Development of plant species and ecotypes tolerant to drought stress as crop plants

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§ 8 Dissertation

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 5.

B: (3)

…vornimmt sowie die individuellen eigenen Beiträge und ggf. die Beiträge weiterer Autoren an den jeweiligen Publikationen darlegt.

Publikation (Kapitel 2):

Guretzki S, Papenbrock J (2013) Comparative analysis of methods analyzing effects of drought on the herbaceous plant *Lablab purpureus*. *Journal of Applied Botany and Food Quality* 86, 47-54 (DOI:10.5073/JABFQ.2013.086.007).

- Etablierung der verwendeten Methoden für *L. purpureus*.
- Zusammenstellung von geeigneten Methoden zur Messung der Auswirkungen von Trockenstress bei *L. purpureus*.
- Durchführung der Experimente.
- Erstellung des Manuskripts für die Veröffentlichung.

Publikation (Kapitel 3):

Hastilestari BR, Mudersbach M, Tomala F, Vogt H, Biskupek-Korell B, Van Damme P, **Guretzki S**, Papenbrock J (2013) *Euphorbia tirucalli* L. – comprehensive characterization of a drought tolerant plant with a potential as biofuel source. *PLoS ONE* 8 (e63501. doi:10.1371/journal.pone.0063501).

- Etablierung verschiedener Methoden für die Messung der Auswirkungen von Trockenstress zur Anwendung bei *E. tirucalli*.
- Etablierung der Methode AFLP
- Unterstützung bei der Durchführung der Trockenstress und AFLP Experimente.
- Mitarbeit bei der Erstellung des Manuskripts für die Veröffentlichung.

Publikation (Kapitel 4):

Guretzki S, Papenbrock J (2014) Characterization of *Lablab purpureus* regarding drought tolerance, trypsin inhibitor activity and cyanogenic potential for selection in breeding programmes. *Journal of Agronomy and Crop Science* 200, 24-35 (DOI:10.1111/ jac.12043).

- Durchführung der Experimente mit den in Kapitel 2 verwendeten Methoden bei Weiteren *L. purpureus* Genotypen.
- Etablierung der Methode und Durchführung der Experimente in Bezug auf die Messung der Trypsininhibitoraktivität bei *L. purpureus*.
- Vorbereitung der *L. purpureus* Proben für die HCNp Messung.
- Erstellung des Manuskripts für die Veröffentlichung.

KURZZUSAMMENFASSUNG

Die globale Klimaveränderung sorgt für eine stetige Zunahme an landwirtschaftlich genutzten Flächen, die von ariden Klimabedingungen aufgrund von verringerten Niederschlagsmengen betroffen sind. In der Folge können von den Bauern durch den Einsatz gängiger Nutzpflanzen nur noch geringere Erträge erwirtschaftet werden. Ein Ziel dieser Arbeit ist es, ein Methodenportfolio für die quantitative Analyse der Auswirkungen von abiotischem, durch Trockenheit verursachten Stress auf Pflanzen zu erarbeiten. Beispielhaft wird dazu eine Auswahl an Genotypen, der als trockentolerant geltenden Spezies *Lablab purpureus* (L.) Sweet (Helmbohne) untersucht. Die verwendeten Methoden basieren auf der Messung verschiedener Parameter und können in Kombination zeiteffizient ein Ranking der untersuchten Genotypen in Bezug auf die stärkste Trockentoleranz ermöglichen. In Züchtungsprogrammen kann dieses Ranking als Selektionskriterium genutzt werden. Es werden traditionelle Messmethoden wie etwa die Bestimmung der Zunahme der Pflanzenhöhe und Biomasse analysiert, wodurch die langfristigen Auswirkungen bei Trockenstress (z.B. Wachstumsinhibition) auf die Pflanzen beobachtet werden. Demgegenüber stehen die moderneren Methoden Chlorophyllfluoreszenz-Messung, Porometrie und Infrarotthermographie für die Analyse von kurzfristigeren Effekten des Trockenstresses auf die Photosynthese und die Spaltöffnungsbewegungen von Pflanzen. Schlussendlich ist es nicht möglich, alle Methoden für eine Spezies zu nutzen. Die Messungen der Chlorophyllfluoreszenz ergaben bei *L. purpureus* keine brauchbaren Ergebnisse. Ebenso sorgte die windende Wuchsform der Pflanze z.B. bei der Bestimmung der Pflanzenhöhe für Schwierigkeiten. Nutzpflanzen enthalten sekundäre Pflanzenstoffe, die beim Verzehr schädlich wirken können (Anti-nutritional factors). Deshalb wurden die Trypsininhibitoraktivität und das cyanogene Potenzial (HCNp) in den Samen von *L. purpureus* analysiert. Beide Faktoren zeigen eine hohe Varianz in den Ergebnissen und können als negative Selektionsfaktoren in Züchtungsprogrammen genutzt werden. Die Ergebnisse zeigen, dass die Trypsininhibitoraktivität einen genetischen Ursprung haben könnte. Für die zweite in dieser Arbeit genutzte Pflanze *Euphorbia tirucalli* L. wurde, basierend auf dem erarbeiteten Methodenportfolio, ebenfalls ein optimiertes Methodenset für die Trockenstressreaktion etabliert und für die Untersuchung von *E. tirucalli* genutzt. Verschiedene *E. tirucalli*-Genotypen konnten mittels Amplified fragment-length polymorphism (AFLP) in zwei Gruppen aus afrikanischen und vorwiegend nicht afrikanischen Genotypen unterteilt werden.

Schlüsselwörter: *Euphorbia tirucalli*, *Lablab purpureus*, Trockenstress

ABSTRACT

Global climate change provides a steady increase in agriculture areas affected by arid climate conditions, due to reduced rainfall. This results in lower yields for the farmers, which can be achieved by the use of common crops.

One aim of this thesis is to develop a method portfolio for the quantitative analysis of the abiotic stress impact on plants due to drought. Exemplary a genotype selection of the species *Lablab purpureus* (L.) Sweet (Helmbohne) is examined. The species *L. purpureus* is described as drought tolerant. The methods used are based on the measurement of various parameters and can offer a ranking on studied genotypes in a time efficient manner with regard to the highest drought tolerance level. In breeding programs, this ranking can be used as a selection criterion. Traditional measurement methods like determination of increase in plant height and biomass are analyzed. Thereby long-term effects on drought stress for example growth inhibition can be observed on plants. In contrast, the more modern methods chlorophyll fluorescence, porometry and infrared thermography are used for the analysis of plant short-term effects towards drought stress on photosynthesis and stomatal behavior. Finally, it is not possible to use all methods for a species, the measurement of chlorophyll fluorescence in *L. purpureus* showed no useful results. Additionally the twining growth habit of *L. purpureus* caused difficulties in measurements like plant height determination. Crops contain secondary metabolites that can be harmful when consumed (anti-nutritional factors). Therefore, the trypsin inhibitor activity and cyanogenic potential (HCNp) were analyzed in the seeds of *L. purpureus*. Both factors show a high variance in the results and can be used as negative selection criterion. The results indicate that the trypsin inhibitor activity could be of genetic origin.

For *Euphorbia tirucalli* (L.) the second plant used in this thesis an optimized method set based on the method portfolio is established. With this set different genotypes were studied. *E. tirucalli* genotypes could be divided by amplified fragment-length polymorphism (AFLP) in two groups of African and mainly non-African genotypes.

Keywords: Drought stress, *Euphorbia tirucalli*, *Lablab purpureus*

Contents

ABBREVIATIONS:

AFLP	-	amplified fragment length polymorphism
ANOVA	-	analysis of variance
approx.	-	approximately
APS	-	ammonium persulfate
ATP	-	adenosine triphosphate
$CaCl_2$ $2H_2O$	-	calcium chloride dihydrate
CAM	-	crassulacean acid metabolism
CE	-	capillary electrophoresis
cg	-	control group
CH_4	-	methane
CIAT	-	International Centre for Tropical Agriculture
CO_2	-	carbon dioxide
CoA	-	coenzyme A
COD	-	chemical oxygen demand
CPI	-	Commonwealth plant introduction
c.q.	-	*casu quo*
cv.	-	cultivar
CWSI	-	crop water stress index
D	-	deficit
dg	-	drought group
DM	-	dry matter
DNA	-	deoxyribonucleic acid
dNTPs	-	deoxynucleotide triphosphates
DTT	-	dithiothreitol
DW	-	dry weight
e.g.	-	*exempli gratia*
Eco	-	*Escherichia coli*
EDTA	-	ethylenediaminetetraacetic acid
et al.	-	*et alii*
ETR	-	electron transport rate
exp.	-	experiment
FAMD	-	fingerprint analysis with missing data
FAO	-	Food and Agriculture Organization of the United Nations
Fig.	-	figure
F_v/F_m	-	maximal PS II quantum yield
FW	-	fresh weight
GC-MS	-	gas chromatography - mass spectrometry
H_2O	-	water
H_2S	-	hydrogen sulfide
HCl	-	hydrogen chloride

HCN	-	hydrogen cyanide
HCNp	-	cyanogenic potential
HPCE	-	high performance capillary electrophoresis
IRD	-	infrared dye
KAc	-	potassium acetate
LSD	-	least significant different
MA-type	-	morpho-agronomic type
MgAc	-	magnesium acetate
Mor	-	Morroco
Mse	-	*Micrococcus* species
NAD-ME	-	nicotinamine adenine dinucleotide-dependent malic enzyme
NADP-ME	-	nicotinamide adenine dinucleotide phosphate dependent malic enzyme
NaN_3	-	sodium azide
NaOH	-	sodium hydroxide
NCBI	-	National Center for Biotechnology
NJ	-	neighbor joining
NMR	-	nuclear magnetic resonance
NPK	-	nitrogen(N)-phosphorus(P)-potassium(K)
NPQ/4	-	non-photochemical quenching/ 4
O_2	-	oxygen
ODM	-	organic dry matter
PAM	-	pulse-amplitude-modulation
PAR	-	photosynthetically active radiation
PCR	-	polymerase chain reaction
PEP	-	phosphoenolpyruvate
ppm	-	parts per million
PSII	-	photosystem 2
PWC	-	plant water content
RL-Buffer	-	restriction ligation buffer
ROS	-	reactive oxygen species
rpm	-	rounds per minute
RT	-	room temperature
RWC	-	relative water content
SEM	-	standard error of mean
Sen	-	Senegal
Taq	-	*Thermus aquaticus*
TBE	-	tris-borate-EDTA
TDR	-	time-domain reflectometry
TI	-	trypsin inhibitor
TIU	-	trypsin inhibitor units
TNAU	-	Tamil Nadu Agriculture University

Tris	-	2-amino-2-hydroxymethyl-propane-1,3-diol
UK	-	United Kingdom
UPGMA	-	unweighted pair group method with arithmetic mean
USA	-	United States of America
USDA	-	United States Department of Agriculture
VWC	-	volumetric water content
Y(II)	-	effective PS II quantum yield
Y(NO)	-	quantum yield of nonregulated energy dissipation
Y(NPQ)	-	quantum yield of regulated energy dissipation

CHAPTER 1:

GENERAL INTRODUCTION

THE CONSEQUENCES OF ARID CLIMATIC CONDITIONS FOR CROP PRODUCTION AND SOCIETY

Changes in the climate conditions result in changes of rainfall quantities, for semi-arid regions a reduction of the amount of rain is expected. In general higher rainfall variability and an increase in extreme weather events is predicted (FAO, 2012). Nowadays drought is already the leading reason for strong food scarcity in developing countries (FAO, 2012). Toulmin (1986) summarizes the impact of drought for farmers (Fig. 1). The effects of drought conditions can cause a reduction of crop yields and correspondingly a reduction in fodder yields. As a result, the farm animals can die. At the same time farm animals must be sold in order to compensate the loss of crop yields, so that new grain can be purchased as food and fodder source. Due to the sale or loss of working animals, a lower number of fields are cultured and the amount of manure is reduced. In the next season this again leads to a reduction of yield.

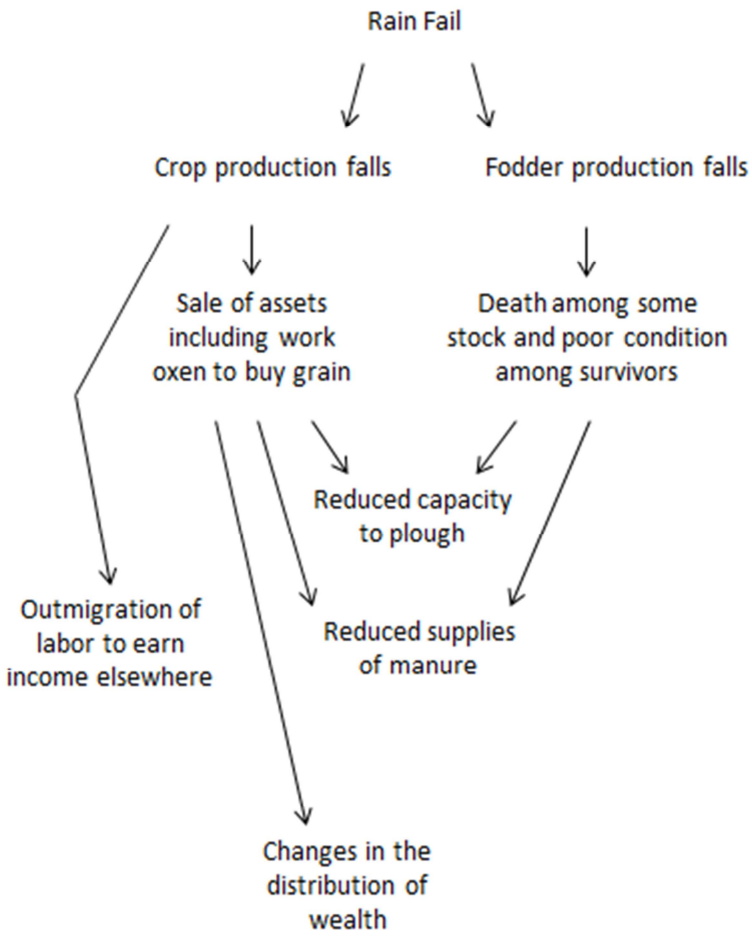

Fig. 1: The impact of drought on the livelihood of farmers (Taken and redrawn from Toulmin (1986)).

Finally, drought ensures for labor migration from the affected regions and for a redistribution of wealth. Each year drought is causing damage both in industrialized countries and in developing countries around the world. Due to their impacts on society the global occurring droughts are in the media spotlight (Fig. 2). For example, in 2012 the United States were affected by the worst drought within the last 25 years. The drought associated with crop losses affected especially the Midwest of the United States and left major damage to the field corn and soybean harvests. As a result of this drought the market prices for agricultural products increased. It is also expected that the consumer prices of processed and packaged products will increase in 2013 (USDA, 2013). The corn yield in Kentucky was affected most (minus 63% in comparison to the last years). Kansas had the largest yield decline in soybean and sorghum harvests. However, most affected by the drought climate conditions was the state Colorado (The guardian, 2013). The insurance costs of the drought will likely exceed $ 20 billion (CNN, 2012).

Fig. 2: The media presence of the topic drought in German newspapers and magazines based on headlines.

One possibility to avoid the shortage of water is the adaptation of crops or crop genotypes to the existing water conditions in the respective region (FAO, 2008). The genetic mechanisms that lead to a high drought tolerance in certain genotypes of a crop have to be

examined. The information gained in combination with classical breeding programs should help to find the right crop genotypes for drought climate conditions (Nature, 2012).

THE EFFECTS OF DROUGHT ON PLANTS

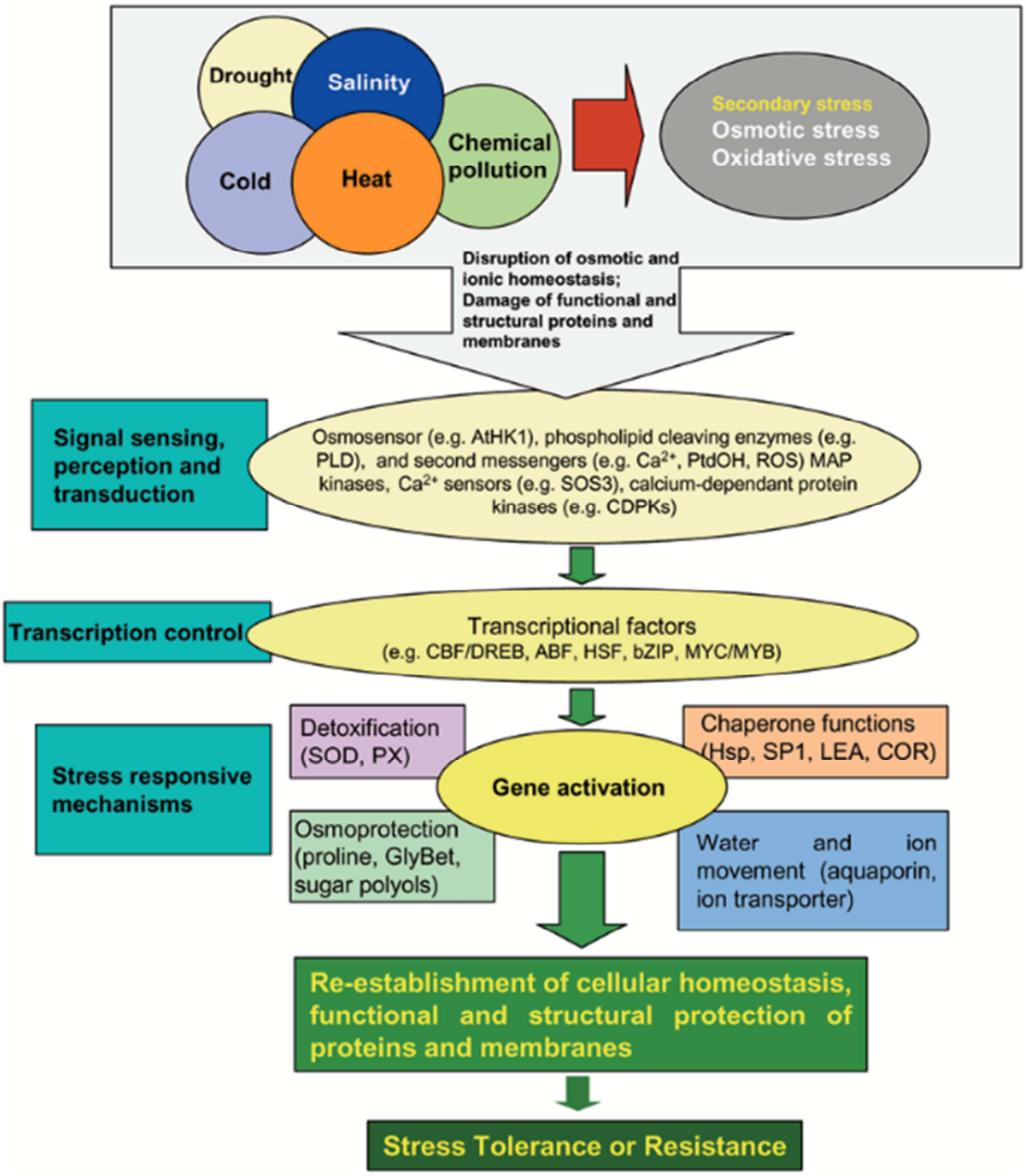

Fig. 3: Reaction chain of plants under the influence of abiotic stress, such as drought. Abiotic stress results in secondary stress and in changes of the ionic and osmotic homeostasis as well as in damage on protein and membrane level. As a result, various reactions e.g. detoxification mechanisms start to regain the unstressed state of the plant (taken from Wang *et al*. 2003).

Drought stress leads to numerous changes in the plant organism. Wang *et al*. (2003) summarizes the response of the plant to abiotic stress (Fig. 3). Abiotic stress initiates "secondary stress", which means osmotic and oxidative stress. As a result, there is a

disturbance of the cell homeostasis as well as damage at the protein and the membrane level. The triggered response of the plant consists of detoxification mechanisms, activation of proteins with chaperone functions, osmoprotection mechanisms and water and ion movement. Objective of all reactions in the plant organism is the restoration of the cellular homeostasis and the repair of damage caused by the abiotic stress.

Zhu (2001) divided the responses of plants under stress in three functional groups, including osmotic homeostasis or osmotic adjustment, growth inhibition as well as damage control and repair. The different signaling pathways (Fig. 4) of the plant ensure the maintenance of osmotic homeostasis in the plant cells. Furthermore the signal pathways regulate the growth adaptation to the prevailing environmental conditions and start the detoxification processes to control and repair the damage caused by drought stress (Zhu, 2002).

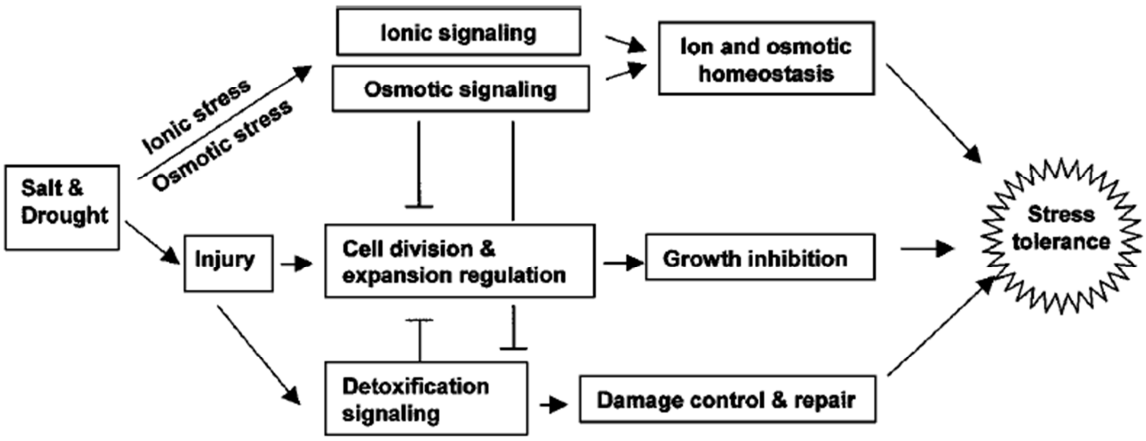

Fig. 4: Functional groups of plant reactions under influence of drought and their interconnection (taken from Zhu, 2002).

There are cross-connections between the functional groups. When the cellular homeostasis is restored, detoxification reactions are stopped. Subsequently, normal growth is initiated again (Zhu, 2002).

The effects under drought can be divided into two categories (Fig. 5). On the one hand plants react with short-term responses such as stomatal closure and inhibition of growth but also with osmotic adjustment. On the other hand, there are long-term responses like shoot growth inhibition, reduced transpiration area and increased root/shoot ratio of the plant under drought environmental conditions. Understanding these mechanisms builds a basis for better crop breeding programs regarding drought tolerance (Chaves *et al*., 2003).

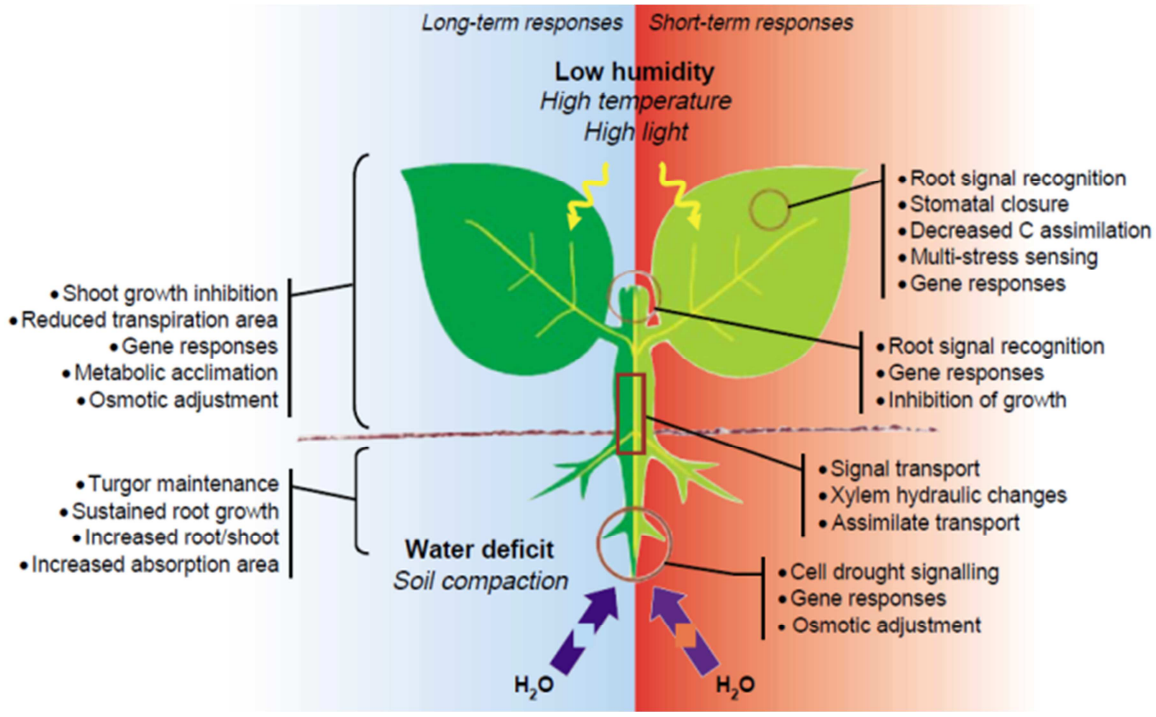

Fig. 5: Short-term and long-term reactions in plants under drought stress conditions (taken from Chaves *et al*., 2003).

METHODS FOR MEASURING THE IMPACT OF DROUGHT ON PLANTS

There are various possibilities to analyze the influence of drought stress on plants. It is possible to use traditional methods and in comparison techniques used only since a couple of years. Different aspects of the impacts of drought stress can be determined by these methods. Traditional methods such as determination of plant height and fresh weight - dry weight measurements are more likely used to analyze long term responses of plants under drought stress. For example the factors shoot growth inhibition and reduced transpiration area are both defined by Chaves *et al*. (2003) as long term response. A newer method to analyze drought stress through chlorophyll fluorescence measurements is Pulse-Amplitude-Modulation (PAM)-Imaging (Baker, 2008). This allows observing the impact of drought stress on photosynthesis. The behavior of the stomata under drought stress can be analyzed by porometry. Stomata closure is a short-term response of plants under drought stress (Chaves *et al*., 2003). By using a thermal imaging camera, the stomata behavior measurement is applicable for groups of plants and even for whole fields at once (Jones *et al.*, 2009). Turgor pressure measurement of leaves is another way to visualize early impacts of drought stress on plants (Zimmermann *et al*., 2008). Jones (2007) summarizes plant measurements that are useful for plant breeding and plant screening in a subjective classification. These include for

example relative water content, turgor pressure and stomatal conductance/infrared thermography.

PLANTS WITH A POTENTIAL AS CROP FOR AREAS WITH DROUGHT CONDITIONS AND THEIR SECONDARY PLANT METABOLITES

The perfect crop for areas threatened by drought conditions should provide high and stable yields with minimal effort. A short growth period is preferred to avoid the consequences of climate condition fluctuations. To increase the value of the perfect crop to the farmers, the perfect crop should also provide additionally benefits. For example, these benefits can result from nutraceutical or pharmaceutical usable compounds or from a potential as energy delivering plant. A possibility to find the perfect crop would be the use of genetic altered plants, adapted to drought conditions. Yang *et al.* (2010) concluded that not all parts of the plant response to stress are known in detail. However, there is some success in terms of improved drought tolerance in for example maize and rice, but studies often show a too low yield for a meaningful use of these altered crops. Another disadvantage of genetically engineered plants is that these plants are partially not accepted by the population (Zeit, 2013). Another method to find an appropriate crop for drought conditions is the screening of species or genotypes of a species regarding their drought tolerance and secondary plant metabolites. In the following sections two promising crop species are shown. Both are drought tolerant and show additional usage potential: *Lablab purpureus* (L.) Sweet and *Euphorbia tiruncalli* (L.).

