leaf number between VWC-levels among all genotypes. From the fifth week all genotypes showed a significantly slight increase in leaf

number at severe stress VWC 5% compared to VWC 25%. Under severe stress conditions genotype Cairo showed a significant lower increase after recovery until week 6 also genotype El-Kharga showed a decrease started in the second week and slowly increased after withholding the water stress.

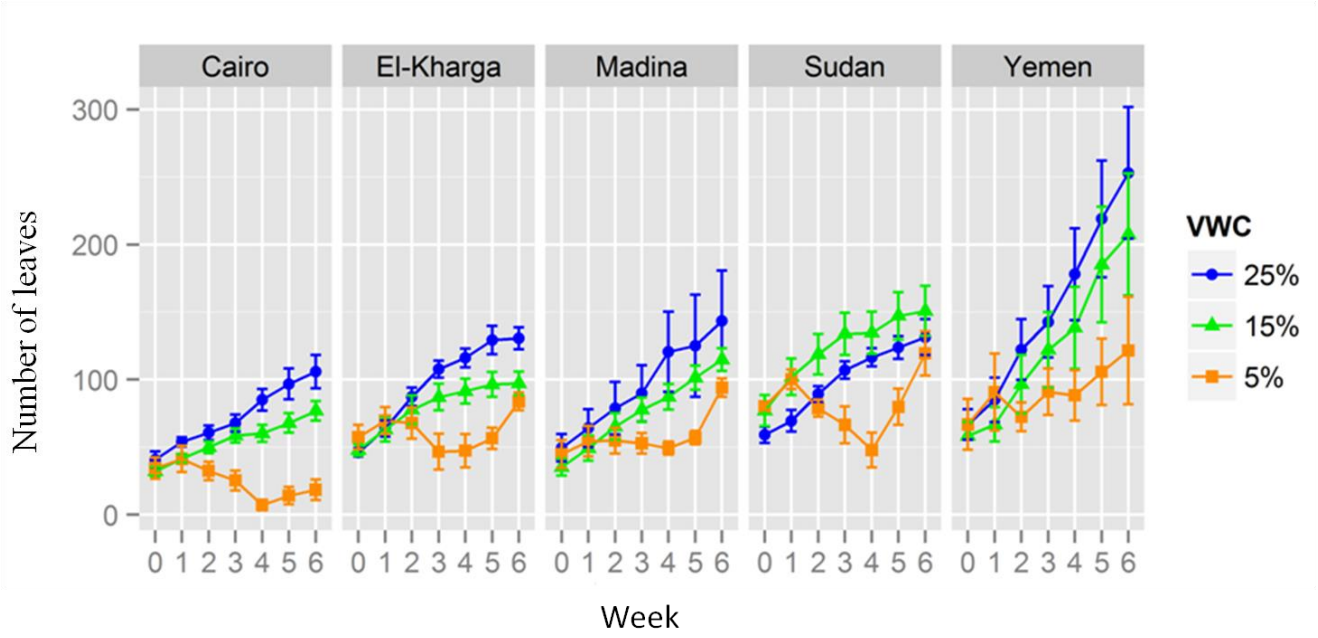

Figure 2. Effect of water stress on leaf number during 4 weeks drought stress (VWC 25%, VWC 15% and VWC5%), followed by 2 weeks recovery under well-watered conditions (VWC 25%) of five *B. aegyptiaca* genotypes. Values are means (± S.E.), n= 5.

Leaf length*width; there were differences in the leaf length*width among the genotypes where genotype Cairo showed the highest value at the sixth week under control treatment (5.7 cm^2) and genotype Yemen showed the lowest leaf length*width (1.06 cm^2) in the sixth week under moderate stress condition. Only genotype El-Kharga and Sudan showed a decrease in leaf length*width under severe stress in the 4th week (Fig. 3a).

Leaf and stem dry mass; under control conditon all genotyes showed the highest production of leaf and stem dry weight (Fig. 3b and 3c) where genotype Cairo showed the highest leaf dry weight (0.7 ± 0.1 g) among the genotype at moderate stress VWC 15% after 4 weeks drought stress where genotype Cairo showed the highest dry weight after recovery (1.9 ± 0.2 g) at VWC 25%. Under severe stress all the genotypes showed significant reduction in leaf dry weight after 4 weeks drought stress which were significantly increased after 2 weeks recovery where the lowest dry weight was obtained from genotype Cairo (0.13 ± 0.1 g) after

2 weeks recovery. After 2 weeks recovery there were highly significant differences between El-Kharga, Medina, Sudan, and Yemen with genotype Cairo in the differences of leaf dry weight at VWC 5% and VWC 25%. Also significant differences were recorded between genotypes Sudan and Cairo in the differences of stem dry weight at VWC 15% and VWC 25%.

Root dry weight; during 4 weeks drought stress *B. aegyptiaca* genotypes showed significant increasing in root dry weight at moderate stress through all genotype compared to VWC 25% and VWC 5% that were lower during drought conditions.

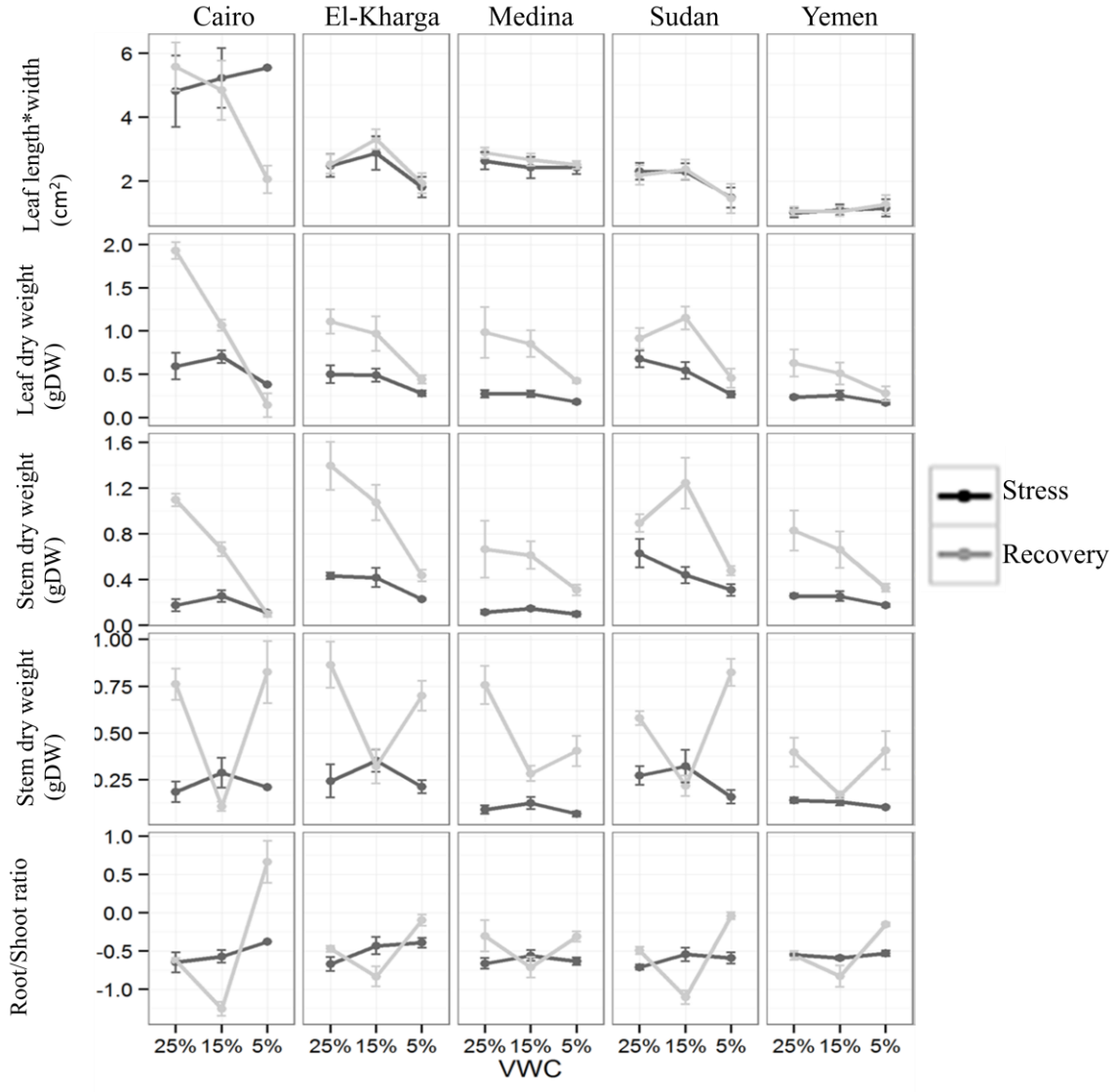

Figure 3. Effect of water stress on (a) leaf area, (b) leaf dry weight, (c) stem dry weight, (d) root dry weight and (e) root/shoot ratio, during 4 weeks drought stress (VWC 25%, VWC 15% and VWC 5%), followed by 2 weeks recovery under well-watered conditions (VWC 25%) for five *B. aegyptiaca* genotypes. Values are means (± S.E.), n =5.

After 2 weeks recovery there were significant increase in all genotypes under VWC 25% and VWC 5% compare to moderate stress VWC 15% (Fig. 3d). After two weeks recovery there were a significant differences between genotype Sudan and Medina in the differences in root dry weight at VWC 5% and VWC 15%. Also at the differences in root dry weight at VWC 5% and VWC 25% for the same genotypes. In General all the genotypes were showed the highest root dry weight at moderate stress VWC 20% during four weeks drought experiment, on the other hand all the genotypes were recorded the lowest root dry weight at moderate stress VWC 20% after two weeks recovery.

Shoot/Root ratio; during 4 weeks drought stress the shoot/root ratio increased (Fig. 3e) significantly among the genotypes by increasing the water stress and genotype Cairo showed the highest ratio (0.45 ± 0.04 g) at VWC 5%. After 2 weeks recovery all the genotypes under moderate stress showed significantly the lowest ratio and genotype Cairo showed the highest ratio (4.1 ± 2.5 g). In genotype Cairo the differences in shoot/root ratio between VWC 5% and VWC 25% were significantly different compared to the differences at the same treatment in genotypes Medina, Sudan, El-Kharga and Yemen. Also the differences in shoot/root ratio at VWC 5% and VWC 15% in genotype Cairo were highly significantly different compared to the differences at the same treatment in genotypes Medina, Sudan, El-Kharga and Yemen.

Leaf and stem water content were significantly different among the genotypes and decreased by increasing water stress where genotype Cairo showed significant steep reduction in leaf water content (51%) under severe drought stress VWC 5% during 4 weeks drought stress. While after 2 weeks recovery a significant increasing in leaf and stem water content were recorded and the highest values obtained from the genotypes was under VWC 5% (Fig. 4a and 4b). The root water content was significantly different under the three level of soil water content after two weeks recovery in each genotype and the highest values were obtained at moderate stress of VWC 15% after the four weeks drought experiment and after two weeks recovery only genotype Cairo showed the highest root water content at VWC 5% after four weeks drought experiment (Fig. 4c).

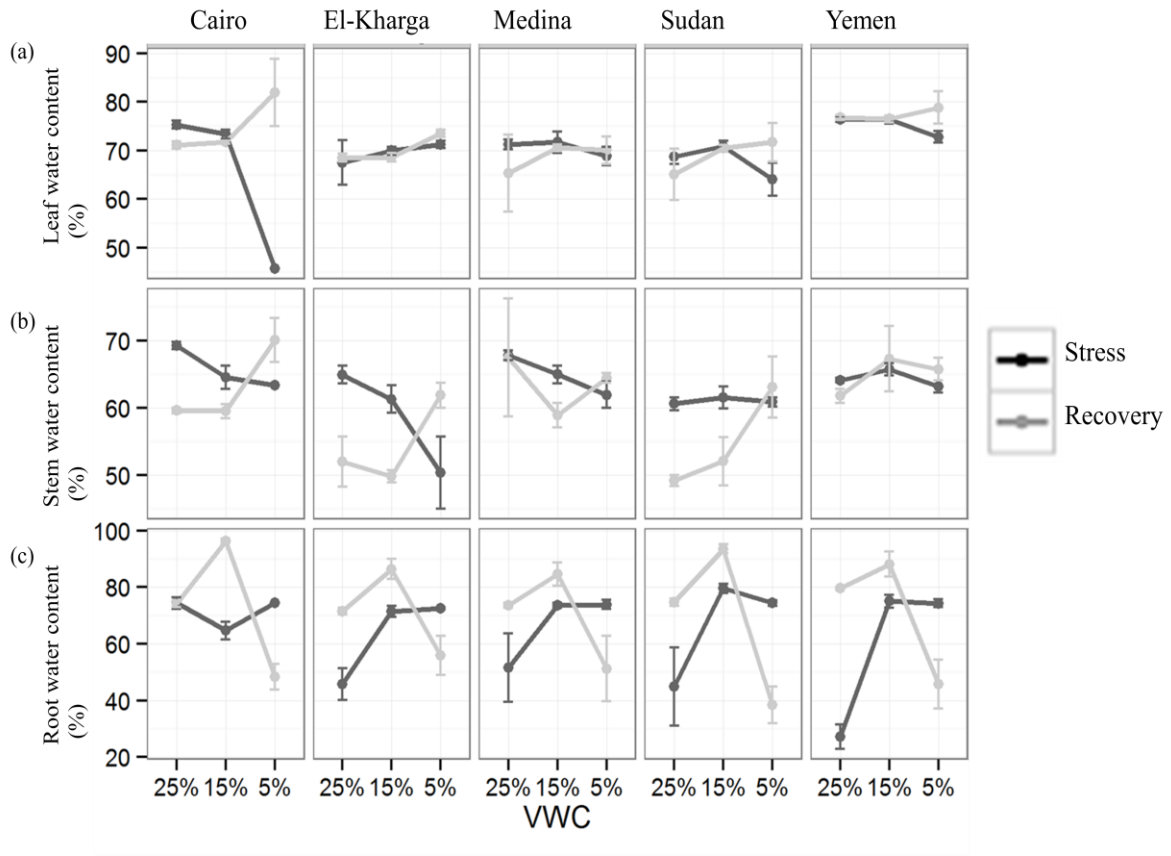

Figure 4. Effect of water stress on (a) leaf water content, (b) stem water content, (c) root water content, during 4 weeks drought stress (VWC 25%, VWC 15% and VWC5%). Followed by 2 weeks recovery under well-water conditions (VWC 25%) for five *B. aegyptiaca* genotypes. Values are means (± S.E.), n =5.

Determination of stomata conductance

The reduction in stomata conductance was highly significant with each genotype under different soil water content during 4 weeks drought stress and after 2 weeks of recovery (Fig. 5a); also there are clear significant differences among genotypes dependent on the genotype-VWC interaction at control and moderate drought stress treatments. Genotype Cairo showed the highest values (282 mmol m^{-2} s^{-1}) after 4 weeks drought stress at VWC 5% and the lowest values obtained from genotype Sudan (31 mmol m^{-2} s^{-1}) at VWC 5% (Fig. 5a). after withholding the water stress the stomata conductance at VWC 5% treatments was significantly increased in genotypes El-Kharga, Medina, Sudan and Yemen, and the highest values were obtained from genotype Sudan (186 mmol m^{-2} s^{-1}) with VWC 5% treatment. The differences in stomata conductance at VWC 5% and VWC 25% in genotype Sudan were significantly different compared to the differences at the same treatment in genotypes Medina and Yemen. Also the differences in stomata conductance at VWC 5% and VWC 15% in genotype Cairo were significantly different compared to the differences at the same treatment in genotypes Medina and Yemen.

Quantum efficiency (Fv/Fm); during 4 weeks drought stress the decreasing in stomata conductance was correlated with a decrease in quantum efficiency (Fv/Fm) in genotypes Medina, Sudan and Yemen (Fig. 5b). There were significant differences among the genotypes at the three level of volumetric water content where the highest value during 4 weeks drought stress obtained under control condition VWC 25% from genotype Cairo (0.836) while the lowest value was obtained for genotype Sudan (0.715) at severe drought stress VWC 5%. After 2 weeks recovery the quantum efficiency (Fv/Fm) were increased for all genotypes under severe stress and genotype Cairo showed the highest value (0.839) at moderate stress VWC 15%.

