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CHARACTERIZATION OF SORGHUM GENOTYPES FOR TRAITS RELATED TO DROUGHT TOLERANCE

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Chapter 1

General introduction

Abstract:

This chapter provides an overview of knowledge on drought, mechanisms and strategies adopted by plants to cope with drought stress and the most common techniques used to screening drought tolerance traits in plants. Particular emphasis was given to *Sorghum bicolor* (Moench L.) as bioenergy crop.

The aim of the thesis, the methodological framework and the overall thesis structure are outlined in this chapter.

INTRODUCTION

1. Research context

In the next 30 years the human population could reach 9 billion people, consequently food, water and energy requests are going to increase (Cohen, 2003; Tilman *et al.,* 2001). Considering that the reserves of non-renewable fossil fuels are limited and that the oil price is rising (http://www.bp.com/en/global/corporate/about-bp/energy-economics/statistical-review-of-world-energy.html), an increasing attention is paid to the utilization of renewable energy resources.

Biofuels, defined as solid, liquid or gas fuels derived from biomass, are today the only direct substitutes for oil in the transport sector according to what has been proposed by Pacala and Socolow (2004).

With the Directive 2003/30/EC of 8th May 2003 "on the promotion of the use of biofuels or other renewable fuels for transport" the European Union laid the foundation for the promotion of alternative fuels in the EU. With the Directive 2009/28/EC of 23rd April 2009 (RES Directive), the EU indicates an updated objective for the reduction of the greenhouse gasses (GHGs) emission in the transport sector: 10% of the final consumption must be covered with renewable energy sources (RES) within 2020. Furthermore the same Directive introduces for the first time a reduction target for the GHGs emissions from fuels: the GHGs saving due to the substitution of current fossil derived fuels with biofuels and bioliquids shall be at least 35% but it will have to be 60% from the 1st of January 2018. In this directive, the criteria of sustainability for biofuels production were listed: the biofuels shall not be made from raw material obtained from land with high biodiversity value and land with high carbon stock. A suitable bioenergy crops should therefore provide high dry matter yield with low input, should be adapted to marginal environments and should limit the depletion of carbon stock in the soil (De Oliveira *et al.*, 2005).

Sweet sorghum has been investigated as a potential source of fermentable sugars for ethanol production due to the high sugar content accumulated in its stalks and its high biomass production, wide geographic and climatic adaptation, and relatively low water and fertilizer requirements befitting the sobriquet of 'smart crop' (Surwenshi *et al.*, 2010). Due to its little breeding history and its high genetic potential, sorghum is a candidate energy crop for bioethanol production. The EC project SWEETFUEL (Sweet Sorghum: An alternative energy crop), in which the present research work is part, had the main objective to optimize yields by

genetic enhancement and to improve cropping and harvest practices in temperate, tropical semi-arid and tropical acid-soil environments with the final goal of bioenergy production.

2. *Sorghum bicolor* as bioenergy crop

Sorghum is the fifth most important cereal crop for planted area and metric tons harvested in the world (FAO 2003). In 2009 Asian and African countries covered 82% of the total area cultivated with sorghum, with yield ranging between 904 and 1096 Kg ha^{-1} respectively. In Europe the total cultivated area is 151526 ha and the yield is 4451 Kg ha^{-1}(FAO, 2011).

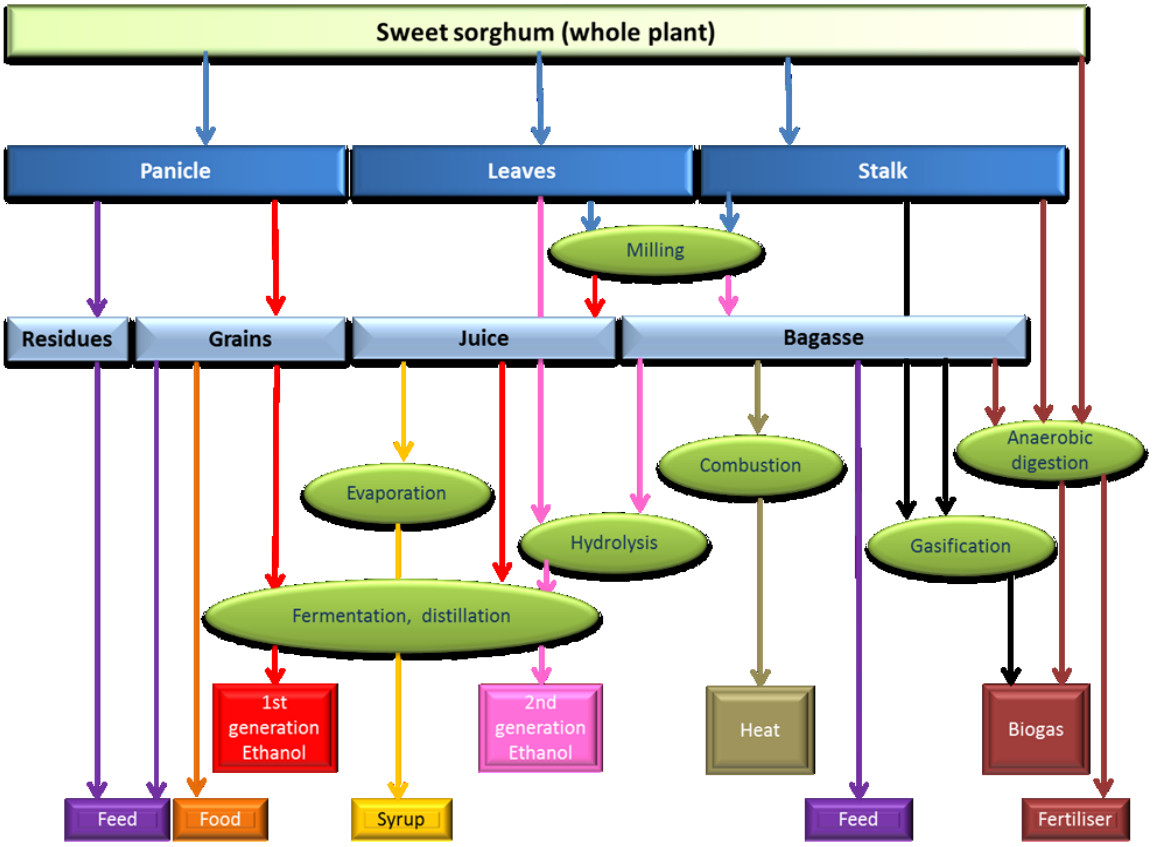
It is appropriate to define sorghum as a multipurpose crop. In fact, it is cultivated for human food, animal feed, as building material, fencing and brooms (Rooney and Waniska, 2000). In human nutrition it is used in fermented and fermented breads, couscous, snacks, alcoholic and non-alcoholic beverages mostly in Africa and Asia or as gluten-free product (Ciacci *et al.*, 2007), especially in USA and Europe, where nowadays sorghum is studied for its healthy properties (high antioxidant content of some varieties) that could have impact on its use in the health food industry (Awika and Rooney, 2004; Dykes and Rooney, 2006).