LABLAB PURPUREUS (L.) SWEET

L. purpureus is one of the plants used in this thesis with crop potential for areas with low precipitation rate (Fig. 6). Fig. 6 B shows a *L. purpureus* plant grown in the greenhouse. *L. purpureus* belongs taxonomically to the Eudicots, Core Eudicots, Fabids order Fabales, family Fabaceae (The Angiosperm Phylogeny Group (APGIII), 2009). Synonyms for these species are *Dolichos purpureus* and *Dolichos lablab* (NCBI taxonomy). The German common name is Helmbohne (Lieberei and Reisdorff, 2012). The plant is found in Africa and Asia, the origin is probably Africa. There exist the subspecies *bengalensis*, *purpureus* and *uncinatus* (Maass *et al*., 2005). The different genotypes of *L. purpureus* show a strong variability in the morphology. *L. purpureus* is a twining plant and appears as bushy, semi-erect and prostate growth types (www.lablablab.org (03/19/2013), University of Agricultural Sciences,

Bangalore). The seeds of the genotypes vary for example in size and color (Fig. 6 C). The wild forms of ssp. *uncinatus* (CPI 69498 and CPI 24973) are smaller than the remaining genotypes. The colors vary between white (CPI 29398), brown (cv. Rongai) and black (TNAU-2). The characteristic white hilum of the seeds can also be seen in Fig. 6 C. Flowers (white, pink, red, purple) and pods (white, green, purple) also vary in color.

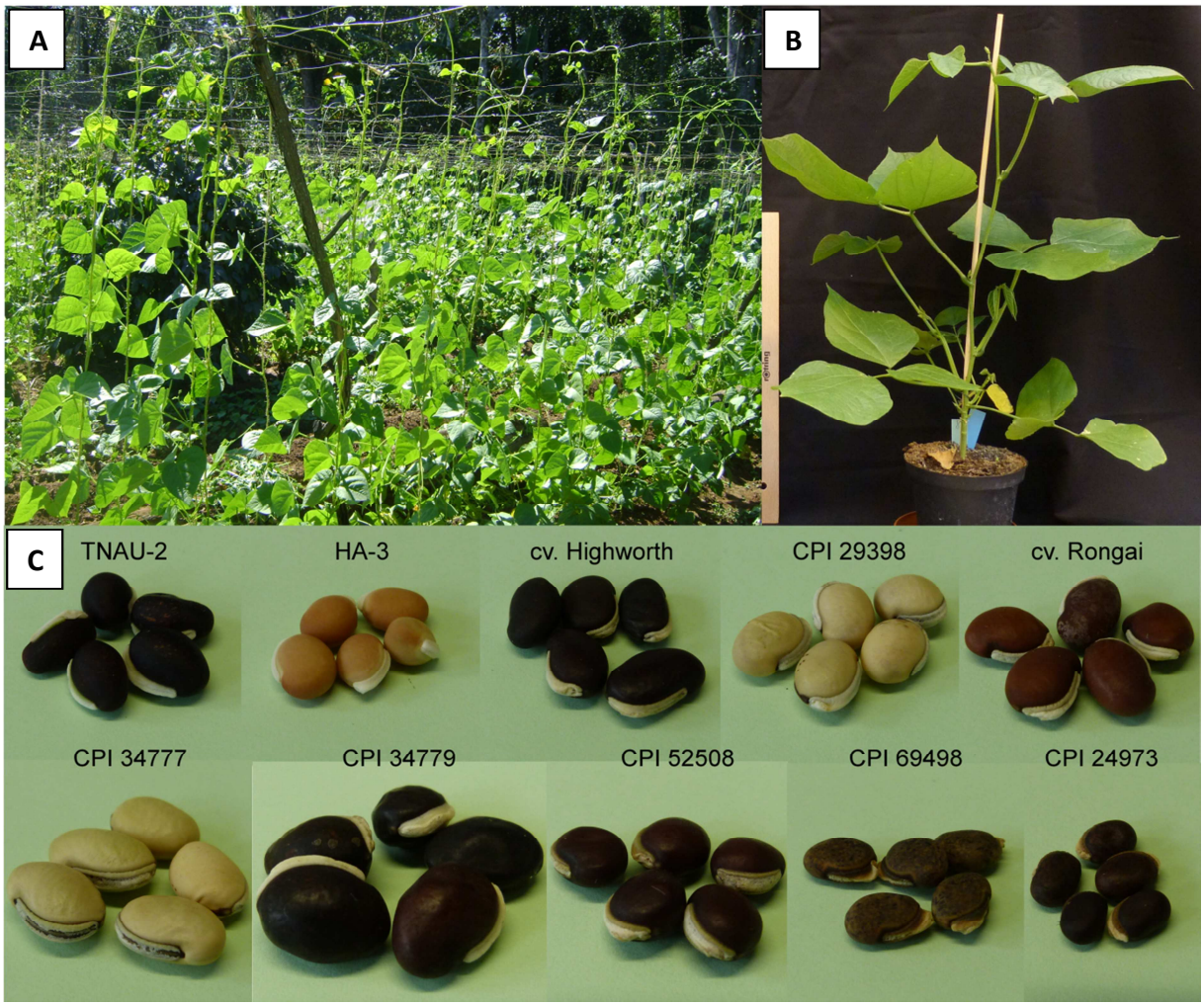

Fig. 6: The species *L. purpureus*. *L. purpureus* plants in combination with coffee plants (dark green plant on the left side) on a field near Tamil Nadu Agriculture University (TNAU) horticultural research station Thadiyankudisai, Lower Pulney hills, Tamil Nadu, India (A). A seven weeks old *L. purpureus* plant of genotype HA-4 (B). The highly varied seed morphology of some *L. purpureus* genotypes used in this thesis (C). (Pictures: Indian student from TNAU (A), Sebastian Guretzki (B+C))

L. purpureus is a perennial plant, but mainly cultivated as annual crop (www.lablablab.org (03/19/2013), University of Agricultural Sciences, Bangalore). Maass *et al*. (2010) summarizes that *L. purpureus* is used in mixed cropping schemes. Fig. 6 A shows the cultivation in combination with coffee plants on a field near Tamil Nadu Agriculture University horticultural research station Thadiyankudisai, Lower Pulney hills, Tamil Nadu,

India. *L. purpureus* secures the daily income of the farmers and thus offers the possibility of ensuring the supply of the more valuable coffee plants. In contrast, *L .purpureus* is cultivated even without further plant species. The plant is considered to be drought tolerant, the degree of tolerance, however, seems to vary within the species, as described in Maass *et al*. (2010).

EUPHORBIA TIRUCALLI L.

E.tirucalli is the second plant used in this thesis with potential as a crop for areas with low rates of rainfall (Fig. 7). *E. tirucalli* belongs taxonomically to the Eudicots, Core Eudicots, Fabids, order Malpighiales, family Euphorbiaceae (The Angiosperm Phylogeny Group (APGIII), 2009). A German common name is Milchbusch (Lieberei and Reisdorff, 2012). Mwine and Van Damme (2011) described the plant as shrub or small tree (4 to 12 m height) with succulent branches and only a few, 1.3 to 2.5 cm long leaves (Fig. 7 B).

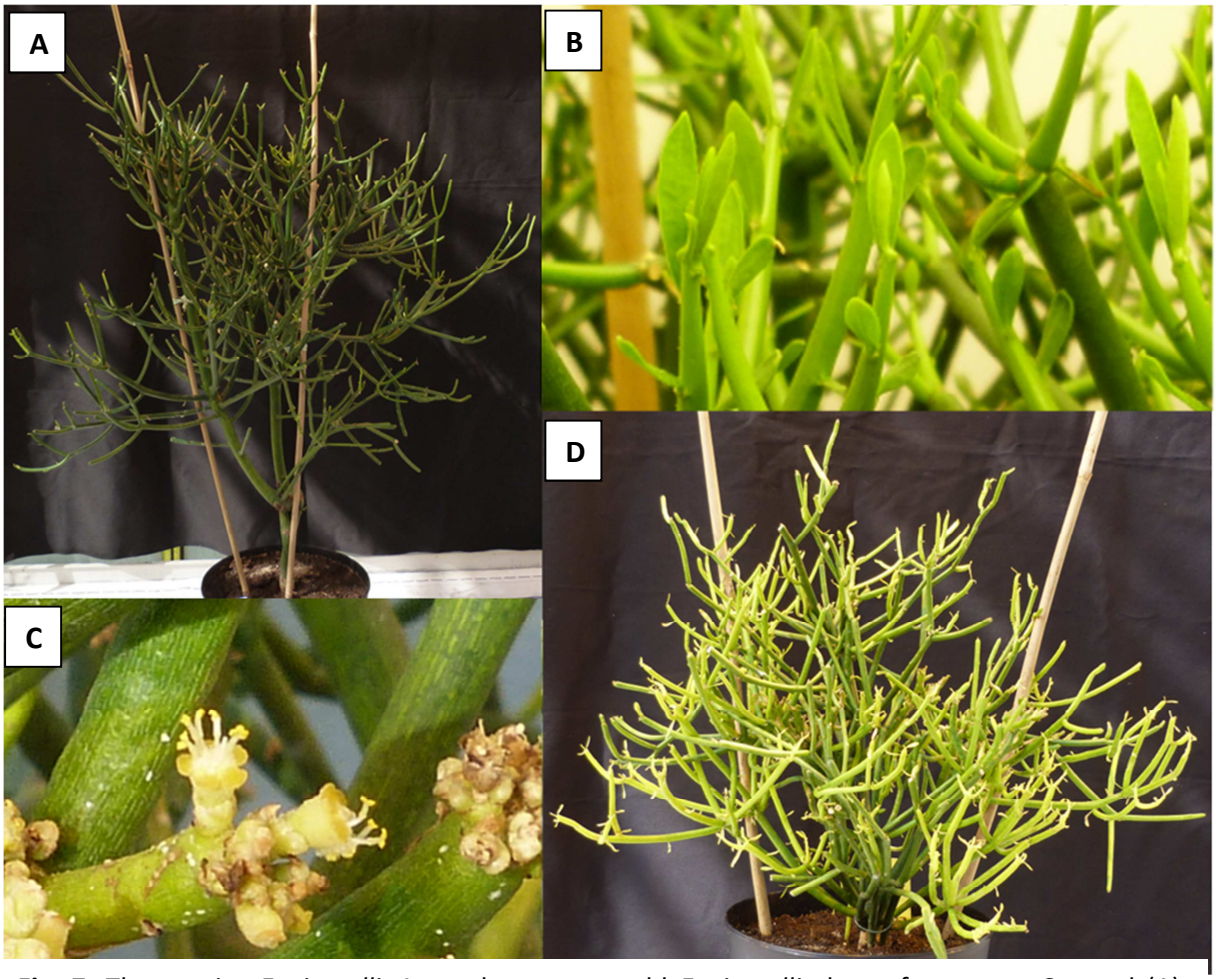

Fig. 7: The species *E. tirucalli*. Around two years old *E. tirucalli* plant of genotype Senegal (A), leaves of genotype Senegal (B). Flowers of a genotype USA female plant (C). About two years old *E. tirucalli* plant of genotype USA (D). (Pictures: Sebastian Guretzki)

Fig. 7 shows about two years old *E. tirucalli* plants of genotype Senegal (A) and USA (D) grown in the greenhouse in Hannover. Both genotypes are used in this thesis. Fig. 7 C shows flowers of a female plant of genotype USA. Genotype USA shows a peculiarity, as the color of the plant is considerably yellowish (golden) in comparison with the other genotypes (Fig. 7 C and 7 D). Van Damme (2001) describes that the origin of the plant is Africa, subsequently the plant has spread out in tropical and subtropical zones of other continents. The drought tolerance of *E. tirucalli* is classified as high (Janssens *et al*., 2009).

THE OCCURRENCE OF ANTI-NUTRITIONAL FACTORS IN PLANTS

Anti-nutritional factors are common secondary metabolites of the plant organism. But these components can have a negative impact on nutrition of other organisms (Kumar, 1992). Protease inhibitors like trypsin inhibitors belong to the anti-nutritional factors. They disturb the process of digestion and can affect growth and development negatively as summarized by Ryan (1990). Glycosides such as saponins and cyanogens are also assigned to the anti-nutritional factors. Saponins are potentially hazardous, because they can lyse erythrocytes (Kumar, 1992). Cyanogenic glycosides contain cyanide; by degeneration this cyanide is released as hydrogen cyanide (Møller, 2010). Plants that contain cyanogenic glycosides are therefore potentially fatal after consumption as summarized by Vetter (2000). Tannins interfere also the digestion (Kumar, 1992). Reddy *et al.* (1982) reported that phosphorus occurs in form of phytic acid for example in legumes. Phytic acid is another anti-nutritional factor because it forms complexes with minerals, such as iron and magnesium, thereby preventing the absorption of these minerals as described by Reddy *et al*. (1982). The species *L. purpureus* contains for example trypsin inhibitors and substances that form hydrogen cyanide (Soetan, 2012).

USEFUL COMPONENTS OF PLANTS AND THEIR APPLICATION POSSIBILITIES

In addition to negatively functioning as anti-nutritional factors, there is also a variety of secondary metabolites in the plant that can be used in a positive way. These phytochemicals increase the economic value of plants for the farmers and give an incentive to cultivate the plants as crop. Morris (2009) summarizes that *L. purpureus* contains secondary metabolites that can be used as pharmaceutical and nutraceutical for example as appetite suppressant, for reduction of blood pressure or they could act preventively against pancreatic cancer. Additionally, the plant is useful to fight against malnutrition because of their nutrient composition as reported by Morris (2009). *E. tirucalli* also contains potential

pharmaceuticals, for example as prostate cancer medicine (Aylward and Parsons, 2008). Today, the energy market is subject to substantial change. Therefore, there is a search for renewable and sustainable sources for energy. Due to its latex content the plant can be used as biofuel source and the biomass of *E. tirucalli* can serve as a source of biogas (Calvin, 1978; Loke *et al*., 2011).

AIMS OF THIS THESIS

- Development of a system of methods for genotype screening of a species with regard to drought tolerance using *L. purpureus* as an example.
- Investigation of drought tolerance differences within *L. purpureus* genotypes.
- Analysis of the content of anti-nutritional factors in different genotypes using *L. purpureus*.
- Adaptation of the methods established for *L. purpureus* on a succulent plant like *E. tirucalli*.
- Determination of the genetic relationship between genotypes of *E. tirucalli* by establishing the method amplified fragment-length polymorphism (AFLP).

CHAPTER 2:

COMPARATIVE ANALYSIS OF METHODS ANALYZING EFFECTS OF DROUGHT ON THE HERBACEOUS PLANT *Lablab purpureus*

Guretzki S, Papenbrock J (2013) Comparative analysis of methods analyzing effects of drought on the herbaceous plant *Lablab purpureus*. *Journal of Applied Botany and Food Quality* 86, 47-54 (DOI:10.5073/JABFQ.2013.086.007)

ABSTRACT

Due to the changing climatic conditions, there is an enlargement of land areas with insufficient rainfall and therefore a reduction in the cultivated area for common crops. Hence, it is now important to find plants that are adapted to these drought conditions. The focus of our research was to apply and compare different methods to quantify the impact of drought stress on plants.

Lablab purpureus is considered to be drought tolerant. Therefore, we used *L. purpureus* genotypes from three continents CPI 36903 (Europe), CPI 52508 (Africa) and HA-4 (Asia) as examples for our study. All genotypes were screened for their tolerance to drought stress by various methods to obtain quantitative data on the drought stress tolerance of individual genotypes and to find out which methods are especially suitable for the measurement of drought tolerance. Classical methods such as leaf size, plant height, biomass, and plant water content were investigated. In addition, by chlorophyll fluorescence measurement effects of drought on the photosynthetic system were examined. Infrared thermography was used in order to make the changes in leaf temperature in plants stressed by drought compared to unstressed plants visible. The methods were complemented by the measurement of leaf conductivity.

Results indicate a difference in the usability of the methods for the determination of drought stress. Finally, a set of methods is assembled based on suitability for drought tolerance analysis in plants. The methods include classical growth parameters, including dry weight biomass, plant water content (PWC) and leaf size determination, as well as height measurements of the plants. The stomata behavior is analyzed by leaf conductivity and infrared thermography, both methods complete the set for drought tolerance identification.

Based on the results of these methods a ranking of the examined genotypes with respect to their drought tolerance is created.

INTRODUCTION

Because of global warming the climate conditions in e.g. Africa, South Asia and East Asia will become more arid (Dai, 2011). However, the precipitation amount is a key factor for the productivity of crops (Rosenzweig et *al*., 2001). Common crops have to deal with these new environmental conditions. The detection of new crop plants or genotypes of crop plants that are particularly adapted to more arid conditions is therefore a major objective to preserve the livelihood of the local populations. *Lablab purpureus* (L.) Sweet (synonyms: *Dolichos purpureus*, *Dolichos lablab* (NCBI-Taxonomy)), the experimental plant in this study, is referred to as drought tolerant in Maass et *al*. (2010). *L. purpureus* belongs to the Eudicots, Core Eudicots, Fabids order Fabales, family Fabaceae (The Angiosperm Phylogeny Group (APGIII), 2009). The herbaceous plant is perennial but is often grown as an annual plant. *L. purpureus* occur as bushy, semi-erect and prostate growth habit types. The stem is twining, the leaves are alternate and trifoliate. Flowers exist in different colors (white, pink, red, purple). Pods and seeds vary in color and size. Various parts of the plant are used as food (flowers, leaves, pods, root tubers, seeds) or fodder (www.lablab.org, University of Agricultural Sciences, Bangalore). *L. purpureus* is cultivated as a component of mixed cropping schemes or home gardens, wherein the plant is known especially in Africa, South Asia and South East Asia (Maass et *al*., 2010). Studies showed that this species is adapted to drought, but there are differences in terms of drought tolerance within the species as summarized in Maass et *al*. (2010). However, the differences in drought tolerance have not been quantified for this species so far.

Also for other herbaceous species studies on the quantitative analysis to measure the impact of drought stress on plants are rare. There are the more traditional parameters measured, including the determination of the fresh weight and dry weight biomass, plant water content, as well as development of plant height and leaf size to investigate the effects of stress on plants. In addition, there are new non-destructive methods which allow a rapid screening of plants. This includes the determination of leaf chlorophyll fluorescence by PAM-Imaging in order to get information about the state of the photosynthetic system under stress conditions (Woo et *al*., 2008; Sperdouli and Moustakas, 2012). The behavior of stomatal con-ductivity can be observed over a large area by infrared thermo-graphy, a further rapid screening method (Grant et *al*., 2006). This makes the method interesting for phenotype screening and breeding programs (Chaves et *al*., 2003). Another option for

stomatal conductivity measurements is the use of a porometer (Jones et *al*., 2002; Grant et *al*., 2006). The combination of infrared thermography and chlorophyll fluorescence for the observation of changes in transpiration rate and photosynthesis can be used for detection of early plant stress and stress tolerance screening (Chaerle et *al*., 2007, 2009). Munns et *al*. (2010) concluded that stomatal conductance is a growth rate indicator for plants under water stress and thermography is a good screening method. Together it is possible to find the best genotypes for different growth conditions. Berger et *al*. (2010) points out that chlorophyll fluorescence is helpful for the detection of servere drought stress. For early drought stress detection chlorophyll fluorescence seems only useful in combination with other methods to gain a more comprehensive picture of the plant stress response. The objective of this study is to find methods that are suitable for recognizing the impact of drought stress on plants and methods that are appropriate in a combined way for a reliable detection of drought-tolerant genotypes in the case of *L. purpureus* as an example for an herbaceous species. More precisely, to compile methods those allow the drought stress detection before the leaves wilt. The drought tolerance results correlated with the results of genetic analyses can be used to find the best genotypes for field experiments within future breeding programs.

MATERIAL AND METHODS

PLANT MATERIAL, GROWTH CONDITIONS AND DROUGHT TREATMENT

Seeds of *Lablab purpureus* (L.) Sweet were originally obtained from Dr. B.L. Maass, International Centre for Tropical Agriculture (CIAT), Nairobi, Kenya (CPI 36903, CPI 52508) and from Dr. M.B. Gowda, University of Agricultural Sciences, Bangalore, India (HA-4). CPI 36903 (Southern Ukraine, Europe) and CPI 52508 (Mozambique, Africa) are semi-domesticated genotypes (Maass and Usongo, 2007). The origin of HA-4 is Karnataka, Southern India, Asia. For germination, seeds were soaked in water at room temperature overnight and transferred on type CL T soil (Einheitserde, Sinntal-Altengronau, Germany) in pots of 12 cm diameter the following day. The soil contains 30% clay. Plants were grown in the greenhouse in a 12 h light/dark rhythm at a temperature of 22°C/22°C. When the outdoor light conditions did not ensure sufficient light intensity inside the greenhouse, additional light was switched on to obtain a constant quantum fluence rate of approx. 350 µmol m^{-2} s^{-1} (sodium vapor lamps, SON-T Agro 400, Philips, Amsterdam, Netherlands). Plants were grown in the greenhouse for five weeks under well-watered conditions.

The drought experiments started in week six under the same greenhouse conditions. On day one, the plants were watered for the last time before the plants were set to the experimental conditions. Experimental groups differ in soil volumetric water content (VWC); the control group (35% VWC) and the drought group (20% VWC) were used to reach a moisture content level near field capacity and near the permanent wilting point of the soil, respectively, in accordance to the manual of the used device. For the measurement of VWC time domain reflectometry (TDR) (Fieldscout, Spectrum Technologies, Plainfield, USA) was used. For daily irrigation, water was added based on water deficit calculation (D) of the Fieldscout, 1 mm = approx. 7.8 ml (experiment (exp.) 1) and approx. 8.0 ml (exp. 2) for a pot with approx. 710 cm^3 (exp. 1) and approx. 750 cm^3 (exp. 2) volume according to the formula of truncated cones. The water contained 0.25% Wuxal Top N fertilizer (Aglukon, Düsseldorf, Germany). Five plants per treatment were grown for every genotype with a distance of approx. 7.5 cm (exp. 1) and approx. 35 cm (exp. 2) between the pots.

GROWTH PARAMETERS

The growth parameters plant height, leaf size, biomass and plant water content (PWC) were determined. The plant height was measured with a folding yardstick; thereby the length of the longest shoot was used. The leaf of *L. purpureus* is divided into three leaflets. Instead of the leaf area, the approximate leaf size was calculated measuring the length of the paired leaflets and the terminal leaflet plus petiole (between paired leaflets and terminal leaflet). After-wards, both values were multiplied to obtain the leaf size. The fresh and dry weight for biomass and PWC were determined by harvesting above-ground plant material, which was then dried in an incubator at approx. 110°C for 48 h. Plant material was weighed before drying for fresh weight (FW) and after drying for dry weight (DW). For biomass data DW was used. PWC was calculated using the formula PWC = (FW - DW)/ FW * 100.

MEASUREMENTS FOR QUANTIFICATION OF THE DROUGHT STRESS EFFECTS

The effect of drought stress was examined by porometry, thermal imaging measurements and PAM-imaging. Porometry and thermal imaging measurements were done on attached leaves as non-destructive methods. Stomatal conductance (mmol m^{-2} s^{-1}) of leaves was determined by the use of the porometer AP4 (Delta-T Devices, Burwell, UK) in the morning. The measurements were performed on either of the two youngest fully expanded leaves, wherein only the higher value was used for further calculations (exp. 2) or one leaf was measured over the entire experimental period (exp. 1).

Thermal imaging investigation was carried out with the camera T360 (FLIR Systems, Wilsonville, USA) in accordance with Grant et *al*. (2006) in the afternoon. To ensure consistent measurements, the camera was turned on at least 30 min before taking the first picture. The youngest fully expanded leaves were examined. One leaflet of the paired leaflets of a leaf was used as dry (T_{dry}) the other one as wet (T_{wet}) reference. Dry reference leaflets were covered with petroleum jelly and wet reference leaflets were wetted with water on both sides. The terminal leaflet of the same leaf was used as sample (T_{leaf}). T_{dry} was measured at least five min after the application of petroleum jelly, T_{wet} immediately after using the water. In addition to T_{leaf} a stomatal conductance measurement was performed on the terminal leaflet. The thermal imaging pictures were analyzed using the software FLIR QuickReport 1.2 SP2 (FLIR Systems, Wilsonville, USA). The object parameters were set for each image to emissivity 0.95, reflected apparent temperature 23°C, atmospheric temperature 23°C, relative humidity 45%/50% and distance 0.2 m. Based on the results the index *I*G was calculated using the formula $IG = (T_{dry} - T_{leaf})/ (T_{leaf} - T_{wet})$. Additionally the crop water stress index (CWSI) was calculated from $CSWI = (T_{dry} - T_{leaf})/ (T_{dry} - T_{wet})$.

Whether there is an influence of drought stress on photosynthesis was investigated by chlorophyll fluorescence using an Imaging PAM M series device and ImagingWin v2.32 software (Heinz Walz, Effeltrich, Germany). The measurements were performed either on a young fully expanded leaf (using cut off leaves; exp. 2) or one leaf was measured over the entire experimental period (using attached leaves; exp. 1) in the morning. For analysis of the photosynthetic system light curves were analyzed as presented by the manufacturer. Through the use of the filter plate IMAG-MAX/F the effective PAR values are about 15% lower. Before the measurement, the plants were dark adapted for 20 min. The parameters F_v/F_m (maximal PS II quantum yield), Y(II) (effective PS II quantum yield), Y(NPQ) (quantum yield of regulated energy dissipation), Y(NO) (quantum yield of non-regulated energy dissipation), NPQ/4 (non-photochemical quenching/4) and ETR (electron transport rate) were analyzed (for background information: Baker, 2008; Sperdouli and Moustakas, 2012). F_v/F_m values were obtained from the false-color images created by ImagingWin software. ETR values were determined using a mean value of PAR 396-801 µmol quanta m^{-2} s^{-1}. The other parameters were analyzed based on the PAR 396 (approx. growth light intensity) and 801 (approx. twice the growth light intensity) results.

STATISTICAL ANALYSIS

All statistical analyzes were conducted with R 2.15.2 (www.r-project.org) in combination with R Studio v0.97.248 (RStudio, Boston, USA). Box plots were drawn using ggplot2 version 0.9.3 (Wickham, 2009). Significant differences (p <0.05) were determined by the Welch t-test analysis.

RESULTS

DEVELOPMENT OF CLASSICAL GROWTH PARAMETERS UNDER DROUGHT STRESS

The classic growth parameters, gain in biomass DW, plant water content, leaf size (exp. 1) and plant height growth (exp. 2) were analyzed (Fig. 8, 9). Drought stress results in a decrease of biomass DW by slower growth in stressed plants (Fig. 8A, 9A). In experiment set up 1 HA-4 was the least affected genotype among the groups. The strongest decrease in exp. 2 was found for genotype CPI 52508 (cg: 5.9 g; dg: 4.7 g), HA-4 showed no impact of drought stress (cg: 4.3 g; dg: 4.3 g). Significant differences (p<0.05) between stressed and unstressed plants for biomass dry weight measurements were found for CPI 36903 and CPI 52508. There is a reduction in plant water content under drought stress conditions, too (Fig. 8B, 9B).