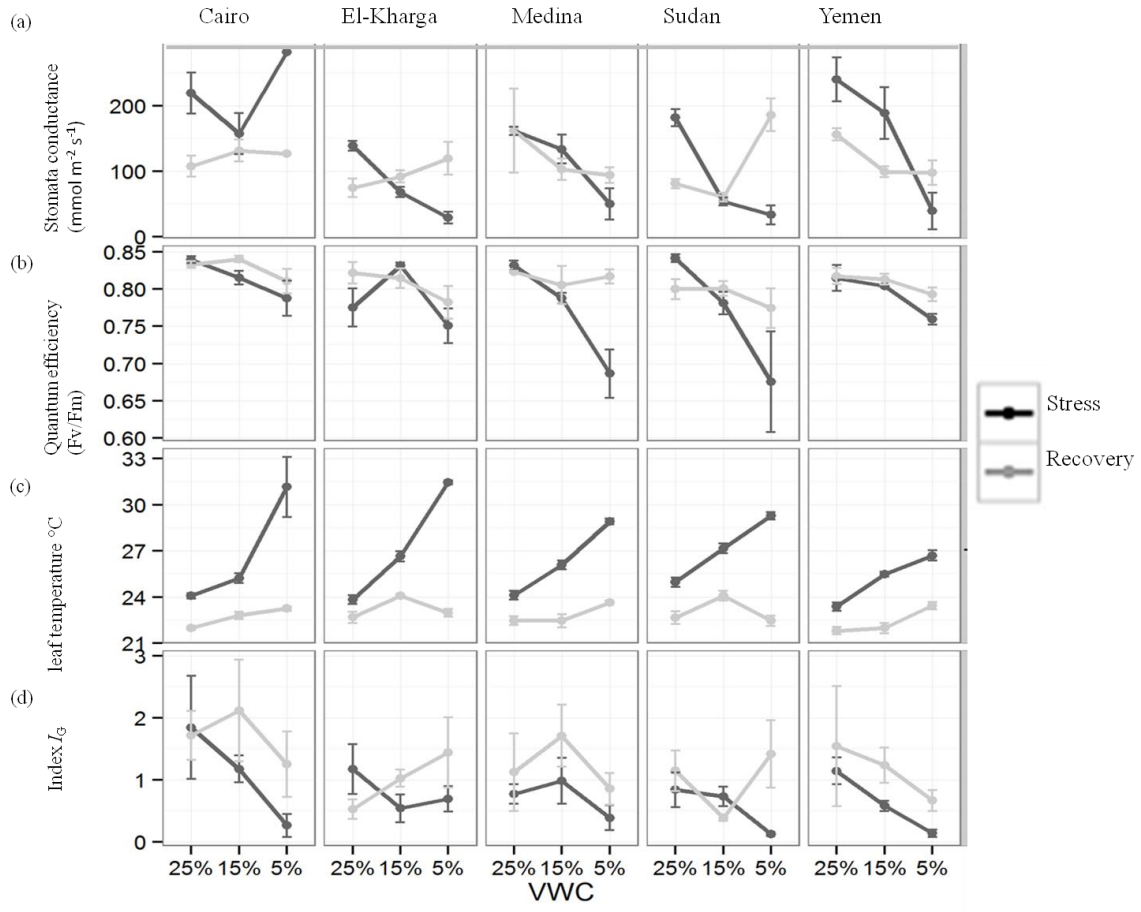

Figure 5. Effect of water stress on (a) leaf conductance, (b) quantum efficiency (Fv/Fm), (c) leaf temperature, (d) IG index = (T_{dry} - T_{leaf})/ (T_{leaf} - T_{wet}), during 4 weeks drought stress (VWC 25%, VWC 15% and VWC 5%). followed by 2 weeks recovery under well-water conditions (VWC 25%) through five *B. aegyptiaca* genotypes. Values are means (± S.E.), n = 5.

Thermal Imaging; during 4 weeks drought stress the reduction in stomata conductance and quantum efficiency (Fv/Fm) by increasing water stress was combined with increasing in leaf temperature among all genotypes. There were significant differences in leaf temperature among the genotypes at different volumetric water content. The highest leaf temperature

under drought conditions was obtained from genotype El-Kharga (31.4°C) at severe drought stress VWC 5% while the lowest temperature was obtained from genotype Yemen (23.3°C) under control treatment VWC 25% (Fig. 5c). After 2 weeks recovery there was a significant decrease in leaf temperature among all genotypes while the values were between (21.8: 24.08°C). The increase in leaf temperature was combined with a decrease in IG index among all the genotypes through different VWC during drought conditions (Fig. 5d).

Generally, in week 4 during drought stress conditions, the leaf temperatures were generally higher (also under VWC 25%), but the increase in leaf temperature from 25% to 15% to VWC 5% is steep in week 4, and not very steep or inconsistent in week 6, this is considered as the Week-VWC-interaction, that were also significant. There were significant differences at the level of VWC-genotype-week interactions, where in week 4 the increase of leaf temperature were steep from 25% to 15% to VWC 5% for genotypes Cairo and El-Kharga and less steep in genotypes Yemen, Medina and Sudan while in week 6 the steepness of increase from 25% to 15% to VWC 5% was low or inconsistent.

Discussion

The goal of this study was to examine and compare the effects of water deficit during 4 weeks drought stress followed by 2 weeks recovery among different genotypes of *B. aegyptiaca* to recommend the most drought-tolerant genotype for cultivation in arid and semi-arid areas for biodiesel production. The results indicate that *B. aegyptiaca* genotypes differed in their morphological and physiological responses to cope with the water shortage during 4 weeks drought conditions while all the genotypes were recovered under well-water condition. Several growth and physiological parameters were examined to compare and explain the effect of water deficit among the genotypes where these parameters were showed significant reduction under severe stress compared to the control treatment.

We consider VWC 25% as control treatment and VWC 15% as moderate drought and VWC 5% as severe drought stress. *Balanites aegyptiaca* genotypes under well-watered conditions produced the highest amount of above soil biomass such as leaf and stem dry weight. Under drought stress conditions there were slight decrease in plant water content in leaves and stems and a decrease in the leaf number and slow increase in stem length that started between the second and third week. According to Chaves et al. (2003) the plant adapt to severe water stress through an avoidance mechanism to stop the tissue desiccation: the plant decreases the

water loss and increases the water absorption and leaf shedding through the older leaves. This reaction is followed by tolerance mechanisms such as osmotic adjustment and production of high levels of solute concentrations in living cells.

The negative influence of drought stress is explained by the reduction in growth parameters by decreasing the frequency of cell division and elongation (Farooq et al. 2009). It was reported that under water stress conditions there was a reduction in growth parameters such as plant height, leaf and stem dry weight and leaf area in the Fabaceae *Vicia faba* (Zabawi and Dennett 2010). A reduction in plant height was also recorded in *Vicia radiata* (Ranawake et al. 2011).

Balanites aegyptiaca genotypes showed higher biomass when cultivated in clay-loam substrate (Chapter 4) compared to the clay-loam mixed with sand in this experiment. The effect of soil type was previously reported by Elfeel et al. (2009). They indicated a wide significant impact of the soil type on the growth parameters and germination of *B. aegyptiaca* seed sources collected from 11 provenances. The root length and biomass had important role in the response of *Balanites* sources to water stress under moderate stress VWC 20%. The root architecture of seedlings of *Balanites aegyptiaca* (L.) Del. *Acacia tortilis* (Forsk.) and *Zizyphus mauritiana* Lam. was investigated under water stress and the results indicated that the total density of the roots of *B. aegyptiaca* is very important to overcome water stress. The rooting depth of *A. tortilis* and *B. aegyptiaca* are different from that of *Z. mauritiana* where *B. aegyptiaca* showed the larger total root length (Logbo et al. 2013).

Balanites aegyptiaca showed a reduction in stomata conductance as avoidance mechanism under severe drought stress among all the genotypes. This decrease in stomata conductance was combined with an increase in leaf temperature resulting in a decrease in quantum efficiency. These non-invasive techniques, such as measurements of stomata conductance and infrared thermometry, were already used to examine the impact of drought stress. Jones (2007) indicated that both methods are sufficient for the investigation of the drought impact through several genotypes. This method enables the investigation of stomata response under water limitations condition. Stomata closure is considered both, as short and long-term plant response to drought stress (Chaves et al. 2003). Arve et al. (2009) reported that stomata closure takes place when the turgor pressure on the guard cells decreased. That is also associated with the ABA signal that affects the stomata closure, causes a decrease in CO_2 uptake, transpiration and photosynthesis, and in addition a decrease in the absorption of nutrients and water. Grant et al. (2006) reported that there was a correlation between stomata

conductance and leaf temperature when the impact of water stress was examined on *Phaseolus vulgaris*.

The small leaf area in genotype Yemen is a distinct feature to adapt to drought stress. The early stomata closure in genotype El-Kharga enabled the plants to overcome the stress by decreasing the losses in plant water content. Thus genotype El-Kharga can be considered as the most drought-tolerant genotypes in this investigation. Otherwise, genotype Cairo was recorded as high stress-tolerant in a previous study (Chapter 4) but in this study Cairo showed the lowest tolerance for drought stress compared to the other genotypes under investigation. It was reported that genotype Cairo had several characters such as large leaf area that increase the transpiration rate in this genotype and also no early stomata closure was reported in this study under severe drought stress resulting in high losses in plant water content. Therefore, we suggest that the soil type plays an important role for the drought tolerance of this genotype. When the plants were grown in high clay soil (Chapter 4) genotype Cairo showed the highest tolerance response to drought stress. Otherwise when grown in clay sandy loam soil genotype Cairo showed the lowest tolerance to drought stress.

Conclusion

In this study the morpho-phosyolgical response to water stress through five different genotypes of *B. aegyptiaca* were investigated during four weeks drought stress followed by two weeks recovery. Under severe drought stress, each genotype showed significant reduction in growth paramters such as leaf and stem dry weight, leaf area, leaf and stem water content beside the leaf number and shoot length, also non-invasive techniques to measure stomata conductance, leaf temperature and quantum efficiency were used. It was reported that the reduction in growth paramters started between the second and the third week among all genotypes. While after 2 weeks recovery all the genotypes showed incresing in growth and physiological paramters. Based on this study several methods could be recommended to examine the effect of water stress in *B. aegyptiaca* such as root related parameters (root length, root water content and root dry weight), leaf temperature, leaf conductance beside the leaf number. Genotypes El-Kharga showed the highest drought tolerance response to cope with drought stress and can be first recommended for further field tests and maybe finally for the cultivation in arid and non-arid regions as a source for biodiesel production.

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Chapter 6

Establishment of an *in vitro* propagation and transformation system of *Balanites aegyptiaca*

Abstract

Balanites aegyptiaca is xerophytic tree species belonging to the family of Balanitaceae and distributed in the tropical and arid lands in North and West of Africa and West of Asia. It is considered as a drought-tolerant plant species and sensitive to salt stress. The tree contains many of secondary metabolites and high percentage of oil in the kernels that was used in biodiesel production, the plant is propagated through vegetative cuttings and seeds which have several limitations. This study aimed to establish an *in vitro* propagation system in two *B. aegyptiaca* provenances (El-Kharga and Wadi El-Alaqi) from nodal and cotyledon explants. The explants were placed on MS medium supplemented with different concentration of cytokinins (BA and TDZ) for shoot induction. Also three different strains of *Agrobacterium tumefaciens* (GV3101, EHA 105 and LBA 4404) harbouring the plasmid of pCAMBIA2301 which contains GUS and *nptII* gene were used to establish first steps towards a transformation system in *B. aegyptiaca* after that the selected *A. tumefaciens* strain was used to introduce the binary vector pBinAR contains (ERD10) to produce salt tolerant *B. aegyptiaca*. The results of regeneration showed that there were no significant differences between two provenances at the level of plant growth regulator concentrations. BA was significantly more effective in shoot induction from nodal explants at 8.8 µM BA where each provenance had different optimum concentration for highest shoot length and number of shoots per explants. TDZ has negative effect on shoot length but was more efficient in number of shoots produced per cotyledon explants. The results of transformation indicate that *A. tumefaciens* GV3101 strain showed the highest survival rate and highest number of explants, positive in the GUS assay and PCR. The *A. tumefaciens* GV3101 strain harbouring binary vector pBinAR contains (ERD10) was used for further transformation experiments of *B. aegyptiaca* to produce salt-tolerant *B. aegyptiaca.* The presences of ERD10 and nptII gene were detected by PCR in the transformed *B. aegyptiaca* plants.

Keywords: *B. aegyptiaca*, regeneration, *A. tumefaciens*, ERD10, salt stress

Introduction

Balanites aegyptiaca belongs to the family of Balanitaceae. The xerophytic tree species is distributed in the tropical and arid lands in Northern Africa, West Asia and Arabia (Hall and Walker 1991). According to the US National Research Council (NRC) report, the species grows naturally in one of the most drought-affected regions in the world. It is considered among the 24 high priority lost crops in Africa providing needs of different food supplies besides medicinal compounds and fuel wood for the people living in these regions; nevertheless the plant is neglected in the scientific communities (NRC 2008).

Balanites aegyptiaca is considered as a drought-tolerant plant species (Hall and Walker 1991) and multi-purpose tree. The oil content in the kernel is approximately 46.7% and has successfully been tested for biodiesel production (Chapagain et al. 2009). Saponins that are used as foaming agents in the detergent production were extracted from kernels and several parts of the plants which also revealed activity in anti-cancer therapies (Mohamed et al. 2002; Chapagain and Wiesmann 2008). Various parts of *B. aegyptiaca* contain beneficial secondary metabolites in roots and fruits that were used as antibacterial and anti-leishmanials for medical treatment and the production of oral contraceptives (El-Tahir et al. 1998).

Genetic improvements of woody tree species are constrained by several factors such as the large size and long life cycle, self incompatibility and high level of heterozygosity. These limitations could be bypassed through biotechnological techniques like *in vitro* multiplication and gene transfer (Merkle and Dean 2000; Peña and Séguin 2001) providing many advantages such as large and fast scale propagation of elite genotypes in a short time with low cost and small space, disease-free plant production, season–independent multiplication besides the preservation and easy exchange of genetic resources (Rathore et al. 2004; Gupta et al. 2014).

The conventional propagation of *B. aegyptiaca* is done through vegetative cuttings, seeds and root suckers but these methods were not efficient and season-reliant, slow growing and required large space, besides that the poor germination rate of seeds and age dependency of root suckers disable the possibility of mass multiplication to produce large numbers of planting stocks (Anis et al. 2010). Also low survival rates and poor root induction from vegetative cuttings were observed. Our data showed that the root induction from vegetative cutting was less than 30% and season-dependent (data not shown).

Several attempts were done for *in vitro* propagation of *B. aegyptiaca* by producing somatic embryos through root explants (Saharan et al. 2011), *in vitro* shoot induction and plant

regeneration from nodal explants (Siddique and Anis 2008), nodal explants with axillary buds (Ndoye et al. 2003; Anis et al. 2010), callus induction through several explants like young thorns, apical buds, root segments and cotyledon explants (Gour et al. 2007), and direct shoot morphogenesis from root segments (Gour et al. 2005; Varshney and Anis 2013). Dawah et al. (2013) showed that kinetin at 1 mg l^{-1} combined with NAA at 0.2 mg l^{-1} mixed with 4 g l^{-1} casein hydrolysate was more efficient for shoot number obtained from nodal explants. On other hand increase the sucrose concentration to 70 g l^{-1} separately or mixed with kinetin and NAA caused suppression of the shoot number per explants. El-Mekawy et al. (2012) kinetin at 1 mg l^{-1} combined with NAA 0.2 mg l^{-1} and 40 mg l^{-1} adenine sulphate gave the highest number of shoots from shoot tip explants, also kinetin at 1 mg l^{-1} + 0.2 mg l^{-1} NAA plus 0.25, 0.50 or 1 mg l^{-1} diphenyl urea gave the highest number of shoot per explants for nodal and shoot tip explants, finally, NAA at 0.2 mg l^{-1} combined with 8 mg l^{-1} pholorglucinol induce the vegetative growth for nodal and shoot explants. Gour and Kant (2011) investigate the effect of low cost gelling agent and carbon source in the *in vitro* rooting of *Balanites aegyptiaca* and *Phyllanthus emblica* microshoots, the results showed that the presence of isabgol as gelling agent in the rooting media for *Balanites aegyptiaca* was equivalent to that from agar and isabgol could reduce the cost of gelling agents by 44%, also sugar table could be used for the *in vitro* rotting of *Balanites aegyptiaca* and *Phyllanthus emblica* microshoots. Also the *in vitro* preservation of shoot culture and nodal explants was performed through encapsulation and the genetic stability of regenerates plantlets was confirmed thought ISSR and RAPD (Hwida and El-Kader 2012; Varshney and Anis 2013, 2014).

Salinity has a negative effect on plants through decreasing growth rate even at low salt concentrations. The reduction in growth is combined with alterations in the ion balance, mineral nutrition, stomata conductance and reduction in the rate of photosynthetic CO_2 assimilation (Brugnoli and Lauteri 1991). *Balanites aegyptiaca* seedlings are sensitive at low levels of salinity (12 dS m^{-1}) which is considered as saline irrigation water. The salinity level at 24 dS m^{-1} was harmful for the growth of the seedlings by reducing the photosynthesis and transpiration rates (Radwan et al. 2000). The effect of salt stress on three varied sources of *Balanites aegyptiaca* was investigated through varied levels of salt concentrations (control, 5, 8 and 10 dS m^{-1}). The results revealed that under salt stress, seedlings exhibited lower biomass and growth. The level of sodium was increased in the leaves by increasing the salt concentration in the soil, also tannin was increased by increasing the salinity and was significantly different among the sources and the three sources showed significant differences in growth parameters and there were variability among different intra-specific sources of

Balanites in response to salinity (Elfeel et al. 2013). In this way it is advantageous to improve the ability of this plant species to overcome salt stress in order to cultivate *B. aegyptiaca* in arid and non-arid land in various soil types.

Dehydrins (DHNs) or Late Embryogenesis Abundant group 2 proteins (LEA group 2) have an important function in plant response and adaptation to abiotic stress. They are listed as RAB proteins (responsive to abscisic acid, ABA) and the expression of many DHNs is raised by the phytohormone ABA, while they accumulate in maturing seeds and vegetative tissues after abiotic stress application (Hanin et al. 2011). Dehydrins are essential for the dehydration tolerance process. Under dehydration stress LEA proteins in their random coil conformation bind water to protect cellular structures where several studies revealed that DHNs are associated with macromolecules like the nucleoprotein complex, endomembranes in the cytoplasm and are attached to the plasma membrane (Caruso et al. 2002). Deng et al. (2005) suggested that the DHN, early responsive to dehydration 10 (ERD10), from *Brassica napus* L. was induced through cold, salt stress and ABA treatment. The highest expression was reported in leaves more than in stem and roots. They suggested that ERD10 has a constitutive expression in leaves as defense mechanism under stress. The role of ERD10 in seed development, germination and plant protection under stress conditions was investigated by Kim and Nam (2010). They studied the physiological functions of ERD10 through the analysis the expression of ERD10 T-DNA insertion mutant under several stress condition in Arabidopsis. They reported a decrease in stress tolerance with ERD10 mutants and the activation of C-repeat binding factor/dehydration-responsive element binding (CBF/DREBs) not encountered under cold stress in comparison to the wild-type. They showed that ERD10 is essential for activation of CBF/DREBs genes in Arabidopsis; also the ERD10 was important for seed development where the ERD10 mutants revealed abnormal shaping and poor germination.

ERD10 has a chaperon activity and could support protection of proteins in response to stress through avoiding the heat-induced aggregation and inactivation of several substrates like alcohol dehydrogenase, lysozyme and citrate synthase. It was also reported that ERD10 is associated to acidic phospholipid vesicles without considerable impact on membrane fluidity (Kovacs et al. 2008).