Sorghum is used for cattle, rabbit and broilers feed, similar to the use of maize (Dowling *et al*., 2002; Muriu *et al*., 2001). Furthermore the improvements in production technology, carried out in USA, to eliminate the prussic acid (containing HCN) could enhance the feed use of sorghum also in Europe (Berenji and Dahlberg, 2004).

The classical use of sorghum for industrial purpose is broomcorn. The recent trend in favouring natural and ecological products have led to renewed interest in biodegradable, old-fashioned hand-made brooms, reflecting a positive impact on broomcorn production (Berenji and Kišgeci, 1996).The fibres of the robust F1 hybrids, obtained by crossing grain sorghum and broomcorn, are used for paper pulping (Amaducci *et al.*, 2004). This improved technology could be an alternative to deforestation in order to have different raw materials for paper production (Dahlberg *et al.*, 2011).

Among the industrial uses of sorghum, the most important one is the renewable energy production (bioethanol and biogas production). The ethanol market is, in fact, one of the fastest growing segments of the sorghum industry in USA and the most implemented technology considering that nowadays equal amount of ethanol are produced starting from the same amount of grain sorghum as well as from maize (Dahlberg *et al*., 2011). Specific sorghum genotypes accumulates high levels of sugars in the stalks of the plant. The total amount of sugars could be converted in bioethanol while biomass and other organic waste (bagasse) could be transformed in biogas. Also the lignocellulose residues could be converted

in ethanol by second generation process after enzymatic hydrolysis (Sipos *et al.*, 2009; Yuan *et al.*, 2008). Furthermore high biomass yield with limited cropping input and efficient production systems make sorghum a very interesting crop for bioenergy production (Monti andVenturi, 2003; Zegada-Lizarazu and Monti, 2012). The recent interest in sorghum is nowadays also fuelled by its non GMO status, its drought tolerance and its wide adaptability (Dahlberg *et al.*, 2011).

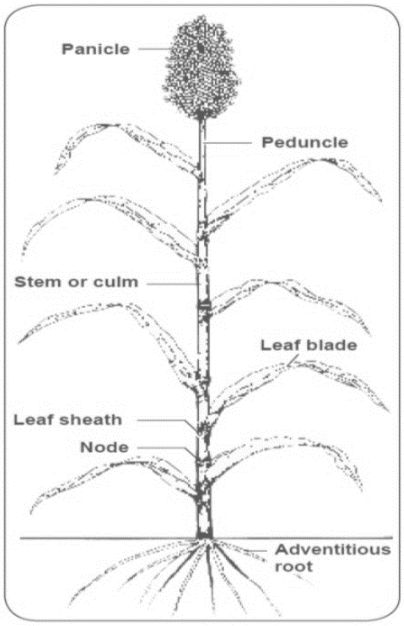


2.1 Sorghum characteristics, botanical classification, morphology and growth

Sorghum bicolor is a photoperiod sensitive plant originated from the tropics that can grow in rainy and semiarid region, also in areas too dry for maize production (Zegada-Lizarazu and Monti, 2013). Being a C4 cereals, is more heat and drought tolerant than C3 plants, like wheat or rice, originated from temperate region (Blum *et al.*, 1990). The optimum temperature range for sorghum is from 21 to 35 °C for seed germination, 26 to 34 ° C for vegetative growth and development, and 25 to 28 °C for reproductive growth (Maiti, 1996) and daytime/night-time temperatures of > 32/22 °C (Prasad *et al.*, 2006).

The origin and the early domestication of sorghum took place in northeaster Africa approximately 5000 years ago (Mann *et al.*, 1983).The sorghum genus is divided into 5 taxonomic subgenera: *Eusorghum*, *Chaetosorghum*, *Heterosorghum*, *Parasorghum* and

Stiposorghum. The *Eusorghum* sections contains all the domesticated and cultivated sorghum races as *Sorghum bicolor* subsp. *bicolo*r that have 2n = 20 chromosomes and is described as annual, with thick culms, often branched and with many tillers. Among the *Sorghum bicolor* subsp bicolor 5 races have been identified on the basis of the spikelet morphology: *bicolor, guinea, caudatum, kafir and durra* (Harlan and deWet, 1972). In order to explain the variation existing within a given race, another classification was proposed by Dahlberg *et al.* (2004) introducing the working groups (sub-races) that integrates the previous classification.