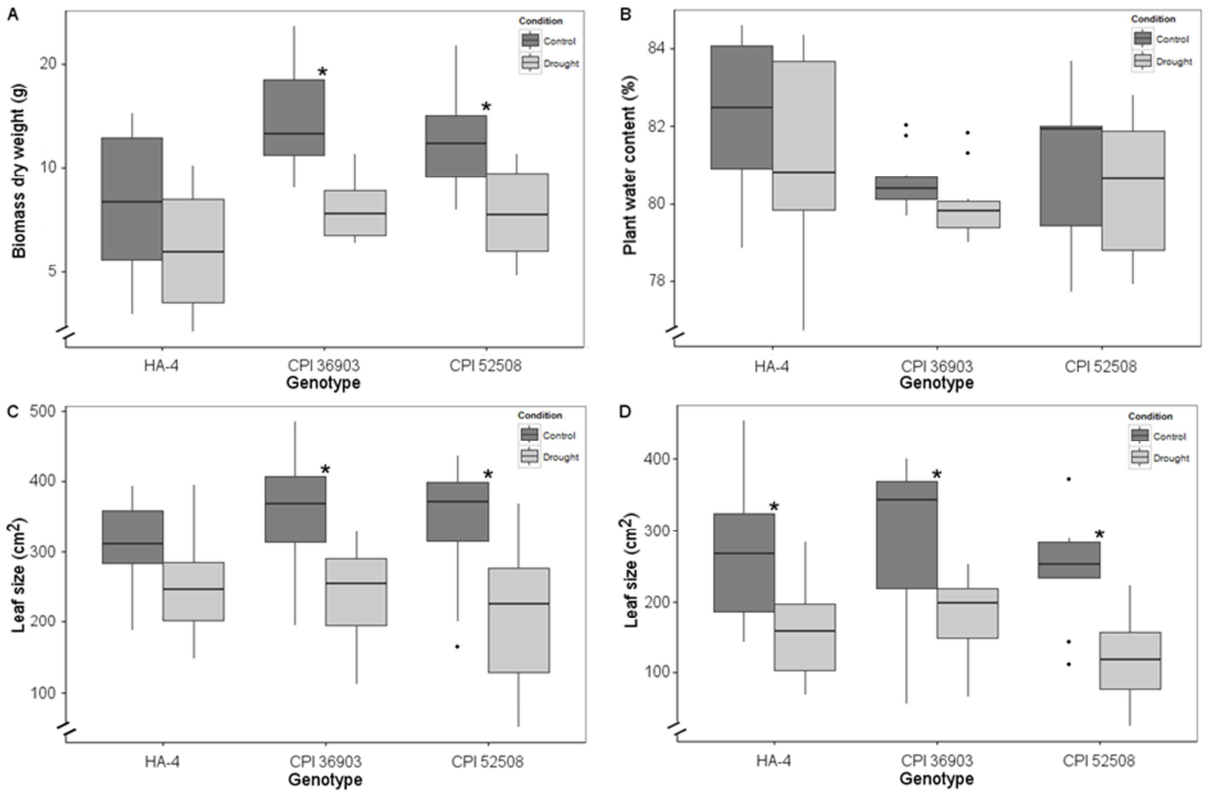

Fig. 8: Effects of water limitation after 15 days on (A) biomass dry weight (g), (B) plant water content (%) and after eight days on (C) size older leaf (cm^2) and (D) size younger leaf (cm^2); n=10, genotypes with * = p<0.05, exp. 1.

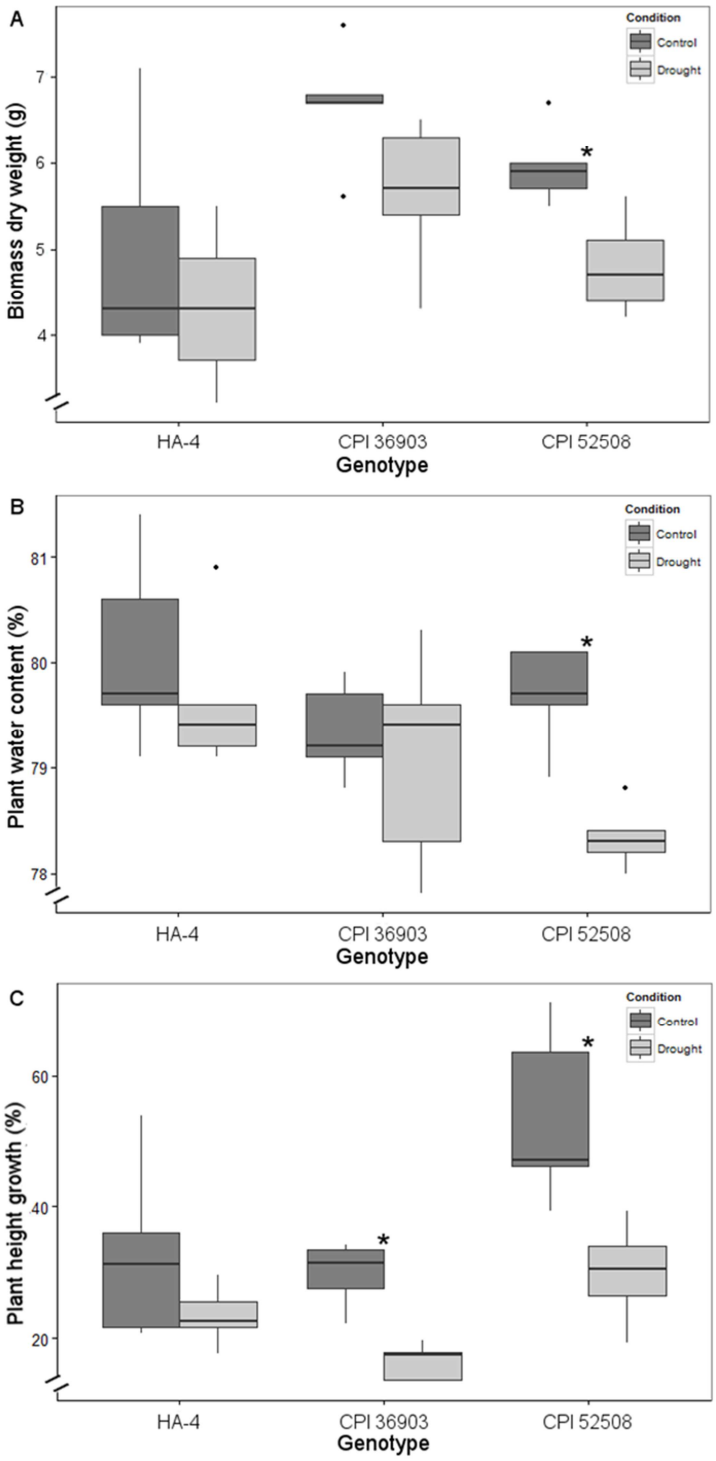

Fig. 9: Effects of water limitation after nine days on (A) biomass dry weight (g) (n=5), (B) plant water content (%) (n=5) and (C) plant height growth (%) (n=4-5); genotypes with * = p<0.05, exp. 2.

Significant differences (p<0.05) among the groups occurred only in genotype CPI 52508 exp. 2 (cg: 79.7%; dg: 78.3%). Generally, there were only small changes in the drought stress plants in comparison to unstressed plants. There was also a considerable decrease in leaf size and plant growth, in the case of drought stressed plants. The strongest difference in leaf

sizes between both groups (Fig. 8D) was found in CPI 52508 (cg: 253 cm^2; dg: 118 cm^2), HA-4 showed a smaller difference (cg: 267 cm^2; dg: 157 cm^2). The reduction of leaf size under drought stress is stronger in younger leaves (Fig. 8D) compared to older leaves (Fig. 8C). The decrease was in younger leaves between 21% (HA-4) and 39% (CPI 52508) and in older leaves between 41% (HA-4) and 53% (CPI 52508). The height growth of the plant was affected particularly in CPI 36903 (cg: 47%; dg: 31%). The slightest effect was found in HA-4 (cg: 31%; dg: 23%). However, it must be considered that *L. purpureus* is a twining plant and thereby the measurement of plant height to obtain the growth rate was impaired. In general, a large mean variation especially of the controls can be observed although the seed material was homogenous and also the plantlets had a homogenous phenotype. The conditions in the greenhouse might have local maxima and minima resulting in a large mean deviation, based on single measuring points collected from five different plants.

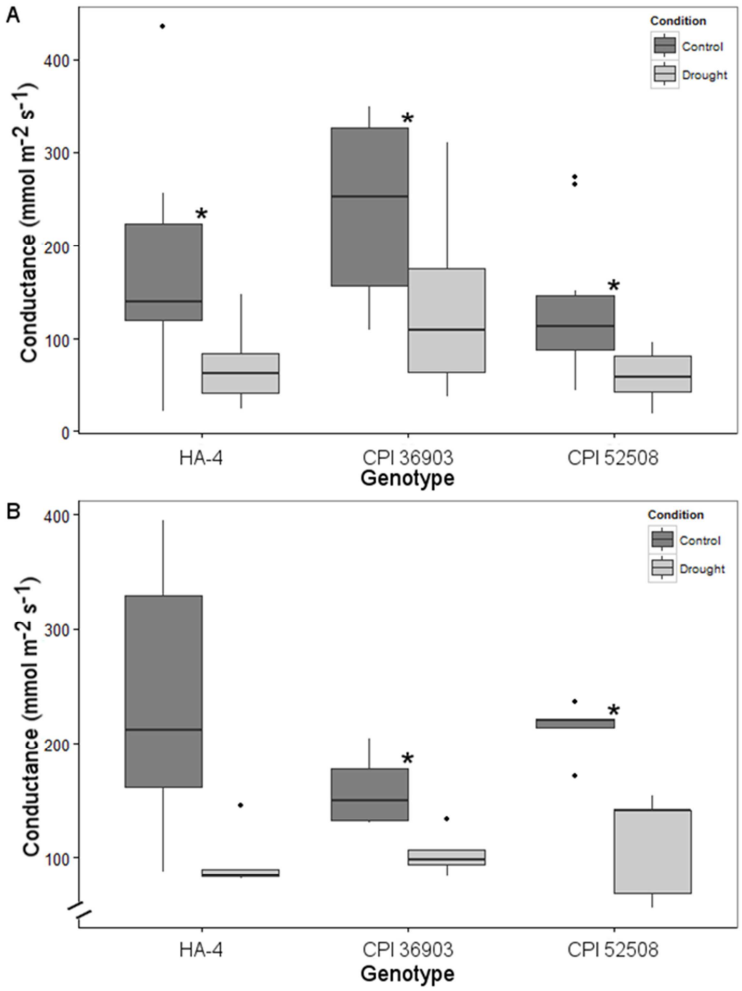

Fig. 10: Effects of water limitation after eight days on stomatal conductance through porometry in mmol m^{-2} s^{-1} (A) exp. 1 (n=10) and (B) exp. 2 (n=5); genotypes with * = p<0.05.

DETERMINATION OF LEAF CONDUCTANCE

The leaf conductivity was measured in the morning by porometry (Fig. 10). Drought stress led to a reduction in leaf conductance. In summary, the lowest difference between control and drought group showed CPI 52508 (exp. 2 - cg: 220 mmol m^{-2} s^{-1}; dg: 141 mmol m^{-2} s^{-1} (Fig. 10B)) in both experiments. HA-4 was most affected by drought stress (exp. 2 - cg: 212 mmol m^{-2} s^{-1}; dg: 84 mmol m^{-2} s^{-1} (Fig. 10B)). CPI 36903 behaved uneven; in exp. 1 the differences among the groups are similar to HA-4 and with larger space avail-able per plant similar to CPI 52508 in exp. 2. Significant differences (p<0.05) among drought stressed and unstressed plants were found for all genotypes.

ANALYSIS OF THE IMPACT OF DROUGHT STRESS ON *L. PURPUREUS* BY INFRARED THERMOGRAPHY

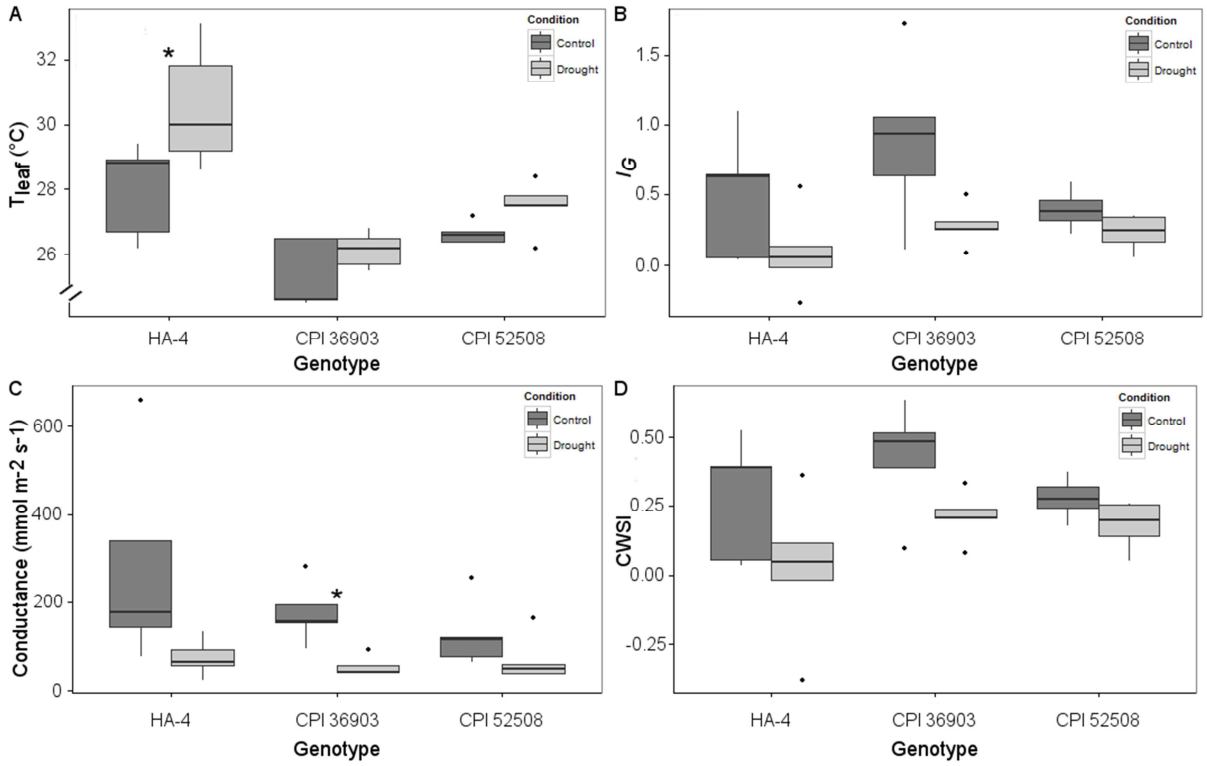

Fig. 11: Effects of water limitation after eight days on (A) leaf temperature (°C), (B) index I_G, (C) stomatal conductance (mmol m^{-2} s^{-1}) and (D) crop water stress index (CWSI) based on infrared thermography analysis; n=5, genotypes with * = p<0.05, exp. 2.

The influence of drought stress on surface temperature of plant leaves was analyzed by using an infrared thermography camera (Fig. 11). Drought stress leads to an increase in leaf temperature by closed stomata (Fig. 11A). Only HA-4 (cg: 30°C; dg: 28.8°C) showed a

significant difference (p<0.05) between drought stressed and unstressed plants. CPI 36903 was stronger affected by drought stress (cg: 26.2°C; dg: 24.6°C). CWSI and *IG* values drop under drought stress, wherein the results of both indices were homologous (Fig. 11B and 11D). The biggest differences between the two groups were measured in HA-4, followed by genotypes CPI 36903 and CPI 52508. The CWSI decrease for HA-4 (cg: 0.39; dg: 0.05) was 88% and in comparison only 28% for CPI 52508 (cg: 0.28; dg: 0.20). There were no significant differences (p<0.05) between the groups. Leaf conductance measured during the same time to substantiate the results of the infrared thermography camera showed lower values under drought stress (Fig. 11C). Drought stress led to a large decrease for CPI 36903 (cg: 158 mmol m^{-2} s^{-1}; dg: 44.5 mmol m^{-2} s^{-1}), the decreases for HA-4 and CPI 52508 were also above 50%. The leaf conductance confirms again that CPI 52508 was least affected by drought stress

MEASUREMENT OF VARIOUS CHLOROPHYLL FLUORESCENCE FACTORS DURING DROUGHT STRESS

Chlorophyll fluorescence was examined by factors ETR, F_v/F_m, NPQ/4, Y(II), Y(NPQ) and Y(NO) through a light curve (Fig. 12, 13).

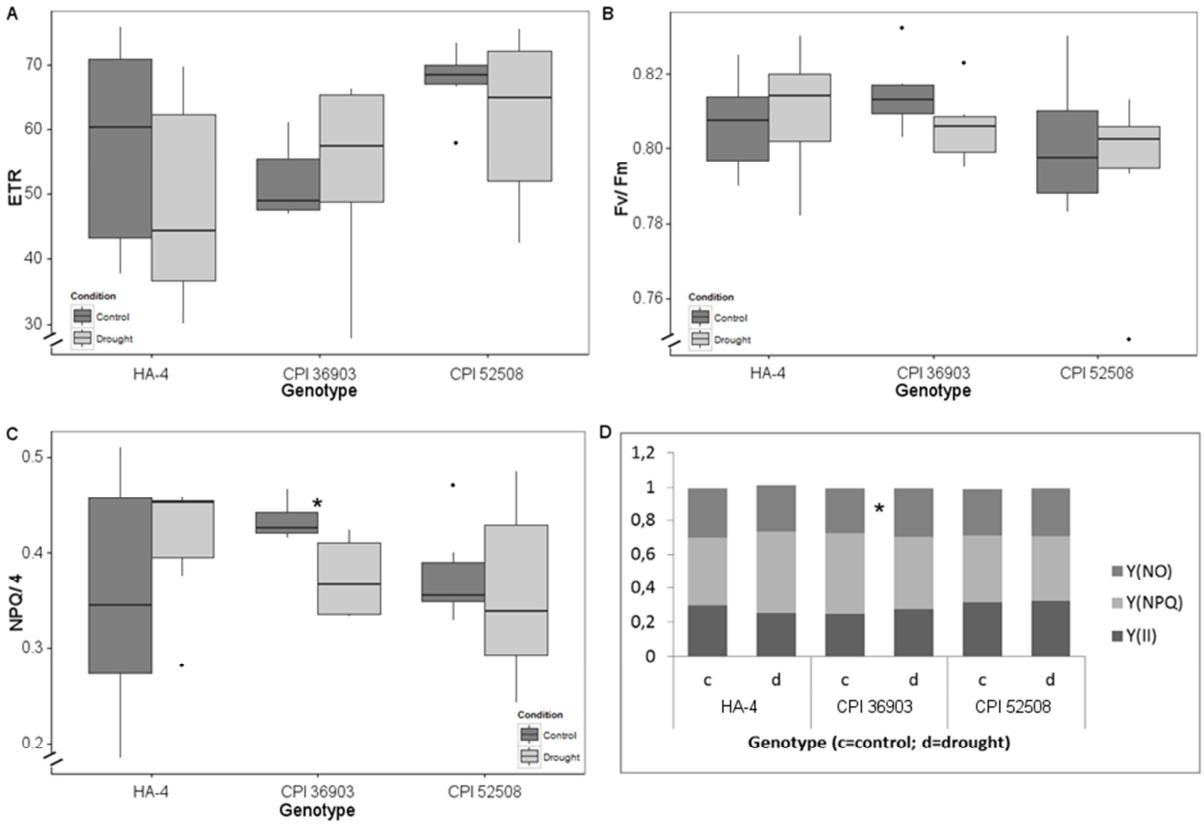

Fig. 12: Effects of water limitation after eight days on (A) ETR, (B) F_v/F_m and under LL conditions through chlorophyll fluorescence measurements (C) NPQ/4, (D) Y(II) Y(NO) Y(NPQ); n=6, genotypes with * = p<0.05, exp. 1.

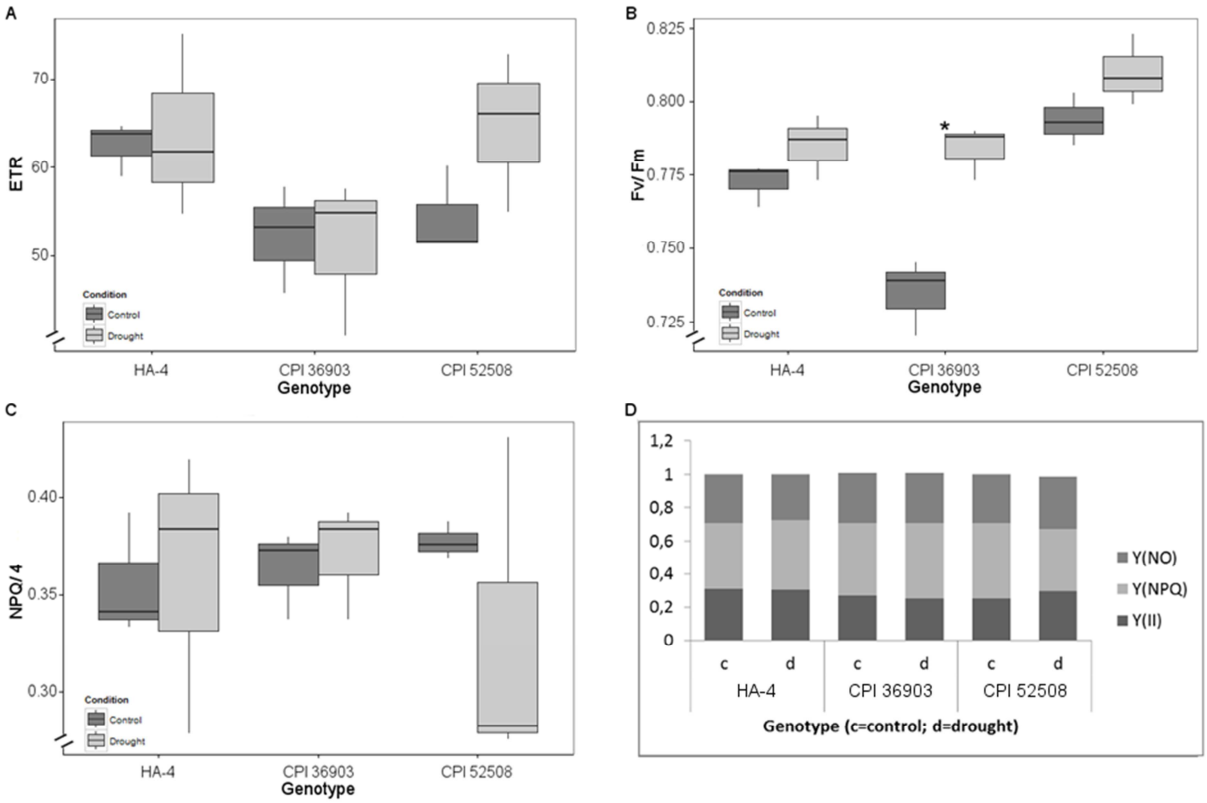

Fig. 13: Effects of water limitation after nine days on (A) ETR, (B) F_v/F_m and under LL conditions through chlorophyll fluorescence analysis (C) NPQ/4, (D) Y(II) Y(NO) Y(NPQ); n=3, genotypes with * = p<0.05, exp. 2.

F_v/F_m showed no notable differences between control and drought groups for all genotypes (Fig. 12B, 13B), except a drought group increase for CPI 36903 in exp. 2 (cg: 0739; dg: 0788). Stress leads to F_v/F_m reduction. Drought stress caused also a decrease in rate of electron transport (Fig. 12A, 13A). ETR decreased under drought stress especially in HA-4 (exp. 1 - cg: 60.28; dg: 44.37; exp. 2 - cg: 63.75; dg 61.82), an increase under drought stress was found for CPI 36903 (exp. 1 - cg: 49.03; dg: 57.44)17%; exp. 2 - cg: 53.18; dg: 54.88). Factor NPQ/4 increased under stress conditions (Fig. 12C, 13C). Also that was particularly apparent for HA-4 (exp. 1 - cg: 0.345; dg: 0.453; exp. 2 - cg: 0.341; dg 0.384). Drought stress indicates a decrease in Y(II) and thereby a change in Y(NPQ) and Y(NO) (Fig. 12D, 13D). HA-4 was again most negatively affected by drought stress. Generally, there were only significant differences in CPI 36903. Based on the results CPI 36903 tends to be classified between the other two genotypes with respect to drought tolerance. HA-4 has a lower tolerance to drought. Overall, the results of chlorophyll fluorescence were very inconsistent and provide only evidences for drought tolerant genotypes.

RESULT SUMMARY OF THE DIFFERENT DROUGHT STRESS MEASUREMENT TECHNIQUES

Table 1 summarizes the results of the stress methods used in this study. A strong negative influence of drought stress on the genotype of a species is represented by a low score. A high score indicates that the influence of drought stress on the genotype is low. Genotypes with high scores are therefore more drought tolerant in comparison to the others. HA-4 (values 6 in the scale) appears to be the least drought tolerant genotype, CPI 36903 (8) is slightly more drought tolerant. CPI 52508 (10) is the most drought tolerant genotype of the three genotypes used in this study. It is noticeable that HA-4 is the most tolerant genotype, if the classical growth parameters are used. In the other methods HA-4 is the least adapted genotype. The results for CPI 52508 are exactly the opposite.

Table 1: Result summary of used stress methods. In this case each genotype is assigned to a value of one to three. Three corresponds to a low negative influence and one represents a strong negative influence of drought for the plants. Genotypes with similar values get the same rating.

Genotype	Growth parameters	Leaf conductance	Infrared thermography	Chlorophyll fluorescence	Sum
HA-4	3	1	1	1	6
CPI 36903	2	2	1	3	8
CPI 52508	1	3	3	3	10

DISCUSSION

THE EFFECTIVENESS OF THE DETERMINATION OF TRADITIONAL GROWTH PARAMETERS RELATING TO DROUGHT TOLERANCE INVESTIGATIONS

The determination of classical growth parameters, e.g. leaf area measurements, is helpful in the screening of drought tolerance (Jones, 2007). The impact of drought leads to a reduced growth of plants. The limited availability of water leads to reduced turgor and restrictions in mitosis. This results in a lower rate of cell division and elongation, and therefore in reduced growth (Farooq et *al*., 2009). Biomass, PWC, leaf size and plant height decreased under drought conditions in *L. purpureus*. Especially genotypes CPI 36903 and CPI 52508 indicated significant differences between the control groups and drought treatment groups. Leaf size and plant height measurements combine the advantages of being non-destructive methods;

biomass and PWC determination belong to the destructive methods. Reduction in leaf dry weight and stem dry weight, plant height and leaf area was already observed in faba bean (*Vicia faba* L.) under drought stress conditions (Zabawi and Dennett, 2010). A study on the effects of drought stress at different growth stages of the mung bean (*Vigna radiata* L.) also showed a reduction in plant height. Drought stress affected the measured plant height in different magnitude depending on the growth stage (Ranawake et *al*., 2011). A comparison of two common bean (*Phaseolus vulgaris* L.) varieties, one heavily influenced and the other one less affected by drought stress in yield, was done. A stronger decrease in relative water content and relative growth rate was shown in the variety that was more influenced by drought stress (Lizana et *al*., 2006). In conclusion, the results suggest that traditional growth factors can help to find drought tolerant genotypes in *L. purpureus*. But some of the techniques belong to the destructive methods. Furthermore results of the classical growth parameters reflect the impact of drought stress on the plant as a whole. It is therefore useful to study further non-destructive and non-invasive methods according to their suitability to detect drought tolerance and indicate specifically the impact of drought on the plants. For this we tested the turgor pressure probes for non-invasive online-monitoring of the water relations of intact leaves Zimmermann et *al*. (2008). However, problems arose in the operation of this system in terms of *L. purpureus*: the leaves were injured because they are too thin and grow very fast. With respect to genotype screening a lot of probes are required or the probes need to be repositioned frequently. This is in contrast to the aim that methods should allow a fast high-throughput screening of genotypes.