This study aimed to establish a regeneration system through nodal explants with axillary buds and cotyledon explants excised from seeds of *B. aegyptiaca* collected from three different provenances. The regeneration efficiency was compared among the three provenances to select the most efficient provenance in the establishment of a transformation system of *B.*

aegyptiaca via *Agrobacterium tumefaciens* harbouring the vector pBinAR with the dehydrin gene (ERD10) to produce more salt-tolerant *B. aegyptiaca* plants.

Materials and methods

Plant material and culture conditions

Balanites aegyptiaca seeds were naturally cultivated and collected from different places in Egypt (El-Kharga, Wadi El-Alaqi and Wadi El-Gemal) (Table 1). The hard, woody endocarp of fruits was mechanically broken by light hammering to release the seeds that were washed thoroughly with running tap water, and then sterilized with sodium hypochlorite (9.4% active chlorine) (Roth, Karlsruhe, Germany) for 20 min. Afterwards these seeds were washed with distilled water four times. For germination the sterilized seeds were placed on Murashige and Skoog (MS) (Murashige and Skoog 1962) medium supplemented with 3% (w/v) sucrose, 0.7% gelrite, and the pH was adjusted to 5.7 using 1 M NaOH. The culture media and instruments were sterilized by autoclaving at 121°C for 20 min. The cultures were incubated at 24°C in a growth chamber under 16:8 h light:dark photoperiod managed through cool white fluorescent tubes with photosynthetic photon flux density (PPFD) of 30 µmol m^{-2} s^{-1} PAR. After four weeks, shoot segments containing one single node and proximal parts of the cotyledons were excised after removing the meristem and used as explants.

Table 1. Provenances, origin and coordinates of the seed collection used in this study.

Provenances	Locations	Coordinates
Wadi El-Alaqi	Park of the Faculty of Sciences, University of South Valley, Aswan, Egypt	24°5'20.177"N; 32°53'59.385"E
Wadi El-Gemal	Park of the Faculty of Sciences, University of South Valley, Aswan, Egypt	24°41'16.573"N; 35°5'1.248"E
		24°41'16.573"N; 35°5'1.248"E
El-Kharga Oasis	Park of the Faculty of Sciences, University of South Valley, Aswan, Egypt	24°5'20.177"N; 32°53'59.385"E

Induction of multiple shoots

Nodal and cotyledon explants were used separately for shoot induction. Explants were placed on MS medium supplemented with the cytokinins 6-benzyladenine (BA) and thidiazuron (TDZ) at different concentrations (2.2, 4.4, 8.8, and 13.2 µM) in case of nodal explants and (4.4, 8.8, 13.2, and 22 µM) in case of cotyledon explants. Each treatment had 25 explants distributed to 5 Petri dishes each one contained 5 cotyledon explants, the nodal explants were cultured in two sterilized boxes approximately 10 cm each containing 12 nodal explants and the experiment were performed with 3 repetitions for both explants types. After 4 weeks for nodal explants and 6 weeks for cotyledon explants (subculture in the same media after three weeks), the explants that produced shoots were transferred to MS medium supplemented with the optimal concentration of cytokinins in combination with α-naphthalene acetic acid (NAA) in different concentrations (1.3, 2.6, and 5.3 µM) for shoot elongation and proliferation for 4 weeks. Then the percentage of explants that produced shoots, number of shoots per regenerating explant and shoot length were recorded. Nodal explants were moved for 2 weeks to plant growth regulator-free MS medium to devoid the effect of cytokinin. Cotyledon explants with small shoots were transferred to MS medium supplemented with 1.5 µM gibberellic acid (GA3) for 4 weeks.

Root induction and acclimatization

Shoots (2 to 3 cm) with 3 to 5 leaves were excised and moved to half strength MS medium supplemented with indole-3-butric acid (IBA) at different concentrations (1.2, 2.4, and 4.8 µM) for 2 weeks, the number of shoots per replicate were approximately 15. After that the explants were subcultured on half strength plant growth regulator-free MS medium for 4 weeks. The percentage of explants producing roots, number of roots per explants and root length were recorded after 4 weeks of rooting stage. Complete plantlets with healthy shoots and roots were transferred to 6 cm pots filled with CL P soil type (natural clay and peat, ratio 2:1) (Einheitserde, Sinntal-Altengronau, Germany) and covered with plastic bags to keep humidity and watered every 2 days with tap water for 2 weeks. Then the cover was removed to adapt the plants for another 2 weeks under greenhouse conditions a temperature of 22°C and at least 12 h light/dark condition. If the outside light conditions did not enclose appropriate light intensity inside the greenhouse, extra light was supplied to obtain a constant quantum fluence rate of approximately 350 mol $m^{-2}s^{-1}$ (sodium vapor lamps, SON-T Agro 400, Philips, Amsterdam, Netherlands).

Establishment of a transformation system in *B. aegyptiaca* via *Agrobacterium tumefaciens*

For transformation of *B. aegyptiaca* the *Escherichia coli* strain DH5α was used to amplify plasmid pCAMBIA2301 (Fig. 1) that was transformed by the freeze/thaw shock transformation method (Cohen et al. 1972) into three different strains of *A. tumefaciens* (GV3101, EHA 105 and LBA 4404). The plasmid pCAMBIA2301 and the *A. tumefaciens* EHA 105 and LBA 4404 strains were kindly provided by Professor Traud Winkelmann, Institute of Horticultural Production Systems, Leibniz Hannover University, Germany. The cells were spread on 2YT (16 g bacto tryptone, 10 g yeast extract, 5 g NaCl, pH 7.0 NaOH) plates supplemented with the antibiotics rifampicin (50 mg l^{-1}) and kanamycin (50 mg l^{-1}) for GV3101 and EHA 105 strains and in case of LBA 4404 rifampicin (25 mg l^{-1}), streptomycin (25 mg l^{-1}) and kanamycin (50 mg l^{-1}) for selection. At the second day of incubation the transformed colonies were taken for plasmid isolation and the presence of 800 bp gene product of kanamycin was detected by PCR.

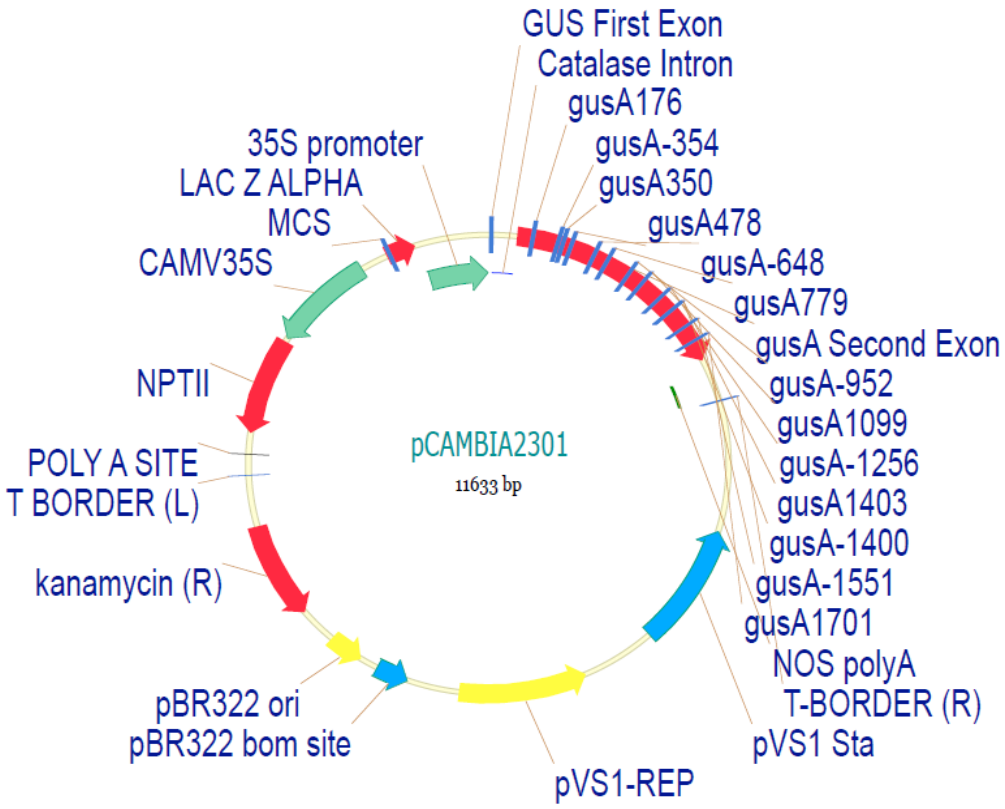

Figure 1. Schematic diagram of pCAMBIA2301 (www.cambia.org).

Agrobacterium tumefaciens (250 µl) cells were inoculated in 100 ml Erlenmeyer flasks containing 10 ml of 2YT medium. The culture was shaken (180 rpm) at 28°C overnight. When an OD_{600} of 0.9-1.0 was reached the bacteria were diluted 10 times and kept on the shaker (180 rpm) at 28°C until an OD_{600} of 0.6. Then the bacteria were harvested by centrifugation (5,000 rpm) at 4°C for 10 min. After discarding the supernatant, the pellet was re-suspended in 100 ml MS medium without any hormones supplemented with 100 µM acetosyringone, and kept on the shaker (180 rpm) at 25°C to obtain a suitable concentration of OD_{600} of 0.5.

Explant preparation and transformation

Nodal and cotyledon segments excised from Wadi-Alaqi 1-month old aseptic seedlings and the survival rate of nodal and cotyledon explants was studied by placing them on MS supplemented with 8.8 µM BA combined with different concentrations of kanamycin (50, 100, 150, 250, and 300 mg l^{-1}). The explants in this study were co-cultivated with the *A. tumefaciens* **s**uspension (OD_{600} 0.5) for 30 min. The nodal and cotyledon explants were removed from the *A. tumefaciens* suspension and maintained under dark condition on co-cultivation medium (MS medium supplemented sucrose 3%, gelrite 0.7%, 2 g l^{-1} MES, 0.8 g l^{-1} glutamine, 1 mg l^{-1} adenine and BA 8.8 µM) with the support of sterile filter paper at 22±2°C for three days. The explants were washed several times with sterilized distilled water. The final wash was done with ticarcillin solution (100 mg l^{-1}) to suppress *A. tumefaciens*. With each *A. tumefaciens* strain treatments, approximately 42 explants were used and distributed as 20 cotyledon explants distributed in 3 Petri dishes and 24 nodal explants were cultured in approximately 10 cm sterilized poxes each one contains 12 nodal explants. The explants were placed on shoot regeneration MS medium supplemented with 8.8 µM BA in case of nodal explants and 13.2 µM BA in case of cotyledon explants for 10 days (3 days under dim light and 7 days in light) provided through cool white fluorescent tubes with irradiance of (30 µmol m^{-2} s^{-1} PAR). The explants were subcultured every 3 weeks on selective medium supplemented with increasing concentrations of kanamycin at each subculture (100, 150, and 200 mg l^{-1}). After two months several factors were recorded, number of explants produced shoots, number of shoots per each explants produced shoots, shoot length and the survival rate after two months of the cultivation on MS supplemented with 100:200 mg l^{-1} kanamycin to finally select *A. tumefaciens* strain to introduce the binary vector containing ERD10.

The coding region of the dehydration protein 10 (ERD10) (Gene bank, AY376669.1, bp 113-928) gene was amplified from cDNA obtained from *Brassica napus.* Total RNA was extracted according to Sokolowsky et al. (1990) and transcribed into cDNA using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Dreieich, Germany). cDNA amplification was performed through primers were used: forward primer 5´-GGTACCATGGCTGAAGAGTACAAG-3´ and reverse primer 5´-GGATCCTCATCCTTCTAAATCATCGG-3´. The amplified cDNA was cloned into the binary vector pBinAR (Höfgen and Willmitzer 1992) and the plasmid was introduced into *A. tumefaciens* GV3101 by the freeze/thaw shock transformation method (Cohen et al. 1972).

Molecular analysis

The histochemical GUS assay according to Jefferson (1987) was performed through 20 explants collected from each treatment with the three *A. tumefaciens*. Screening for transformed plants containing the nptII gene was conducted by PCR. The leaves of regenerated plants were harvested and total DNA was extracted using the Plant Nucleospin II Kit (Macherey & Nagel, Düren, Germany) as described by the manufacturer. The cycling condition for PCR was 94ºC 3 min, then 1 min at 94ºC, 45 s at 39ºC and 1 min at 72ºC for 28 cycles finishing with a final step of 10 min at 72ºC. For PCR amplification following primers were used: NPTII-pCambia 5‘-ATGGCTAAAATGAGAATA-3‘ as forward primer and NPTII-pCambia 5‘-CTAAAACAATTCATCCAG-3‘ as reverse primer to amplify a 750 bp kanamycin PCR fragment.

Screening for transformed plants containing the ERD10 gene was conducted by PCR. The leaves of regenerated plants were harvested after acclimatization of transformed plants in the greenhouse and total DNA was extracted using the Plant Nucleospin II Kit (Macherey & Nagel, Düren, Germany) as described by the manufacturer. Four PCR programs were used to detect (1) presence of ERD10+nptII gene, the cycling condition for PCR was 95ºC 10 min, then 1 min at 94ºC, 1 min at 42ºC and 1 min at 72ºC for 28 cycles finishing with a final step of 10 min at 72ºC. For PCR amplification the following primers were used 5‘-GGTACCATGGCTGAAGAGTACAAG-3‘as forward primer for ERD10 and 5‘-CTAAAACAATTCATCCAG-3‘ as reverse primer for *nptII* gene to amplify a 1550 bp PCR fragment (2) presence of *nptII* gene+ERD10, the cycling condition for PCR was 95ºC 10 min, then 1 min at 94ºC, 1 min at 42ºC and 1 min at 72ºC for 28 cycles finishing with a final step of 10 min at 72ºC. For PCR amplification the following primers were used 5‘-

ATGGCTAAAATGAGAATA-3‘ as forward primer for nptII gene and 5‘-GGAT**CC**TCATCCTTCTAAATCATCGG-3‘as reverse primer for ERD10 to amplify a 1550 bp PCR fragment (3) presence of ERD10 gene, the cycling condition for PCR was 95ºC 10 min, then 1 min at 94ºC, 1 min at 57ºC and 1 min at 72ºC for 28 cycles finishing with a final step of 10 min at 72ºC. For PCR amplification the following primers was used 5‘-GGTACCATGGCTGAAGAGTACAAG-3‘as forward primer and 5‘-GGAT**CC**TCATCCTTCTAAATCATCGG-3‘as reverse primer to amplify a 800 bp PCR fragment (4) presence of *nptII* gene, the cycling condition for PCR was 95ºC 10 min, then 1 min at 94ºC, 1 min at 39ºC and 1 min at 72ºC for 28 cycles finishing with a final step of 10 min at 72ºC. For PCR amplification the following primers was used 5‘-ATGGCTAAAATGAGAATA-3‘ as forward primer and 5‘-CTAAAACAATTCATCCAG-3‘ as reverse primer to amplify a 750 bp PCR fragment

Statistical analysis

Between 20 and 25 nodal and cotyledon explants were used in each treatment and all the experiments from provenances El-Kharga and Wadi El-Alaqi were repeated thrice and provenance Wadi El-Gemal was only repeated once. To analyze significant effects for single variables, two-factorial analysis of variances (ANOVA) was performed, with main effects for provenance, plant growth regulator concentrations and provenance-plant growth regulator concentrations interaction and all pair-wise means were compared using Tukey test with ($P < 0.05$).

Results

Shoot induction and multiplications

Sterilized seeds of *B. aegyptiaca* collected from three provenances (100 seeds from each provenance) were inoculated directly on MS basal medium without plant growth regulators and showed different germination percentage. After 4 weeks seeds from Wadi El-Alaqi gave the highest germination percentage (66%) (Table 2).

Table 2. The germination rate of seeds from three different *B. aegyptiaca* provenances (Wadi El-Gemal, El-Kharga and Wadi El-Alaqi) after 4 weeks on MS medium.

Provenances	Germination rate (%)
Wadi El-Gemal	60
El-Kharga	50
Wadi El-Alaqi	66

In this study the regeneration experiments for provenances El-Kharga and Wadi El-Alaqi were repeated three times and one time for Wadi El-Gemal. So this study will mainly focus on the results of provenances El-Kharga and Wadi El-Alaqi. Cotyledon explants from all provenances failed to develop shoots in a MS medium without plant growth regulator (PGR), on the other hand nodal explants produced shoots when placed on MS medium free of PGR. Both nodal and cotyledon explants from each provenance were responding with shoot formation when inoculated on MS medium supplemented with various concentrations of cytokinins. Nodal explants exhibited shoot induction and axillary bud breaking after 2 weeks of inoculation where cotyledon explants showed shoot induction after 3 weeks. Shoot proliferation was obtained through the second subculture for another 3 weeks. There were no significance differences between the two provenances, Wadi El-Alaqi and El-Kharga, or on the level of provenances-PGR interaction for both explants.

The effect of both cytokinins BA and TDZ on shoot induction was significantly different with BA being more effective in the response of nodal explants for shoot induction. Provenance El-Kharga showed the highest percentage of nodal explants producing shoots at 13.2 µM BA (85% ± 6.8), where Wadi El-Alaqi provenance showed the highest percentage of nodal explants producing shoots at 8.8 µM BA (89 ± 5.68), and provenance El-Kharga and Wadi El-Alaqi respectively, showed the highest number of shoots (3.7 ± 1.8 and 3.4 ±1.4) per regenerating nodal explant (Table 3) at 8.8 µM BA. The shoot length of regenerated nodal explants was decreased by increasing the cytokinin concentrations in both provenances. In contrast, the nodal explants placed on MS medium free of PGR exhibited the highest shoot length for both provenances (1.61 ± 1.1 and 1.39 ± 0.4 cm) for provenance Wadi El-Alaqi and El-Kharga respectively. Provenance El-Kharga showed the highest percentage of cotyledon explants producing shoots (49%) at 13.2 µM BA and 46% at 22 µM BA for provenances Wadi El-Alaqi (Fig. 4). The cytokinin TDZ was more effective than BA in the number of shoots produced per regenerated cotyledon explants, and provenance Wadi El-Alaqi exhibited the highest value (3.6 ± 1.9) at 22 µM TDZ while El-Kharga showed the

highest value (3.3 ± 2.1) at 13.2 µM TDZ (Table 4). Shoot length was affected under TDZ concentrations where the highest shoot length (Table 4) was obtained with provenance Wadi El-Alaqi (1.00 ± 0.7) at 13.2 µM BA and at 22 µM BA (0.78 ± 0.5) for El-Kharga. Also the higher concentrations of TDZ induced the callus formation at the base of the explants and the regenerated shoots exhibited a degree of hyperhydricity.