Sweet sorghum seeds can germinate in 3 to 5 days at optimum temperature (25 to 30 °C) and moisture emitting a small coleoptiles and a primary root that is going to die after the emission of the secondary or adventitious roots that are the main responsible of the anchorage of the plant to the soil and supply of nutrients.The vegetative stage, that has the optimum temperature for vegetative grow that 33-34 °C, begins when the growth starts above the soil surface and the shoot system starts to form. It consists of the stem (stalk), leaves, nodes, and internodes (Figure 1.2). During this period the plant grows rapidly producing all the leaf area that will be important during the grain- filling period. In many sorghum cultivars leaves and stem surface is covered with thick waxy coatings that prevents water losses and protects the plant in case of drought stress. The boot stage follows the vegetative growth: the sheath of the flag leaf swells and in 6 to 10 days the peduncle grows rapidly pushing up the inflorescence from the boot (flowering stage). Between flowering and the final stage of growth, the grain filling period is the most important period for grain production and the most affected by drought. At physiological maturity the grain has a moisture content that ranges between 25 to 40%.

Figure 1.2: Botanical parts of a sorghum plant (Source: Murty et al. 1994).

2.2 Cultivation

Density of plantation depends on variety, earliness, plant size, environmental conditions, etc and varies between 110,000 to more than 400,000 plants per hectare. Generally, planting in wider rows are recommended for the low rainfall areas and on soils with a poor water-holding capacity. Planting depth is also determined by soil type and should not exceed 25 mm. After sowing it is essential to maintain good soil moisture conditions to ensure the emergence. Taking into account the cycle length and the fact that the stage of sugar accumulation is affected by low temperatures, in the Mediterranean climates sowing should be performed at the beginning of May so that sorghum can be able to complete its cycle. The irrigation requirements depend on the site and the irrigation system used, besides the intrinsic factor of the variety requirements and could range between 500 to 1000 mm in a growing season. Till the canopy closure, sorghum is sensitive to weed completion. So it is better to apply herbicide in pre-emergence, immediately after sowing.

2.3 The *Sorghum bicolor* genome

Paterson *et al.*(2005) estimated that sorghum genome is about 730 mega base pair (Mbp), 60% larger than rice genome but only ¼ of maize genome. Sorghum has a small diploid genome and low level of gene duplication. DNA transposons constitute 7.5% of the sorghum genome, intermediate between maize (2.7%) and rice (13.7%).

The recent sequencing of the *Sorghum bicolor* genome provided a great boost in the knowledge of the evolution of grasses genome (Paterson *et al.*, 2009). Gene order and density are similar to those of rice and the retrotransposon accumulation explain more than 75% of the whole genome size compared to rice. The number and size of sorghum gene families are similar to those of Arabidopsis, rice and poplar. The 58% of sorghum gene families were shared among all species and at least the 93% with only one species. Nearly 94% of high-confidence sorghum genes have orthologues in rice, Arabidopsis and polar, and all together these gene set define 11502 ancestral angiosperm gene families. About 24% gene families have members only in sorghum and rice, while only 7% are sorghum specific.

The conservation of grass gene structures and order facilitate the development of DNA markers in order to perform crop improvement. About 71000 simple-sequence repeats (SSRs) were identified in sorghum while conserved- intron scanning primers for 6760 genes provide DNA markers useful across many monocotyledons (Lohithaswa *et al.*, 2007). For example, the characteristic adaptation of sorghum to drought may be partly related to expansion of the

miRNA and several gene families. In particular, rice miRNA169g, up regulated during drought stress (Zhao *et al*., 2007), has five sorghum homologues, or cytochrome P450 domain- containing genes, involved in scavenging toxins accumulated in response to stress, are more abundant in sorghum than in rice (326 versus 228).

Thanks to the similarity of sorghum genome to other crops, genetic maps were constructed anchoring the sorghum maps to those of sugarcane, maize and rice(Bowers *et al.*, 2003; Ming *et al.*, 1998; Paterson *et al.*, 2004). Physical map of sorghum bicolor and *Sorghum propinquum* have been constructed and subsequently anchored to the mapped sequenced-tagged sites (STS) loci (Bowers *et al.*, 2005). A consensus map, representing the integration of the whole genome sequence of sorghum with several QTLs studies, was built by Mace and Jordan (2011).

A whole transcriptome exon array for Sorghum bicolor was designed by Chromatin and Affimetrix: Sorgh-WTa520972F. The genechip contains 1,026,373 probes covering 27577 genes across the 10 chromosomes, chloroplast and mitochondria of sorghum bicolor. In order to study the sorghum transcriptome and build a gene expression atlas, Shakoor *et al.* (2014) collected 78 sorghum samples from different developmental stages and tissue types of three major sorghum ideotypes (grain, sweet and bioenergy sorghum). In this study it was observed a tissue and genotype-specific expression pattern of some relevant metabolic pathways (sugar metabolizing enzymes, sucrose transporters and phenylpropanoid- monolignol pathway) highlighting the significance of intraspecies variation existing in sorghum.

3. Drought stress

Plants are sessile organism that spend all their life on a substrate and are extremely dependent from their habitat. For this reason they are forced to detect different stress conditions during the entire duration of their life. These factors could have adverse effects on plant growth and development or could decrease yield production as final consequence. Nevertheless during century of domestication, plants showed a great capability to growth also in drought prone and hostile areas revealing strong adaptative skills to adverse conditions. Growth and development are affected by the genotype interaction with several external factors such as light intensity, temperature, water and mineral availability, etc.