INFRARED THERMOGRAPHY AS A VALUABLE ADDITION TO THE MEASUREMENT OF STOMATAL CONDUCTANCE

Stomatal conductance and infrared thermography are techniques that can be useful in an analysis of drought tolerances (Jones, 2007). Under drought stress conditions stomata 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 possible only punctually on a leaf. This results in a large mean variance of the data (Fig. 3). In contrast, it is possible to investigate the stomata behavior and thereby temperature changes of a leaf or even of complete plants with infrared thermography. The differences in stomatal conductance between drought and control groups in *L. purpureus* are

significant. The differences in *T*leaf measurements were significant, the thermal indices *I*g and CWSI showed non-significant differences between stressed and unstressed plants in *L. pupureus*. A study by Grant et *al*. (2006) proves that the results of stomatal conductance measurements using a porometer correlate with the results of infrared thermography indices. For this grapevines (*Vitis vinifera* L.), french beans (*P. vulgaris*) and lupins (*Lupinus albus* L.) were examined. For *P. vulgaris* differences between well watered plants and drought stress plants were found for *T*leaf and the thermal indices *I*G and CWSI. It is suggested that measurements of *T*leaf are probably sufficient for the comparison of different genotypes. For the calculations of the thermal indices *I*G and CWSI measurements of the minimum temperature for a leaf (in this study *T*wet), and the maximum achievable leaf temperature (in this study *T*dry) is required at the same time as *T*leaf measurements. The consequence is that the thermal indices are less susceptible to fluctuations in ambient conditions over a specific time period (Idso et *al*., 1981; Jones, 1999). Otherwise, any changes in surface temperature may originate from changing environmental conditions. Thus, the thermal indices *I*G and CWSI should be used to observe the stomatal behavior in long time experiments (Grant et *al*., 2006). In conclusion, measurements of stomatal conductance in combination with *T*leaf are the best for the identification of drought tolerant genotypes.

THE SUITABILITY OF CHLOROPHYLL FLUORESCENCE IN DROUGHT TOLERANCE SCREENINGS

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 analysis of chlorophyll fluorescence in both experiments led to no meaningful results. The measurements of F_v/F_m, ETR, NPQ/4, Y(II), Y(NO) and Y(NPQ) showed almost no significant differences. Some of the drought stressed groups showed even better values in comparison to the control groups of the same genotypes. For example, F_v/F_m is considered as fast-measuring factor in the case of stress for plants. For non-stressed C3 plants values of about 0.83 (Björkman and Demmig, 1987) are expected. These approx. values were obtained with one exception in the analysis of the two experiments done here by both control groups and drought groups of the genotypes. Chlorophyll fluorescence does not appear to be sensitive enough to detect early symptoms of drought stress, at least in *L. purpureus*. This assumption is supported by a study with *Arabidopsis thaliana* (L.) Heynh. plants. Here, drought stress was initiated by complete withheld of water. A change in the measured values of F_v/F_m, NPQ

and Y(II) occurred only after long-term (more than ten days) drought stress. ETR and Y(NO) measurements behaved similarly, at first there was no impact of stress and then both factors reflected strong signs of stress (Woo et *al*., 2008). Only a slightly decrease of F_v/F_m was found for *P. vulgaris* seven days after stopping irrigation (Miyashita et *al*., 2005). Another study with *A. thaliana* compared the behavior of the photosynthetic system under mild, moderate and severe drought stress. In this experiment, water was withheld until the soil water content reached 66-68% for mild drought stress, 50-52% for moderate drought stress and 43-45% for severe drought stress in comparison to the soil water content of the control group. Severe drought stress caused the strongest changes in comparison to the control group. But mild drought stress led in comparison to moderate drought stress to larger photosynthesis modifications. It was concluded that the response of the plant matches with the "Threshold for Tolerance Model" (Sperdouli and Moustakas, 2012). According to this model, tolerance mechanisms are started with lag time or induced by threshold concentrations (Barceló and Poschenrieder, 2002). Moderate stress caused less damage to the plant, because stress adaptation processes and repair mechanisms started in the plant whereas during mild drought conditions the stress threshold was not reached. Therefore, the plants were 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). The results of our study showed only small differences in ETR, F_v/F_m, NPQ/4, Y(II), Y(NO) and Y(NPQ) between the control and drought stress groups indicating a moderate stress level of the plant. Important for the characterization of drought tolerance in different genotypes is therefore the correct strength of drought stress, then chlorophyll fluorescence measurements might be used efficiently. New measurement protocols could provide more reliable data in early symptoms of drought stress on the photosynthetic system. Burke et *al*. (2010) measured F_v/F_m at two time points by harvesting leaf punches. The chlorophyll fluorescence measurement thereby loses the advantage of being a non-destructive method. In summary, measurements of chlorophyll fluorescence in the case of *L. purpureus* are not advised without reservation. This is demon-strated by the measurement results: the differences between the unstressed and stressed plants are usually too low in order to make statements about the drought tolerance of the tested genotypes.

CONCLUSION

For the screening of drought-tolerant genotypes under moderate drought stress traditional methods such as leaf size measurements and biomass determination as well as new techniques like infrared thermography are suitable. Chlorophyll fluorescence is only appropriate to examine the impact of severe drought stress conditions or in recovery experiments. Because *L. purpureus* is a twining plant, the measurement of the more traditional growth parameter plant height is difficult. Overall, the combination of several methods is recommended. Based on the results a combination of infrared thermography and porometer measurements in conjunction with traditional growth parameter like biomass investigations is advisable for *L. purpureus* under greenhouse conditions. These methods are also suitable for other 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. It must be considered that the selected genotypes of these greenhouse experiments have to be tested under field conditions. Finally, the yield of the required plant product of the selected genotypes in field conditions is most important for the growers.

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CHAPTER 3:

EUPHORBIA TIRUCALLI L. – COMPREHENSIVE CHARACTERIZATION OF A DROUGHT TOLERANT PLANT AND ITS POTENTIAL AS BIOFUEL SOURCE

Hastilestari BR, Mudersbach M, Tomala F, Vogt H, Biskupek-Korell B, Van Damme P, **Guretzki S**, Papenbrock J (2013) *Euphorbia tirucalli* L. – comprehensive characterization of a drought tolerant plant with a potential as biofuel source. *PLoS ONE* 8 (e63501. doi:10.1371/journal.pone.0063501).

ABSTRACT

Of late, decrease in mineral oil supplies has stimulated research on use of biomass as an alternative energy source. Climate change has brought problems such as increased drought and erratic rains. This, together with a rise in land degeneration problems with concomitant loss in soil fertility has inspired the scientific world to look for alternative bio-energy species. *Euphorbia tirucalli* L., a tree with C_3/CAM metabolism in leaves/stem, can be cultivated on marginal, arid land and could be a good alternative source of biofuel.

We analyzed a broad variety of *E. tirucalli* plants collected from different countries for their genetic diversity using AFLP. Physiological responses to induced drought stress were determined in a number of genotypes by monitoring growth parameters and influence on photosynthesis. For future breeding of economically interesting genotypes, rubber content and biogas production were quantified.

Cluster analysis shows that the studied genotypes are divided into two groups, African and mostly non-African genotypes. Different genotypes respond significantly different to various levels of water. Malate measurement indicates that there is induction of CAM in leaves following drought stress. Rubber content varies strongly between genotypes. An investigation of the biogas production capacities of six *E. tirucalli* genotypes reveals biogas yields higher than from rapeseed but lower than maize silage.

Keywords: Biogas, CAM*, Euphorbia tirucalli* L., drought stress, malate, NMR, rubber.

INTRODUCTION

Agriculture faces a range of serious environmental problems such as soil salinisation and depletion of water resources. Additionally, agricultural production and unsustainable human intervention often leave the land under stress, leading to an increase in non-arable land area (Dai, 2012). The supply of fossil fuel in future will also soon start decreasing. Therefore, efforts are made to find substitute sources of energy. One such source is solar energy, which is unlimited. Plants capture this energy through photosynthesis. Faced with a decrease in arable land and crude oil supply, it is important to find species for growing in marginal, non-arable land. These plants should have high drought and salinity tolerance as well as contain compounds that could be used in phytochemical, pharmaceutical or nutraceutical applications.

Euphorbia tirucalli L. belongs to the dicotyledonous order Euphorbiales, family Euphorbiaceae, subsection *tirucalli* (Bruyns *et al*., 2006). The natural distribution of *E. tirucalli* comprises the Paleotropical region of Madagascar, the Cape region (South Africa), East Africa, and Indochina (Van Damme, 2001). This plant is also grown as garden plant in numerous tropical countries, also in America. *E. tirucalli* seems to have high salinity and drought tolerance (Janssens *et al.*, 2009) and survives in a wide range of habitats even under conditions in which most crops c.q. plants cannot grow. These include tropical arid areas with low rainfall, poor eroded or saline soils and high altitudes but *E. tirucalli* cannot survive frost (Van Damme, 2001). Its high stress tolerance can be explained at least in part by its photosynthetic system. The family of *E. tirucalli*, the Euphorbiaceae, consists of five subfamilies (Webster, 1975) and its species have C_3, C_4, intermediate C_3-C_4 and/or Crassulacean Acid Metabolism (CAM) photosynthetic systems dependent on the ecological conditions (Batanouny *et al*., 1991). Batanouny *et al*. (1991) reported that *Euphorbia* species having the C_3 photosynthetic pathway grow under conditions of better water resources and lower temperature, whereas CAM and C_4 plants grow under high temperature. The photosynthetic system of *E. tirucalli* stems has been identified to follow CAM (Bender, 1971). It has been classified based on the C-isotope ratio. The range of values -8 to -18 are characteristic of plants with C_4 or CAM (Pearcy, 1975), while “Kranz” anatomy provides strong evidence of C_4 system. Meanwhile Ting *et al*. (1985) described values in the range of -15.4 to -16.2 were classified as CAM plants, whereas -12.6 and -11.3 as C_4. Bender (1971) showed $^{13}C/^{12}C$ ratios of *E. tirucalli* was -15.3. This value indicated that *E. tirucalli* did not

follow C_4; this was also supported that there was no Kranz syndrome in *E. tirucalli* stem (Smith, 1982). Its photosynthetic system followed C_3 in non-succulent leaves and CAM pathway in succulent stems based on gas exchange observations (Van Damme, 2001). In CAM plants one can observe an opening/closure of stomata during night/day allowing nightly CO_2 uptake accompanied with malate oscillation that follows stomatal opening and closure (Nuernbergk, 1961; Osmond, 1978). Hence, malate presence confirms CAM photosynthetic pathway in *E. tirucalli.* Under unfavorable conditions, its non-succulent C_3 leaves soon die and the plant will then continue its metabolism via the CAM photosynthetic pathway in the stem. The combination of C_3 leaves and CAM stems can explain *E. tirucalli*`s fast accumulation of biomass since C_3 maximizes growth during favorable conditions and CAM during drought to reduce water loss and maintain photosynthetic integrity (Cushman and Borland, 2002). C_3 photosynthetic pathway takes place when leaves are present and in combination with CAM stem, whereas CAM stem takes up CO_2 when conditions deteriorate. However, to date there is no evidence that there is a change from C_3 and CAM at leaf level following drought events, a mechanism that has been evidenced in *Mesembryanthemum crystallinum* L. (Holtum and Winter, 1982) and the genus *Sedum* (Gravatt and Martin, 1992).

E. tirucalli has been reported to present numerous pharmacological activities. The species has been patented for modern drugs such as prostate cancer medicine (Aylward and Parsons, 2008) and has a very high ethnomedicinal value (Duke, 1983; Kumar, 1999; Schmelzer and Gurib-Fakim, 2008; Van Damme, 1989). *E. tirucalli* produces and stores abundant amounts of latex in so-called laticifers (Uchida *et al.*, 2009). *E. tirucalli* latex contains high amounts of sterols and triterpenes (Nielsen *et al*., 1979) and might be used for rubber fractionation and has been investigated for its diesel oil properties (Duke, 1983; Furstenberger and Hecker, 1977; Calvin, 1978, 1980; Kalita, 2008; Mwine and Van Damme, 2011). Through the hydrocarbons of its latex, the species was documented in 1978 to produce the equivalent to 10-50 barrels oil L ha^{-1} (Calvin, 1978), whereas its biomass can yield 8,250 m^3 ha^{-1} biogas (in the tropical, subhumid conditions of Colombia (Loke *et al*., 2011). Furthermore, *E. tirucalli* latex has pesticidal properties against such pests as mosquitoes (*Aedes aegypti* and *Culex quinquefasciatus*) (Rahuman *et al.*, 2008), bacteria (*Staphylococcus aureus*) (Lirio *et al*., 1998), molluscs (*Lymnaea natalensis*) and nematodes such as *Haplolaimus indicus*, *Helicotylenchus indicus* and *Tylenchus filiformis* (Vassiliades, 1984). *E. tirucalli* latex can also be used as glue and adhesive (Murali and Mwangi, 1998).

The morphological characteristics of different *E. tirucalli* accessions do not allow differentiating them amongst themselves, except for one US accession that has yellow tips and has been promoted for ornamental uses. Hence, classification of *E. tirucalli* based on its genetic characteristic will be more precise than using morphological descriptors. Until now, genetic diversity between *E. tirucalli* genotypes from different areas has not been investigated. Analysis of genetic diversity among genotypes is also a prerequisite if one wants to start selecting and/or breeding for increased drought tolerance, gain in biomass, rubber content and biogas production. Our final aim is to recommend the best genotypes first for field research experiments and then for initiating commercial *E. tirucalli* plantations in arid areas for the respective applications.

MATERIAL AND METHODS

PLANT MATERIAL, PROPAGATION AND GROWTH CONDITIONS

Mother plants of genotypes Morocco, Senegal, Burundi, Rwanda, Kenya and USA were collected by Van Damme over the last 20 years from wild individuals and grown in greenhouses at Ghent University, Department of Plant Production, Laboratory for Tropical and Subtropical Agriculture and Ethnobotany, Belgium. Genotype India was collected in Ajmer and Jaipur from naturalized plants but genotype Jaipur could not be propagated as it died after delivery. Genotype Indonesia was collected in Yogyakarta from a wild-grown individual, genotype Italy was collected in Calabria from a cultivated ornamental, genotype Togo was collected in Togo from wild plants by Torsten Schmidt (Hannover, Germany), whereas genotype Hannover was an ornamental specimen of unknown origin. No specific permissions were required for collecting on these locations because the plants grow like weed on locations that are not privately-owned or protected in any way and the *E. tirucalli* species does not belong to endangered or protected species.

Propagation for our experiments was done vegetatively by cuttings taken on no predefined part of the respective mother plants. The 10 – 15 cm cuttings obtained from healthy plants and planted in pots with volume of 436 cm^3 according to the formula of truncated cones that contained a mixture of clay-loam:sand (2:1). These cuttings were cultivated in the greenhouse of Institute of Botany, Leibniz University Hannover, for six months at 14 h/24°C (day) and 10 h/22°C (night) with a light intensity of 350 µmol m^{-2} s^{-1}; and watered once every two days. In control conditions fertilizer Wuxal Top N (Aglukon, Düsseldorf, Germany) consisting of 0.6% NPK and 99.4% water was applied once every two days (about 8.6 ml per

pot). For the water stress conditions the same concentration of fertilizer was added in a smaller volume of water.

MOLECULAR ANALYSIS THROUGH GENETIC MARKER

DNA extraction and quantification

DNA was extracted from twelve genotypes of the *E. tirucalli* collection. DNA isolation procedure using NucleoSpin® Plant II Kit (Macherey & Nagel GmbH & Co. KG, Düren, Germany) was used to extract genomic DNA from 60 mg of young leaf samples. Freshly extracted DNA was quantified photometrically using an Uvikon xs photometer (Biotek Germany, Bad Friedrichshall, Germany). Quantification was done by measuring 2 µl of non-diluted DNA sample at 260 nm wavelength. Extracted DNA was stored at -20°C until use.

Amplified Fragment Length Polymorphism (AFLP)

AFLP analysis was performed essentially as described by Vos *et al*. (1995). Restriction fragments were produced by digestion of 250 ng genomic DNA for 1 h at 37°C with 0.5 µl *Eco*RI (10 U/µl) and 0.3 µl *Mse*I (10 U/µl) in a total volume of 25 µl containing 2.5 µl 10 x RL Buffer, 100 mM Tris HCl, 100 mM MgAc, 500 mM KAc, 50 mM DTT, pH 7.5, and H_2O. The digestion was followed by ligation of specific *Mse*I (50 pmol) and *Eco*RI (5 pmol) adapters (MWG Biotech Eurofins, Ebersberg, Germany) with 5 µL reaction mix (0.5 µl of *Eco*RI adapter, 0.5 µl of *Mse*I adapter, 0.6 µl of 10 mM ATP, 0.5 µl 10 x RL-Buffer, 0.05 µl of T4-DNA-Ligase (1 U μl^{-1}), and 2.85 µl H_2O) which was added to the restricted DNA and incubated for 3.5 h at 37°C.

For the pre-amplification a reaction mix (5 µl of digested and ligated DNA, 1.5 µl *Eco*RI+0 (5' GACTGCGTACAA TTC 3') and *Mse*I+0 (5' GATGAGTCCTGAGTAA 3') or *Eco*RI+A/*Mse*I+A primer combinations (50 ng μl^{-1}), 5 µl dNTPs (2 mM), 5 µl 10 x Williams Buffer (100 mM Tris/HCl, pH 8.3; 500 mM KCl; 20 mM $MgCl_2$; 0.01% gelatine; H_2O), 1 µl *Taq* polymerase (5 U μl^{-1}) and 31 µl H_2O) was amplified in a thermocycler with 94°C/ 5 min, then 20 cycles of 94°C/ 30 s, 60°C/ 30 s, 72°C/60 s and finally 72°C/ 10 min. Selective amplifications were performed using primer pairs containing three selective nucleotides. For selective amplification, 2.5 µl of a 20-fold diluted pre-amplification mixture with reaction mix (2.5 µl *Eco*RI-IRD primer (2 ng μl^{-1}), 0.3 *Mse*I primer (50 ng μl^{-1}), 1 µl dNTPs (2 mM), 0.05 µl *Taq* polymerase (5 U μl^{-1}), 1 µl 10 x Williams Buffer and 2.65 µl H_2O) was amplified consisting of 94°C/ 5 min, one cycle of 94°C/ 30 s, 65°C/ 30 s and 72°C/ 60 s, then lowering the annealing temperature to about 0.7°C reduction per cycle for next 11 cycles, thereafter 24 cycles of 94°C/ 30 s, 56°C/ 30 s, 72°C/ 60

s and lastly 72°C/ 10 min. IRD 700 labelled *Eco*RI primers and *Mse*I primers with three selective nucleotides at their 5' end was used (Table 2). After PCR, an equal volume of sequencing loading buffer (98% formamide, 10 mM EDTA, pararosaniline 0.05%) was added. The mixture was heated to 90°C for 3 min and then cooled on ice.

Table 2: Primer combinations for selective amplification.

Primer combination	***Eco*RI 700**	***Mse*I**
1	GACTGCGTACAA TTC ACA	GATGAGTCCTGAG TAA ACT
2	GACTGCGTACAA TTC ACA	GATGAGTCCTGAG TAA ACT
3	GACTGCGTACAA TTC ACA	GATGAGTCCTGAG TAA ACA
4	GACTGCGTACAA TTC ACC	GATGAGTCCTGAG TAA ATTA
5	GACTGCGTACAA TTC ACC	GATGAGTCCTGAG TAA ATGG
6	GACTGCGTACAA TTC ACA	GATGAGTCCTGAG TAA ATGG
7	GACTGCGTACAA TTC ACA	GATGAGTCCTGAG TAA ACAT

Marked fragments were separated over 6% polyacrylamide gel from Sequa gel X® (16 ml of monomer solution, 4 ml of complete buffer and 160 µl of 10% APS) with 1X TBE buffer. A sizing standard was labeled with IRD 700 at their 5' end (MWG Biotech Eurofins). Samples were analyzed on a LICOR Gene Reader 4300 automated sequencer (LI-COR Biosciences, Lincoln, USA), at condition 1500 V, 35 A, 40 W, 45°C, slow scan speed and 30 min pre-run.

PCR product detection and phylogenic analysis

Detection of AFLP products and phylogenetic analysis of DNA AFLP fingerprints was conducted based on the number, frequency and distribution of amplified DNA fragments. AFLP product diversity was determined from the difference in gel migration of PCR products from each individual sample. Based on the presence or absence of AFLP bands, band profiles were translated into binary data. Data were analyzed using fingerprint analysis with missing data 1.0 (FAMD) (program available from http://homepage.univie.ac.at/philipp.maria.schlueter/famd.html) (Schlüter and Harris, 2006). The tree was generated using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The tree was visualized using the TreeView program version 1.6.6 (Page, 1996).

INVESTIGATION OF DROUGHT TOLERANCE

Investigation of drought effects was conducted based on Jefferies (1994) with some modifications. Six month old *E. tirucalli* plants from Morocco and Senegal with a height of 27-29 cm were selected. This experiment was conducted in a climatic chamber for 8 weeks with condition 24/20°C day (14 h)/night (10 h), at light intensity 155 µmol m^{-2} s^{-1} and 60% humidity. Twenty plants from each genotype were grown in clay-loam and sand substrate with four different volumetric water contents (VWC) 25%, 15%, 10% and 5% monitored using Fieldscout® based on time domain reflectometry (TDR) (Spectrum Technologies, Plainfield, USA). Dry set value was 1% below and wet value was 1% above the respective VWCs. According to the manual of this instrument, sandy-clay-loam substrate has water holding capacity of 25% VWC, and a wilting point at 15% VWC. Soil moisture was measured based on water deficit (D) values which indicate the amount of irrigation water necessary to raise the soil water content to the target point. Water was added based on calculation of D values times 8.66 ml for a pot with 7 cm height.

As *E. tirucalli* grows in semi-arid and arid areas, two VWC points below 15% were investigated for their effect on the species' physiology. Selected VWC points were 10% and 5%. Growth parameters such as plant height, root length, dry matter production, and water content were measured. Plant height and tap root length were measured with a scale. For fresh and dry biomass determination shoots and root of plants were harvested separately and measured after 8 weeks of treatment. Shoots and roots were dried in an incubator at 90°C for 36 h. Investigation on whether there was an effect of drought on photosynthesis during drought application, chlorophyll fluorescence measurements were conducted every week during 8 weeks during drought treatment using the non-invasive method of Imaging PAM (M series, Heinz Walz GmbH, Effeltrich, Germany). Hence, quantum efficiency (F_v/F_m) was measured at leaves having C_3 photosynthetic pathway and stems having CAM photosynthetic pathway.

INVESTIGATION OF THE C_3 AND CAM PHOTOSYNTHETIC PATHWAYS: MALATE DETERMINATION

Stems and leaves of genotypes Morocco and Senegal were harvested at the end of the dark period (5 am) and the end of the light period (7 pm). The end of the dark period is the phase where malate concentration is highest, whereas the end of the light period is the phase where this value is lowest (Kluge, 1971). Harvested material with 3 replications was put in liquid nitrogen and stored in the freezer at -80°C before malate extraction.

Malate was extracted by putting 60 mg of leaves and stems of each genotype separately in 1.4 ml H_2O and vortexing the mixture for 1 min; the mixture as then kept at room temperature for 10 min and mixed again for 1 min. A centrifugation by 13,000 rpm at 4°C for 10 min followed whereupon the supernatant was pipetted into new tubes and centrifuged again at 13,000 rpm for 10 min at 4°C. The supernatant was then pipetted into new tubes and kept at -20°C until measurement by capillary electrophoresis (CE). A P/ACE™ MDQ capillary electrophoresis system (Beckman Coulter, Krefeld, Germany) was used for CE analyses. Separations were performed in a eCAP™ CE-MS capillary (fused silica, 75 µm i.d., 57 cm total length, 50 cm effective length, Beckman Coulter). Before starting the analyses the capillary was equilibrated with the background electrolyte Basic Anion Buffer for HPCE (Agilent Technologies, Waldbronn, Germany) at 14.5 psi for 4 min. Injection was done by applying 0.7 psi for 3.5 s. Separation of the samples was performed by applying 14 kV for 10 min at 22°C. After each run, the capillary was washed with the background electrolyte for 4 min. Buffer was changed after 8 to 10 runs. Samples were detected at 235 nm with a bandwidth of 10 nm. Calibration graphs were generated with 0.313 to 10 mM malic acid. Elaboration of the electropherograms was done using Karat 32 7.0 software (Beckman Coulter).

LATEX ANALYSIS

E. tirucalli latex consists of 2.8% to 8.3% rubber and 50.4% to 82.1% resin (Duke, 1983). Latex of *E. tirucalli* has attracted a lot of attention because it has an economical potential as source of rubber. Therefore, rubber content was investigated in different genotypes. Rubber content analysis was conducted by LipoFit Analytic GmbH (Regensburg, Germany) using nuclear magnetic resonance (NMR, 600 MHz Bruker Avance$^+$ spectrometer, Bruker Daltonic GmbH, Bremen, Germany). Samples were taken from Burundi, Hannover, Kenya, Morocco, Rwanda, Senegal, Togo and USA genotypes. The input material was 100 to 500 mg fresh weight of stems.

To fresh plant material, 1.5 ml water p.a. (0.03% NaN_3) and a sharp aglet were added. By shaking 10 min the material was mechanically milled. The aglet was extracted from the suspension by a magnet. The suspension was centrifuged (20 min; 14,500 rpm; 20°C) to separate cell debris. Sodium phosphate buffer pH 6.8 (final concentration 100 mM), D20 (5%) and sodium trimethyl silyl propionate (0.1 mM) were added to the supernatant. The suspension was then transferred to 5 mm-NMR-tubes.

Relative rubber concentrations refer to the average of the spectra measured in the *E. tirucalli* samples. The average is calculated out of the integral from all the spectra which are expected to contain rubber signals. The reference for the absolute concentrations was 1,4-polyisoprene with a molar mass of 47,300 g mol^{-1}. The reference was also measured by NMR. In reference to polyisoprene, only the spectra with the same pattern as the reference were calculated.