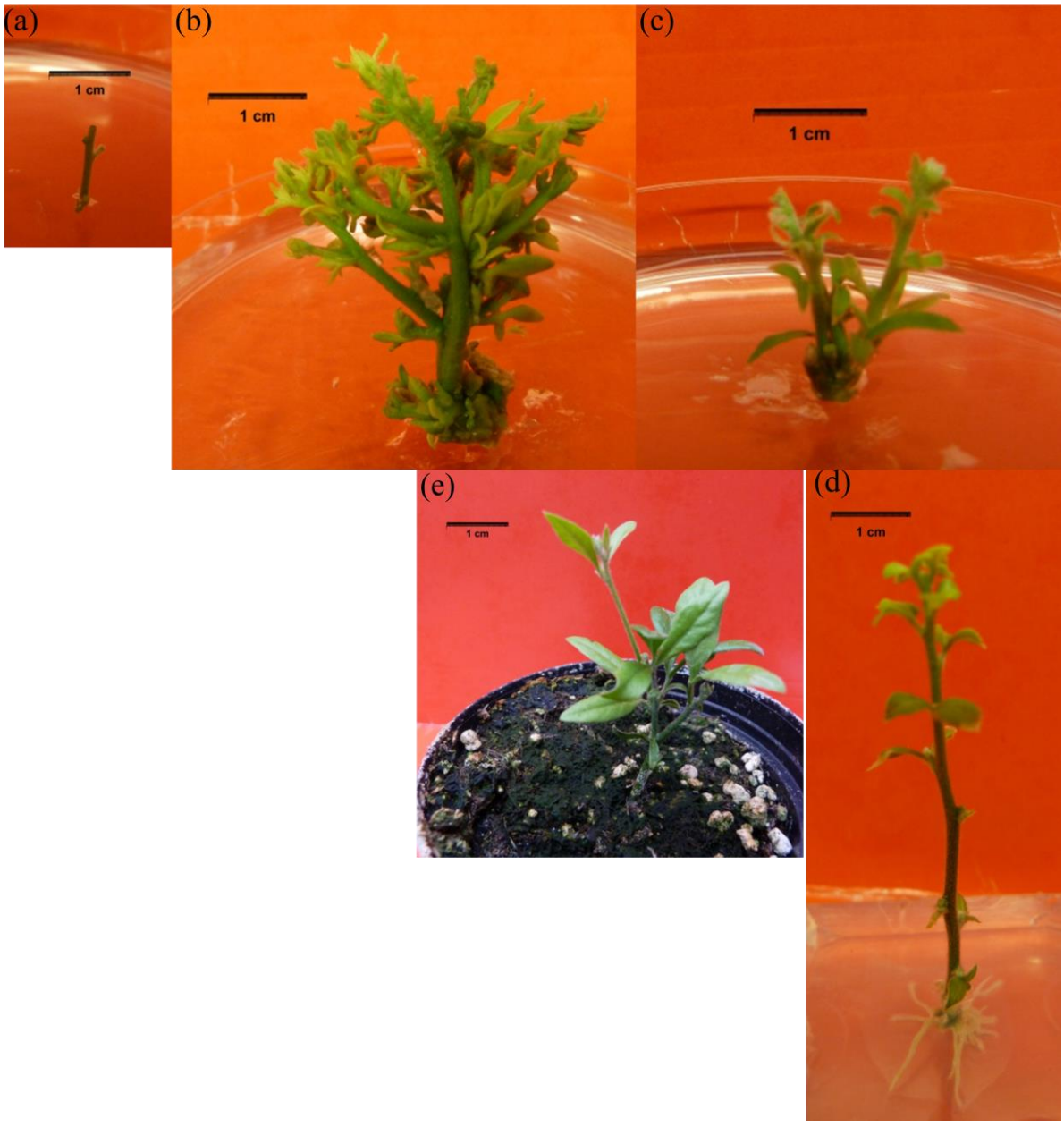

Figure 2. Multiple shoot formation in *B. aegyptiaca* using nodal explants with axillary bud obtained from Wadi El-Alaqi provenance. (**a)** Nodal explants, **(b)** multiple shoot induction on MS + 8.8 µM BA after 4 weeks **(c)** elongation and proliferation of multiple shoots on MS + 8.8 µM BA+ 1.3 µM NAA after 8 weeks, **(d)** *in vitro* rooted plantlet on MS + 1.2 µM IBA, **(e)** and a regenerated, acclimatized plant in soil.

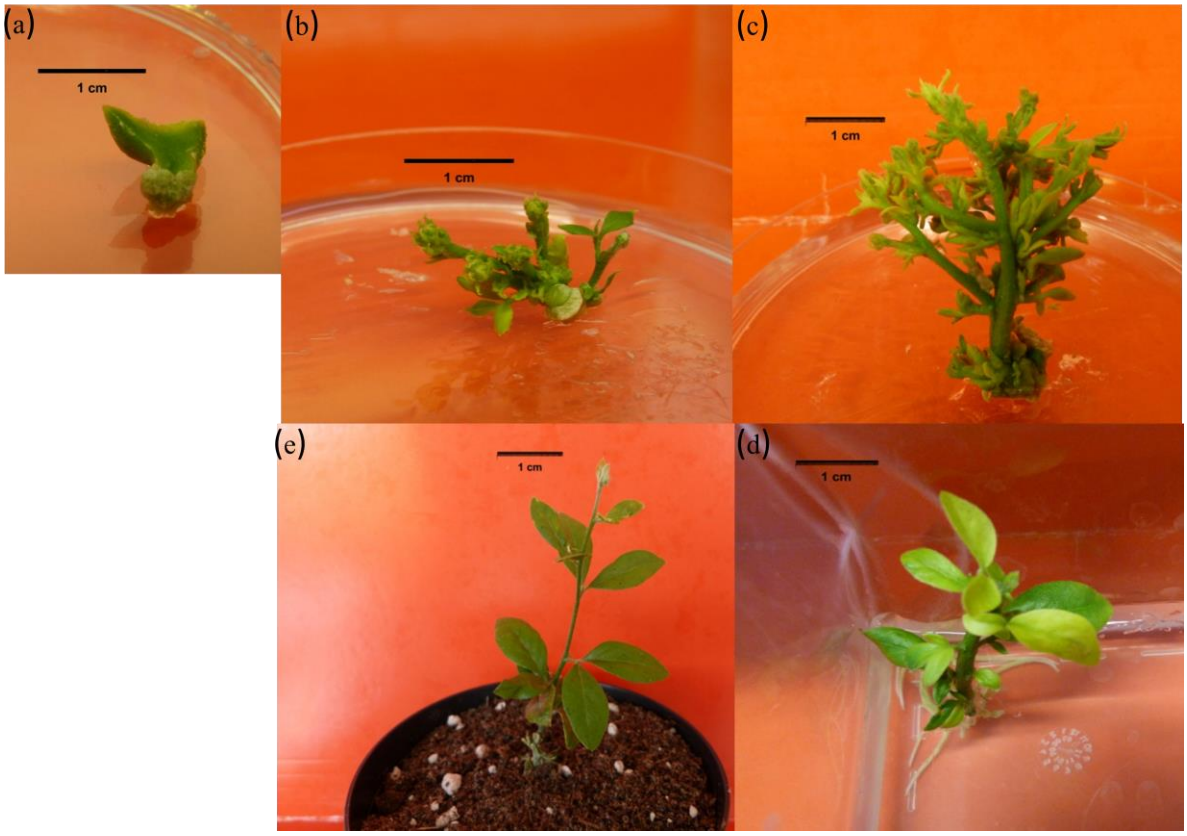

Figure 3. Shoot regeneration of *B. aegyptiaca* from cotyledon explants obtained from Wadi El-Alaqi provenance. **(a)** Cotyledon explants in first day, **(b)** shoot induction after 6 weeks on 13.2 µM BA, **(c)** shoot elongation and proliferation after 4 weeks on 1.3 µM NAA, **(d)** rooting after 1 month on 2.4 µM IBA, **(e)** and a regenerated, acclimatized plant in soil.

Table 3. Effect of different concentrations of cytokinins BA and TDZ (2.2, 4.4, 8.8, and 13.2 µM) on shoot formation from nodal explants from different provenances of *B. aegyptiaca* after 4 weeks of culture.

Provenance	Plant rowth regulators (µM)		No. of explants produced shoots (%)	No. of shoots per explant forming shoots	Shoot length (cm)
	TDZ	BA			
El-Kharga	0		57^{ab}	1.5 ± 0.7^{a}	1.39 ± 0.4^{b}
	2.2		49 ± 11.5^{ac}	1.7 ± 0.8^{a}	0.24 ± 0.1^{a}
	4.4		59 ± 2.6^{ab}	2.1 ± 0.8^{ab}	0.37 ± 0.2^{a}
	8.8		35 ± 3.7^{a}	2.3 ± 1.4^{ab}	0.31 ± 0.2^{a}
	13.2		35 ± 8.3^{a}	1.8 ± 1.0^{ab}	0.32 ± 0.1^{a}
		2.2	78 ± 3.2^{bc}	2.3 ± 1.0^{ab}	1.27 ± 0.6^{b}
		4.4	80 ± 11.01^{b}	3.5 ± 2.1^{ab}	1.20 ± 0.7^{b}
		8.8	78 ± 16.5^{b}	3.7 ± 1.8^{b}	1.18 ± 0.9^{b}
		13.2	85 ± 6.8^{b}	3.3 ± 1.4^{ab}	1.08 ± 0.6^{b}
Wadi	0		85	1.0 ± 0.6	2.33 ± 0.6
	2.2		43	1.8 ± 0.2	0.55 ± 0.0
	4.4		43	2.7 ± 0.5	0.47 ± 0.1
	8.8		37	2.3 ± 0.8	0.59 ± 0.1
	13.2		37	2.0 ± 0.2	0.37 ± 0.0
		2.2	82	2.1 ± 0.2	1.07 ± 0.1
		4.4	60	2.2 ± 0.3	1.14 ± 0.2
		8.8	62	3.0 ± 0.4	1.04 ± 0.1
		13.2	68	2.5 ± 0.4	0.92 ± 0.1
Wadi Alaqi	0		56 ± 12.1^{c}	1.6 ± 0.8^{a}	1.61 ± 1.1^{d}
	2.2		41 ± 18.0^{a}	1.8 ± 0.8^{ab}	0.30 ± 0.1^{a}
	4.4		34 ± 13.6^{a}	1.9 ± 0.6^{a}	0.28 ± 0.1^{a}
	8.8		34 ± 13.6^{a}	1.9 ± 0.6^{ab}	0.28 ± 0.1^{a}
	13.2		31 ± 8.0^{a}	2.1 ± 1.1^{abc}	0.33 ± 0.3^{a}
		2.2	79 ± 9.0^{bc}	2.5 ± 1.2^{ad}	1.09 ± 0.5^{bc}
		4.4	$83 \pm 10.^{c}$	2.9 ± 1.4^{bd}	1.30 ± 1.0^{c}
		8.8	89 ± 5.6^{c}	3.4 ± 1.5^{d}	0.82 ± 0.5^{bc}
		13.2	78 ± 7.2^{bc}	3.2 ± 1.4^{cd}	0.76 ± 0.5^{b}

Values represent means ± SD from 3 repetitions. Means followed by the same letters within a column are not significantly different after Tukey test at ($P < 0.05$).

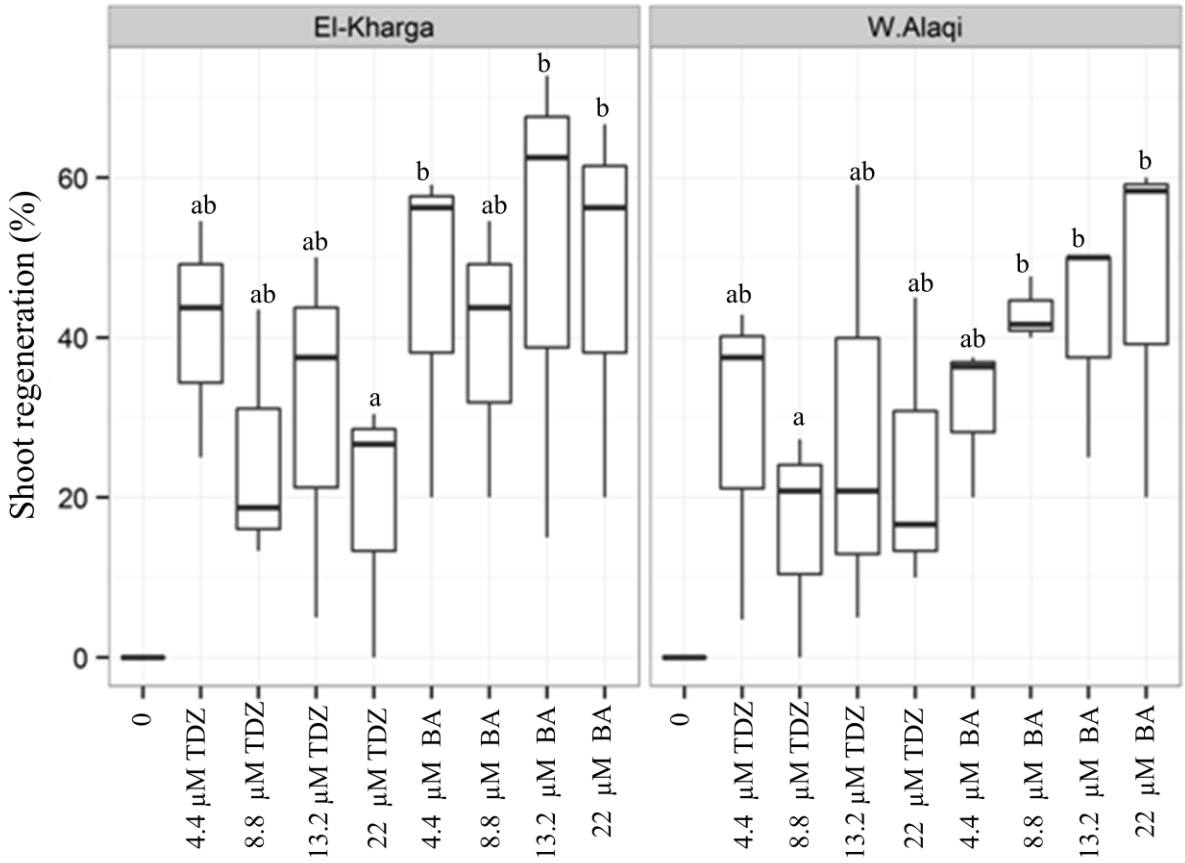

Figure 4. Effect of different concentrations of the cytokinins BA and TDZ (4.4, 8.8, 13.2 and 22 µM) on the percentage of cotyledon explants regenerating shoots from different provenances of *B. aegyptiaca* after 6 weeks of culture, n = 3, supplementary tables (8 and 9).

Table 4. Effect of different concentrations of the cytokinins BA and TDZ (4.4, 8.8, 13.2, and 22 µM) on shoot regeneration from cotyledon explants from different provenances of *B. aegyptiaca* after 6 weeks of culture.

Provenances	**Plant growth regulators (µM)**		**No. of shoots per regenerating explant**	**Shoot length (cm)**
	TDZ	**BA**		
El-Kharga	0		0	0
	4.4		3.0 ± 1.9^{ab}	0.57 ± 0.5^{ab}
	8.8		3.1 ± 2.2^{ab}	0.25 ± 0.1^{a}
	13.2		3.3 ± 2.1^{b}	0.33 ± 0.2^{ab}
	22		3.1 ± 2.2^{ab}	0.33 ± 0.1^{ab}
		4.4	1.7 ± 0.8^{a}	0.77 ± 0.5^{b}
		8.8	3.1 ± 1.4^{b}	0.76 ± 0.4^{b}
		13.2	2.8 ± 1.0^{b}	0.73 ± 0.4^{b}
		22	2.3 ± 1.0^{ab}	0.78 ± 0.5^{b}
Wadi	0		0	0

	4.4		1.0 ± 0.0	0.30 ± 0.0
	8.8		1.0 ± 0.0	0.30 ± 0.0
	13.2		2.0 ± 0.0	0.30 ± 0.0
	22		1.0 ± 0.0	0.80 ± 0.0
		4.4	1.4 ± 0.2	3.45 ± 0.7
		8.8	2.6 ± 0.6	1.16 ± 0.2
		13.2	3.3 ± 0.6	0.81 ± 0.1
		22	3.5 ± 0.9	2.34 ± 0.9
Wadi Alaqi	0		0	0
	4.4		2.5 ± 1.4^{a}	0.32 ± 0.2^{abc}
	8.8		2.8 ± 0.8^{a}	0.25 ± 0.2^{a}
	13.2		3.0 ± 1.8^{a}	0.23 ± 0.1^{ab}
	22		3.6 ± 1.9^{a}	0.24 ± 0.1^{ab}
		4.4	2.1 ± 1.0^{a}	0.52 ± 0.3^{abc}
		8.8	3.1 ± 1.6^{a}	0.72 ± 0.3^{bd}
		13.2	2.6 ± 1.1^{a}	1.00 ± 0.7^{d}
		22	2.9 ± 1.2^{a}	0.74 ± 0.4^{cd}

Values represent means ± SD from 3 repetitions. Means followed by the same letters within a column are not significantly different after Tukey test at ($P < 0.05$) supplementary tables (10, 11 and 12).