According the Shorter Oxford English Dictionary (1983), stress can be defined as any uncontrollable pressure exert by a force or an influence that tends to inhibit the normal functional systems (Jones *et al.,* 1989). Jones and Qualset (1984) defined stress as any environmental conditions, or combination of environmental conditions, that impedes to a

given plant to achieve their own genetic potential for growth, development and reproduction. Taiz and Zeiger (2006) defined stress as an internal factor that exerts a disadvantageous influence on a plant and its effects are measured on the basis of the plant's survival, growth (biomass accumulation), yield, or the primary assimilation process, which are related to overall growth.

The performance of a plant under stress are influenced by stress (severity, duration and combination of stress) and plants characteristics (organ or tissue in question, stage of development and genotype).

Boyer and Westgate (2004) assessed that drought stress is one of the most significant causes of crop yield loss. In facts, water plays in plants vital roles acting as solvent, transport medium, biochemical reactant, creating turgor pressure and evaporative coolant. Any water limitation will result in a decrease in whole plant photosynthesis and growth due to stomatal closure with associated changes in carbon and nitrogen metabolism (Sanchez *et al.*, 2002). The responses trigged by drought stress are several and detectable at different scales (Figure 1.3). For these reasons the drought stress have been often labelled as "complex trait" by genetists (Blum, 2011).

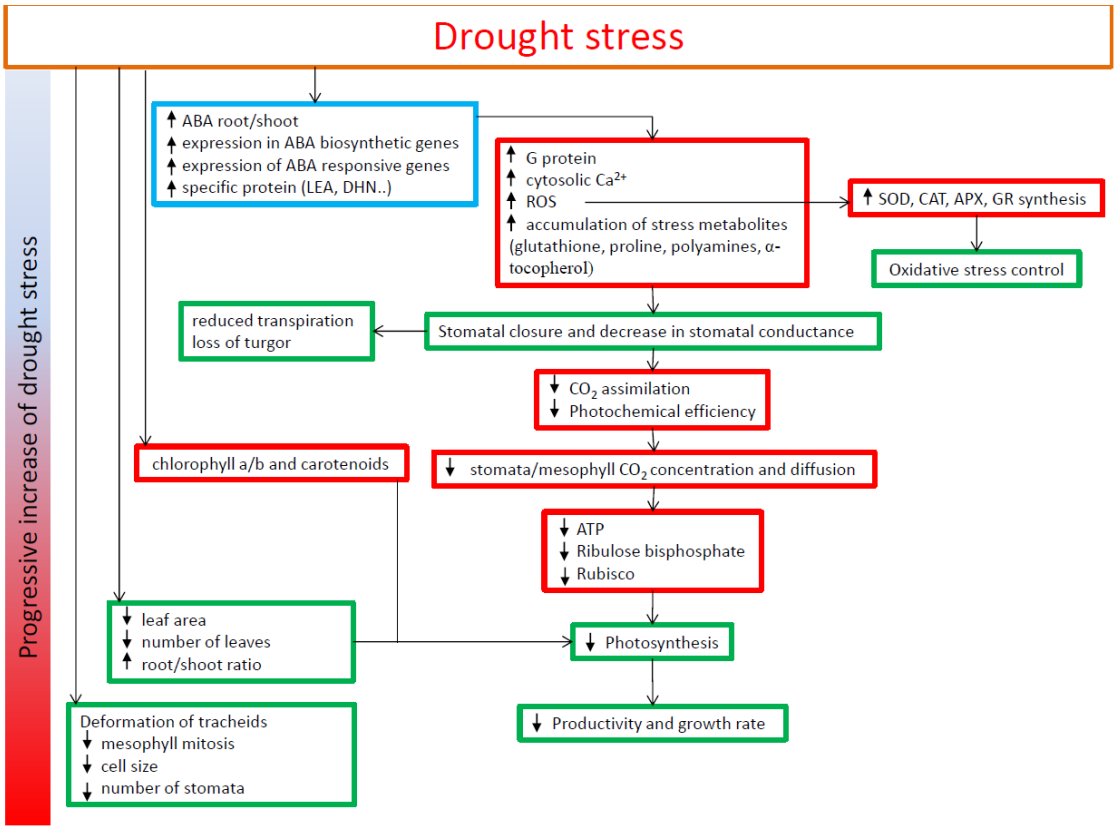


Figure.1.3: different responses triggered by drought stress. The blue boxes represent molecular responses, the red ones the biochemical and the green ones the resulting physiological responses. The different height of the boxes represents the progressive level of drought stress reached. ABA = Abscisic Acid, LEA= Late Embryogenesis Abundant protein, DHN= dehydrin protein, ROS= Reactive Oxygen Species, SOD=

Superoxide dismutase, CAT=catalase, APX= Ascorbate peroxidase, GR= glutathione reductase, ATP= adenosine triphosphate.

3.1 Plant response to drought stress

Plants can respond to water stress with three different mechanisms:

- *Drought avoidance* - the plants maintain cell turgor and high cell water content under water limiting conditions. It involves rapid phenological changes, such as leaf rolling and shedding, reduction in leaf area in order to reduce water loss by transpiration, and increase in stomatal and cuticular resistance (Morgan, 1984, Turner, 1986). Most sorghum genotypes have a thick waxy cuticle that limits water loss during drought stress period. On the other hands, a deep root system able to explore deep soil layers is associated to the conservation of water supply during period of water stress. The cell architecture of mesophyll tissue of C4 plants allow them to accumulateCO2 in the bundle sheath cells, reducing photorespiration, reducing stomatal conductance to preserve water without decreasing carbon fixation rates. Leaf abscission, dormancy, and any other mechanisms that reduce water loss by transpiration are considered drought avoidance mechanisms.
- *Drought escape*- is generally referred to the early completion of plant's life cycle, for example flowering before the onset of water stress. Some early maturing sorghum genotypes adopt this strategy to avoid water deficit periods that could occur during the growing season in some regions. From a molecular point of view, drought induces the expression of genes encoding for proteins involved in protection and signal transduction (Mundree *et al*., 2002). Several drought responsive genes are induced by exogenous treatment with ABA (abscisic acid), the hormone involved in water deficit signalling, but an additional gene set is also induced by drought in an ABA-independent signal transduction pathway (Mundree *et al.*, 2002). The promoters of these genes contain sequence specific ABA- responsive cis-elements (called ABRE). The same cis-elements were found in sorghum genes responding to ABA (Buchanan *et al.*, 2005). ABA-independent pathway genes contain other nine characteristic base pair defined as dehydration responsive elements (DRE). Proteins that can bind DRE include the ethylene-responsive element binding proteins, AP2 proteins, and DRE binding factor 1 and 2 that can activate the transcription of genes containing the DRE sequence (Mundree *et al.*, 2002).
- *Drought tolerance* allows the plant to maintain metabolic activity under water limited conditions though osmotic adjustment and antioxidant capacity. In order to lower the

osmotic potential and maintain turgor, the plants can accumulate compatible solutes including sugars, organic acids, amino acids, sugar alcohols or ions. Osmotic adjustment is important in the drought tolerance of many C4 species growing in arid environments and allows the growth of sorghum when leaf water potential is low (Girma and Krieg, 1992; Jones, 1978). In particular *Sorghum bicolor* accumulates glycine betaine and proline in response to water deficit (Buchanan *et al.,* 2005). On the other hand the antioxidant capacity consist in the ability of the plants to detoxify reactive oxygen species (ROS) that can cause cell damage by lipid peroxidation or protein and nucleic acid modification (Scandalios, 2005). Some plants are able to prevent ROS damage by using superoxide dismutase, catalases, or peroxidase and by using free radical scavengers as carotenoids, ascorbate, proline, tocopherols, and glutathione (Mundree *et al*., 2002). Reduced number of ROS and the prevention of oxidative stress is a proxy of abiotic stress tolerance such as drought stress.

4. Screening for drought tolerance traits in sorghum

Drought, as other abiotic stresses, elicits a wide range of responses addressed to avoid or tolerate the water loss through physiological, biochemical and molecular processes (Verslues *et al.*, 2006). Understanding how plants cope with water stress and which are the drought stress responses at physiological and molecular level is still one of the most important topics in plant science (Shao *et al.*, 2007).

Several methods, both in field or in controlled-environment, are nowadays commonly used for screening drought tolerance. Field studies have more advantages than those performed in controlled environments because they represent the true nature of the farmer's and breeder's field conditions. The major limitation of field experiment is the lack in control of the environmental conditions that make the screening process very long and difficult.

Leaf area (Karamanos and Papatheohari, 1999; Tsuj *et al.*, 2003),vegetative growth (Younis *et al.*, 2000), root dry matter (Giuliani *et al*., 2005; Huang and Gao, 2000),whole plant transpiration rate (Luquet *et al*., 2008; Xin *et al.*, 2008), photosystem II energy use and non-photochemical quenching (Cousins *et al.*, 2002; Jagtap *et al.*, 1998) have been used as physiological traits to screen genotypes for drought tolerance. On the other hand cDNA libraries (Pratt *et al.*, 2005), microarrays (Buchanan *et al.*, 2005; Pasini *et al.*, 2014) and RNA-Seq experiment (Dugas *et al.*, 2011) were used to evaluate the stress response of sorghum to drought at transcriptional level.

4.1 Physiological screening for drought tolerance traits in sorghum

Drought is the most important abiotic stress limiting crop productivity. The natural selection has favoured mechanisms of adaptation and tolerance, while breeding programs have generally targeted the improvement of crop productivity and economic yield.

Screening for drought tolerance is nowadays accomplished by selecting genotypes under field or greenhouse conditions using laboratory test. Measurements of different physiological processes of plant response to drought is important to understand how the plant reacts to remove or reduce the harmful effects of water deficit (Grzesiak *et al.*, 2003). Carrying out screening experiment in field conditions implies a lot of methodological problems related to the control of water content in the soil and also the necessity to verify the results with laboratory tests. Physiological traits relevant to plant response to drought are often related to vital processes: stomatal conductance and leaf temperature, osmotic adjustment, membrane composition, antioxidative defence and stay green. As a consequence there is not a single response pattern that is highly correlated with yield under drought environments. The most widespread parameters used as functional tools in screening programs for traits related to drought are listed below.

4.1.1 Cell division, vegetative growth and biomass partitioning

The difficulty in identifying a physiological parameter to be used as a reliable indicator of yield performance under drought has suggested that yield performance over a range of environments should be used as the main indicator for drought tolerance (Voltas *et al.*, 2005). The yield capacity has been expressed in relation to an environment- related physiological trait (canopy temperature or water potential) or on specific environmental factors (weather, soil water availability, vapor pressure deficit, VPD). All these different approaches, based on the yield as function of an environmental index, lead to compare genotype performances under different degrees of water limitation (Araus *et al.*, 2003). So an ideal genotype should show the highest yield combined with the lowest sensitivity to water stress (Cattivelli *et al.*, 2008), even if very often high yield in wet and dry condition are associated to high sensitivity to water stress.

It should be considered that the biomass yield is only the last step of a series of mechanisms and processes that involve the whole plants. The leaf apparatus is usually the one most affected by drought. Reduction in leaf area in response to drought can occur either through hastened leaf senescence or a decline in leaf expansion and extent of reduction appears to be dependent on relative tolerance of sorghum varieties (Ashraf and Ahmad, 1998).