BIOGAS PRODUCTION

Plant material of genotypes Kenya, Morocco, Rwanda, Senegal, Togo, and USA was harvested from the greenhouse (Hannover, Germany), dried, and chopped into 0.5 to 4 cm pieces before being used in biogas batch tests. Biogas yields of the selected genotypes were determined through anaerobic batch digestion tests according to the German Standard Procedure VDI 4630 (2006). The inoculum was biogas slurry from an agricultural biogas plant mainly fed with maize silage. Organic dry matter (ODM), density and chemical oxygen demand (COD) were determined for all samples and the inoculum according to standard methods. Based on results, the weighted samples of the substrates and the inoculum were balanced to obtain a Slurry Loading Rate (SLR; $ODM_{substrate}$ to $ODM_{inoculum}$) of 0.3 as recommended by VDI 4630 (2006). Each substrate and one control without the addition of substrate, was incubated in triplicate in gas-tight 1,250 ml dark DURAN glass bottles. Experiments were conducted for 28 days at 38°C in a warming cupboard. Biogas yields (L kg^{-1} ODM) were calculated based on the pressure in the bottles following biogas production. Rise in pressure was recorded with LabView software connected to the batch plant. After tests were finished, the concentration of CH_4 in the biogas produced were analyzed as follows: In each bottle, 20 ml of a 10 molar NaOH solution were injected through the septum with the help of a syringe. The NaOH solution fixes the CO_2 in the biogas by reacting to sodium carbonate which precipitates in the liquid phase. As a result, in the bottles a decrease in pressure occurs and on the basis of this data, the methane ratio in the produced biogas can be calculated. H_2S in biogas samples of genotypes Morocco, Kenya and USA were quantified using gas chromatography.

STATISTICAL ANALYSIS

All statistical analysis was conducted with Statistix 8 version 2 (Analytical software, Tallahassee, USA). Interaction between means was calculated by the least significant

different (LSD) at $p<0.05$. Graphs were drawn using SigmaPlot Version 12.2 (Systat Software Inc., San Jose, USA).

RESULTS

GENETIC MARKER ANALYSIS

AFLP technique was used as a tool for assessing species relationships within the *E. tirucalli* collection. Seven primer combinations were selected for AFLP analysis (Table 2). Total number of polymorphic bands was 243 with a mean of 34.7. We were able to derive two main groups from the phylogenetic analysis of the 12 accessions of *E. tirucalli* cluster analysis using UPGMA with 1000 bootstrap replicates (Fig. 14).

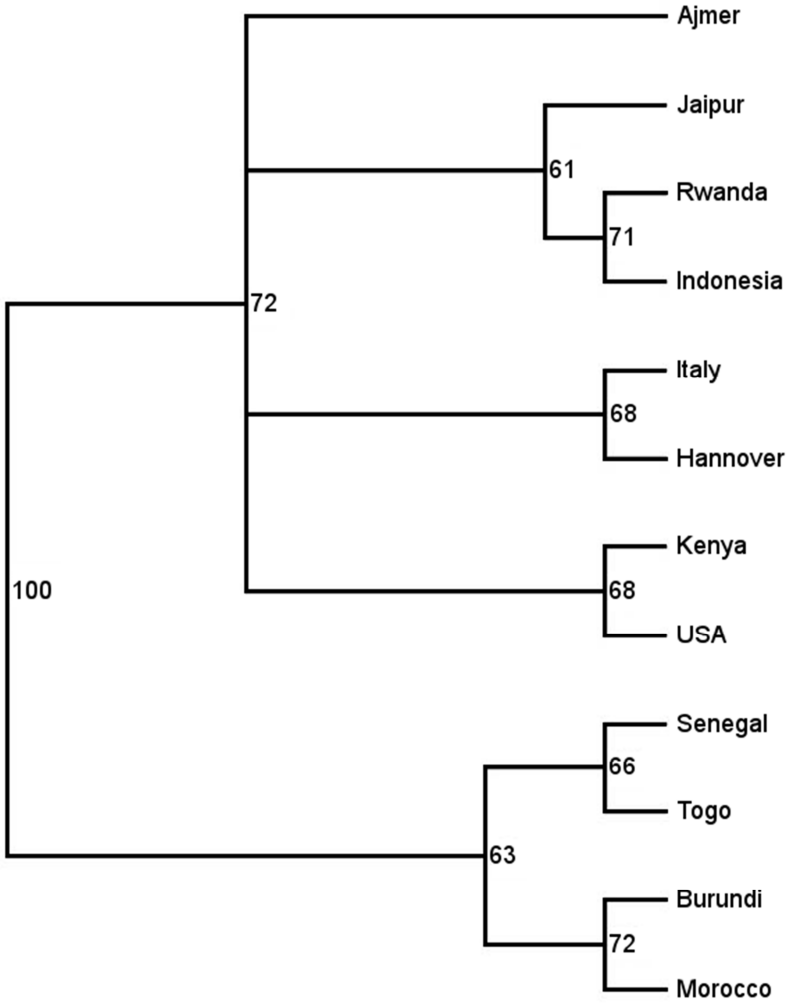

Fig. 14: Dendrogram of twelve *E. tirucalli* genotypes calculated with UPGMA showing the phenetic relationships within the colletion. Bootstrap values ≥ 50% are above the branches.

Nevertheless, the genotypes tested share a lot of similarities as evidenced from the low bootstrap values. The first group consists of two clades and comprises mainly genotypes from Africa: Burundi, Morocco, Senegal and Togo accessions that are clustered with a

bootstrap value of 63. The second group consists of four clades with mainly non-African genotypes (except Kenya and Rwanda): Ajmer (India), Hannover (Germany), Indonesia, Italy, Jaipur (India), Kenya, Rwanda and USA with a bootstrap value of 72. A dendrogram derived from NJ calculation showed the same pattern (data not shown). All genotypes have been propagated by cuttings and cultivated in the greenhouse since a long time or at least for a couple of years. Therefore they should have the same amount of endophytes, if any. In our AFLP analysis the genotypes differ in several hundred bands. In case there are some bands originating from endophytes they would not influence the results significantly.

STRESS TOLERANCE

We were interested to analyze physiological differences among members of the genetically quite homogeneous African group. Therefore the response to different soil water contents of *E. tirucalli* genotypes Morocco and Senegal that were grown on clay-loam:sandy soil type after eight weeks of treatment was evidenced through the measurement of growth parameters.

Plant height was significantly reduced by applying drought stress in the experiment (Fig. 15A). It decreased in line with the decrease in VWC (%). Average plant height before treatment was 29.06 cm for Morocco and 27.93 cm for Senegal. After eight weeks the highest height of genotype Morocco was with plants grown in VWC 25% (54.30±1.48 cm) whereas lowest values were obtained in VWC 5% (40.30±1.89 cm). Genotype Senegal had the highest (54.91±3.45 cm) and the lowest (32.80±0.86 cm) heights in the same respective VWCs. Plant height decreased linearly with decrease in water content. Thus, genotype Morocco grew by 86.85% at normal water content and 38.67% at high water limitation. Meanwhile, growth in genotype Senegal was 96.59 % at VWC 25% and 17.43% at VWC 5%. Growth percentage showed that genotype Senegal grew faster than genotype Morocco when water was well available, but that drought highly decreased the growth rate.

Increased water limitation caused reduction of dry weight (Fig. 15C) and water content (Fig. 15D) in both genotypes. Genotype Senegal had higher biomass accumulation at VWC 25% (12.74±0.51) than genotype Morocco (10.53±0.54). The first genotype also had higher yield at the lowest VWC (6.71±0.39 g) than genotype Morocco (5.74±0.22 g). Decrease in water content percentage was small due to water limitation: genotype Senegal was 88% and Morocco 84% at VWC 25%, and 84% and 79% at VWC 5%, respectively.

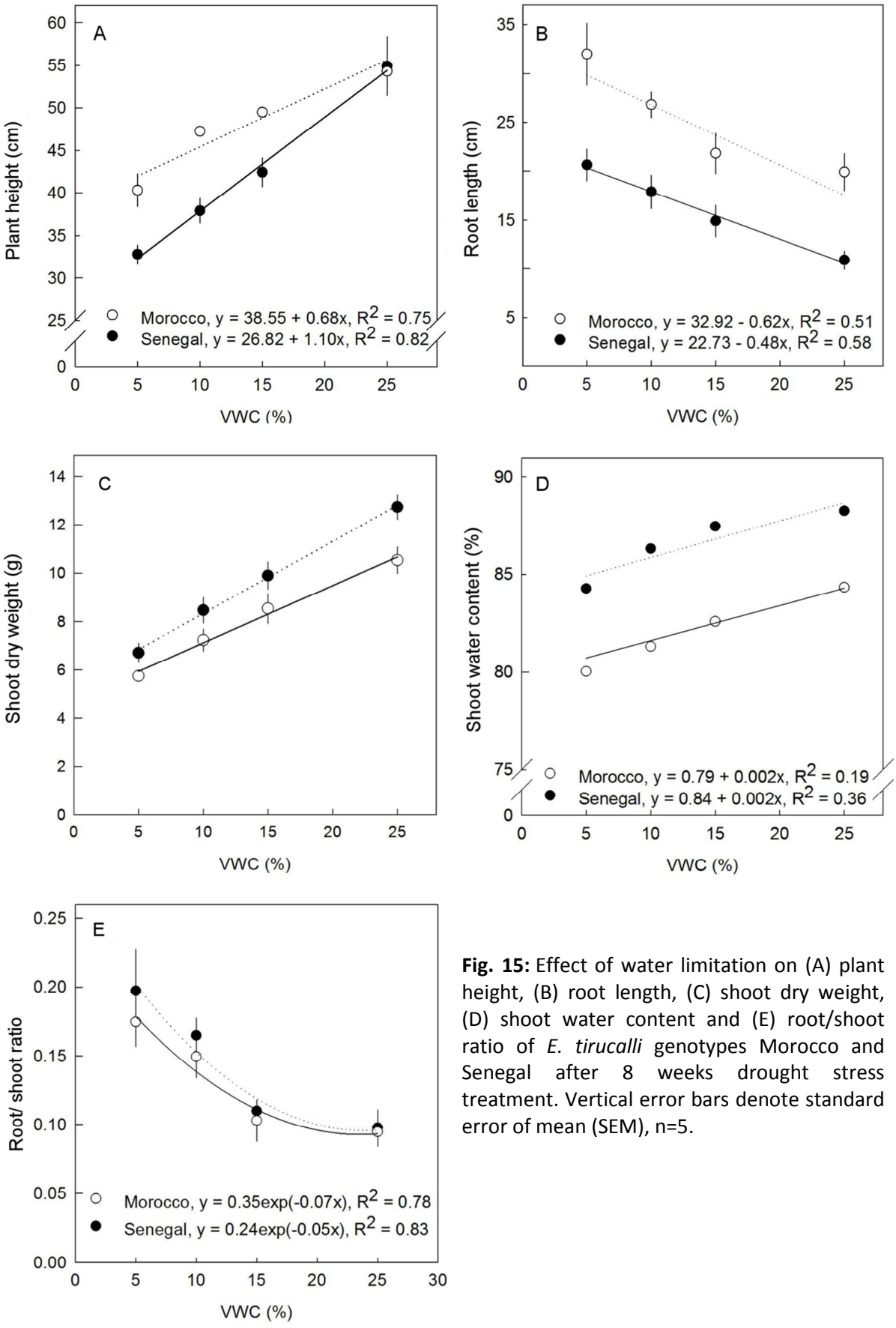

Fig. 15: Effect of water limitation on (A) plant height, (B) root length, (C) shoot dry weight, (D) shoot water content and (E) root/shoot ratio of *E. tirucalli* genotypes Morocco and Senegal after 8 weeks drought stress treatment. Vertical error bars denote standard error of mean (SEM), n=5.

Drought stress increased tap root length (Fig. 15B) and root/shoot ratio (Fig. 15E) in both genotypes. Genotype Senegal showed a ratio of 0.09±0.01 at VWC 25% and 0.19±0.03 at

VWC 5%, genotype Morocco 0.09±0.01 - 0.17±0.02 in VWC (%) 25 to 5, respectively. The result implies that both genotypes partitioned photosynthetic products more in root biomass following drought stress. Plant height, dry weight, water content percentage and root/shoot ratio of genotypes Morocco and Senegal showed a significant reduction when plants were subjected to a drought stress of eight weeks. The stress responses of both genotypes differed indicating differences in phenotypic plasticity.

CHLOROPHYLL FLUORESCENCE

Quantum efficiency of genotypes Morocco and Senegal in the photosystems of leaves and stems over eight weeks decreased linearly with water limitation (Fig. 16). Stems (Fig. 16B, 16D) of both genotypes showed higher quantum efficiency than leaves (Fig. 16A, 16C).

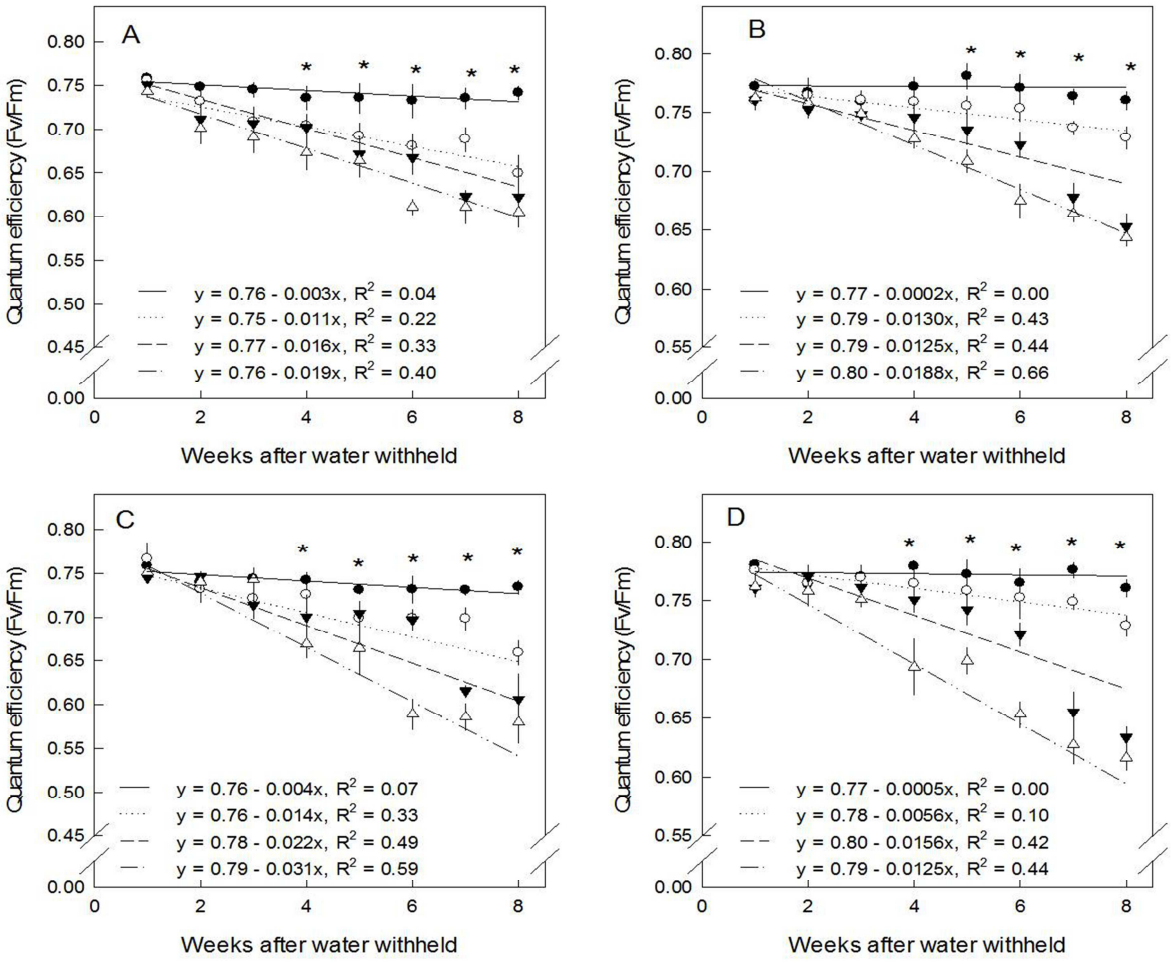

Fig. 16: Effect of water limitation on quantum effciency during 8 weeks drought stress treatment, n=5 (A) Morocco leaves, (B) Morocco stem, (C) Senegal leaves (D) Senegal stem, (●) VWC 25%, (○) VWC 15 %, (▼) VWC 10% and (Δ) VWC 5%, n=5. Vertical error bars denote the standard error of mean (SEM). Stars above the point denote significant difference between VWC in each week treatment following the Tukey procedure (p<0.05).

Quantum efficiency of Morocco leaves for all VWCs (%) was in a range of 0.757 – 0.605. These values were higher than those for genotype Senegal (0.758 – 0.579) at similar VWCs. Genotype Morocco also had higher values at stem level (0.780 – 0.643) than genotype Senegal (0.780 – 0.616). In the leaves of both genotypes, there was no significant difference between different VWCs in the first three weeks, but there was a significant difference from week four onwards. When considering stems, however, genotypes performed differently. In genotype Morocco, significant differences between VWCs started to develop in week five, while in genotype Senegal (Fig. 16D) changes started in week four. This shows that genotype Morocco had higher drought tolerance than genotype Senegal.

MALATE CONTENT

Differences in photosynthetic pathways were ascertained by comparing malate content of leaves and stems before drought stress and after exposure to drought stress. Our results show that before drought exposure, there was malate content oscillation between day and night in both genotypes' stems (Fig. 17). In genotype Morocco, malate content of stems at the end of light period was 58.9% lower than that at the end of dark period. Meanwhile, decrease in genotype Senegal was only 17.4%.

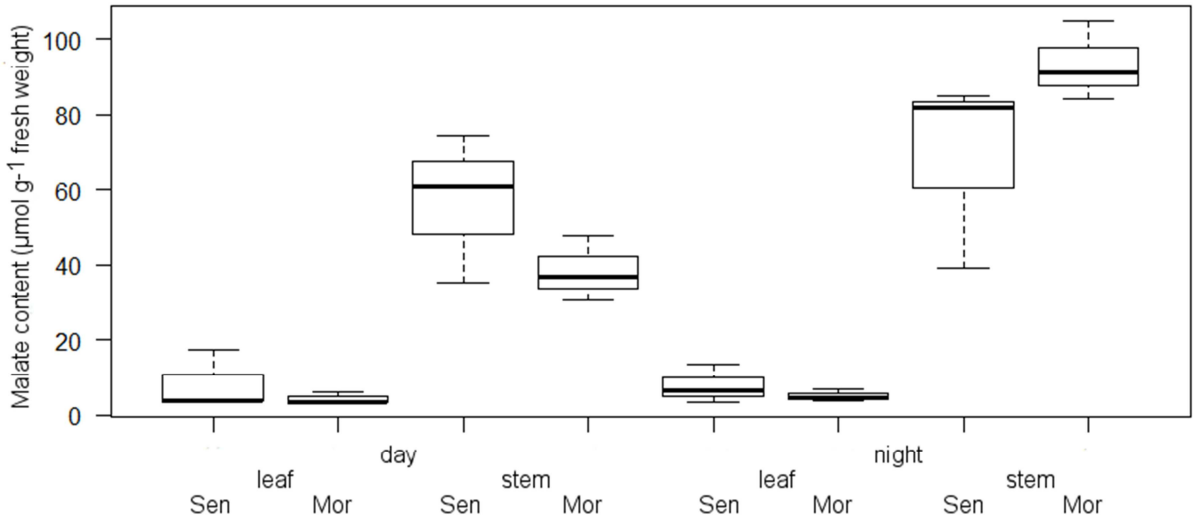

Fig. 17: Box plot (n=3) of malate contents of stems and leaves (µmol g^{-1} fresh weight) of *E. tirucalli* genotypes Morroco (Mor) and Senegal (Sen).

With increasing drought stress, malate content increased in stems of both genotypes (Fig. 18). We noted a significant difference in malate content in stems and leaves of the plants, but there was no significance difference between genotypes. The highest malate oscillation between day and night at stem level for genotype Morocco was 68.75% in VWC 15% whereas for genotype Senegal it was 69.55% at VWC 10%.

In leaves, there were significant differences between day and night malate content at VWCs 10% and 5%. In VWC 10%, malate content was 48.22% and 33.16% lower during the day than during the day for genotypes Morocco and Senegal, respectively. In VWC 5%, we only evidenced a significant different in genotype Senegal. At this VWC, day-time malate content was 50% lower than that at night. These values would indicate that there is CAM induction in leaves following drought stress which strength might be genotype-dependent.

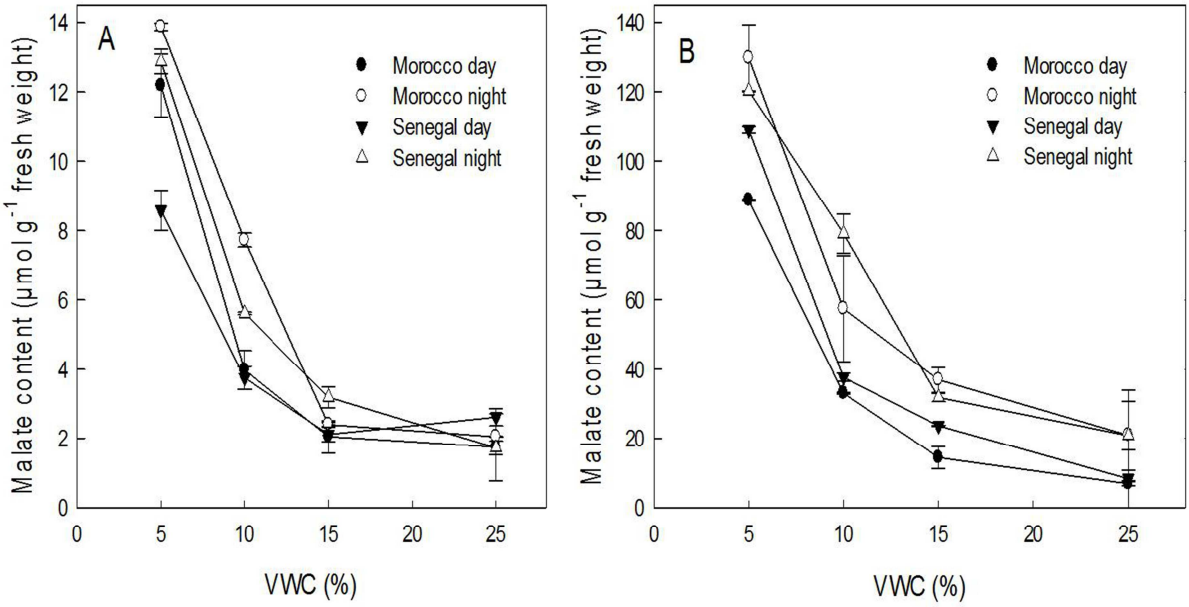

Fig. 18: Malate content of (A) leaves (B) stem of genotypes Morocco and Senegal at day and night on different VWC after eight weeks of drought stress treatment, Vertical error bars denote standard error of mean (SEM), n=3.

RUBBER CONTENT

E. tirucalli can be a source of rubber. The rubber content analysis was done by NMR for eight genotypes in our collection, including Morocco and Senegal. The analysis showed strong differences in the concentration of rubber between the genotypes (Fig. 19). Senegal, with 10.74 mg g^{-1} fresh weight, had the highest amount of rubber among genotypes tested, followed by USA 8.80 mg g^{-1} fresh weight. The lowest rubber concentration was found in genotype Togo which had 1.42 mg g^{-1} fresh weight. There is no correlation of rubber content and genotype classification (Fig. 14 and Fig. 19), at least in greenhouse conditions.

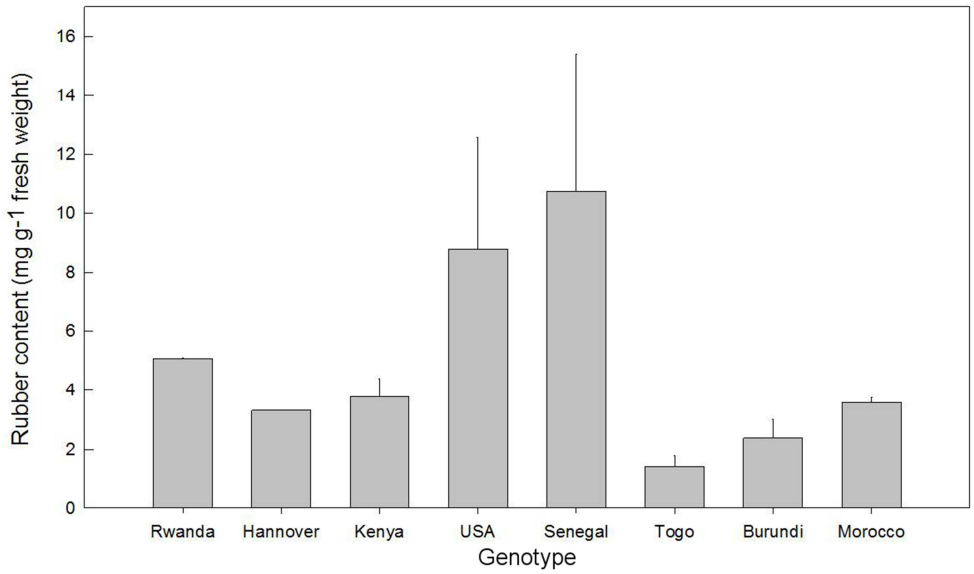

Fig. 19: Rubber content of eight *E. tirucalli* genotypes. Each bar illustrates the mean (n=3). Vertical error bars denote standard error of mean (SEM).

BIOGAS PRODUCTION

The results of the mesophilic anaerobic digestion of dried samples of six different genotypes of *E. tirucalli* indicate a promising potential with regard to the use of dried biomass of this species as a feedstock for biogas production. Specific biogas production (L biogas kg^{-1} ODM) was in the range of 114 for genotype Togo and 637 for genotype Kenya.

Table 3: Specific biogas production (L biogas kg^{-1} ODM) and gas composition in the biogas produced.

Genotype	Biogas production	CH_4 (%)	H_2S (ppm)
Togo	114	69	n.a.
USA	367	44	~1350
Morocco	435	43	~1630
Senegal	440	54	n.a.
Rwanda	522	41	n.a.
Kenya	637	50	~1750

n.a., not analyzed. In case standard deviations could be calculated, they were always less than 10%.

Both genotypes which has been investigated in more detail in the drought stress experiments show values around 440 L biogas kg^{-1} ODM, about 70% of the highest value. The methane concentrations lie between 43% and 69%, depending on the genotype. These are preliminary results based on two independent experiments. Not for all genotypes data for all the three replicates in each experiment could be obtained due to initial technical problems

with our bench-scale biogas plant. Therefore, we are currently not able to calculate any reliable standard deviations. The experiment will be repeated shortly for all genotypes with optimised equipment. Remarkable are the high amounts of H_2S which reached up to 1,750 ppm (Table 3).

DISCUSSION

MOLECULAR ANALYSIS THROUGH GENETIC MARKERS

The division in two groups as presented in Fig. 14 is congruent with the geographic division in an African group and a mostly non-African group (except for Kenya and Rwanda). More samples have to be collected for example from Pakistan, Egypt, and Somalia to analyze whether they belong to the non-African group. Analysis of the genotypes from Brazil might help to estimate the phylogenetic position of the USA genotype, if this is domestic species. Genotypes of *E. tirucalli* are propagated vegetatively since many years in the greenhouse. Therefore the genotype originally collected is not changed since the cultivation due to pollination of flowers. Therefore the genetic drift between generations is low. This vegetative propagation also occurs naturally and/or is conducted by man because this plant seldom produces viable seeds (Van Damme, 2001). The dendrogram shows that there is no correlation between morphological characters, as genotype Kenya and USA that have different stem color are clustered as a monophyletic group. Genotype USA has the most distinctive morphological character, i.e. yellow tips. This morphological character is useful for marketing purposes as this accession is sold as an ornamental. The division in two groups within the collection may indicate the breeding potential for different utilizations that can be explored. Generally, genotypes in the African group grow faster and produce more biomass than those in the non-African group (data not shown). It indicates that genotypes in the African group may be suitable as source of biomass and therefore bioenergy, while genotypes in the other group may be suitable for other purposes such as ornamental plant.