Combinations of cytokinins and auxin were verified for further shoot proliferation and multiplication (Fig. 2c and 3c). To increase the shoot multiplication and to decrease the impact of high concentration of cytokinins on shoot length, 8.8 µM BA and 4.4 µM TDZ were selected with different concentrations of NAA (1.3, 2.6 and 5.3 µM). There was a significant difference between both cytokinins in shoot multiplication at different concentrations of NAA. The highest number of shoots per shoot forming nodal explant (4.78 ± 2.3) was obtained for provenance El-Kharga at 8.8 µM BA with 2.6 µM NAA and for provenance Wadi Alaqi was (4.17 ± 1.9) at the same concentration (Table 5) so that 2.6 µM NAA could be the optimum concentration for shoot multiplication through nodal explants in both provenances. But the highest shoot length (2.28 ± 1.33 and 1.8 ± 1.1) was obtained from regenerated nodal explants of provenance Wadi Alaqi and El-Kharga respectively, when inoculated on MS without PGR. The combination of cytokinins and auxin increased the percentage of cotyledon explants produced shoots where the percentage was increased in provenance El-Kharga (57%) at 8.8 µM BA with 1.3 µM NAA and in provenance Wadi Alaqi (56%) at 8.8 µM BA with 2.6 µM NAA (data not shown). Despite the impact of TDZ on shoot length, provenance El-Kharga exhibited the highest number of shoots per regenerated cotyledon explant (6.1 ± 3.1) at 4.4 µM TDZ with 5.3 µM NAA while provenance Wadi Alaqi exhibit the highest number of shoots per regenerated cotyledon explants (4.4 ± 3) at 4.4 µM TDZ with 1.3 µM NAA. Provenance El-Kharga showed the highest shoot length (1.10 ± 0.5) from regenerated cotyledon explants at 8.8 µM BA with 2.6

µM NAA while the highest shoot length for provenance Wadi Alaqi (0.95 ± 0.5 cm) was obtained at 8.8 µM BA with 5.3 µM NAA (Table 6). So that the optimum concentration for shoot multiplication through cotyledon explants in El-Kharga was 4.4 µM TDZ with 5.3 µM NAA and 4.4 µM TDZ with 1.3 µM NAA in Wadi Alaqi. While the optimum concentration for shoot length through cotyledon explants was in El-Kharga 8.8 µM BA with 2.6 µM NAA and 8.8 µM BA with 5.3 µM NAA in Wadi Alaqi.

Table 5. Effect of optimal concentration of BA and TDZ with different concentrations of NAA on shoot multiplication of nodal explants from different provenances of *B. aegyptiaca* after 8 weeks of culture.

Provenances	**Plant growth regulators (µM)**			**No. of shoots per explant**	**Shoot length (cm)**
	TDZ	**BA**	**NAA**		
El-Kharga		0	0	1.88 ± 1.2[a]	1.80 ± 1.1[c]
	4.4		1.3	2.60 ± 1.2[a]	0.27 ± 0.1[a]
	4.4		2.6	2.76 ± 1.2[a]	0.29 ± 0.1[a]
	4.4		5.3	2.10 ± 1.0[a]	0.32 ± 0.2[a]
		8.8	1.3	4.60 ± 2.8[b]	1.18 ± 0.5[b]
		8.8	2.6	4.78 ± 2.3[b]	1.09 ± 0.5[b]
		8.8	5.3	4.30 ± 2.0[b]	1.32 ± 0.6[b]
Wadi		0	0	1.00 ± 0.0	3.14 ± 0.6
	4.4		1.3	3.16 ± 0.4	0.36 ± 0.0
	4.4		2.6	3.30 ± 0.8	0.47 ± 0.0
	4.4		5.3	3.27 ± 0.4	0.29 ± 0.0
		8.8	1.3	3.06 ± 0.3	1.25 ± 0.1
		8.8	2.6	2.66 ± 0.2	1.26 ± 0.1
		8.8	5.3	3.15 ± 0.4	1.35 ± 0.1
Wadi Alaqi		0	0	1.68 ± 0.8[a]	2.28 ± 1.3[c]
	4.4		1.3	2.52 ± 1.3[a]	0.32 ± 0.3[a]
	4.4		2.6	2.48 ± 2.0[a]	0.30 ± 0.2[a]
	4.4		5.3	2.23 ± 1.4[a]	0.20 ± 0.0[a]
		8.8	1.3	3.85 ± 2.0[b]	1.32 ± 0.7[b]
		8.8	2.6	4.17 ± 1.9[b]	1.06 ± 0.5[b]
		8.8	5.3	4.04 ± 1.8[b]	1.20 ± 0.5[b]

Values represent means ± SD from 3 repetitions. Means followed by the same letters within a column are not significantly different after Tukey test at ($P < 0.05$), supplementary tables (1, 2, 3 and 4).

Table 6. Effect of optimal concentrations of BA and TDZ with different concentrations of NAA on shoot multiplication of cotyledon explants from different provenances after 10 weeks of culture.

Provenances	Plant growth regulators (µM)			No. of shoots per explant	Shoot length
	TDZ	BA	NAA		
El-Kharga	4.4		1.3	3.8 ± 2.1^{a}	0.35 ± 0.2^{a}
	4.4		2.6	4.5 ± 3.0^{ab}	0.40 ± 0.2^{a}
	4.4		5.3	6.1 ± 3.1^{b}	0.40 ± 0.3^{a}
		8.8	1.3	3.1 ± 1.6^{a}	0.93 ± 0.4^{b}
		8.8	2.6	3.2 ± 2.0^{a}	1.10 ± 0.5^{b}
		8.8	5.3	3.3 ± 1.7^{a}	0.90 ± 0.4^{b}
Wadi	4.4		1.3	3.2 ± 0.48	0.24 ± 0.02
	4.4		2.6	1.4 ± 4.79	0.22 ± 0.041
	4.4		5.3	2.0 ± 0.1	0.30 ± 0.18
		8.8	1.3	2.3 ± 0.33	1.81 ± 0.34
		8.8	2.6	4.2 ± 1.24	1.34 ± 0.24
		8.8	5.3	2.2 ± 0.75	1.02 ± 0.26
Wadi-Alaqi	4.4		1.3	4.4 ± 3.0^{a}	0.28 ± 0.1^{ab}
	4.4		2.6	3.7 ± 2.1^{a}	0.26 ± 0.1^{a}
	4.4		5.3	2.7 ± 1.0^{a}	0.28 ± 0.1^{a}
		8.8	1.3	3.2 ± 1.0^{a}	0.81 ± 0.4^{bc}
		8.8	2.6	2.9 ± 1.0^{a}	0.85 ± 0.6^{c}
		8.8	5.3	3.0 ± 1.0^{a}	0.95 ± 0.5^{c}

Values represent means ± SD from 3 repetitions. Means followed by the same letters within a column are not significantly different after Tukey test at ($P< 0.05$), supplementary tables (13, 14, 15, 16 and 17).

Root formation and acclimatization

In both provenances shoots from nodal explants produced roots on MS basal medium without PGR. There was no significant difference between the provenances or among the different concentrations of IBA. There was only a significance difference in provenance El-Kharga between the control treatments and the different concentrations of IBA. The highest percentage of shoots from nodal explants producing roots was obtained from provenance El-Kharga at 1.2 µM IBA also from provenance Wadi Alaqi at the same concentration (Fig. 5), the highest number of roots per shoots (5.6 ± 4.3) was observed for provenance Wadi Alaqi

(Fig. 2d) at 4.8 µM IBA while provenance El-Kharga exhibit the highest number of roots per shoots (4.2 ± 2.8) at 1.2 µM IBA (Table 7). The highest root length (1.4 ± 1.5 cm) was obtained from provenance El-Kharga at 0 µM IBA, while provenance Wadi Alaqi showed the highest root length (0.55 ± 0.4 cm) at 2.4 µM IBA (Table 7).

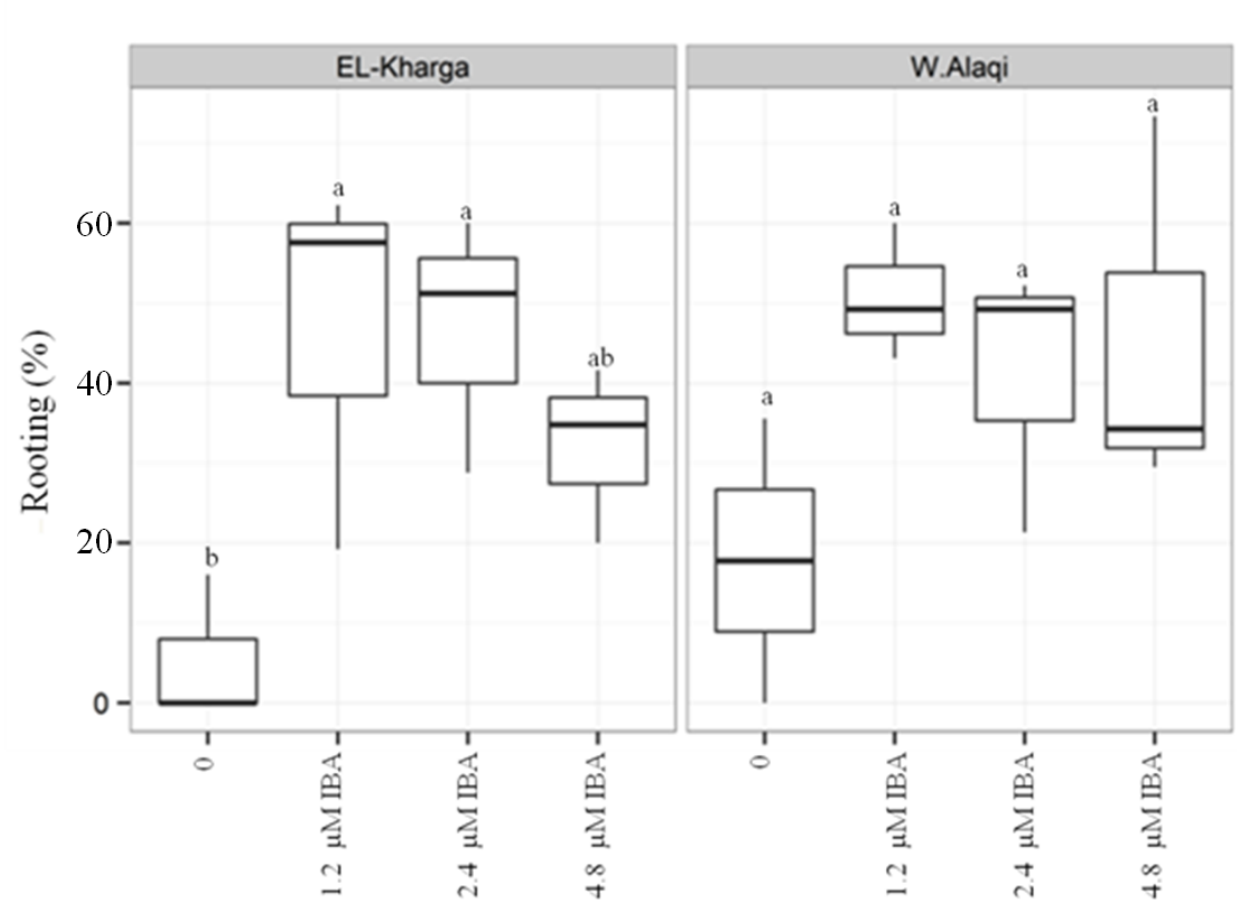

Figure 5. Effect of different concentrations of IBA on the percentage of shoots from nodal explants producing roots for two provenances of *B. aegyptiaca* after 6 weeks of culture supplementary tables (5 and 6), number of replicate = 3, number of shoots per replicate was approximately 15 for each concentration.

Table 7. Effect of different concentrations of IBA on root formation of shoot from nodal explants from different provenances of *B. aegyptiaca* after 6 weeks of culture.

Provenances	Plant growth regulator IBA (µM)	No. of roots per rooted shoot	Root length (cm)
El-Kharga	0	3.0 ± 1.4[a]	1.40 ± 1.5[a]
	1.2	4.2 ± 2.8[b]	0.88 ± 0.8[a]
	2.4	3.5 ± 2.0[b]	0.70 ± 0.4[a]
	4.8	3.4 ± 2.5[b]	0.80 ± 0.7[a]
Wadi	0	0.0	0.0
	1.2	6.8 ± 0.8	0.40 ± 0.0
	2.4	6.1 ± 1.8	0.21 ± 0.0
	4.8	3.0 ± 0.0	0.26 ± 0.0
Wadi-Alaqi	0	4.4 ± 4.1[a]	0.19 ± 0.2[a]
	1.2	5.0 ± 4.4[b]	0.52 ± 0.4[a]
	2.4	5.1 ± 3.3[b]	0.55 ± 0.4[a]
	4.8	5.6 ± 4.3[b]	0.52 ± 0.4[a]

Values represent means ± SD from 3 repetitions. Means followed by the same letters within a column are not significantly different after Tukey test at (P< 0.05), supplementary tables (7).

On the other hand the highest percentage of shoots from cotyledon explants producing roots (71%) was obtained from provenance Wadi Alaqi at 2.4 µM IBA and (64%) for provenance El-Kharga at the same concentration (Fig. 3d and 6). The highest number of roots produced per cotyledon explant (6.6 ± 4.0) was obtained from provenance Wadi Alaqi at 4.8 µM IBA and (5.7 ± 2.0) from provenance El-Kharga at 1.2 µM IBA. The longest roots (1.09 ± 1.0 cm) obtained from provenance Wadi Alaqi at 2.4 µM IBA and (1.07 ± 1.0 cm) from provenance El-Kharga at same concentration (Table 8).

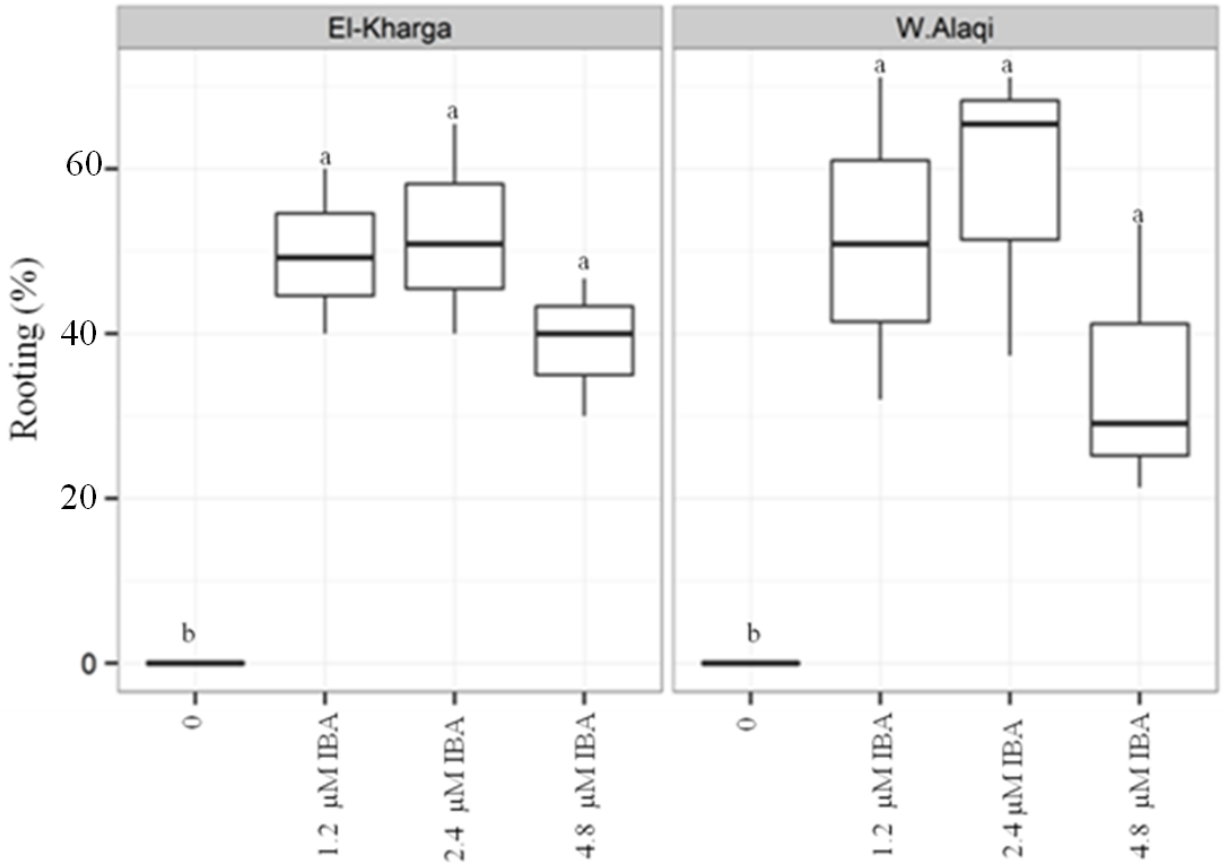

Figure 6. Effect of different concentrations of IBA on the percentage of shoots from cotyledon explants produced roots through different provenances of *B. aegyptiaca* after 6 weeks of culture, number of replicate = 3, number of shoots per replicate was approximately 15 for each concentration.

Table 8. Effect of different concentrations of IBA on root formation of shoots from cotyledon explants collected from different provenances of *B. aegyptiaca* after 6 weeks of culture.

Provenances	Plant growth regulator IBA (µM)	No. of roots per rooted explants	Root length (cm)
El-Kharga	1.2	5.7 ± 2.0[a]	0.89 ± 0.6[a]
	2.4	4.7 ± 3.0[a]	1.07 ± 1.0[a]
	4.8	4.4 ± 2.0[a]	0.56 ± 0.3[a]
Wadi	1.2	7.0 ± 3.63	1.05 ± 0.42
	2.4	5.5 ± 2.50	1.13 ± 0.93
	4.8	3.0 ± 0.0	0.20 ± 0.0
Wadi-Alaqi	1.2	6.4 ± 4.0[a]	0.96 ± 0.9[a]
	2.4	4.9 ± 3.0[a]	1.09 ± 1.0[a]
	4.8	6.6 ± 4.0[a]	0.68 ± 0.5[a]

Values represent means ± SD from 3 repetitions. Means followed by the same letters within a column are not significantly different after Tukey test at ($P < 0.05$).