Cell division and cell growth are the primary process involved in plant growth. Being the leaves the centres of photosynthesis and performing a fundamental role in plant growth and development, leaf expansion is the most sensitive process to drought (Alves and Setter, 2004). A decrease in leaf area expansion due to drought stress results in a decrease of transpiration surface, which is reached through the reduction in cell size and in numbers of cells produced by leaf meristem in order to limit further water depletions (Tardieu *et al.*, 2000). Cell size and development contribute in different ways to leaf area reduction depending on the developmental stage at which the plant, and its leaves, were stressed. The older leaves diminish leaf area by reducing mature cell size, while in the younger leaves the inhibition of cell division resulted in fewer cells per leaf (Alves and Setter, 2004). Drought stress affects leaf area by reducing leaf numbers, leaf expansion rate and the final leaf size, but could also accelerate leaf senescence leading to the death of leaf tissue (Brevedan and Egli, 2003). Loss in leaf area could be interpreted as drought avoidance mechanism limiting further water losses by transpiration, while a decrease in leaf senescence, especially in post-flowering during grain-filling stages, is defined as drought tolerance mechanisms.

Total leaf area, specific leaf weight and specific leaf area are decreased all under water stress while the development of the root system is usually less inhibited, and sometimes may even be promoted (Sharp and Davies, 1979): as a consequence the root/shoot ratio is increased under drought stress conditions (Munamava and Riddoch, 2001). An important feature of the root system response to soil drying is the ability of some roots to continue elongation at water potentials that are low enough to inhibit shoot growth completely. Maintenance of root growth during water deficits could be interpreted as a benefit to maintain an adequate plant water supply, and is under genetic control (Sponchiado *et al*., 1989).

4.1.2 Photosynthesis, transpiration and stomatal conductance

Photosynthesis, transpiration, and stomatal conductance are considerably affected by drought stress either from a physiological and biochemical point of view, either at molecular level through gene expression. At the onset of water stress, the initial effects are the closure of stomata opening and a consequent lower photosynthetic rate (Cornic, 2000), followed by an increase of intercellular CO_2 concentration (C_i) (Siddique *et al.*, 1999), the flow of CO_2 into the mesophyll tissue decrease (Chaves *et al.*, 2003; Perez-Martin *et al.*, 2014) leading the decline in regeneration of ribulose bisphoshate (RuBP) and in ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) content and activity (Bota *et al.*, 2004; Parry *et al.*, 2002). When the stress becomes more severe, it will affect the photosynthetic capacity of the

mesophyll causing decreases in carboxylation, electron transport chain, inducing ultra-structural changes in chloroplasts.

Stress intensity and/or duration affect the rate and the extent of recovery of photosynthesis after water recovery (Miyashita *et al.*, 2005). The photosynthetic recovery rate plays an important role in terming plant's tolerance to drought and avoiding dramatic losses in crop yield (Chaves *et al*., 2009). The recovery could be divided into two stages: the first stage occurs few hours after re-watering and corresponds to an improvement of leaf water status and stomata re-opening (Pinheiro *et al.*, 2005) while the second stage, lasting several days, requires synthesis of fresh photosynthetic proteins (Kirschbaum, 1988).

4.1.3 Chlorophyll fluorescence

In physiological conditions, photon energy absorbed by photosynthetic pigments (chlorophyll and carotenoids) drives primary photochemical reactions. Irradiation excites Chlorophyll (Chl) molecules to a first excited singlet state stable for less than 10^{-8} seconds (Briantais *et al*, 1979) and charge separation at the reaction centre (RC). If the charge separation does not occur, excited pigments return to ground level and absorbed energy is released as heat and/or red and far-red Chl fluorescence (Krause and Weis, 1991). The deleterious effects of water stress, involving mechanical damage to the membrane structure and function due to shrinking of the vacuole, result in reduction of turgor, cell expansion inhibition, vegetative growth retardation and carbon gain reduction (Sayed, 2003). The stomatal closure induced by drought stress depletes internal CO_2 leading to accumulation of energy-containing products of electron transport, free radicals formation, light-harvesting complexes perturbation and photoinhibition. In water stress conditions, the photosynthetic quantum conversion declines, with a concomitant increase in red and far-red chlorophyll fluorescence (Lichtenthaler and Miehé, 1997). Measuring the Chl fluorescence gives insight on the efficiency of the photochemical processes and the technique is nowadays widespread used as probe of photosynthesis in vivo.

Kautsky *et al.* were the first to identify changes in the yield of Chl fluorescence after transferring photosynthetic material from the dark into the light (1960). The rise in the yield of Chl fluorescence is the consequence of reduction of electron acceptors in the photosynthetic pathway downstream of photosystem II (PSII). In fact, when PSII absorbs light and Q_A accepts an electron, it is not able to accept another one until it has passed the first to the following electron carrier Q_B. During this period the reaction centre is not able to accept other electron and it is said to be "closed". The presence of closed reaction centres leads to an

overall reduction in the efficiency of photochemistry and a corresponding increase in the yield of fluorescence till a maximum peak (Maxwell and Johnson, 2000) (Figure 1.4). After this peak, the fluorescence level starts to decrease again on a time-scale of few minutes; this phenomenon is called "fluorescence quenching" and is due to the increased rate at which electrons are transported away from PSII (due to the light activation of enzymes involved in carbon metabolism and the opening of the stomata- "photochemical quenching", qP) and to the increased efficiency with which energy is converted to heat ("non-photochemical quenching", qNP) (Table 1.1). A large number of different coefficients have been calculated to quantify photochemical and non-photochemical quenching. The calculation of fluorescence parameters can be easily explained referring to a typical experiment (Maxwell and Johnson, 2000).