RESPONSE OF PLANTS TO DIFFERENT DROUGHT TREATMENTS

Variation in drought tolerance within a genotype collection is important for subsequent selection work. Analysis of physiological parameters shows that plant height, dry weight and water content decreased with higher drought stress. Research on other plant species, such as *Amaranthus* and wheat, showed also that there is reduction in plant height and biomass with increase in drought stress in the soil (Liu and Stützel, 2004; Zhang *et al*., 2011). In

general, decrease in biomass production rate due to stress exposure has been found to be associated with cessation of photosynthesis, metabolic dysfunction and damage of cellular structure (Krasensky and Jonak, 2012). Further, in response to drought stress, *E. tirucalli* genotypes Morocco and Senegal altered their root dry mass ratio and root length as one of the mechanisms to adapt to drought stress. Root dry mass in drought conditions is higher than in normal condition; this is in accordance with early studies (Liu and Stützel, 2004; Dias *et al.*, 2007) and in line with the theory of functional balance which indicates that plants will respond to low water contents with a relative increase in the flow of assimilates to roots and increased root dry mass (Brouwer, 1963). The root grows longer which enables the plant getting to deeper water layers thus escaping from water deficits near the surface (Schenk and Jackson, 2002). Root elongation reduces shoot dry weight as photosynthesis yield is used for root development at the expense of shoots. Our results of responses to different water content RWC showed that 15% VWC was a critical threshold, below which plants partitioned assimilates to roots which might reduce stem yield.

C_3 leaves wither and die quickly after the onset of stress, and also *E. tirucalli* becomes leafless. CAM stems can proceed with photosynthesis with closed stomata during the day. This provides an ecological advantage of CAM as it allows supplying CO_2 (Martin and Jackson, 1986) through decarboxylation of malate; hence it can prevent photorespiration damage during stress (Borland *et al*., 2006). However, during prolonged drought stress, CO_2 release from decarboxylation may be insufficient to protect chloroplast membranes from oxidative stress. This oxidative stress derives from partially reduced forms of atmospheric O_2 and influences the repair of PSII during stress (Nishiyama *et al*., 2006). Cessation of photosynthesis is supported by a decline in F_v/F_m along with prolonged drought in both genotypes. The decline of F_v/F_m becomes higher at lower VWCs, whereby VWC 5% shows the highest decline. The decrease of F_v/F_m at high water limitation has been related to a decline in functioning of primary photochemical reactions, primarily involving inhibition of PSII that is located in the thylakoid membrane system (Souza *et al.*, 2003). The values between leaves and stems are not significantly different in the three first weeks of the experiments, during which stress symptoms such as leaf senescence did not appear yet. After prolonged stress, values at stems of both genotypes are higher than at leaves. Quantum efficiency values for all VWC values of genotype Morocco at leaf (0.757 – 0.605) and stem (0.780 – 0.643) levels were higher than in genotype Senegal for both leaf (0.758 – 0.579) and stem (0.780 – 0.616)

levels, respectively. This indicates that quantum efficiency difference is also determined genetically. Drought significantly decreases quantum efficiency at week five for stems of genotype Morocco and at week four for stems of genotype Senegal. Lower photosynthetic efficiency under stress is associated with a damaged photosystem due to stress and reflects a certain degree of environmental stress (Maxwell and Johnson, 2000). The CAM photosynthetic pathway in the stem provides an ecological advantage by supplying CO_2 through decarboxylation of malate (Herrera, 2008); hence, it can prevent formation of reactive oxygen species (ROS) and limit photorespiration during stress (Borland *et al*., 2006). However, during prolonged drought stress or higher water limitation, the release of CO_2 from decarboxylation may be insufficient to protect chloroplast membranes from oxidative stress, which affects the repair of PSII during stress (Nishiyama *et al*., 2006).

Stomatal conductance and infrared thermography measurements are suitable for genotype screening towards their drought tolerance. However, due to the cylindrical morphology of the *E. tirucalli* stem it is impossible to use a regular porometer. We obtained some results using a thermography camera T360 (FLIR Systems, Wilsonville, USA). In several parameters determined we observed differences in drought tolerance among the two genotypes supporting the data shown in Fig. 15 to 18. However, due to the *E. tirucalli* morphology the results could not be exactly calculated and compared. In summary, the genotype Morocco is more tolerant to drought than genotype Senegal.

Water use efficiency, and assimilation rate to transpiration rate ratio increase in CAM is higher than in C_3 and C_4 (Maxwell and Johnson, 2000). However, biomass accumulation in CAM plants is usually very low, so that growth rate of plants that only rely on CAM is often limited (Heldt and Piechulla, 2011). However, in some species such as *M. crystallinum*, a plant with facultative CAM, photosynthetic rate is higher than that C_3 species due to a high CO_2 fixation rate at night which contributes for a great part to biomass production (Bloom and Troughton, 1979).

E. tirucalli genotypes Morocco and Senegal were both shown to tolerate severe drought stress (VWC 5%) without causing any plant death. Thus, our result confirms that the species has very good potential to be grown in arid area. Genotype Morocco had 84% water content and 16% dry weight in VWC 25%; those values decreased down to 79% and 21% in severe drought stress. Meanwhile, genotype Senegal had 88% water content and 12% dry weight, those values decreased down to 84% and 16% at the same VWCs. *E. tirucalli* water content

and dry weight differs between studies: 76.6% water content and 23.4% dry weight (Loke *et al*., 2011), 88.33% water content and 11.67% dry weight (Orwa *et al*., 2009), or 90% water content and 10% dry weight (Van Damme, 2001). Different percentages of water content and dry weight might be due to differences in genotypes and growth environment.

CAM AND C_3 PHOTOSYNTHETIC PATHWAYS IN *E. TIRUCALLI*

The analysis of malate content in two genotypes of *E. tirucalli* shows that there are significant differences in leaves and stem. This clearly indicates that there is a difference in photosynthetic pathways between both parts. This result confirms the findings of Van Damme (1990) evidenced by gas exchange experiments that there are two photosynthetic pathways allowing to distinguish C_3 leaves from CAM. Malate content before exposure to water limitation shows that the highest content is in nocturnal stems which confirms dark nocturnal CO_2 uptake (Kluge and Heinigner, 1973). More gas exchange experiments are needed to quantify the CO_2 uptake. We observed open stomata at night and closed stomata during the day. Wax patches appear as a dotted white line along the stem axis in a magnified view and surround the stomata (data not shown). These epicuticular wax patches do not melt in greenhouse conditions to seal or block the stomata. Therefore CO_2 influx at night is not hindered by melted wax. Malate content under higher water limitation increases both in stems and leaves, maybe as an indication of CAM induction in the latter. In stems, the highest percentage of malate day – night oscillation of genotype Morocco is at VWC 15% whereas for genotype Senegal we evidenced it at VWC 10%. Malate might be transported from the stem into the leaves. However, so far it was not reported that malate or other water-soluble compounds are transported via the non-articulate laticifers from organ to organ. Phosphoenolpyruvate (PEP) carboxylase enzyme activity and gene expression could be investigated in stems and leaves to prove our hypothesis that there might be CAM induction in leaves under drought stress.

Photosynthesis in non-succulent leaves of *E. tirucalli* is reported as C_3 and CAM in succulent stems (Van Damme, 2001). Having two photosynthetic pathways in two very distinct plant parts is reasonable as it is supported by different anatomy. In genotype Morocco, we evidenced a significant difference in malate content (in µmol g^{-1} fresh weight) at VWC 10% between 3.9 (day) and 7.7 (night) and at VWC 5% between 12.2 (day) and 13.9 (night) while genotype Senegal shows differences at VWC 10% of 3.7 (day) and 5.5 (night) and at VWC 5% of 8.0 (day) and 12.9 (night). This result, however, reveals that there may be an induction of

CAM in leaves due to drought stress as there is oscillation in nocturnal and diurnal malate content. This result which may seem at odds with previous results needs further investigation because anatomically leaves of *E. tirucalli* are non-succulent, in contrast to the stems. It is thereby tempting to question whether the leaves are really non-succulent. Indeed, CAM is a syndrome that implies a certain degree of succulence based on the presence of large vacuoles for malate storage (Nuernbergk, 1961). We therefore recommend *E. tirucalli* leaves would be anatomically investigated for large vacuoles for supporting malate storage. Species such as *Tillandsia usneoides* L. that perform CAM with non-succulent anatomy still have large vacuoles (Kluge *et al*., 1973; Loeschen *et al*., 1993).

Environmental conditions can influence the plasticity of photosynthetic pathways. Strong stress leads to conversion of C_3 to CAM photosynthetic pathway, for example in the genus *Clusia* (Taybi *et al*., 2004). Change of C_3 to CAM has been documented in other, succulent, species such as *M. crystallinum* (Holtum and Winter, 1982), genus *Sedum* (Gravatt and Martin, 1992), and some species of *Peperomia* and *Clusia* (Ting *et al*., 1993; Borland *et al*., 1998). CAM induction during stress positively influences the activities of enzymes involved in malate metabolism (Holtum and Winter, 1982; Ostrem *et al*., 1990; Cushmann, 1992).These enzymes are nicotinamine adenine dinucleotide-dependent malic enzyme (NAD-ME) (Dittrich *et al*., 1973), nicotinamide adenine dinucleotide phosphate dependent malic enzyme (NADP-ME) (Holtum and Winter, 1982), and PEP carboxylase (Ting, 1968).

With two photosynthetic pathways present at leaf and stem levels, and certain plasticity in switching between C_3/CAM metabolism in *E. tirucalli*, it is not surprising that this plant is recommended as source of biomass for biofuel production that can be grown in marginal conditions. Loke *et al*. (2011) mentioned the prospect of planting *E. tirucalli*; they are already monitoring plantations in Colombia, and are planning to have more in Somalia and other dry African countries. The species can yield 22-25 t dry weight biomass ha^{-1} y^{-1} under optimal conditions whereby optimal planting density is estimated at 14,000 plants ha^{-1}. However, the data presented by the latter authors are not complemented by detailed information on cropping conditions such as irrigation, planting density, and genotypes used. In addition, Van Damme (unpublished data) was able to show that a 3 years' old plantation in Kenya was able to fetch around 500 t ha^{-1} of fresh material.

POTENTIAL USE AS SOURCE OF RUBBER AND BIOGAS

Our results indicate that rubber content varies between genotypes, independently of the affiliation to one AFLP group. This result is supported by a study with several other genotypes: rubber content was different in each genotype depending on soil, climate and year (Uzabakiliho *et al*., 1987), whereas it is not clear whether this is due only to genetic determinants or whether there are also some environmental influences that intervene. Akpan *et al*. (2007), who analysed latex yield of *Hevea brasiliensis* L. found that rubber yield was influenced by clone and soil type. The authors revealed that when soil fertility was better, rubber (latex) yield was also higher. We evidenced the highest rubber content in genotype Senegal. This result supports Van Damme (1990) who mentioned that the Senegal genotype was promising as a source of rubber.

Latex of *E. tirucalli* has drawn a lot of attention because it contains high levels of rubber. It has been used as such since the early 20th century (Scasselati-Sforzolini, 1916). The type of rubber of *E. tirucalli* is a mixture of long chain ketones and cis-1,4 polyisoprene, and is slightly soluble in hot alcohol (Uzabakiliho *et al*., 1987; Blaschek *et al*., 1998). Beside rubber, the latex of this plant also consists of a resin which prevents long-term stability of latex (Orwa *et al*., 2009). Although the rubber has lower quality than that of *H. brasiliensis*, its properties should be further explored in order to fully exploit its potential as a naturally occurring polymer. The detailed composition of sterols and triterpenoids in greenhouse-grown plants and field-grown plants has to be analyzed by GC-MS in the future. Also the expression of the rate limiting enzyme of the mevalonate pathway, 3-hydroxy-3-methylglutaryl-CoA reductase, should be analyzed for its expression in different *E. tirucalli* genotypes to analyze the genetic dependency of the biosynthesis of latex components.

The use of *E. tirucalli* as a source of energy is promising because it grows fast whilst having at the same time low water requirements and a low demand for nutrients (Van Damme, 2001). It was stated that this species could be used for biofuel production due to its high latex content (Calvin, 1978). Our results indicate that the biogas production in our batch tests varies among genotypes (Table 3). The results also show that *E. tirucalli* definitely has potential to serve as a feedstock for the production of biogas.

To date only a few experimental results concerning the biogas production potential of *E. tirucalli* have been published. Sow *et al.* (1989) reported a potential annual methane production of around 3,000 m^3 ha^{-1} per year based on research carried out in Kenya with a stand density of 80,000 plants per hectare and a biomass yield of 20 t ha^{-1} y^{-1} (DM). In field

experiments in Colombia, 30 t ha^{-1} y^{-1} (DM) of *E. tirucalli* biomass brought about 8,250 m^3 ha^{-1} biogas (Loke *et al*., 2011). Assuming a methane content of approx. 50% (Table 3), the methane yield of *E. tirucalli* seems to be smaller compared to the yields of maize silage (5,800 m^3 ha^{-1} y^{-1}) and forage beet plus leaves (5,800 m^3 $ha^{-1}y^{-1}$); however, its yield exceeds that of wheat (2,960 m^3 ha^{-1} y^{-1}) and rapeseed (1,190 m^3 ha^{-1} y^{-1}) (Weiland, 2003).

In the results presented here it is remarkable that the H_2S concentrations are the comparatively high in the *E. tirucalli*-derived biogas. H_2S contents are indeed lower than those from the fermentation of manure, biowaste and food waste which are in the range of 2,000–6,000 ppm due to a high content of sulfur-containing proteins (Schieder *et al*., 2003), but higher than those of maize silage-derived biogas with approx. 500 ppm. H_2S can impair the utilization of biogas, as it has the ability to corrode the metal parts of the fermenting installation and can cause health problems in high doses and long exposures (Binder *et al*., 2009). To decrease H_2S content during processing, different techniques are available, such as biofilters consisting of phototrophic (*Cholorobium limicola*) or chemotrophic bacteria (*Thiobacillus* spp.) (Syed *et al*., 2006). In order to improve the reliability of the method, further biogas batch tests with *E. tirucalli* should comprise a systematical variation of the following parameters: age of plant material (because the older the plant, the higher the lignin content), particle size of the substrate in order to investigate its influence on biodegradability of feedstock, optimization of choice and pre-treatment of the inoculum (Tomala, 2012), and last but not least genotype-dependent differences.

The presented data are based on lab-scale experiments. Further field experiments will be necessary before a specific *E. tirucalli* genotype can be proposed for practical application. Among the genotypes tested, Kenya has the highest yield in biogas per organic dry matter and should be further analyzed for its biomass gain during drought stress conditions in the greenhouse and in the field. Senegal is promising as a source of biomass and biogas as well. When water availability is limited, using genotype Morocco with higher drought tolerance as a source of bio-energy is recommended, because biogas production using genotype Morocco is as high as with genotype Senegal. Genotype USA is promising as an ornamental plant and source of biogas, but its drought tolerance is not yet known. Combining these valuable characteristics through breeding may bring more benefit. Stocked genotypes could be distributed to interested farmers and researchers in arid areas for performing field experiments and challenge the greenhouse results by natural conditions.

CONCLUSION

E. tirucalli has a high potential as drought-tolerant crop plant because of its unique combination of photosynthetic pathways and as source of biofuel, rubber and maybe even phytochemicals. The genetic relationship within the collection was analyzed by AFLP. There may be induction of CAM in leaves due to stress. Despite these substantial results, several questions remain to be addressed. The confirmation of *E. tirucalli* photosynthetic pathways' plasticity at leaf level, that may play an important role to survive during drought stress, needs to be investigated in more detail. Thus, it will be interesting to analyze how enzymes influence metabolic adjustment to stress conditions in leaves and stem. To explore the use of *E. tirucalli*, determination of rubber composition in different genotypes, and quality and technical optimization of fermentation processes for the production of biogas need to be performed. The characterized genotypes from our greenhouse should be used in field experiments in tropical regions to verify and extend the data obtained in greenhouse conditions.

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CHAPTER 4:

CHARACTERIZATION OF LABLAB PURPUREUS REGARDING DROUGHT TOLERANCE, TRYPSIN INHIBITOR ACTIVITY AND CYANOGENIC POTENTIAL FOR SELECTION IN BREEDING PROGRAMMES

Guretzki S, Papenbrock J (2014) Characterization of *Lablab purpureus* regarding drought tolerance, trypsin inhibitor activity and cyanogenic potential for selection in breeding programmes. *Journal of Agronomy and Crop Science* 200, 24-35 (DOI:10.1111/jac.12043).

ABSTRACT

Climate change is responsible for the decrease in rainfall in many regions. One consequence is a reduction in arable land for traditional crops. Therefore, we are looking for drought-tolerant crops from many regions to replace sensitive crops currently in use. *Lablab purpureus* (L.) Sweet, which is grown mainly in Africa and South Asia, is considered to be drought tolerant. The species *L. purpureus* is a member of the Fabaceae family and has multiple functions, for example as food or forage. In this study, *L. purpureus* genotypes were compared to find the best genotypes adapted to the new climate conditions and with the highest benefits as food and fodder. The drought tolerance of four *L. purpureus* genotypes was examined. Classical growth parameters, infrared thermography and stomatal conductance were measured after induction of drought stress to monitor the impact of drought on plant growth and development. Significant differences among the genotypes were found in the tested growth stage. The ranking of the most drought-tolerant genotype was method dependent. To find potentially usable genotypes, the trypsin inhibitor activity was determined and an analysis of the cyanogenic potential (HCNp) was performed. Both trypsin inhibitor activity and HCNp showed significant differences among the genotypes without showing a correlation to each other. In summary, we recommend as selection criteria of the best genotypes for future breeding programmes: (i) a combination of at least two, better three, independent methods for the determination of drought effects on *L. purpureus* and (ii) the chemical analysis of compounds which are important for the nutritional value.

Keywords:

Drought stress, drought tolerance, HCNp, infrared thermography, *Lablab purpureus* (L.) Sweet, trypsin inhibitor activity.

INTRODUCTION

Global warming since the 1970s gives rise to an increase in land areas affected by drought. Africa, South and East Asia, but also Southern Europe are areas influenced by these more arid climate conditions (Dai 2011). For crop production, drought is a major problem (Boyer 1982). Common crops have to deal with these new environmental conditions to continue to serve as a basis of life for the population in these regions. Today, it is important to find new crop plants or genotypes of crops that are particularly adapted to more arid conditions. Additionally, drought tolerant crops should offer a wide range of applications for livelihood of farmers and for commercial use. A supposedly drought-tolerant plant is *Lablab purpureus* (L.) Sweet (synonyms: *Dolichos purpureus, Dolichos lablab* (NCBI taxonomy)) in the Fabaceae family. The herbaceous plant is perennial and occurs as bushy, semi-erect and prostate growth habit type with white, pink, red or purple flowers. *L. purpureus* is a twining plant with alternate and trifoliate leaves. Pods and seeds vary considerably in colour and size. Flowers, leaves, pods, root tubers and seeds are used as food; for example, younger, fully expanded leaves can be prepared like spinach and root tubers can be baked after cooking. The plant is also a forage crop (www.lablablab.org (accessed 22 April 2013), University of Agricultural Sciences, Bangalore).

The result of a genetic analysis of different *L. purpureus* genotypes by amplified fragment length polymorphism (AFLP) concludes that landraces from Asia or Africa have a lesser genetic diversity than African wild forms. Additionally, it is assumed that the dissemination occurred from Africa to Asia (Maass et al. 2005). Today, this plant is grown mainly in South Asia, South East Asia and Africa. *Lablab purpureus* is used in mixed cropping schemes or as part of home gardens. Studies showed that the species is adapted to drought, but there are differences in terms of drought tolerance within the species as summarized in Maass et al. (2010). *Lablab purpureus* contains several secondary metabolites that can be used as nutraceuticals or pharmaceuticals, such as lauric acid and brassinolide, and can assist against malnutrition (Morris 2009). Trypsin inhibitors inhibit the activity of trypsin; thus, they have an impact on the digestive process and can affect growth and development of humans and farm animals (Ryan 1990). Therefore, they belong to the antinutritional factors. Correct pre-treatment of food and fodder can significantly reduce the trypsin inhibitor activity. Different cooking methods like boiling in water and roasting ensure a reduction in the activity from *L. purpureus* seeds (Devaraj and Manjunath 1995, Osman 2007). For the human diet, the

trypsin inhibitor activity is not relevant. When feed is concerned, the treatment can be uneconomic (Grosjean et al. 1989), and therefore, studying different genotypes in terms of trypsin inhibitor activity is important for plant breeders and animal feed manufacturers (Bacon et al. 1995). The same applies for the cyanogenic potential (HCNp), which is referred as the sum of cyanide-containing compounds in an examined tissue (Ballhorn et al. 2005). Over 2500 plants possess cyanogenic glycosides (Vetter 2000). Cyanide is stored in form of cyanogenic glycosides and is released as hydrogen cyanide (HCN) due to degradation (Møller 2010). The consumption of cyanide-containing plants can cause poisoning or even death of animals and humans (Vetter 2000). Within *L. purpureus* genotypes, the HCN content can vary in seeds (Soetan 2012). HCN is considerably reduced in *L. purpureus*, for example, by soaking in water because the released HCN is soluble in water (Vijayakumari et al. 1995).

The objective of this study is the identification of the most remarkable genotypes in relation to high drought tolerance. Results can be combined with the results of genetic analyses, so that the most promising genotypes can be studied in field experiments within future breeding programmes. The analysis of the trypsin inhibitor activity and HCNp should serve as additional criterions for the selection of appropriate *L. purpureus* genotypes.

MATERIAL AND METHODS

PLANT MATERIAL

Lablab purpureus seeds were originally obtained from Dr. B.L. Maass, Georg-August-University Göttingen, Germany (CPI 24973, CPI 60216, cv. Rongai, CPI 29398, cv. Highworth, CPI 52508, CPI 34779, CPI 52535, CPI 36903, CPI 69498, CPI 76996, CPI 34777), from Dr. M. Byre Gowda, University of Agricultural Sciences, Bangalore, India (MAC-7 (GL 414), HA-3, HA-4) and collected with permission from a research field of the Tamil Nadu Agricultural University, India (TNAU-2). Table 4 lists additional information of the genotypes used. For the analysis of the chemical composition of the seeds, plants were grown fully watered in greenhouse conditions or in the field (MAC-7 (GL 414), HA-3, HA-4). For comparison, the chemical composition of commercially available seeds of *Pisum sativum* L. cv. Kleine Rheinländerin was analysed.

Differences in the response of four *L. purpureus* genotypes (TNAU-2, HA-3, CPI 34777, CPI 34779) in relation to mild drought stress have been investigated to identify genotypes with high drought stress tolerance. Four genotypes from India were selected to examine the potential of the genotypes within a country in relation to drought tolerance.

GROWTH CONDITIONS AND DROUGHT TREATMENT

The *L. purpureus* seeds were soaked in water overnight at room temperature (RT) for the purpose of germination. On the next day, the seeds were transferred on type CL T soil (Einheitserde, Sinntal-Altengronau, Germany), a soil with 30% clay content, in pots of 12 cm diameter. Plants were grown in the greenhouse for 5 weeks in a 12-h light/dark rhythm and at a temperature of 22 °C/22 °C under well-watered conditions. When the outdoor light conditions did not ensure sufficient light intensity inside the greenhouse, additional light was provided to obtain a constant quantum fluence rate of approximately. 350 µmol m^{-2} s^{-1} (sodium vapour lamps, SON–T Agro 400, Philips, Amsterdam, Netherlands).

In week six, the drought experiment was started. The plants were watered for the last time on day one before they were set to the experimental irrigation conditions till the end of the experiment, 8 or 9 days later. Experimental groups differed in soil volumetric water content (VWC); growth conditions of the control group (35% VWC) and the drought group (20% VWC) were chosen to reach a moisture content level near field capacity and near the permanent wilting point of the soil, respectively. This experimental design was well-founded on several pre-tests using different VWC. For the measurement of VWC time domain, reflectometry (TDR) (Fieldscout, Spectrum Technologies, Plainfield, USA) was used. Water containing 0.25% Wuxal Top N fertilizer (Aglukon, Düsseldorf, Germany) was added daily, based on water deficit calculation (D) of the Fieldscout, 1 mm = approximately 8.0 ml for a pot with 750 cm^3 volume according to the formula of truncated cones. Five plants per treatment were grown for each genotype in a distance of approximately 35 cm between the pots. The plants of one genotype grew in a random distribution side by side. The relative humidity between the plants was 40-50% measured by the porometer AP4 (Delta-T Devices, Burwell, UK).

GROWTH PARAMETERS

The growth parameters plant height, biomass and plant water content (PWC) were examined 9 days after starting the experiment. Thereby, the length of the longest shoot was measured (n = 4–5). Above-ground plant material was cut off to obtain biomass (n = 5) and PWC (n = 5) of the experimental plants, which was then dried in an incubator at 110 °C for 48 h. Plant material was weighed on a precision balance before drying for fresh weight (FW) and after drying for dry weight (DW). For biomass data, DW was used. PWC was calculated using the formula PWC = (FW–DW)/FW * 100.

Table 4: General information about the *Lablab purpureus* genotypes used in this study. This information includes subspecies, origin of the genotype, form, morpho-agronomic type (MA type) and genetic classification (AFLP group), and results of trypsin inhibitor (TI) activity (Fig. 24) and HCNp (Fig. 25) investigation (Pengelly and Maass 2001, Hall and Naidu 2004, Maass et al. 2005, Maass 2006, Maass and Usongo 2007, Venkatesha et al. 2007, D'souza and Devaraj 2011)

Genotype	Subspecies	Origin	Form	MA-type	AFLP-group	Rank TI activity analysis	Rank HCNp analysis
Pisum sativum	/	Germany	/	/	/	1	6
CPI 24973	*uncinatus*	Zimbabwe[2]	Wild	/	w4	2	16
CPI 60216	*purpureus*	Uganda[2]	Wild	RB-10	w4	3	15
TNAU-2	/	India	/	/	/	4	1
MAC-7 (GL414)[1]	/	India	/	/	/	5	4
cv. Rongai	*purpureus*	Kenya[2]	Cultivated	RB-2	A	6	3
HA-4	*purpureus*	India	Cultivated	/	/	7	2
CPI 29398	*purpureus*	ex Indonesia[2]	Cultivated	RB-2	A	8	8
cv. Highworth	*purpureus*	ex India[3]	Cultivated	RB-2	/	9	10
CPI 52508	*purpureus*	Mozambique[2]	Semi-domesticated	RB-8	A	10	13
CPI 34779	*bengalensis*	ex India[2]	Cultivated	RB-7	B	11	/
CPI 52535	*purpureus*	ex India[2]	Cultivated	RB-3	B	12	9
HA-3	*purpureus*	India[4]	Cultivated	/	/	13	7
CPI 36903	*purpureus*	ex USSR[2]	Semi-domesticated	RB-8	B	14	14
CPI 69498	*uncinatus*	Zimbabwe[2]	Wild	RB-9	w2	15	5
CPI 76996	*purpureus*	Zambia[2]	Cultivated	RB-6	A	16	12
CPI 34777	*purpureus*	ex India[2]	Cultivated	RB-3	B	17	11

[1]Personal correspondence: Dr. M. Byre Gowda, University of Agricultural Sciences, Bangalore, India, [2]Maass et al. (2005), [3]Maass (2006), [4]Venkatesha et al. (2007).