After acclimatization the survival rate of plantlets obtained from Wadi El-Alaqi was 69% and 87% for plantlets produced from nodal and cotyledon explants respectively. The survival rate of plantlets obtained from El-Kharga was 77% and 95% for plantlets produced from nodal and cotyledon explants respectively

Transformation of *B. aegyptiaca* via *A. tumefaciens* harbouring pCAMBIA2301

The capability of nodal explants to survive in different concentration of kanamycin (50, 100, 150, 250, and 300 mg l^{-1}) was investigated for two months (Fig. 7) the percentage of dead nodal explants was increased by increasing the kanamycin concentration and at 100 mg l^{-1} 30 explants were dead from 42 explants was used (67%).

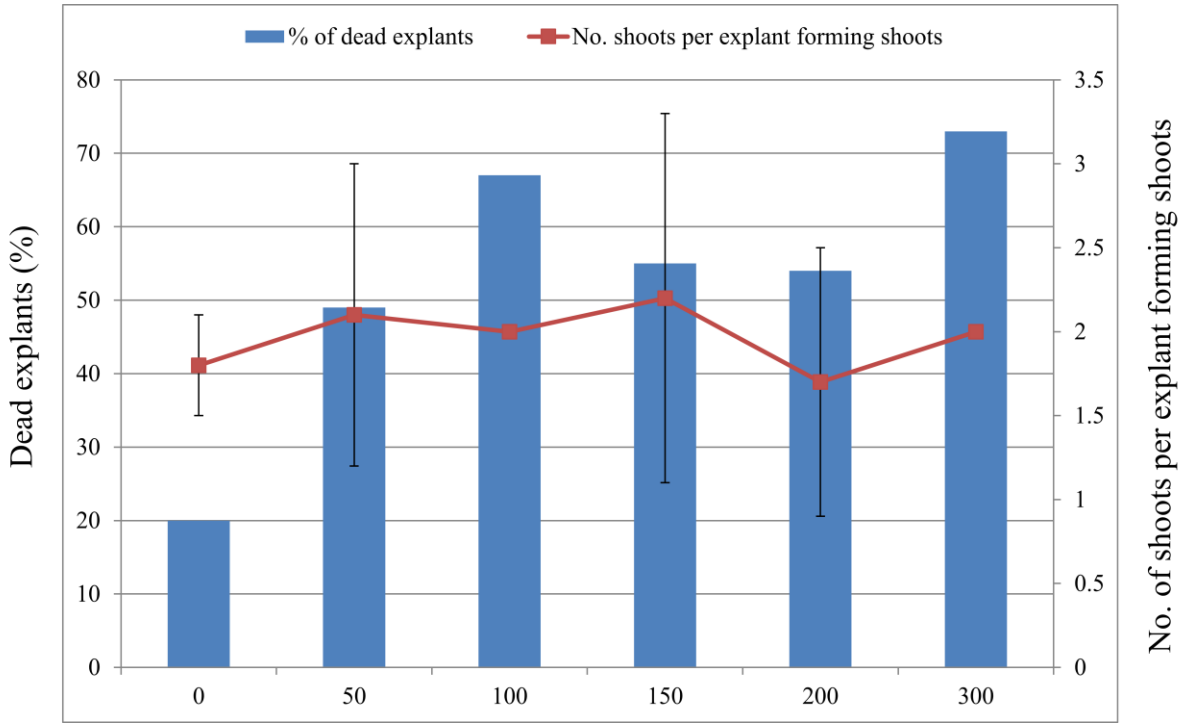

Figure 7. The percentage of dead nodal explants and number of explants producing shoots after two months survive in different concentration of kanamycin (50, 100, 150, 250, and 300 mg l^{-1}) where 42 nodal explants were used for each treatment.

After that the percentage was decreased by increasing the concentration at 150 and 200 mg l^{-1} and increased again at 300 mg l^{-1} where 73% of the explants were dead. Based on these results the nodal explants were placed on 100 mg l^{-1} kanamycin in the beginning of the experiment and the concentration was increased through each subculture. The presence of kanamycin in the medium affected the shoot length and growth, and the leaves turned white in control plants compared to the transformed plant (Fig. 8)

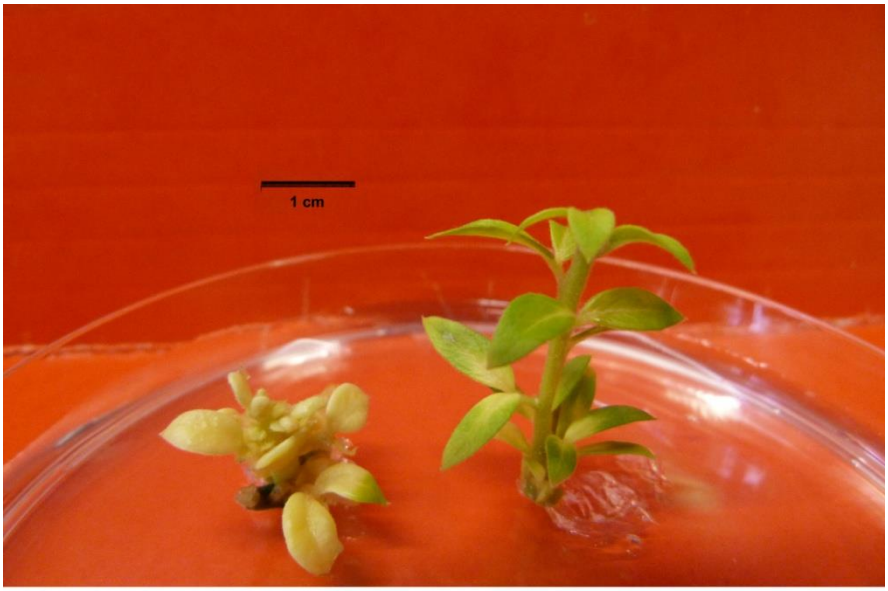

Figure 8. Nodal explants from provenances Wadi El-Alaqi transformed with *A. tumefaciens* GV 3101 strain (left) compared to non transformed nodal explants (right) after two months placed on MS supplemented with 8.8 µM and 200 mg l^{-1} kanamycin.

Three different *A. tumefaciens* strains (EHA 105, GV 3101, and LBA 4404) harbouring plasmid pCAMBIA2301 containing the GUS intron as a reporter gene and aminoglycoside 3'-phosphotransferase (*nptII*) as a selectable marker gene were used for transformation. Transformed nodal explants were placed on MS medium supplemented with 100 mg l^{-1} kanamycin as a selective agent. At each subculture the kanamycin concentration was increased by 50 mg l^{-1} up to 200 mg l^{-1}. After 2 months, the regeneration percentage from 42 explants were used in each treatments were (48, 45 and 39%) for the transformed explants through GV3101, LBA 4404 and EHA 105 *A. tumefaciens* strains respectively and 6 out of 22 explants were regenerated from control treatment. The number of shoots per explant was 2.9 ± 0.8, 2.6 ± 1 and 1.9 ± 0.8 for LBA 4404, GV3101 and EHA 105 *A. tumefaciens* strains, respectively.

The histochemical GUS assay (Fig. 9) was conducted on 20 explants from each treatment and the results showed that two nodal explants were positive from the *A. tumefaciens* GV3101 strain and one nodal explants was positive from *A. tumefaciens* EHA105 strain.

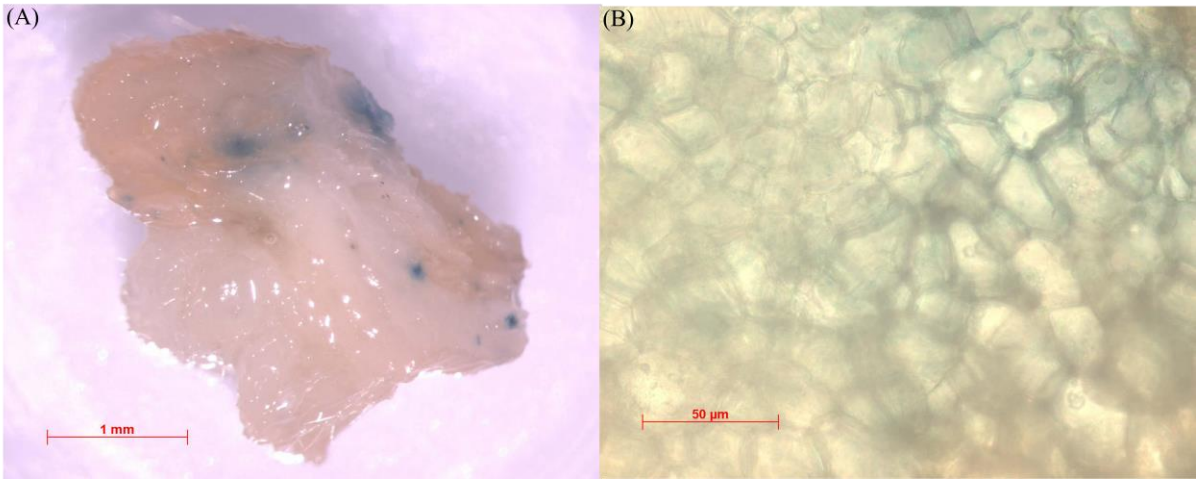

Figure 9. Histochemical GUS assay in **(a)** callus and **(b)** leaf produced from nodal explants of *B. aegyptiaca* after two months of transformation via *A. tumefaciens* GV3101 strain containing pCAMBIA2301.

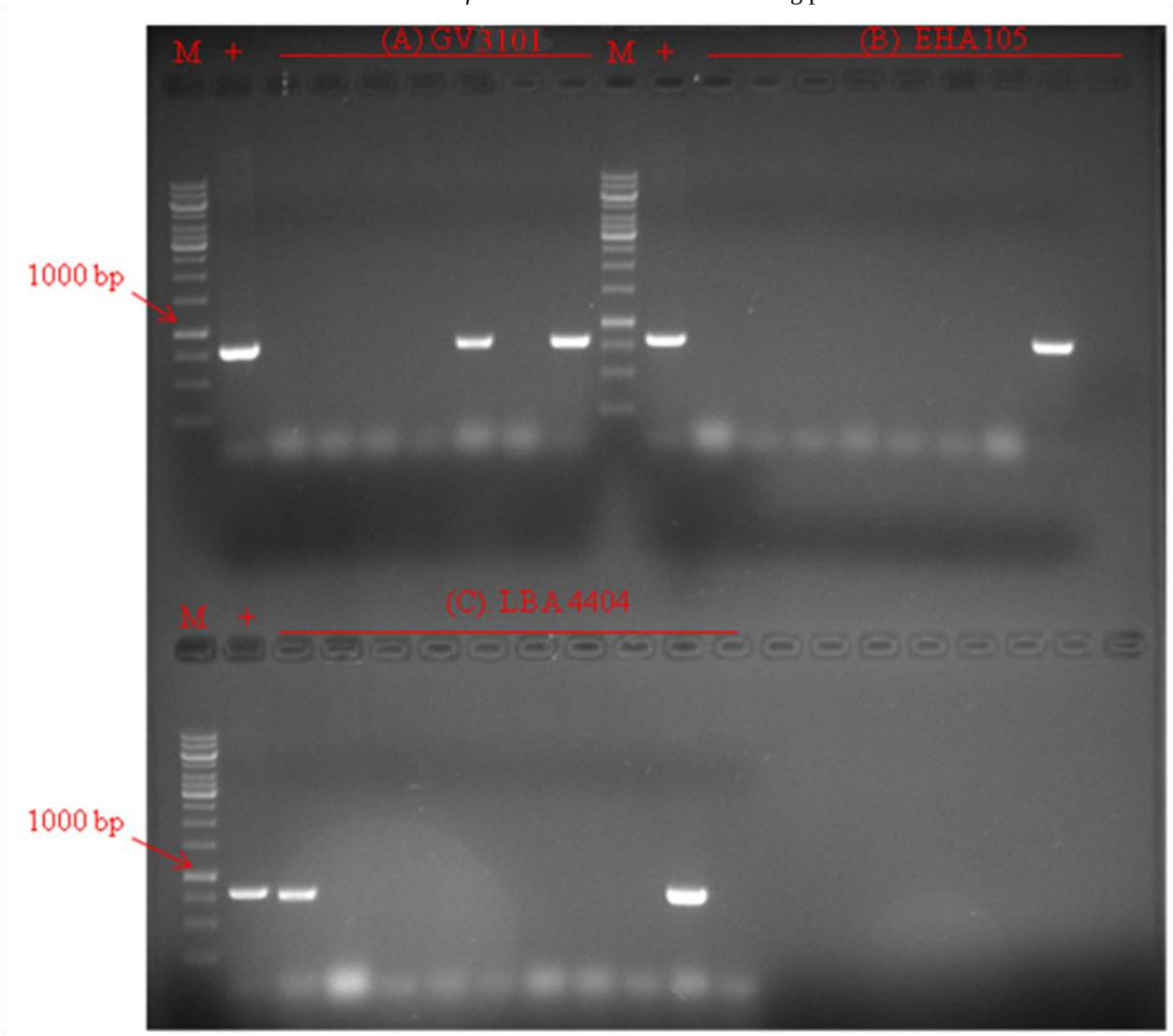

Figure 10. PCR products of *nptII* gene (800 bp). (M). One kbp DNA ladder. (+). Plasmid pCAMBIA2301, **(a)** leaves excised from different nodal explants after 2 months of transformation via *A. tumefaciens* GV3101 strain *containing* pCAMBIA2301, **(b)** leaves excised from different nodal explants after 2 months of transformation via *A. tumefaciens* EHA105 strain containing pCAMBIA2301, and **(c)** leaves excised from different nodal explants after 2 months of transformation via *A. tumefaciens* LBA4404 strain containing pCAMBIA2301.

Leaves from 8 transformed nodal explants from each treatment were taken to detect the presence of the *nptII* gene by PCR. The results indicated that the *A. tumefaciens* GV3101 and LBA4404 strain gave two positive PCR results (Fig. 10a and 10c) and one positive PCR product obtained with *A. tumefaciens* EHA105 strain (Fig. 10b). *A. tumefaciens* GV3101 strain showed the highest regeneration percentage and numbers of positive histochemical GUS assay and PCR for the presence of *nptII* gene while transformed explants with LBA4404 strain failed to gave positive expression for GUS gene.

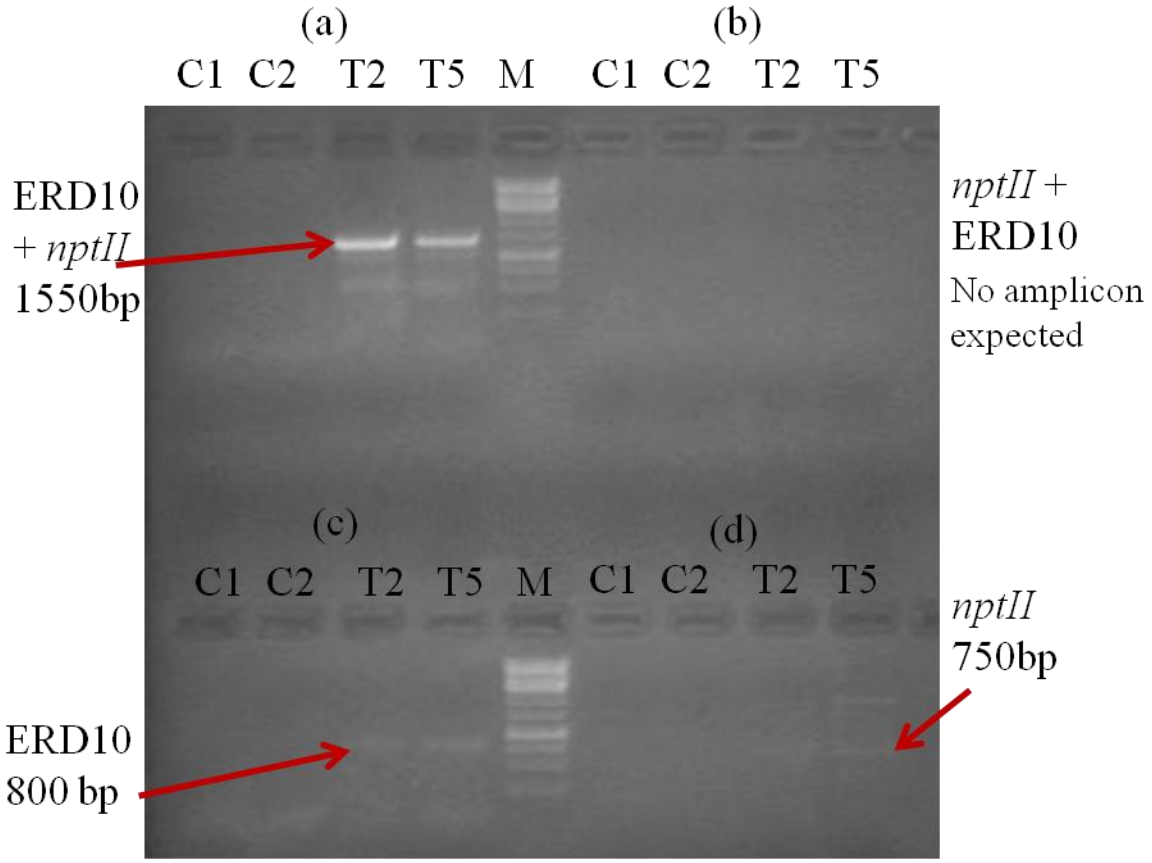

Figure 11. PCR products of (a). ERD 10+*nptII* gene (1550 bp), (b). *nptII* gene+ERD 10 (no amplicon expected), (c). ERD 10 (800 bp) and (d). *nptII* gene (750 bp). (M). One kbp DNA ladder. (C1 and C2) leaves excised from non transformed *B. aegyptiaca* plants after acclamtization in greenhouse. (T2 and T5) leaves excised from *B. aegyptiaca* plants transformed via *A. tumefaciens* GV3101 strain harbouring binary vector pBinAR contains (ERD10) after acclamtization in greenhouse. A positive control (plasmid DNA) and a no template control (water) have bene done but not shown in this figure..