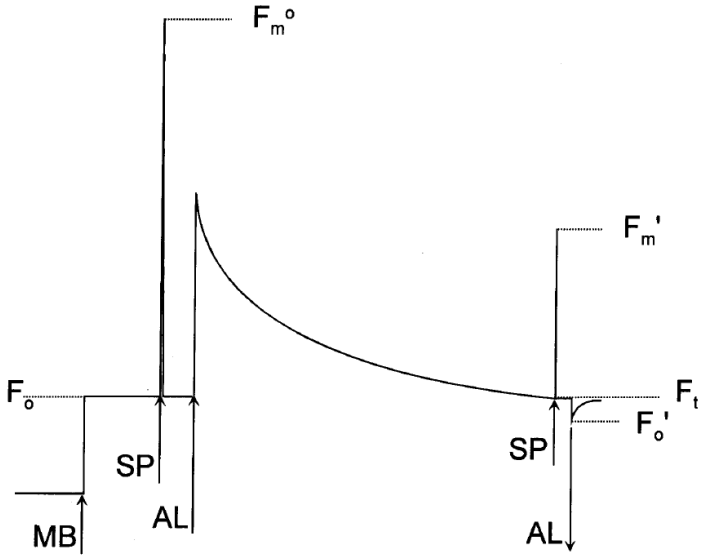


Figure1.4:Sequence of a typical fluorescence trace. The zero fluorescence level F_0 is measured after switching on a measuring light (MB). The application of a saturating light pulse (SP) allows to determine the maximum fluorescence level (F_m^0). A light source able to drive photosynthesis (AL) is applied and after a period of time (crop specific) another flash of saturating light (SP) is applied to measure the maximum fluorescence in the light (F_m'). The level of fluorescence after the last light flash is termed Ft. Turning off the actinic light (AL) the zero level of fluorescence in the light (F_0') can be estimated.

Table 1.1: commonly used fluorescence parameter

Photochemical quenching parameters:		
ϕ_{PSII}	Quantum yield of PSII	$(F'_m - F_t) / F'_m$
qP	Proportion of open PSII	$(F'_m - F_t) / (F'_m - F'_0)$
F_v/F_m	Maximum quantum yield of PSII	$(F_m - F_0) / F_m$
Non photochemical quenching parameter:		
NPQ	Non photochemical quenching	$(F_m - F'_m) / F'_m$

The most useful parameter is the efficiency of the PSII photochemistry (ΦPSII) (Genty *et al.,* 1989) that measures the proportion of the light absorbed by Chl associated to PSII that is used in photochemistry. Under laboratory conditions, a strong linear relationship exists between this parameter and the efficiency of the carbon fixation but a discrepancy occurs, in general, under stress conditions due to the changes in the rate of photorespiration or pseudocyclic electron transport (Fryer *et al*., 1998). Also the qP is widely used to measure photochemistry giving the insight of the proportion of PSII reaction centres that are open. ΦPSII and qP are interrelated by a third parameter, the maximum quantum yield of PSII (F_v/F_m), that measures the intrinsic efficiency of PSII and it is used as sensitive indicator of plant photosynthetic performance, having optimal values around 0.83 for most plant species (Maxwell and Johnson, 2000).

Chlorophyll fluorescence emitted from the chloroplast thylakoid membrane is often used as a very sensitive intrinsic indicator of the photosynthetic reaction in photosystem II (Ahmed *et al.*, 2002) and the flow of electrons through PSII is indicative of the overall rate of photosynthesis giving the potential to estimate photosynthetic performance in a non-invasive and instantaneous manner. Being PSII the most vulnerable part of the photosynthetic apparatus, damage to PSII are often the first manifestation of stress in a leaf. Analysis of chlorophyll fluorescence and measurement of the Fv/Fm ratio under drought stress are used to determine damage to light reaction systems in photosynthetic mechanisms. It is well known that drought stress affects Fv/Fm, decreases the electron transport rate (ETR) and the effective quantum yield of photosystem II (ΦPSII) (Zlatev and Yordanov, 2004). The decreased CO2 assimilation and electron transport rate due to drought stress are reflected also in reduced Chl fluorescence measurements in assessing water stress-induced effects in sorghum (Masojídek *et al.*, 1991).

The most powerful application of Chl fluorescence is to combine it with gas exchange measurements in order to obtain a full picture of the response of plants to their environment.

4.2 Molecular screening for drought tolerance traits in sorghum

Drought tolerance is a typical quantitative trait. The molecular approach available leads to identify genes and drought related QTLs allowing the development of non-conventional breeding techniques to improve crops yields in drought prone environments. The good performance of sorghum under water stress, the high genetic variance among genotypes and the relatively small size of sorghum genome, make this cereal an ideal crop for the

identification of drought related genomic regions and genes necessary to unravel the highly complex drought tolerance trait (Paterson *et al.*, 2009; Sanchez *et al.*, 2002).Several sorghum linkage maps were built using different type of DNA markers (Mace *et al.,* 2009; Rami *et al.*, 1998) reaching high density level (Ashraf, 2010). Different genomic regions related to drought tolerance in pre-flowering and post-flowering stage were identified recently (Kebede *et al.*, 2001).The recent availability of the sorghum genome allows monitoring the genome-wide gene expression profiling at a single time in response to several abiotic stresses through microarray or RNA-Seq analysis (Buchanan *et al.*, 2005; Dugas *et al.*, 2011; Yazawa *et al.*, 2013) permitting to identify drought stress responsive genes, their relationship and their regulatory elements as well as the post-transcriptional modifications due to small RNAs. These small RNAs (including microRNAs, miRNAs, and short-interfering RNAs, siRNAs) are able to silence genes by driving target mRNAs to degradation or repressing their translation (Ambros, 2004; Bartel, 2004). MiRNAs were found in plants, animals and other eukaryotes as well as in DNA virus. In plants, miRNAs are 20-24 nucleotides long non-coding RNAs complementing their mRNAs target inducing their cleavage and their silencing (Khraiwesh *et al.*, 2012). Considering their importance in post-transcriptional gene silencing, their involvement in stress regulated gene expression seemed likely and was confirmed in several studies as important "players" in plant resistance to biotic and abiotic stresses (Navarro *et al.*, 2006; Pasini *et al.*, 2014; Sunkar *et al.*, 2007).