QUANTIFICATION OF DROUGHT STRESS EFFECTS BY POROMETRY AND THERMAL IMAGING

Drought stress effects were examined by porometry (n = 5) and thermal imaging measurements (n = 4–5) 8 days after starting the experiment. This was carried out using attached leaves as non-destructive methods. Stomatal conductance (mmol m^{-2} s^{-1}) of leaves was determined in the morning by the use of the porometer AP4 (Delta-T Devices, Burwell, UK). The measurements were performed on either of the two youngest fully expanded leaves; only the higher value was used for further calculations.

Thermal imaging investigation was carried out with the camera T360 (FLIR Systems, Wilsonville, USA) according to Grant et al. (2006). The youngest fully expanded leaves were analysed. For optimal signal-to-noise rations, the camera was turned on at least 30 min before the first thermographic picture was taken. While one of the paired leaflets was used to measure T_{dry}, the other one was used for measuring the T_{wet} reference. Dry reference leaflets were covered with petroleum jelly, and wet reference leaflets were wetted with water on both sides. The terminal leaflet of the same leaf was used as sample (T_{leaf}). T_{dry} was measured not earlier than five min after the application of petroleum jelly, T_{wet} immediately after using the water. In addition to T_{leaf}, a stomatal conductance measurement was performed on the terminal leaflet. FLIR QuickReport 1.2 SP2 (FLIR Systems, Wilsonville, USA) was used for analyzing the taken pictures. The object parameters were set for each image to emissivity 0.95, reflected apparent temperature 23 °C, atmospheric temperature 23 °C, relative humidity 45%/50% and distance 0.2 m. Based on the results, the index I_G, which is proportional to stomatal conductance, was calculated using the formula $I_G = (T_{dry} - T_{leaf})/(T_{leaf} - T_{wet})$. Finally, the crop water stress

DETERMINATION OF DROUGHT STRESS BY CHLOROPHYLL FLUORESCENCE MEASUREMENTS

Drought stress impact on photosynthesis was analysed by chlorophyll fluorescence measurements using an Imaging PAM M series device and ImagingWin v2.32 software (Heinz Walz, Effeltrich, Germany). Measurements (n = 3) were performed 9 days after the onset of drought treatments on a young fully expanded leaf in the morning shortly after the leaves were cut off. Light curves using different photosynthetically active radiations (PAR) as recommended in the manufacturer's handbook were examined. The effective PAR values were about 15% lower due to the utilization of the filter plate IMAG-MAX/F. Before the measurement, the plants were dark adapted for 20 min. The parameters F_v/F_m (maximal PS II quantum yield), Y(II) (effective PS II quantum yield), Y(NPQ) (quantum yield of regulated energy dissipation), Y(NO) (quantum yield of non-regulated energy dissipation), NPQ/4 (nonphotochemical quenching/4) and ETR (electron transport rate) were analysed (for detailed information, see Baker 2008, Sperdouli and Moustakas 2012). F_v/F_m values were obtained from the false-colour images created by Imaging-Win software. ETR values were determined using a mean value of PAR 396-801 µmol quanta m^{-2} s^{-1}. The other parameters were analysed based on the PAR 396 (approximately growth light intensity) and 801 (approximately twice the growth light intensity) results.

DETERMINATION OF THE TRYPSIN INHIBITOR ACTIVITY AND CYANOGENIC POTENTIAL

The trypsin inhibitor activity was analysed by a microassay according to Bacon et al. (1995) with several modifications. The trypsin solution was prepared with 84.78 mg (4.6 U mg^{-1}) of trypsin 1 : 250 from bovine pancreas (Serva Electrophoresis, Heidelberg, Germany) in 500 ml 1 mM HCl. The substrate solution was prepared according to Kakade et al. (1974). One hundred mg N-α-benzoyl-DL-arginine 4-nitroanilide hydrochloride (Sigma-Aldrich, St. Louis, USA) was dissolved in 5 ml dimethyl sulfoxide and incubated for 30 min at RT. Aliquots of 1 ml were stored at -20 °C. One aliquot was mixed with 50 ml 50 mM Tris–HCl, pH 8.2, and 20 mM $CaCl_2$ 2 x H_2O, preheated to 37 °C and freshly prepared daily. For extraction of the trypsin inhibitor, 10 mg plant material was mixed with 10 ml of 9 mM HCl and shaken for 60 min at 30 rpm and RT on an orbital shaker (Polymax 2040, Heidolph Instruments, Schwabach, Germany). The extract was centrifuged for 20 min (10.140 g) at RT and afterwards diluted with dest. H_2O as required. During the assay, a water bath was used heated to 37 °C for the incubation steps. After each addition of reagents, the 96-well plate was fixed and shaken for 10 s with 500 rpm on a thermo shaker (Thermomixer compact, Eppendorf, Hamburg, Germany). Per 96-well microtiter plate, four samples were loaded in three replicates. The samples were analysed photometrically in a reader (Synergy Mx, BioTek Germany, Bad Friedrichshall, Germany) at 410 nm. For the determination of the cyanogenic potential (HCNp), the method of Ballhorn et al. (2005) was used exactly as described. Three replicates per genotype were performed for trypsin inhibitor activity and cyanogenic potential (HCNp) measurements.

STATISTICAL ANALYSIS AND CALCULATION ON THE IMPACT OF STRESS

For statistical analyses, R 2.15.2 (www.r-project.org (01/09/2013)) in combination with R Studio v0.97.248 (RStudio, Boston, USA) was used. Box plots were chosen because this graphical display contains much information on data's symmetry, and also outlier and data sets can be easily compared. Box plots were drawn with ggplot2 version 0.9.3 (Wickham 2009). Significant differences ($P < 0.05$) were determined by ANOVA plus Tukey's multiple comparisons of means (trypsin inhibitor activity and HCNp test) and Welch's *t*-test (drought stress investigation) analysis. Drought stress susceptibility of each genotype was calculated using the median of control and treated groups. The larger the difference in%, the stronger the impact of drought stress. A low total score represents a strong influence and a high score a small effect on the genotype by drought stress.

RESULTS

DETERMINATION OF GROWTH PARAMETERS

The classic growth parameters, gain in biomass DW (g), plant water content (%) and plant height (%), were analysed (Fig. 20). The gain in biomass DW for plants under drought stress is lower as compared with the gain of unstressed plants (Fig. 20a). The most affected genotype was TNAU-2 (cg: 6.1 g; dg: 4.1 g). For CPI 34779, a similar difference in biomass gain among stressed and unstressed plants was measured. The smallest difference was found in genotype CPI 34777 (cg: 5.3 g; dg: 4.3 g). Overall, biomass DW showed no significant differences ($P < 0.05$) between both treatments. There was a reduction in plant water content under drought stress conditions (Fig. 20b). Significant differences ($P < 0.05$) occurred in genotype HA-3 (cg: 79.8%; dg: 77.6%) and TNAU-2 (cg: 79.5%; dg: 78.3%) between stressed and unstressed plants. Genotypes CPI 34777 and CPI 34779 showed smaller but insignificant differences. There was also a considerable decrease in plant height of drought stressed plants (Fig. 20c). For genotype CPI 34777, a significant difference ($P < 0.05$) was between treatments. However, CPI 34779 (cg: 6.6%; dg: 1.9%) and TNAU-2 (cg: 12.6%; dg: 5.5%) showed larger, but not significant differences among control and drought treatments. The least affected genotype was HA-3 (cg: 21.8%; dg: 14.6%).

MEASUREMENT OF LEAF CONDUCTANCE

Stomatal conductance was measured in the morning by porometry (Fig. 21). In general, drought stress leads to a reduction in conductance because a first response to drought stress is stomata closing. The strongest difference between control and drought group was found in TNAU-2 (cg: 183 mmol m^{-2} s^{-1}; dg: 49 mmol m^{-2} s^{-1}), but HA-3 was similarly affected by drought stress (cg: 197 mmol m^{-2} s^{-1}; dg: 57 mmol m^{-2} s^{-1}). CPI 34779 showed a considerably smaller difference between treatments (cg: 107 mmol m^{-2} s^{-1}; dg: 61 mmol m^{-2} s^{-1}). Significant differences ($P < 0.05$) between stressed and unstressed plants were found for TNAU-2, HA-3 and CPI 34777 indicating differences in the regulation of stomatal closure among genotypes.

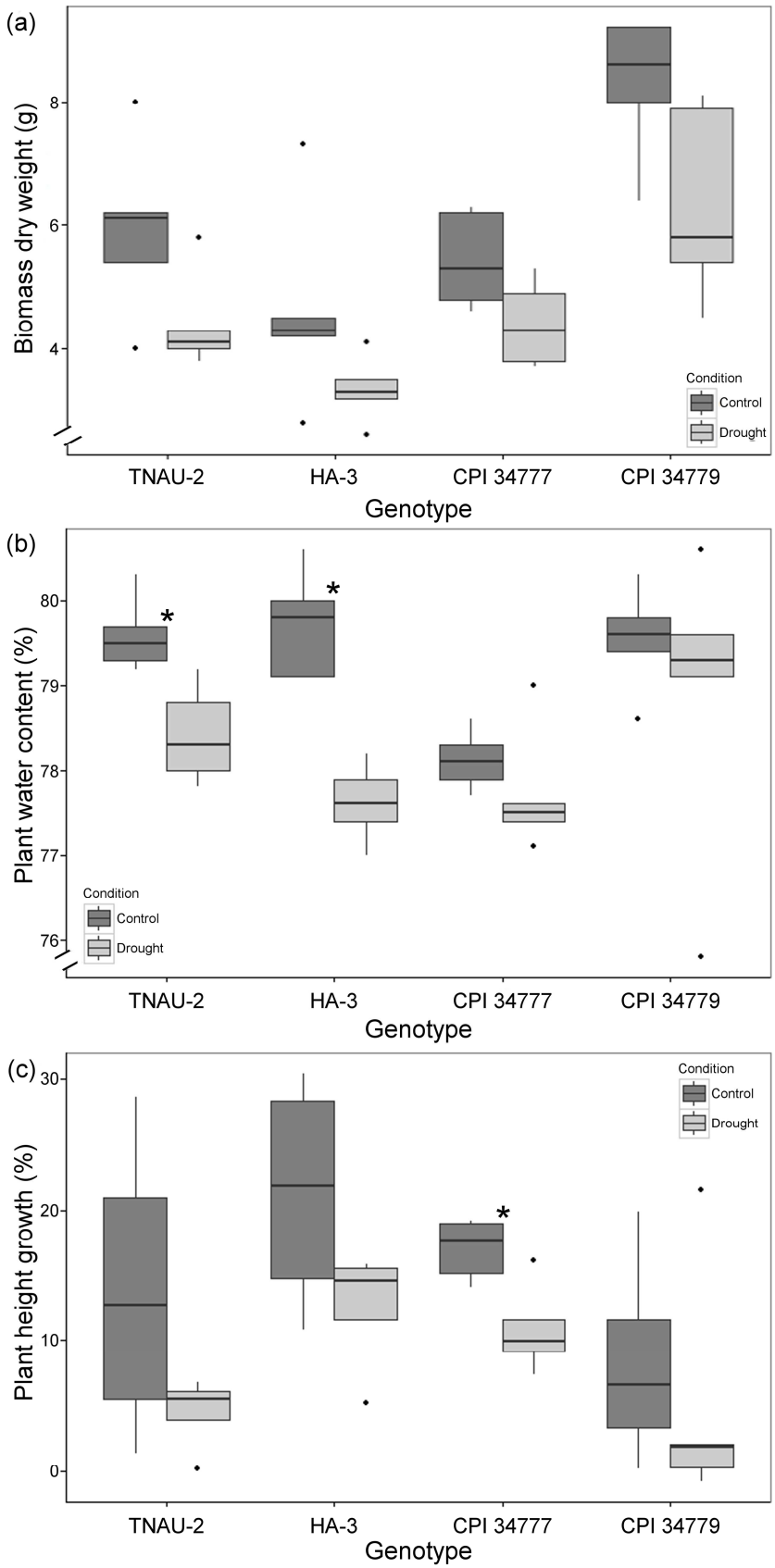

Fig. 20: Effects of water limitation after 9 days on (a) biomass dry weight (g) (n = 5), (b) plant water content (%) (n = 5) and (c) plant height (%) (n = 4–5); genotypes with *P < 0.05.

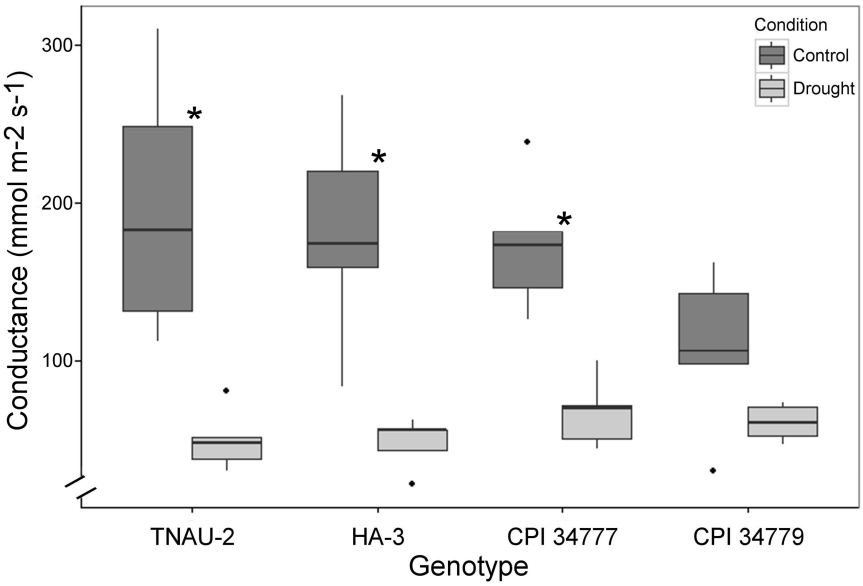

Fig. 21: Effects of water limitation after 8 days on stomatal conductance through porometry measurement (mmol m^{-2} s^{-1}); n = 5, genotypes with *P < 0.05.

LEAF SURFACE TEMPERATURE ANALYZED BY INFRARED THERMOGRAPHY

The effect of drought stress on the surface temperature of plant leaves was studied by infrared thermography (Fig. 22). Drought stress led to an increase in leaf temperature (Fig. 22a). The drought group of HA-3 showed the strongest increase in temperature as compared with the control group (cg: 24.8 °C; dg: 26.4 °C). The measured temperature increase in CPI 34777 and TNAU-2 was on an equal level. CPI 34779 was least affected by drought stress; the genotype showed only a small difference between control and drought group (cg: 25.4 °C; dg: 25.8 °C). CWSI and IG values dropped under drought stress, wherein the results of both indices were parallel (Fig. 22b & d). The biggest influence of drought stress was measured for TNAU-2 (CWSI – cg: 0.24; dg: -0.04), followed by genotypes HA-3 and CPI 34777. CPI 34779 was least affected under drought stress (CWSI – cg: 0.29; dg: 0.13).

The stomatal conductance measured simultaneously to verify the infrared thermography results by a second method confirmed the results obtained (Fig. 22c). Drought stress led to a large decrease in stomatal conductance for CPI 34777 (cg: 128 mmol m^{-2} s^{-1}; dg: 23 mmol m^{-2} s^{-1}) and HA-3 (cg: 131 mmol m^{-2} s^{-1}; dg: 24 mmol m^{-2} s^{-1}). CPI 34779 was again the least affected genotype by drought stress. Infrared thermography results showed significant differences (P < 0.05) in all leaf temperature factors between stressed and unstressed plants of genotypes HA-3 and CPI 34777.

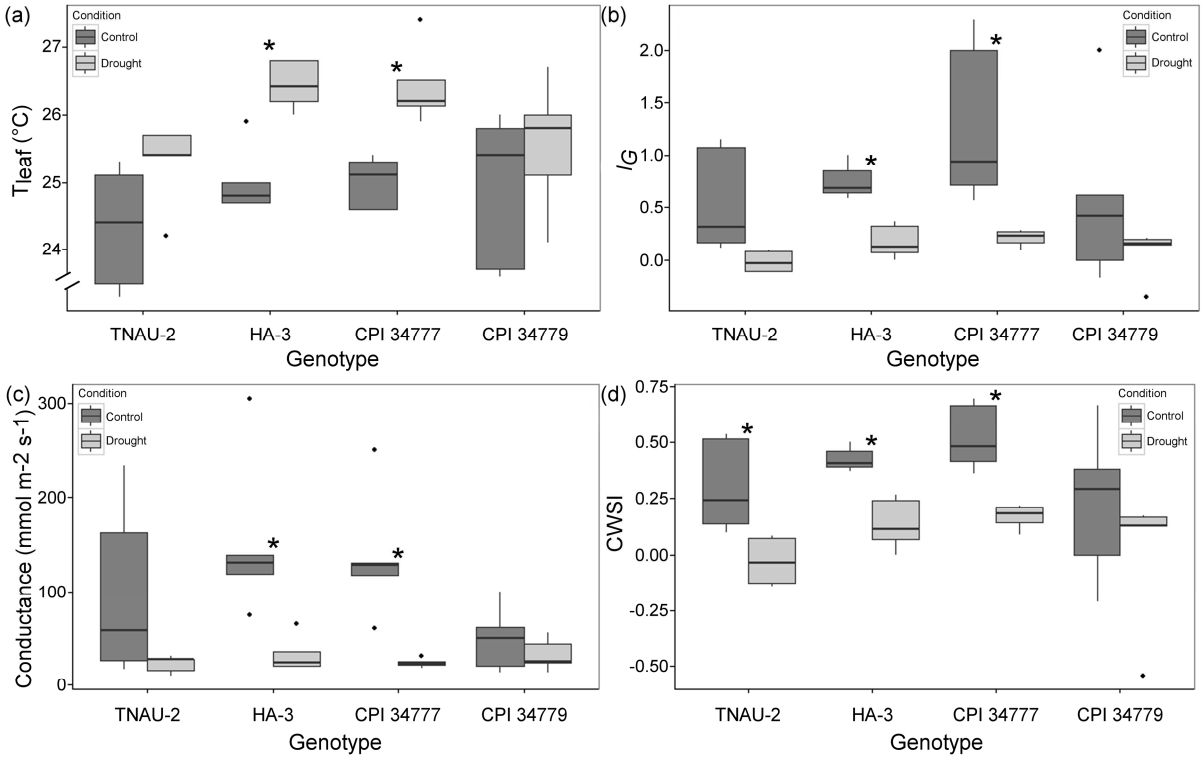

Fig. 22: Effects of water limitation after 8 days on (a) leaf temperature (°C), (b) index I_G, (c) stomatal conductance (mmol m^{-2} s^{-1}) and (d) crop water stress index (CWSI) based on infrared thermography analysis; n = 4–5, genotypes with *P < 0.05.

ANALYSIS OF PHOTOSYNTHETIC PARAMETERS BY CHLOROPHYLL FLUORESCENCE MEASUREMENTS

Chlorophyll fluorescence was examined by the factors ETR, Fv/Fm, NPQ/4, Y(II), Y(NPQ) and Y(NO) using light curves (Fig. 23) (see method part for abbreviation and details). In general, drought stress caused a decrease in the rate of electron transport (Fig. 23a). ETR decreased under drought stress especially in HA-3 (cg: 59; dg: 41), followed by genotype TNAU-2 (cg: 48; dg: 37), whereas an increase was found for genotype CPI 34779 (cg: 35; dg: 41). In none of the genotypes, Fv/Fm showed differences among control and drought stress treatments (Fig. 23b). Factor NPQ/4 increased under stress conditions (Fig. 23c). HA-3 was most negatively affected by drought stress (cg: 0.333; dg: 0.565). CPI 34779 even showed a decrease and was therefore the least affected genotype (cg: 0.333; dg: 0.302). Drought stress is indicated by a decrease in Y(II) and thereby a change in Y(NPQ) and Y(NO) (Fig. 23d). This pattern is reflected by HA-3 (Y(II) -28.1%; Y(NPQ) +28.9%; Y(NO) -20.6%). For CPI 34779, a contrary behaviour was measured (Y(II) +13.6%; Y(NPQ) -6.5%; Y(NO) ±0.0%). Significant differences (P < 0.05) in chlorophyll fluorescence measurements were detected only for HA-3 in case of NPQ/4, Y(NPQ) and Y(NO).

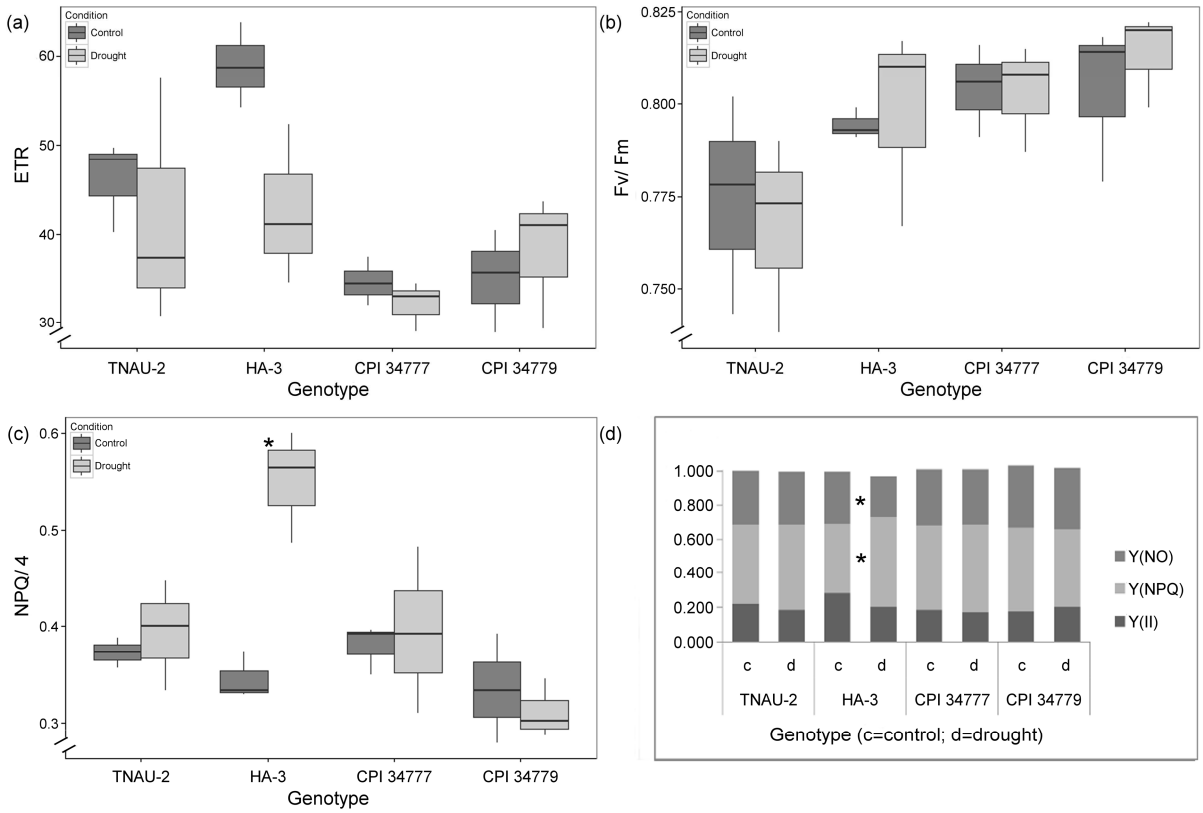

Fig. 23: Effects of water limitation after 9 days on (a) ETR, (b) Fv/Fm and (c) NPQ/4 (d) Y(II) Y(NO) Y(NPQ) through chlorophyll fluorescence measurement at 396 µmol quanta m^{-2} s^{-1}; n = 3, genotypes with *P < 0.05.

SUMMARY OF THE RESULTS OBTAINED BY DETERMINATION OF DIFFERENT STRESS PARAMETERS

Table 5 summarizes the results of the used stress detecting methods (growth parameters, leaf conductance, infrared thermography and chlorophyll fluorescence). We are aware the ranking does not reflect that the traits are not of equal weight. It is an attempt to synthesize the data obtained by different approaches in a simple matrix. Genotypes TNAU-2 (5) and HA-3 (8) were stronger negatively affected under drought conditions. In comparison to these genotypes, CPI 34777 (13) and CPI 34779 (14) were less affected under 20% VWC soil conditions considering all results. Concerning methods that show significant differences in our experiment, the focus should lie on stomatal conductance, infrared thermography (T_{leaf}) as well as plant water content. The combined overall result of these methods revealed that CPI 34779 and CPI 34777 are less influenced by drought stress than TNAU-2 and HA-3.

Table 5: Summary of measured plant responses. Each *Lablab purpureus* genotype is assigned to an arbitrary value of one to four. Four corresponds to a low negative influence and one represents a strong negative influence of drought on plant growth and development

Genotype	Growth parameters	Leaf conductance	Infrared thermography	Chlorophyll fluorescence	Sum
TNAU-2	1	1	2	1	5
HA-3	3	2	1	2	8
CPI 34777	4	3	3	3	13
CPI 34779	2	4	4	4	14

TRYPSIN INHIBITOR ACTIVITY

The level of the trypsin inhibitor activity was examined in sixteen genotypes of *L. purpureus*. *Pisum sativum* was included for comparison with the *L. purpureus* genotypes. Results show a wide range in the trypsin inhibitor concentrations with strong and significant differences ($P < 0.05$) (Fig. 24). There are significant differences in means of trypsin inhibitor concentrations among *L. purpureus* genotypes and between *L. purpureus* genotypes and *P. sativum*.

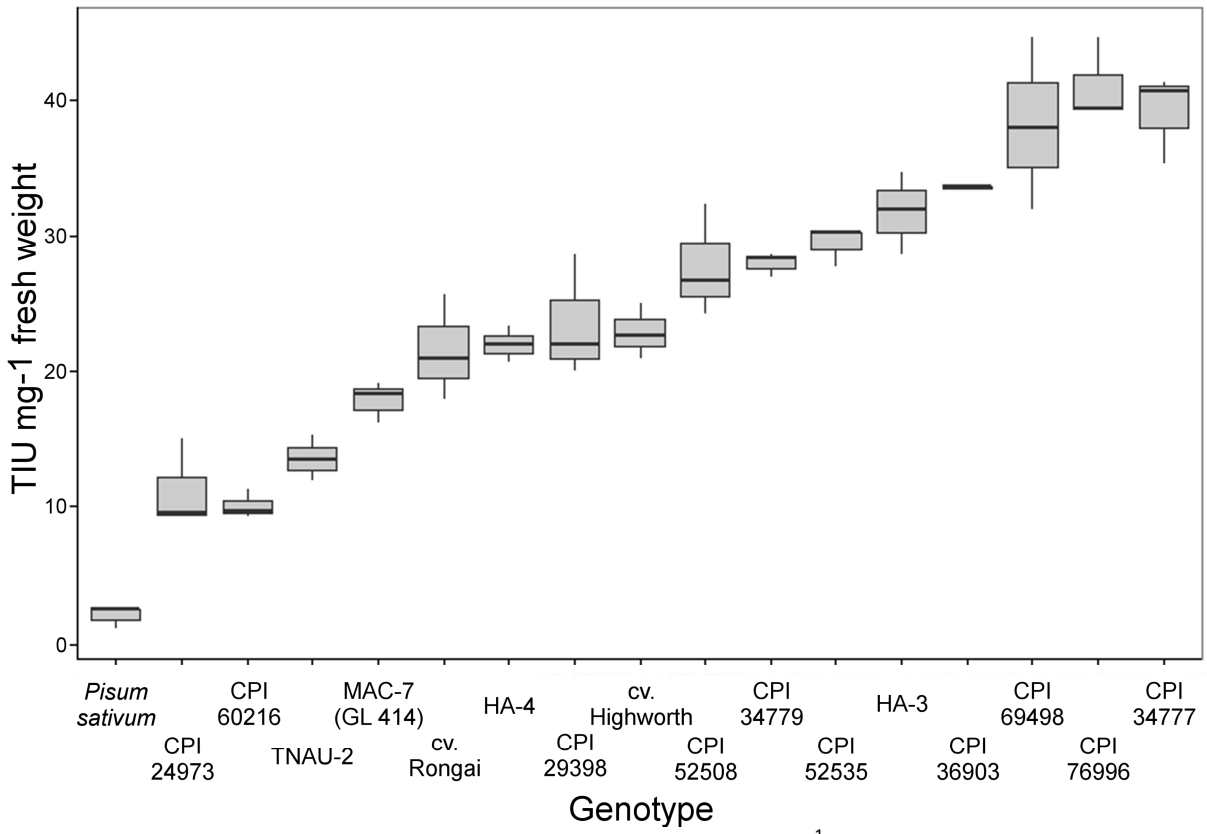

Fig. 24: Box plot (n = 3) of trypsin inhibitor contents (TIU mg^{-1} fresh material) of different *L. purpureus* genotypes and *P. sativum* ($P < 0.05$).