Leaves from 2 transformed (T2 and T5) and non transformed (C1 and C2) *B. aegyptiaca* plants were taken for further PCR analysis. The presence of ERD 10 gene+*nptII* gene was detected through forward primer of ERd10 and reverse primer of *nptII* gene, where the transformed plants (T2 and T5) gave 1550 bp PCR fragment and negative PCR with non

transformed plants (Fig. 11a). The presence of *nptII* gene+ERD 10 gene was detected through forward primer of *nptII* gene and reverse primer of ERD 10 gene, where both transformed and non transformed plants gave negative PCR (Fig. 11b). Presence of ERD 10 gene was detected and the transformed plant (T2 and T5) gave 800 bp PCR fragment and negative PCR with non transformed plants (Fig. 11c). The presence of *nptII* gene was detected and the transformed plants (T2 and T5) gave 1550 bp PCR fragment and negative PCR with non transformed plants (Fig. 11d). The ERD10 was succesfully sequenced from the transgenic plant but the identity of the other sequences still need to be clarified.

Discussion

In the present study seed sources of *B. aegyptiaca* were collected from three provenances in Egypt (El-Kharga, Wadi El-Gemal and Wadi-Alaqi) to establish an efficient and simple in vitro system for *B. aegyptiaca* through nodal and cotyledon explants obtained from in vitro raised seedlings. Different concentrations of cytokinins (BA and TDZ) alone or in a combination with different concentrations of auxin (NAA) were used for shoot induction and proliferation. There were no significant differences between the two provenances with both explants.

There were significant differences between the two cytokinins at different concentrations alone or combined with auxin where each provenances showed different optimum concentration of cytokinins alone or combined with auxin for shoot induction and multiplication through both explants. Cytokinins at different concentrations are effective PGR for propagation and proliferation of axillary shoots. The major functions of cytokinins in DNA synthesis and cell division could explain their role in shoot multiplication (Khan et al. 2011). Cytokinins were suggested as an efficient PGR for shoot proliferation in several species and enhance the average of shoot multiplications when combined with low concentrations of auxin (Kataria et al. 2005; Xu et al. 2007; Khan et al. 2011; Gupta et al. 2014).

In this study BA was more efficient than TDZ for axillary bud sprouting and induced shoot proliferation in nodal explants and accelerated the number of shoots per regenerating explants and shoot length in nodal and cotyledon explants when used alone or combined with different concentrations of NAA. This is consistent with previous studies in *B. aegyptiaca* (Ndoye et al. 2003; Siddique and Anis 2008; Anis et al. 2010) where it was reported that TDZ was inferior to BA in the in vitro induction of axillary shoots in this species (Table 3). BA showed

efficient impact in multiple shoot bud proliferations in many conditions through using various types of explants (Anis et al. 2009; Husain and Anis 2009; Siddique and Anis 2009; Khan et al. 2011). Otherwise TDZ was more effective than BA to induce a high number of shoots per cotyledon explants (Table 4) in both provenances (El-Kharga and Wadi El-Alaqi). TDZ was more efficient to increase the number of shoots per explant in several woody plant species such as *Fraxinus pennsylvanica* Marsh (Kim et al. 1997), *Anacardium occidentale* L. (Shirly and Sadhana 2002), *Vitex negundo* (Ahmad and Anis 2007) and *Cotoneaster wilsonii* (Sivanesan et al. 2010). In contrast, TDZ inhibited the shoot elongation and decreased the number of nodal and cotyledon explants producing shoots when compared to BA alone or when combined with different concentrations of NAA. Also the combinations of TDZ with NAA accelerated the axillary shoots multiplications more than TDZ alone.

It was reported that TDZ enhance the axillary shoot proliferation in several woody plant species at low concentration (less than 1 µM) alone or in combination with another cytokinins or auxin (Huetteman and Preece 1993). The higher concentrations of TDZ more than 1 µM induced callus formation in silver maple and prevented the axillary shoot growth (Ashby et al. 1987). It was reported that TDZ inhibited root formation while Gray and Benton (1991) showed that the rooting percentage was 8% when the shoots of muscadine grape placed on medium contains 0.5 µM TDZ compared to 34% rooting when the shoots placed on medium contained 5 µM BA. Also the higher concentrations induced the formation of adventitious shoot (Chalupa 1988). The formation of callus and adventitious shoots through high concentration of TDZ increase the chance of somaclonal variation that is not preferred for the clonal uniformity, so it's recommended to care about the concentration of TDZ, which enhance only the proliferation of axillary shoot (Huetteman and Preece 1993).

The negative effect of TDZ on shoot length was also reported in *Cotoneaster wilsonii*, while the higher concentration of TDZ showed a negative impact on the rate of shoot induction and induced callus at the base of the explants also the regenerated shoots exhibited a degree of hyperhydricity (Sivanesan et al. 2010). It was reported that the high cytokinin activity of TDZ enhance the shoot proliferation and multiplication but exhibit a negative effect on shoot elongation resulting in shoot-bud stunted that might be related to the existence of phenyl group in TDZ (Huetteman and Preece 1993; Sivanesan et al. 2010). In woody plant species cotyledon from immature or mature seeds was the most common explants for produce adventitious shoots with TDZ. That was reported in white ash through using 10 µM TDZ

(Bates et al. 1992) and in *Prunus domestica* L. at 5-12.5 µM TDZ (Mante at al. 1988). Adventitious shoots formation through cotyledons increase the possibility for somaclonal variation and not sufficient to guarantee the uniformity (Huetteman and Preece 1993).

In our experiment, to increase the shoot length, the explants were transferred to MS medium without TDZ and sub-cultured every 2 weeks to accelerate the shoot elongation. The percentage of cotyledon explants producing shoots was increased through transfer the explants to a new fresh medium supplemented with cytokinins alone or combined with auxin. The repeating subculture of the explants on fresh culture medium was recommended for efficient multiplication of shoot clumps in woody plants (Phulwaria et al. 2012; Gupta et al. 2014). The efficiency of IBA in root induction was reported in several woody plant species (Distabanjong and Geneve 1997; Sivanesan et al. 2010; Khan et al. 2011). In this study both shoots from explants producing roots when placed on MS medium gave different root numbers and length at different concentration of IBA where provenance El-Kharga and Wadi El-Alaqi showed the highest rooting percentage through nodal explants at 1.2 µM IBA and at 2.4 µM IBA through cotyledon explants for both provenances. Each provenance showed different optimum concentration to produce the highest root length and number of roots per shoots (Table 7 and 8). IBA was used for root induction in several programs for *in vitro* root induction in *B. aegyptiaca* through various types of explants where the highest percentage of root formation from nodal explants was (68%), number of roots (5.3 ± 0.32) and root length (4.1 ± 0.38 cm) obtained through half strength MS medium containing 1.0 µM IBA (Anis et al. 2010). High rooting percentage (97.3%), root length (1.56 ± 0.13) and number of roots per shoots (6.18 ± 0.67) was obtained after 4-5 weeks on B5 medium supplemented with 9.84 µM IBA also it was reported root induction in medium without auxin (Gour et al. 2005; Gour et al. 2007). Shoots obtained from nodal explants was produced roots after 10 days when placed on medium supplemented with high concentration of auxin (20 mg/ml IBA) and the rooting percentage was 48.5%, root numbers was 12.5, and root length was 4.5 cm (Ndoye et al. 2003). The highest percentage of root formation from nodal explants (80 ± 2.6) was obtained at 1.0 µM IBA with activated charcoal (0.5%) with a number of roots of (8.5 ± 0.7) and mean root length of (5.3 ± 0.4 cm) (Siddique and Anis 2008).

Both nodal and cotyledon explants were used to establish an in vitro propagation system in *B. aegyptiaca* where the propagation from nodal explants was faster (4 months) than the propagation through cotyledon (5 months), the plantlet produced from cotyledon have a

thicker stem and bigger leaves compared to the plantlet produced through nodal explants. Cotyledon explants produced a higher number of shoots per explants. The shoots produced from cotyledons were grown slowly compared to shoots produced from nodal explants

To improve the resistance of *B. aegyptiaca* to some abiotic stress like salinity and maximize the capacity to cultivate this plant in large scale in non-arable land for biodiesel productions, it was recommended to establish a transformation system in *B. aegyptiaca* that was performed through three strains of *A. tumefaciens* strains (EHA 105, GV 3101, and LBA 4404) containing plasmid pCAMBIA2301. The results showed that nodal explants treated with GV 3101 strain represent the lowest mortality 48% when placed on MS medium containing high concentration of kanamycin up to 200 mg l^{-1}. Also the explants treated with this strain showed the highest number of positive GUS assay test and the presence of *nptII* gene through PCR. Chetty et al. (2013) examined the transformation efficiency in tomato (*Solanum lycopersicum* L. cv. Micro-Tom) they used different *A. tumefaciens* strains AGL1, EHA105, GV3101, and MP90, containing the plasmid pBI121 which harbouring the *nptII* and *uidA* genes, the presence of both genes was investigated in T0 plants by PCR, Southern blotting and GUS histochemical assay, where the transformation efficiency calculated as the percentage of inoculated explants producing positive PCR (transgenic plants) and *Agrobacterium* strain GV3101 exhibited the highest transformation rate (65%) and the cotyledon transformed with this strain showed the lowest mortality rate compare to the other strains. Bakhsh et al. (2013) indicated that, five *A. tumefaciens* strains (GV2260, LBA4404, AGL1, EHA105, and C58C1) containing pBin19 that harbouring beta-glucuronidase uidA and kanamycin genes, were used to investigate the transformation efficiency in *Nicotiana tabacum* L. cultivar Samsun. *Agrobacterium* strain LBA4404 showed the highest transformation efficiency with 20% then EHA105, GV2260, C58C1, and AGL1 strains, respectively. To establish a transformation system in grapevine, different *A. tumefaciens* strains were used (EHA105, AGL0, AGL1, and LBA4404) harbouring pBINm-gfp5-ER binary vector, besides that different nutrition medium were examined to improve the transformation through embryogenic callus. EHA105 *A. tumefaciens* showed the highest transformation rate, they reported that the *Agrobacterium* strain, the grapevine genotype and culture medium are factors affected the transformation rate and it was recommended to replace 2,4-D (2,4-dichlorophenoxyacetic acid) with NOA (ß-naphthoxyacetic acid) before the co-culture with *Agrobacterium* (Torregrosa et al. 2002). Thus in our study and based on several factors such as the highest survival rate, the number of explants gave positive GUS

assay and the presence of *nptII* gene through PCR, *A. tumefaciens* GV3101 strain was selected and used for further transformation experiments of *B. aegyptiaca*.

Conclusion

Establishment of an in vitro propagation system of tree woody plant species is necessary for clonal propagation and conservation of this plant species and genetic improvements through biotechnological approaches and gene transfer. The present study established an *in vitro* propagation system of two *B. aegyptiaca* provenances (El-Kharga and Wadi El-Alaqi) from nodal and cotyledon explants and this was taken 4 months and 5 months for nodal and cotyledon explants respectively. There were no significant differences between two provenances in the level of plant growth regulator concentrations and provenance-plant growth regulator concentrations interaction. BA was significantly more effective in shoot induction from nodal explants at 8.8 µM BA where as each provenance had different optimum concentrations for highest shoot length and number of shoots per explants. TDZ was more efficient in number of shoots produced per cotyledon explants and provenance El-Kharga exhibited the highest number of shoots per regenerated cotyledon explants at 4.4 µM TDZ combined with 5.3 µM NAA in the second subculture, while provenance Wadi Alaqi exhibited the highest number of shoots per regenerated cotyledon explant at 4.4 µM TDZ with 1.3 µM NAA in the second subculture. But using TDZ with cotyledon may enhance the possibility for somaclonal variation. Optimum concentration for root induction through nodal explants was 1.2 µM IBA and 2.4 µM IBA through cotyledon explants for both provenances. The in vitro propagation system could maximize the potential to cultivate *B. aegyptiaca* through non-arable land for biodiesel production. Also based on the highest survival rate and highest number of explants, positive GUS assay and PCR the *A. tumefaciens* GV3101 strain was used for further transformation experiments of *B. aegyptiaca* and could be used to produce salt-tolerant *B. aegyptiaca*.

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Supplementary tables

Table 1. ANOVA mixed model all data for shoot length from nodal.

	Num. D.F.	**Den,D.F.**	**F-value**	***P*-value**
(Intercept)	1	514	1697.29	0
Replicate	2	30	33.9072	2E-08
Genotypes	1	30	1.799025	0.189897
PGR Conc	7	30	40.07494	1.62E-13
Genotypes: PGR Conc.	7	30	0.994542	0.454159

Table 2. Analysis of deviance GLM for all data for shooting from nodal explants.

	D.f	**Deviance**	**Resid. D.f**	**Resid. Dev**	**Pr(>Chi)**
NULL	#N/A	#N/A	40	361.5967	#N/A
Replicate	2	6.44699	38	355.1497	0.039816
Genotypes	1	1.366487	37	353.7832	0.242416
PGR. Conc.	6	300.6486	31	53.13467	5.94E-62
Genotypes:PGR. Conc	6	9.927125	25	43.20755	0.127755

Table 3. Letter of significant for shooting obtained from El-Kharga genotype.

Letters	**PGR Conc.**
A	BA+NAA: 1.3 µM NAA
A	BA+NAA: 2.6 µM NAA
A	BA+NAA: 5.4 µM NAA
Ab	0
B	TDZ+NAA: 1.3 µM NAA
B	TDZ+NAA: 2.6 µM NAA
B	TDZ+NAA: 5.4 µM NAA

Table 4. Letter of significant for shooting obtained from Wadi-Alaqi genotype.

Letters	**PGR Conc.**
B	BA+NAA: 1.3 µM NAA
Ab	BA+NAA: 2.6 µM NAA
A	BA+NAA: 5.4 µM NAA
C	0
D	TDZ+NAA: 1.3 µM NAA
D	TDZ+NAA: 2.6 µM NAA
D	TDZ+NAA: 5.4 µM NAA

Table 5. ANOVA mixed model all data for rooting (%) from nodal explants.

	D. f	**Deviance**	**Resid. Df**	**Resid. Dev**	**F**	**Pr.(>F)**
NULL	#N/A	#N/A	22	82.06968	#N/A	#N/A
Replicate	2	6.063579	20	76.0061	0.894502	0.432543
Genotypes	1	5.971704	19	70.0344	1.761896	0.207228
PGR Con.c	3	17.96762	16	52.06678	1.76706	0.202934
Genotypes: PGR Conc.	3	4.664607	13	47.40217	0.45875	0.715761

Table 6. Letter of significant for rooting % from nodal explants obtained through El-Kharga genotype.

Letters	PGR Conc.
A	1.2 µM IBA
A	2.4 µM IBA
Ab	4.8 µM IBA
B	0

Table 7. ANOVA mixed model all data for root length from nodal explant nodal.

	numDF	denDF	F-value	*P*-value
(Intercept)	1	158	191.0995	0
Replicate	2	10	1.18436	0.345443
Genotypes	1	10	3.902669	0.07644
PGR Conc.	2	10	0.0752	0.928077
Genotypes: Conc.	2	10	0.065705	0.936808

Table 8. Analysis of deviance GLM for all data through regeneration from cotyledon explants.

	D.f	Deviance	Resid. D.f	Resid. Dev	Pr(>Chi)
NULL	#N/A	#N/A	53	224.6222	#N/A
Replicate	2	97.43652	51	127.1857	6.95E-22
Genotypes	1	2.898393	50	124.2873	0.088668
PGR Conc.	8	87.31257	42	36.97474	1.63E-15
Genotypes: PGR Conc.	8	5.604807	34	31.36994	0.691403

Table 9. Letters of significant for all regeneration data from cotyledon.

Letters	PGR Conc.
Ac	4.4 µM TDZ
A	8.8 µM TDZ
Ab	13.2 µM TDZ
A	22 µM TDZ
Ac	4.4 µM BA
Bc	8.8 µM BA
Bc	13.2 µM BA
C	22 µM BA

Table 10. ANOVA mixed model for all data through shoot length from cotyledon explants

	Num. DF	**Den DF**	**F-value**	***p*-value**
(Intercept)	1	290	607.8306	0
Replicate	2	44	11.94069	7.2E-05
Genotypes	1	44	0.229804	0.634043
PGR.Conc.	7	290	9.864277	4.86E-11
Genotypes: PGR Conc.	7	290	0.617425	0.741445

Table 11. Letters of significant for shoot length obtained from cotyledon of El-Kharga genotype.

Letters	PGR Conc.
Ab	4.4 µM TDZ
A	8.8 µM TDZ
Ab	13.2 µM TDZ
Ab	22 µM TDZ
B	4.4 µM BA
B	8.8 µM BA
B	13.2 µM BA
B	22 µM BA

Table 12. Letters of significant for shoot length obtained from cotyledon of Wadi-Alaqi genotype.

Letters	**PGR Conc.**
Abc	4.4 µM TDZ
A	8.8 µM TDZ
Ab	13.2 µM TDZ
Ab	22 µM TDZ
Abc	4.4 µM BA
Bd	8.8 µM BA
D	13.2 µM BA
Cd	22 µM BA

Table 13. Analysis of deviance of GLM for all data for the number of shoots from cotyledon explants.

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
NULL	#N/A	#N/A	41	147.6604	#N/A
Replicate	2	32.25299	39	115.4074	9.92E-08
Genotypes	1	5.97176	38	109.4357	0.014537
HC	6	92.75066	32	16.68501	8.12E-18
Genotypes:HC	6	3.171603	26	13.51341	0.787021

Table 14. Letters of significant for shoot length obtained from cotyledon of El-Kharga genotype.