4.2.1 RNA-Seq technology

Only a decade ago, gene expression studies were restricted to small scale quantitative PCR analysis of candidate genes or relied on cross-species hybridization on microarrays (Naurin *et al.,* 2008). The rapid development of massive parallel sequencing (next-generation sequencing, NGS) and the implementation of bioinformatics analytical tools in the last few years, changed dramatically the global scenario (Margulies *et al.*, 2005). The whole genome, or the whole transcriptome analyses are nowadays a realistic option also for individual laboratories and for studies on non-model organism.

The RNA-Seq, called also whole transcriptome shotgun sequencing, refers to the use of the high-throughput sequencing technologies for characterizing the RNA content and composition of a given sample. The sequence information from transcripts cannot be read as a whole, but are randomly decomposed into short reads of several hundred base pairs. If genome or transcriptome information are available, the reads are aligned directly onto the reference (genome or transcriptome). If the reference genome (or transcriptome) is not available, the

reads have to reconstruct the transcripts using the so called *de novo* assembly (Wolf, 2013). Counting the reads that fall onto a given transcript provides also a quantitative information on the transcript abundance, the starting point for all biological inference. Before the advent of NGS technologies and RNA-Seq, microarrays were the most used tool for gene expression studies; the decreased costs, increased yield and improved bioinformatics data processing makes it possible to obtain sequence information and gene expression data by sequencing (Wolf *et al.,* 2010) and to overcome the microarray technology. Compared to microarrays, RNA-Seq has also the advantage to capture a wider range of expression values providing also information on RNA splice events (Mortazavi *et al.*, 2008) and to avoid biases in gene expression measurements. On the other hand, the limitations of this new and promising technique should also be considered. First of all, RNA-Seq data collection and statistical analysis are still under development and there are not benefit of decades of experience available for microarray analysis. Second, when the mRNA levels are used as proxy of protein abundance estimation, we should consider also the stability and turnover rates because a gene expression level alone can be a poor predictor of protein abundance (Vogel *et al.*, 2010). Third, the gene expression is highly tissue specific, so exceptional caution is needed in the data interpretation process. Fourth, in the analysis high attention must be given to not lump together genes encoded on different strands.

The transcriptome sequence constitutes an astonishing resource of information to develop a large number of popular molecular markers such as single-nucleotide polymorphisms (SNPs) and microsatellites (Wolf, 2013), and it provides a useful functionally relevant subset of the genome (Lamichhaney *et al.,* 2012). Furthermore, the great advantage of this technology is the possibility to investigate differences in gene expression patterns between population in speciation or adaptation studies (Wolf *et al.*, 2010). Studies carried out on the comparison of gene expression techniques highlighted that RNA-Seq technology appears to be comparable, and in some ways superior, to the existing array-based approaches (Marioni *et al.*, 2008). Considering also the rapidly falling costs of sequencing, it seems only a matter of time before the NGS techniques are widely adopted for this purpose.

Several studies were carried out on sorghum using RNA-Seq technique to explore the gene expression profile in response to osmotic stress and abscisic acid (Dugas *et al.,* 2011) or to provide a sorghum bicolor expression atlas on the dynamic genotype-specific expression profiles for different sorghum genotypes (Shakoor *et al.*, 2014), or for the identification of genome-wide SNPs that can potentially enhance genetic analysis and the application of molecular markers in sorghum genomics and breeding (Bekele *et al.*, 2013).

Aim of the work

The goal of this work was to dissect the complex trait of drought tolerance and to unravel the mechanisms and strategies adopted by plants to cope with drought stress, enhancing physiological and molecular information on drought stress. The further step to achieve an integrated view of plant system biology under drought stress conditions is in progress. It is a huge job that was quite hard to complete in just three years of work.

Sorghum bicolor was selected as candidate crop for bioenergy production for its capacity to provide raw material (sugars, starch and fibre) for the production of bioethanol or biogas.

The specific objectives were to:

- Evaluate the biomass production and composition of sorghum in field trials under rainfed and well watered conditions to assess the suitability of newly developed genotypes to be cultivated for bioenergy production.
- Evaluate drought tolerance of a selection of sorghum genotypes from a physiological and a molecular point of view, and to combine these two approaches in an integrated view of drought response in sorghum.
- Evaluate the diversity existing in the sorghum transcriptome that could be related to drought tolerance, then identify candidate genes that could be used as potentially marker for marker assisted selection.

Outline of the thesis

The thesis consists of five chapters. Chapter 1 provides a general overview on drought stress and the common techniques for screening genotypes for the identification of drought tolerance traits. Chapter 2 deals with evaluation of drought tolerance traits in agronomic field trials and the evaluation of chemical biomass composition for bioenergy production. Chapter 3 provides a combined approach in screening genotypes for drought tolerance. Physiological measurements were combined with gene expression analysis in order to identify candidate genes drought related that could be used as marker for further genetic improvement of sorghum germoplasm. Chapter 4 provides the preliminary results of a detailed analysis carried out using the modern next generation sequencing techniques. The RNA-Seq analysis performed on two genotypes, selected in the previous experiments, highlighted genotypic differences related to the response to drought. Chapter 5 provides a synthesis of the results reported in previous chapters and discusses the overall contribution of this thesis to the current knowledge on drought stress and tolerance.

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