The average median of all *L. purpureus* genotypes was 24.7 trypsin inhibitor units (TIU) mg^{-1} fresh material. The lowest amount was found in CPI 24973 (9.5 TIU mg^{-1} fresh material), whereas an approximately four times higher amount was measured in CPI 34777 (41.0 TIU mg^{-1} fresh material), the genotype with the highest trypsin inhibitor amount. TNAU-2 is another genotype with low trypsin inhibitor concentration (13.5 TIU mg^{-1} fresh material). Both, CPI 34779 (28.0 TIU mg^{-1} fresh material) and HA-3 (32.0 TIU mg^{-1} fresh material) showed trypsin inhibitor concentrations above average median. For *P. sativum*, the least trypsin inhibitor content was measured (2.1 TIU mg^{-1}fresh material), <20% in comparison with the *L. purpureus* genotype CPI 24973 with the lowest amount.

CYANOGENIC POTENTIAL (HCNP)

There are significant differences in means among *L. purpureus* genotypes and between *L purpureus* genotypes and *P. sativum*, respectively (Fig. 25). The lowest HCNp was shown for genotype TNAU-2 (0.9 µmol g^{-1} FW); CPI 24973 showed the highest HCNp (14.9 µmol g^{-1} FW). For *P. sativum*, a cyanogenic potential of 2.4 µmol g^{-1} FW was determined, a value below *L. purpureus* average median, which stands at 3.2 lmol g^{-1} FW. HA-3 (2.4 µmol g^{-1} FW) showed an HCNp below and CPI 34777 (4.7 µmol g^{-1} FW) above average median.

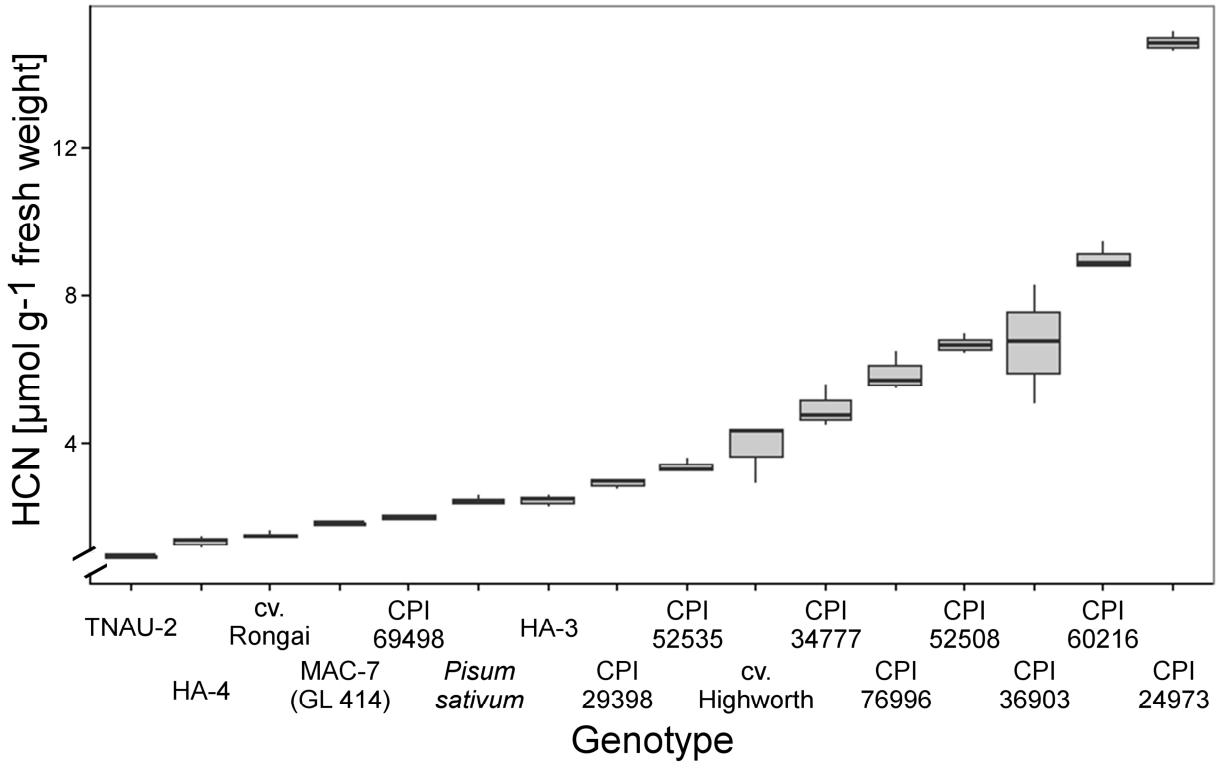

Fig. 25: Box plot (n = 3) of the cyanogenic potential (HCNp) given as HCN contents in µmol g^{-1} fresh weight of different *L. purpureus* genotypes and *P. sativum* ($P < 0.05$).

DISCUSSION

DIFFERENCES IN DROUGHT TOLERANCE MEASUREMENTS WITHIN *L. PURPUREUS*

In this study, the four tested genotypes TNAU-2, HA-3, CPI 34777 and CPI 34779 showed a reduction in the classical growth parameters dry mass, plant water content and plant growth when subjected to drought stress during the early vegetative stage. Many values of the control plants showed a higher 1.5 interquartile range than the treated plants indicating a high variability of conditions in the greenhouse with local maxima and minima. And evenmmore remarkable, the variability of the stress responses induced by the plants was very narrow, indicating various kinds of bottlenecks in their physiological reactions to stress conditions. Significant differences ($P < 0.05$) were found for TNAU-2, HA-3 and CPI 34777 indicating appropriate adaptive reactions to the changed environmental conditions.

We would like give an overview about the few results concerning drought stress experiments with *L. purpureus* obtained so far; however, due to differences in the experimental set up in most cases, direct comparisons remain difficult. The effects of drought stress have been measured for *L. purpureus* genotype HA-4 by examining the parameters of the leaf, shoot and root length, as well as dry and fresh weight for whole plants with comparable results to our study (D'souza and Devaraj 2011). Drought stress has been initiated 10 days after germination by water withheld resulting in a reduction in all measured variables. The reason for the slower growth is a decreased cell elongation, due to reduced turgor pressure, as well as minor cell volume and cell growth in comparison with well-watered plants according to Farooq et al. (2009). The effects on the antioxidant system under drought stress were analysed in the same study in leaves and roots of *L. purpureus*. For leaves, it was concluded that an increase in, for example, guaiacol peroxidase, ascorbate peroxidase and glutathione, helps under drought conditions to ensure that *L. purpureus* is tolerant against drought (D'souza and Devaraj 2011). In another study, the classical growth parameter relative water content (RWC) was measured comparing 15 genotypes including cv. Rongai, cv. Highworth, CPI 24973, CPI 34777 and CPI 60216 with regard to drought tolerance and recovery therefrom (Hall and Naidu 2004). The plants used in that study were 4 weeks old before the water was withheld for 12 days. The RWC varied thereafter between 64.1% and 77.3% in leaves. The leaf survival rate in% was also measured about 12 weeks after water stop and used as an indication of drought tolerance. Eight of the 15 genotypes survived the experiment, including cv. Highworth with the second highest leaf survival rate

of 51.3%. Fourteen weeks after water withheld drought tolerance was also analysed by recovery studies. The result did not differ from the leaf survival rate analysis. It was concluded that methods such as RWC require high experimental effort and are therefore only of limited use for predicting drought tolerance for larger genotype collections (Hall and Naidu 2004). The same authors found an osmoprotectant, which probably contributes to the protoplasmic dehydration tolerance of the more drought-tolerant genotypes. The nature and composition of this osmoprotectant has to be investigated in more detail before it can be included into drought tolerance breeding programmes. More *L. purpureus* genotypes need to be included into a quantitative drought tolerance screening programme under standardized conditions to use the broad genetic variety for further breeding.

By both stomatal conductance and infrared thermography measurements, the stomatal behaviour was investigated. Plants close their stomata during water shortage periods to protect themselves against water losses. One has to keep in mind that leaf temperature is rising due to thermal effects related to illumination. Under physiological conditions, leaf temperature is controlled by transpirationmediated cooling effects. Both methods are suitable for drought tolerance screening of genotypes (Jones 2007) but measure slightly different effects of drought. The advantage of infrared thermography in contrast to stomatal conductance is the possibility to examine entire plants at the same time. Therefore, the results of infrared thermography are less prone to local variations within plants. In our study, significant differences ($P < 0.05$) were found especially between genotypes HA-3 and CPI 34777. Of particular interest is the difference in T_{leaf} among stressed and unstressed plants because T_{leaf} is probably sufficient to make a statement about the drought tolerance of individual genotypes (Grant et al. 2006). Differences among stressed and unstressed plants for T_{leaf} were also found in other bean species, for example common beans (*Phaseolus vulgaris* L.) (Grant et al. 2006).

With respect to the analysis of chlorophyll fluorescence, only in the case of HA-3, significant differences were found between stressed and unstressed plants under mild drought stress. For example, only little differences were determined in the F_v/F_m measurements. A drought stress study with *Arabidopsis thaliana* (L.) Heynh. as model plant showed that changes in chlorophyll fluorescence measurements (F_v/F_m, Y(II), NPQ) arise more than 10 days after water withheld (Woo et al. 2008). The factor F_v/F_m was investigated in connection with *P. vulgaris* in a drought stress experiment (Miyashita et al. 2005). First differences between

control and stressed group could be observed after 1 week without irrigation. Therefore, chlorophyll measurements for *L. purpureus* were probably not sensitive enough to measure the impact of mild drought in an early stage of stress symptom development. Chlorophyll fluorescence measurements are more suitable under severe stress (Sperdouli and Moustakas 2012). Generally, it was possible to find differences in the drought tolerance of the tested genotypes in about 7–8 weeks old plants. However, advantages and disadvantages of the methods have to be considered in terms of suitability for the determination of drought tolerance. The use of the classical methods is recommended here as well as newer techniques, such as infrared thermography. Chlorophyll fluorescence measurements are probably more suitable for the study of severe drought stress. For the selection of the most drought-tolerant *L. purpureus* genotypes in field conditions, the reliability and suitability of the methods should be tested with the most promising genotypes from this (CPI 34779) and other studies (cv. Highworth) also at different developmental stages to test the intermittent and terminal drought stress.

To obtain a full impression of the drought tolerance of a genotype, further factors should be included in an analysis. The large number of genes that can be involved in the mechanisms of abiotic stress tolerance, depending on environmental conditions, should be examined. These gene products can be divided into three groups: signal cascades and transcriptional control, direct protection of membranes and proteins, and involvement in water and ion uptake and transport, based on their biological function as summarized in Wang et al. (2003). The ability for detoxification of reactive oxygen species is another factor to be included into the determination of drought-tolerant genotypes. The detoxification ability is controlled by the activity of substances such as components of the ascorbate–glutathione cycle, catalases and superoxide dismutases (Chaves et al. 2003).

ANTI-NUTRITIONAL FACTORS AS SELECTION CRITERIA IN BREEDING PROGRAMS

Lablab purpureus exhibited a high bandwidth of trypsin inhibitor activity among the examined genotypes. The trypsin inhibitor activity in the tested *P. sativum* was the lowest. Compared with a study with four pea genotypes by Bacon et al. (1995), the activities of the cultivars used in this study were in the lower range. Values varied from 2.5 to 15.3 (TIU mg^{-1} dry matter). Ten *L. purpureus* genotypes were tested by Devaraj and Manjunath (1995). They showed a trypsin inhibitor activity ranging from 11.8 to 27.7 TIU mg^{-1} (Original data: TIU g^{-1}) when using dried seeds with an average activity of 20.2 TIU mg^{-1}. The trypsin inhibitor

activity of this study with 16 *L. purpureus* genotypes is therefore comparable. Raw seeds of *L. purpureus* showed activities between 19.2 and 29.0 TIU mg^{-1} (Ramakrishna *et al*. 2006, Osman 2007). For food production, the trypsin inhibitor activity can be reduced strongly or completely by proper treatment (Devaraj and Manjunath 1995, Ramakrishna et al. 2006, Osman 2007). The trypsin inhibitor activity could be merely genetically regulated. An AFLP analysis examined 103 *L. purpureus* genotypes from around the world (Maass et al. 2005). The genotypes were classified based on the results in the major groups A and B, as well as, for example, w2 and w4. The AFLP groups A and B contain mainly cultivated genotypes of subspecies *purpureus* and *bengalensis*. The genotypes of group A have their origin more likely in Africa, group B genotypes rather in Asia. The AFLP groups w2 and w4 only contain wild African genotypes and genotypes of subspecies *uncinatus*. Eleven of sixteen *L. purpureus* genotypes (Table 4; Fig. 24) used in this study were included in this AFLP investigation. The two genotypes of group w4 (CPI 24973 and CPI 60216) had the lowest trypsin inhibitor activity with 9.5 and 9.7 TUI mg^{-1} fresh material, followed by the AFLP genotypes of group A (cv. Rongai, CPI 29398 and CPI 52508) with activities between 21.0 and 26.7 TUI mg^{-1} fresh material. CPI 34779, CPI 52535, CPI 36903 and CPI 34777 are part of the AFLP group B; the activities were between 28.3 and 40.7 TUI mg^{-1} fresh material. The genotype CPI 69498 is assigned to AFLP group w2, and the trypsin inhibitor activity was 38.0 TUI mg^{-1} fresh material. The only genotype that does not fit into this pattern is CPI 76996 (AFLP group A) with an activity of 39.3 TUI mg^{-1} fresh material; CPI 76996 would be assigned to AFLP group B. Therefore, the results suggest a correlation between genetic background and trypsin inhibitor activity. To confirm this connection, it is now important to examine more genotypes grown under different environmental conditions. Like trypsin inhibitor activity, the variety in HCNp is high within the tested genotypes. Soetan (2012) compared genotypes of *L. purpureus* and showed that cv. Rongai has a lower proportion of cyanogenic glycosides (mg kg^{-1}) than cv. Highworth. This agrees with the findings in this study regarding HCNp. In a study with raw seeds, the HCN concentration is 1.62 mg 100 g^{-1} seeds. When testing which treatment reduces the HCN concentration most, Vijayakumari et al. (1995) conclude that autoclaving has the strongest effect. However, a reduction of more than 50% of the HCN concentration occurred as well after nine hours soaking in distilled water or cooking for 90 min. Contrary to the trypsin inhibitor activity, HCNp is a multifactorial trait under the control of environmental factors, including pathogens. When comparing the

results with the AFLP analysis of Maass *et al*. (2005) (Table 4; Fig. 25), there is no apparent connection between the AFLP groups and HCNp concentrations. The two major AFLP groups A and B cannot be separated from each other on the basis of the HCNp concentrations among the genotypes. Genotype cv. Rongai is just like CPI 52508 part of group A although the HCNp concentrations varied between 1.4 and 6.7 µmol g^{-1} FW. CPI 52535 and CPI 36903 belong to AFLP group B. The concentration ranged between 3.2 and 6.8 µmol g^{-1} FW. It is noticeable that the two genotypes of group w4, CPI 24973 with 14.9 µmol g^{-1} FW and CPI 60216 with 8.9 µmol g^{-1} FW showed by far the highest HCNp values; both are African wild forms. CPI 69498, another wild form from Africa, is part of AFLP group w2 with a relatively low HCNp concentration of 2.0 µmol g^{-1} FW. This wide range in the HCNp results is also consistent with the summary of Vetter (2000) for plants. The amount of HCN varies within genotypes or among genotypes: The amount depends on the environmental conditions under which the plants grew or is attributable to physiological factors. The HCNp investigation should be continued with the genotypes grown under arid environmental conditions to see how this affects the plants.

CONCLUSION

For the determination of drought tolerance, a combination of different methods such as classical growth parameters and infrared thermal imaging is useful to examine the various aspects of the effects of drought. Thereby, the results of one method can confirm or complement the results of another method. Finally, it is possible to identify the most drought-tolerant genotypes in 7–8 weeks old *L. purpureus* plants. Nevertheless, it has to be additionally tested whether these genotypes have the highest drought tolerance during their whole plant life cycle or how they react on intermittent or terminal drought stress.

A major factor in future breeding programmes for arid places is drought tolerance of genotypes. In addition depending on purpose, other factors, for example the amount of antinutritional factors, can be helpful to identify adequate genotypes. The results of trypsin inhibitor activity and HCNp show a wide variety which could be taken into consideration as further selection traits. There could be a link between genetic origin and trypsin inhibitor activity, which must be further explored. Summarizing the results of drought stress experiments and trypsin inhibitor activity, CPI 34779 is the most promising genotype for a breeding programme. CPI 34779 showed the highest drought tolerance and the second lowest trypsin inhibitor activity of the four tested genotypes.

Because HCNp is influenced by the environment, these data should not be included in a ranking in terms of suitability for breeding programmes. In the end, it is important that drought-tolerant genotypes provide products for the specific needs of farmers, for example, in terms of yield, taste or storage suitability, so people will use these adequate genotypes as crop for arid environments.

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CHAPTER 5:

GENERAL DISCUSSION

DEVELOPMENT OF A SYSTEM FOR MEASURING THE IMPACT OF DROUGHT STRESS

The major thesis aim was to develop a combination of methods for the determination of especially drought tolerant genotypes under mild drought stress conditions. For this purpose *L. purpureus* was used as an example crop (Chapter 2). Various methods including destructive and non-destructive methods have been compiled for the investigation of drought tolerant genotypes. On the one hand the methods consist of more traditional growth parameters like biomass and leaf area determinations which can be used to examine the impact of drought stress on plants (Jones, 2007). The impact of drought stress leads to reduced growth as it was shown for other members of the Fabaceae. *V. faba* shows a reduction in leaf and stem dry weight, plant height as well as leaf area (Zabawi and Dennett, 2010) and *V. radiata* shows a reduction in plant height (Ranawake *et al*.,2011). The reasons for these reductions are a lower rate of cell division and elongation (Farooq *et al*., 2009). Newer methods like determination of stomatal conductance and infrared thermometry are also used to measure drought stress effects. According to Jones (2007) both methods are suitable for the screening of genotypes in regard to drought stress. Both methods allow measuring the behavior of the stomata under drought stress conditions. Investigating *P. vulgaris* Grant *et al*. (2006) shows that there exists a correlation between stomatal conductance and infrared thermography. Infrared thermography in contrast to conventional porometers allows the investigation of plant groups or whole fields at once (Jones *et al.*, 2009). The combination of the used methods allows the study of both long-term and short-term effects on the plants under drought stress conditions (Chaves *et al*., 2003).

It is important to assemble the methods for each species individually, because methods can be more or less applicable for a certain species. The results obtained with *L. purpureus* indicate that the consequences of drought stress on photosynthesis cannot be determined by the analysis of chlorophyll fluorescence measurements under mild drought stress. Generally, chlorophyll fluorescence seems to be useful under severe drought conditions, for example withheld of water for several days (Miyashita *et al*., 2005; Woo *et al*., 2008). However, also traditional methods can be problematic. *L. purpureus* is a twining plant and thereby the plant height measurement is impeded. In the case of *E. tirucalli* (Chapter 3), there are difficulties in the use of other methods due to the morphology of leaves and stems. Therefore, the application of a conventional porometer is not possible and the calculation of thermography results is more difficult. In general, the combination of various

methods such as biomass and infrared thermography is recommended in order to examine and quantify the diverse aspects of drought stress impacts even on different species. Subsequently, it is possible to find the best genotypes in regard to areas affected by low precipitation.

DETERMINATION OF MOST DROUGHT TOLERANT *L. PURPUREUS* AND *E. TIRUCALLI* GENOTYPES

Selections of four *L. purpureus* (TNAU-2, HA-3, CPI 34777 and CPI 34779) and two *E. tirucalli* (Morocco and Senegal) genotypes, both species are postulated as drought tolerant (Janssens *et al*., 2009; Maass *et al*., 2010), are examined to find most drought tolerant genotypes (Chapter 3, 4). *L. purpureus* as well as *E. tirucalli* show a decline in traditional growth parameters like plant growth, dry mass and plant water content. When using traditional measuring methods, a decrease has also been observed in other studies with amaranth, wheat and also with *L. purpureus* in e.g. plant height and biomass (Liu and Stützel, 2004; D'souza and Devarj, 2011; Zhang *et al*., 2011). A reason is a cessation of photosynthesis, metabolic dysfunction and damage of cellular structure under stress (Krasensky and Jonak, 2012). For *E. tirucalli* drought stress led to an increase in root dry mass in accordance with the theory of functional balance (Brouwer, 1963). The observed increase of root length enables *E. tirucalli* plants reach deeper soil layers for needed water (Schenk and Jackson, 2002).

Stomatal conductance and infrared thermography measurements show significant differences among control and drought stress groups in the tested *L. purpureus* genotypes. The thermography results for *E. tirucalli* indicate that genotype Morocco is more drought tolerant than genotype Senegal. Plants close their stomata to prevent water loss. Subsequently an increase in leaf temperature can be observed. Grant *et al.* (2006) shows these temperature differences among well-watered and drought stressed plants of *V. vinifera*, *P. vulgaris* and *L. albus* under greenhouse or controlled chamber conditions. Field experiments with *V. vinifera* and *Oryza sativa* L. are reported by Jones *et al*. (2009). The measurement of chlorophyll fluorescence leads to non-relevant results in case of *L. purpureus*. Perhaps, a reason is that the method is not suitable for early drought stress effects under mild drought stress. In studies with *A. thaliana* or *P. vulgaris*, relevant changes in chlorophyll measurements appear after one week or even more days without irrigation

(Miyashita *et al*., 2005; Woo *et al*., 2008). *E. tirucalli* showed a decline in quantum efficiency (F_v/F_m). The decrease is stronger in leaves than in stems. A damaged photosystem explains the decrease in photosynthetic efficiency (Maxwell and Johnson, 2000). In *E. tirucalli* stems the impact of drought stress is lower because of the CAM pathway (Borland *et al*., 2006; Herrera, 2008).

In summary it was possible to put in order the tested genotypes with regard to drought tolerance. The results for the four *L. purpureus* genotypes indicate a higher drought tolerance for CPI 34777 and CPI 34779 in comparison to TNAU-2 and HA-3. Experiments with *E. tirucalli* demonstrate that genotype Morocco is more drought tolerant than genotype Senegal.

EXAMINATION OF SECONDARY PLANT METABOLITES AND GENETIC RELATIONSHIP

Plants contain some secondary metabolites which ideally should not occur in plants. These include trypsin inhibitors and cyanogenic glycosides; both can be found in *L. purpureus* and can be harmful for the consumer (Ryan, 1990; Vetter, 2000). An aim of breeding programs would be the breeding of plant genotypes with low concentrations of harmful secondary plant metabolites. The results in this thesis indicate a high bandwidth in trypsin inhibitor activity and HCNp (Chapter 4). Based on the comparison with an AFLP analysis (Maass *et al*., 2005), it is possible that the trypsin inhibitor activity is of genetic origin. *L. purpureus* groups depending on trypsin inhibitor activity show a considerable overlap in comparison with the classification of this AFLP-analysis. The HCNp examination shows no such accordance and is thus not of genetic origin. Vetter (2000) described that HCN concentration depends on environmental conditions. The AFLP analysis of *E. tirucalli* divides the tested genotypes in an African and mostly non-African group (Chapter 3). The rubber content measured so far in *E. tirucalli* (Chapter 3) indicates no correlation with the AFLP result. This is supported by experiments from Uzabakiliho *et al*. (1987) and it is not known whether the differences in concentration have a genetic or environmental background.

FURTHER EXPERIMENTS TO IMPROVE THE SELECTION OF GENOTYPES FOR BREEDING PROGRAMS

In the end only the yield of favoured product provided by the plants in arid areas will matter to the farmer. Therefore it is necessary to test the most promising genotypes of *L. purpureus*

and *E. tirucalli* with regard to drought tolerance grown in the greenhouse in field experiments under arid conditions to confirm and expand the previous results. Additional methods for the study of drought tolerance could be used, for example gas exchange tests to investigate the plant response to drought stress in a more comprehensive way. To confirm the possible genetic origin of trypsin inhibitor activity, further genotypes of *L. purpureus* must be examined. On the other hand it should be investigated whether the trypsin inhibitor activity of the seeds changes when the plants are grown under different environmental conditions. The same should be done with regard to the HCNp amount in order to find the reasons that ensure a low concentration. To obtain a more complete result of the genetic relationship for *E. tirucalli*, further genotypes from around the world need to be investigated.

CONCLUSION

The main objective of this thesis is to study the impact of drought stress on plant species using several genotypes of *L. purpureus* and *E. tirucalli* as examples. The combination of traditional and modern measurement techniques that examine different plant response aspects to drought stress makes it possible to identify more drought tolerant genotypes of both species. The trypsin inhibitor activity and HCNp results of *L. purpureus* show a wide bandwidth in concentration. By the use of AFLP the genetic relationship between genotypes can be determined. The results of this thesis can be used for genotype selection in breeding programs. Drought tolerant plants can provide a stable income for the farmers in regions with arid climatic conditions. The presence of specific substances in drought tolerant species, such as rubber in E. tirucalli strengthens the plant economic value for farmers and increases the willingness to grow them.

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LIST OF PUBLICATIONS:

PUBLICATIONS IN JOURNALS

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Hastilestari BR, **Guretzki S**, Papenbrock J (2012) Influence of water limitations on photosynthetic parameters in *Euphorbia tirucalli*, a plant with CAM and C_3 metabolism. Annual Main Meeting of the Society for Experimental Biology, Salzburg, June 2012.

Hastilestari BR, **Guretzki S**, Mudersbach M, Biskupek-Korel B, Van Damme PJL, Papenbrock J (2012) *Euphorbia tirucalli* L. -- physiological and genetical characterization of a drought tolerant plant and its potential as a source of bioenergy production. Tropentag, Göttingen, September 2012.

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