Letters	PGR Conc.
A	BA+NAA: 1.3 µM NAA
Ab	BA+NAA: 2.6 µM NAA
A	BA+NAA: 5.4 µM NAA
C	0
Ab	TDZ+NAA: 1.3 µM NAA
Ab	TDZ+NAA: 2.6 µM NAA
B	TDZ+NAA: 5.4 µM NAA

Table 15. ANOVA mixed model for all data for shoot length in cotyledon explants.

	Num. D.F	Den. D.F	F-value	*p*-value
(Intercept)	1	346	617.7778	0
Replicate	2	22	3.281665	0.056592
Genotypes	1	22	0.995998	0.329132
HC	5	22	18.7744	2.8E-07
Genotypes:HC	5	22	0.56337	0.726946

Table 16. Letters of significant for shoot length obtained from cotyledon of El-Kharga genotype.

Letters	PGR Conc.
B	BA+NAA: 1.3 µM NAA
B	BA+NAA: 2.6 µM NAA
B	BA+NAA: 5.4 µM NAA
A	0
A	TDZ+NAA: 1.3 µM NAA
A	TDZ+NAA: 2.6 µM NAA

Table 17. Letters of significant for shoot length obtained from cotyledon of Wadi-Alaqi genotype.

Letters	PGR Conc.
Bc	BA+NAA: 1.3 µM NAA
C	BA+NAA: 2.6 µM NAA
C	BA+NAA: 5.4 µM NAA
Ab	0
A	TDZ+NAA: 1.3 µM NAA
A	TDZ+NAA: 2.6 µM NAA

Chapter 7

General discussion

With the reduction of the energy resources around the world, many countries search for other alternative solutions such as bioenergy through the cultivation of oil-producing plants for biodiesel or biogas production. However, with the reduction in the arable land around the world and the competition between the food crops and bioenergy plants in the availability of arable land a major ethic dilemma is caused. There is a demand to search for plant species that could be cultivated in non-arable land. The main purpose of this study was to investigate the possibility of the cultivation of a drought-tolerant species mainly for the production of bioenergy in non-arable and rural regions. For this purpose *Balanites aegyptiaca* was suggested as drought-tolerant plant and a source of biodiesel. Therefore, investigation of the genetic variation among *B. aegyptiaca* sources collected from different regions was performed and quantitative method to analyse drought tolerance mechanisms in *B. aegyptiaca* to finally select the most drought-tolerant genotype. In parallel, propagation and first steps towards a transformation system for the most drought-tolerant genotype was conducted for cultivation and improvement of other abiotic stress tolerances, such as salt tolerance.

The relation between the genetic diversity and geographic distribution

A genetic characterization of the *B. aegyptiaca* sources which were collected from different provenances was performed and the relation between the genetic diversity and geographical distribution through amplified fragment length polymorphism (AFLP) investigated. For this purpose seed from 12 *B. aegyptiaca* sources were collected from different provenances. The results indicated that there is no clear relation between the high genetic variation and the geographic distribution of the samples. The relation between genetic variation and geographic distribution was recorded in *B. aegyptiaca* by Chamberlain (1992) through isoelectric focusing of peroxidase isozymes. Also the relation between the phenotypic variation and geographic distribution was investigated by Abasse et al. (2010). They reported that there were morphological variations among four natural populations of *B. aegyptiaca* collected from different regions in Eastern Niger while in some parameters significant geographic variations were recorded within populations, for example the trees grown in drier parts exhibited heavier fruits and kernels. The same results were obtained by Elfeel et al. (2009). They recorded a large significant influence of the soil type on the germination and growth parameters of *B. aegyptiaca* seed sources collected from 11 provenances. On the other hand, the cluster analysis and grouping of all provenances was not related to the soil type or level of

rainfall in the provenances but was related to overall growth parameters. Dependent on these results the hypothesis of the relation between the genetic variation and geographic distribution in *B. aegyptiaca* is still not clarified.

In this study 477 (93.5%) polymorphic bands were obtained from 12 *B. aegyptiaca* individuals collected from different provenances. The samples based on this data can be considered as different genotypes. The large percentage of polymorphism was also recorded for other woody plant species (Tang et al. 2003; Paz et al. 2005; Paz et al. 2012). The clustering and principal coordinate analysis distributed the 12 genotypes into three clades. The two genotypes collected from Wadi-El-Gemal located in one clade and genotype El-Kharga was separated in the second clade. Based on the slight decrease in growth parameters and consuming high concentration of total ascorbic acid under severe stress (Chapter 4) and the high potential to recover after withholding water stress (Chapter 5) genotype El-Kharga was considered as the most drought tolerant among several *B. aegyptiaca* genotypes that were exposed to severe water stress conditions. The other genotypes were grouped in the third clade (Wadi-El-Alaqi, Cairo, Sudan, Togo, Ethiobia, Ghana, Medina, Jazan and Yemen). This result showed that the genotypes collected from the same provenances were genetically more closely related than the genotypes collected from different provenances in the same country or from other countries, but one individual from each provenances not enough for better understanding the genetic characterization of *Balanites* sources and there is a demand to increase the size of population and number of individuals.

The influence of outcrossing in the high genetic variation within population in woody plant species was recorded by Hamrick et al. (1992). The woody plant species *B. aegyptiaca* is autocompatible and showed approximately 37% of allopollination. This outcrossing is obtained through insects like Dipterae and Halictidae or the wind (Ndoye et al. 2004; Dubey et al. 2011). This percentage of outcrossing pollination in the species can explain the genetic variation within individuals which was not correlated with the geographic distribution. Also the dispersion of the seeds through the mobility of animal-intake of seeds could explain the genetic variation across various geographic regions.

Identification of the most drought-tolerant *B. aegyptiaca* genotypes

This study was aimed to determine the most drought-tolerant *B. aegyptiaca* genotypes under severe drought stress. For this goal six different *B. aegyptiaca* genotypes collected from various regions (El-Kharga, Wadi El-Alaqi, Wadi El-Gemal, Cairo, Sudan and Togo) were

examined and compared for their morpho-physiological responses to water limitations among the genotypes. The impact of water stress was recorded through classical growth parameters and some more other techniques like the determination of stomata conductance, photochemical efficiency and metabolite contents. The mechanisms plants used to overcome the water limitation can be divided into three levels; escape, avoidance and tolerance (Chaves et al. 2003). To cope with the stress under moderate water stress conditions, *B. aegyptiaca* genotypes decreased the plant water content as avoidance mechanism, while under severe water stress there was a drastic reduction in growth parameters and leaf shedding as tolerance mechanism. These responses were significantly different for each genotype and among the genotypes under control, moderate and severe stress. This was reported also in previous studies on *B. aegyptiaca* by Elfeel et al. (2007) while under water stress conditions there were reductions in shoot and leaf weight. Also Zarad and Hasanin (2011) showed that under water stress *B. aegyptiaca* plants overcome the stress through reduction in the biomass, leaf length* width and relative growth rate. This was also reported in almond cultivars by Gikloo et al. (2012) where the leaf length* width and leaf dry weight were decreased under water stress. The reduction in growth parameters might be caused by the decrease in the frequency of cell division and elongation under water stress (Farooq et al. 2009).

The change in growth parameters as a long term response to drought stress was combined with a short term response through changing stomata conductance (Arve et al. 2009). Stomata closure is considered as an early plant response to water stress (Cornic and Massacci 1996), there were highly significant reductions in stomata conductance in each genotype under different soil water contents observed. There were also clear significant differences among genotypes at control and moderate drought stress treatments. These significant differences were smaller under severe drought stress where El-Kharga and Sudan significantly differed from Togo and Wadi El-Gemal. Genotypes Cairo and El-Kharga exhibited a water saving strategy under severe drought stress through stomata closure that maintained the leaf and stem water content at the save level (Chapter 4). The role of early stomata closure under control treatment and under severe water stress was recorded in several plant species, such as in *J. curcas*. The plants under water stress exhibited avoidance mechanisms through early stomata closure resulting in a reduction of the transpiration level with a decrease in the losses of plant water content (Díaz-López et al. 2012; Fini et al. 2013). The avoidance mechanism through stomata closure under water stress was also reported in the deciduous species of *Cordia africana* Lam. and *Croton macrostachyus* Del. (Gindaba et al. 2004).

Data obtained from photochemical efficiency (ΔF⁄Fm') showed that only genotype Wadi El-Alaqi exhibited a significant reduction in photochemical efficiency at the second week of stress treatment, while other *B. aegyptiaca* genotypes showed a slight decrease in the photochemical efficiency under water stress. The slight decrease in photochemical efficiency among genotypes through different levels of water stress was recorded in *J. curcas* (Maes et al. 2009; Fini et al. 2013) and was also recorded with four genotypes of *Lablab purpureus* (Guretzki and Papenbrock 2013). *Balanites aegyptiaca* produced a high concentration of total ascorbic acid (TAA) compared to other plant species (445, 228, and 53 mg 100^{-1} g FW) in Indian gooseberry, Guava and Orange, respectively (USDA 2007). Where under well-watered conditions genotypes El-Kharga and Togo produced high concentrations of TAA (608.9 and 527.9 mg 100 g^{-1} FW, respectively)**.** We suggested that total ascorbic acid was decreased and consumed under severe drought stress (288.9 and 276.2 mg 100 g^{-1} FW) in genotypes El-Kharga and Togo, respectively, to cope with water stress as tolerance mechanism where TAA showed an important role as electron donor in the scavenging of reactive oxygen species (Zhang 2013).

In summary, *B. aegyptiaca* genotypes exhibited several avoidance and tolerance mechanisms to cope with severe water stress through early stomata closure resulting in a drastic reduction in growth biomass with leaf shedding and produced high concentrations of TAA. The genotypes with the highest drought tolerance from this study were El-Kharga and Cairo. Genotypes El-Kharga and Cairo showed significantly the smallest reduction in biomass from control to severe water stress, also the reduction in stomata conductance for both genotypes from control to severe stress was significantly higher in comparison to the other genotypes, resulting in maintenance of the plant water content in the save level between 72.4 and 53.7% in stems, and 40.7% and 51.5% in leaves for genotypes Cairo and EL-Kharga, respectively, under severe water stress. Also genotype El-Kharga produced the highest amount of TAA under well-water conditions (608.9 mg 100 g^{-1} FW) which was consumed under severe stress (288.9 mg 100 g^{-1} FW) to cope with the stress. Only one plant from each genotype died under severe stress. Therefore both genotypes can be recommended for further field studies in arid and semi-arid regions.

Establishment of a regeneration and transformation system of *B. aegyptiaca*

Establishment of *in vitro* propagation system in *B. aegyptiaca* is a powerful tool for mass micropropagation. So the purpose of this study was to establish a regeneration system

through nodal and cotyledon explants obtained from *B. aegyptiaca* four weeks old seedlings from two genotypes (El-Kharga and Wadi El-Alaqi). For shoot induction and proliferation two different cytokinins, 6-benzyladenine (BA) and thidiazuron (TDZ) at different concentrations were used. At 8.8 µM BA Wadi El-Alaqi genotype showed the highest percentage of nodal explants producing shoots (89%) and genotype El-Kharga showed the highest percentage of cotyledon explants producing shoots (49%) at 13.2 µM BA. The optimal concentration of cytokinins were combined with the auxin α-naphthalene acetic acid (NAA) for shoot multiplication where the highest number of shoots per regenerated nodal explant (4.78 ± 2.3) was recorded for genotype El-Kharga at 8.8 µM BA with 2.6 µM NAA. Genotype El-Kharga showed also the highest number of shoots per regenerated cotyledon explants (6.1 ± 3.1) at 4.4 µM TDZ with 5.3 µM NAA. The results for both explants showed that there were no significant differences between responses of the genotypes under investigation to the level of plant growth regulator (PGR) concentrations or the genotype-PGR interaction. Significant differences were related to the effect of the cytokinins that were recorded between the BA and TDZ at different concentrations separately or combined with auxin. The effects of BA in shoot induction, number of shoot per regenerated explant, and shoot length for both explants were significantly higher than that obtained in the presence of thidiazuron separately or in a combination with NAA. On the other hand TDZ had a negative impact on the shoot length with both explants. The superiority of BA compared to TDZ in shoot induction and sprouting the axillary bud was previously reported in *B. aegyptiaca* (Ndoye et al. 2003; Siddique and Anis 2008; Anis et al. 2010). Also the effectiveness of BA compared to TDZ in shoot multiplication and proliferation by using several types of explants in this species was reported previously (Anis et al. 2010; Husain and Anis 2009; Siddique and Anis 2008; Khan et al. 2011). The impact of cytokinins in shoot multiplication is related to their role in DNA synthesis and cell division (Khan et al. 2011). The negative effect of TDZ on shoot length may be related to the existence of a phenyl group in TDZ that affects the shoot elongation and bud sprouting (Huetteman and Preece 1993; Sivanesan et al. 2010). On the other hand, TDZ was more efficient than BA in the shoot multiplications and number of shoots per cotyledon explants but induced negative effect on shoot length. In vitro rooting was performed through different concentrations of indole-3-butric acid (IBA), which was also observed in various woody plant species (Sivanesan et al. 2010). IBA was used for root induction in several programs for *in vitro* root induction in *B. aegyptiaca* through placed the explants on half strength MS medium containing 1.0 µM IBA (Anis et al. 2010) or on B5

medium supplemented with 9.84 µM IBA also it was reported root induction in medium without auxin (Gour et al. 2005, 2007).

Propagation of *B. aegyptiaca* through the conventional methods has several limitations. Our results showed that root induction from cuttings is very low, less than 30% and dependent on the season, more efficient in the summer than in the winter (data not shown). Also the low survival rate of seeds in soil or sand and the time needed for germination are restrictions. Besides that there is a demand for expanding the cultivation in the non-arable land to provide more seeds for biodiesel production in the economical level and the reservation of the most drought-tolerant genotypes. In vitro propagation provides fast and efficient results for large scale micropropagation, while a complete plant needs 4 months from nodal segment and 5 months from cotyledon explants. These provide more plants for the cultivation in the non-arable regions that are difficult to provide through conventional propagation.
The establishment of a regeneration system provides a chance to establish a transformation system in *B. aegyptiaca* to produce salt stress tolerant plant. Three strains of *Agrobacterium tumefaciens* harbouring the plasmid pCAMBIA2301 were used to select the most efficient strain in transformation. *A. tumefaciens* GV3101 strain based on the highest survival rate and highest number of explants gave positive GUS assay and PCR was used for further transformation experiments of *B. aegyptiaca* to produce transformed *B. aegyptiaca* that might be more tolerant to salt stress.

The micropropagation provides an efficient tool for reservation and maximizes the cultivation of wild and forest trees in t he arid and semi-arid regions as source for biodiesel production or secondary metabolites. This was already done for several plant species: *J. curcas* as source for biodiesel production in arid and semi-arid lands was micropropagated from bud aggregates (Daudet et al. 2013), *E. tirucalli* was used for biogas production and was in vitro regenerated through internodal explants (Uchida et al. 2004), a regeneration system was established through nodal segments for the cultivation of Jaal (*Salvadora persica*) in arid forest areas (Phulwaria et al. 2011), and the multi-purpose medicinal tree *Acacia auriculiformis* was in vitro propagated through nodal stem segments (Length 2011).

Outview and future perspective

The genetic variation among of *B. aegyptiaca* genotypes through AFLP indicated that the genotypes from the same provenances were genetically more closely than the genotypes from different provenances in the same country or from other countries but there was no relation between the high genetic variation within population and the geographic distribution of *B. aegyptiaca* genotypes collected from different regions. One individual from each provenance not enough to investigate the genetic characterization among *Balanites* sources so for better understanding this relation, increase the population size and the distance between the tress and collect more samples from different provenances in Egypt and other countries is a prerequisite to build an overview about the genetic characterization of *Balanites* sources. So we need more experiment to finally provide a strategy to select and cultivate the most drought-tolerant genotype for biodiesel production. Where the combination of genetic markers and the determination of the oil content will identify the most promising genotypes and investigate the variation among different populations.

Under severe drought stress *B. aegyptiaca* genotypes showed several morpho-physiological responses to adapt the stress through early stomata conductance, drastic reduction in growth and production of high concentrations of TAA. Studying the molecular mechanism of plants use to cope with the stress could provide better understanding for the mechanism used by *B. aegyptiaca* genotypes to overcome water stress. Several molecular approaches are recommended such as AFLP-cDNA finger print, DNA methylation, deep sequencing and genome wide association studies (GWAS) and segregating populations that will help to investigate the up-regulated and down regulated genes under drought stress and discover the genes responsible for the tolerance mechanisms to water stress in *B. aegyptiaca*. While the drought experiment through vegetative cuttings showed that genotype El-Kharga is considered as the most drought-tolerant, so there is demand to study the effect of water stress through *Balanites* seedlings to make a recommendation for further field trials in arid and semi-arid regions that might be through vegetative cutting which have limitation in root induction or through plantlets produced from *in vitro* propagation. For the mass micropropagation of *Balanites* sources a regeneration system was established for *B. aegyptiaca* through nodal and cotyledon explants while BA was more efficient in shoot induction and multiplication with both explants. *Balanites* sources were sensitive to salt stress and showed reduction in growth parameters under low level of salt stress (Radwan 2000; Elfeel et al. 2013). Based on the highest survival rate and highest number of explants gave

positive GUS assay and PCR from *Agrobacterium tumefaciens strain* GV 3101, this strain was used to introduce binary vector pBinAR into *B. aegyptiaca* with ERD10 to produce transgenic *B. aegyptiaca* plants more resistant to salt stress. The performance of the transformed plants need to be examined through several concentrations of salt to finally select the most tolerant *B. aegyptiaca* lines that can be cultivated in non-arable land for biodiesel production.

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	National Institute of Laser Enhanced Science (NILES). Cairo University, Cairo

Publications

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Erklärung:

Hiermit erkläre ich, dass ich die Dissertation selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfsleistungen herangezogenen Institutionen vollständig angegeben habe. Ich erkläre auch, dass die Dissertation nicht schon als Masterarbeit, Diplomarbeit oder ähnliche Prüfungsarbeit verwendet worden ist.

Hannover, 05.01.2015