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Physiological and productive responses of *Miscanthus* genotypes to different climatic constraints in Mediterranean environment

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TABLE OF CONTENTS **Pag**

ABSTRACT 1

1 Energy sources 3
1.1 Political and economic motivations for using alternative energy 5
1.2 Current legislation on renewable energy 6
1.3 Reducing global carbon emissions 8

2 Concerns about bioenergy 10
2.1 Benefits of using perennial grasses for energy 12
2.2 Why use *Miscanthus* 14
- *2.2.1 Potential contribution of Miscanthus to reducing carbon emissions* 16
- *2.2.2 Positive and negative impacts of Miscanthus on the environment* 16
- *2.2.3 Positive and negative impacts of Miscanthus on ecology* 17

3 *Miscanthus*
3.1 Botanical description of *Miscanthus* 18
3.2 Agronomic characteristics and bioprocessing 21
- *3.2.1 Site preparation and planting* 22
- *3.2.2 Fertilization* 22
- *3.2.3 Propagation* 22
- *3.2.4 Crop protection* 23
- *3.2.5 Harvest frequency and time* 23
- *3.2.6 Flowering time* 23
- *3.2.7 Biomass production* 24

3.3 End uses of *Miscanthus* 25

4 Response of *Miscanthus* to stress conditions 28
4.1 Mediterranean environment 28
4.2 Morphological and physiological adaptations of plants to the Mediterranean environment 29
4.3 Traits of interest in *Miscanthus* in Mediterranean environment 30
- *4.3.1 Heat stress* 30
- *4.3.2 Drought stress* 32
- *4.3.3 Composition* 33

EXPERIMENTAL PART

1 Purpose of the work 36

2 Materials and methods
2.1 Evaluating wild *Miscanthus* germplasm for biomass potential in Southern Europe 38
2.2 *Miscanthus* biomass yield and biomass quality as affected by harvesting dates 41
2.3 Effect of heat stress on the biomass production and physiology in *Miscanthus* genotypes 44

3 Results and Discussions
3.1 Evaluating wild *Miscanthus* germplasm for biomass potential in Southern Europe
- *3.1.1 Meteorological data* 51
- *3.1.2 Phenology* 52
- *3.1.3 Morphological traits* 54
- *3.1.4 Productive traits* 55
- *3.1.5 Water Use Efficiency (WUE)* 60

3.2 *Miscanthus* biomass yield and biomass quality as affected by harvesting dates
- *3.2.1 Harvest time effect on above-ground dry biomass yield* 62
- *3.2.2 Fertilization effect on the above-ground dry biomass yield* 64
- *3.2.3 Biometric measurements at different harvest dates and year of harvest* 66

- *3.2.4 Raw material composition of Miscanthus x giganteus* 67
- *3.2.5 Ash content in leaves and stems of Miscanthus x giganteus* 70

3.3 Effect of heat stress on the biomass production and physiology in *Miscanthus* genotypes

a) Morphological traits of the studied *Miscanthus* accessions
- *3.3.1 Stem height* 71
- *3.3.2 Stem and leaf number per plant* 72
- *3.3.3 Leaf surface area* 74
- *3.3.4 Stem diameter* 77

b) Productive traits of the studied *Miscanthus* accessions
- *3.3.5 Fresh and dry above-ground biomass* 78
- *3.3.6 Dry above-ground biomass partitioning* 81
- *3.3.7 Fresh and dry below-ground biomass* 83
- *3.3.8 Fresh and dry below-ground biomass partitioning* 86

c) Physiological traits of the studied *Miscanthus* accessions
- *3.3.9 Response of CO_2 assimilation rate (A) to photon flux density (Q)* 90
- *3.3.10 Response of CO_2 assimilation rate (A) to leaf internal CO_2 mole fraction (Ci)* 96
- *3.3.11 Chlorophyll fluorescence* 100
- *3.3.12 Stomata number and size* 108
- *3.3.13 Leaf absorbance* 112
- *3.3.14 Relative chlorophyll a estimation (SPAD values)* 113

4 Conclusions

4.1 Evaluating wild *Miscanthus* germplasm for biomass potential in Southern Europe 116

4.2 *Miscanthus* biomass yield and biomass quality as affected by harvesting dates 116

4.3 Effect of heat stress on the biomass production and physiology in *Miscanthus* genotypes 117

REFERENCES

Abstract

Strong global growth and development has increased demand for energy to refine, manufacture and transport products to support the lifestyles of an increasingly developing and globalized world. In recent decades, fossil fuels have become important sources of energy. However, with increasing demand, there has been developing concern over the sustainability of fossil fuels relating to their potential future sources and harmful byproducts of use, specifically large net carbon releases, which has spurred interest towards the use of alternative renewable energy sources. Potential alternatives are available including wind, solar, hydro, and biomass, all of which are touted to have greater environmental benefits relative to fossil fuels. There has been increasing interest in the use of perennial grasses as energy crops in the US and Europe since the mid-1980s. The characteristics which make perennial grasses attractive for biomass production are their high yield potential, the high contents of lignin and cellulose of their biomass and their generally anticipated positive environmental impact. Energy crops are crops which are produced with the express purpose of using their biomass energetically. There are many ecological benefits expected from the production and use of perennial grasses. The substitution of fossil fuels or of raw materials based on fossil fuels by biomass is an important contribution to reduce anthropogenic CO_2 emissions. Compared to other biomass sources, like woody crops and other C_3 crops, C_4 grasses may be able to provide more than twice the annual biomass yield in warm and temperate regions because of their more efficient photosynthetic pathway. There is big concern for farming systems in the Mediterranean Area. The Mediterranean climate is in fact characterized by hot and dry summers, and most of the global warming models show that the water supply will be much lower and the air temperatures significantly higher in short term, especially during the summertime. In general, perennial grasses are drought resistant crops and recently have been attracting growing interest due to their extensive environmental benefits both at global- and agricultural community-scale. Compared to traditional row crops, perennial grasses generally require lower energy inputs (fertilizers, pesticides etc.), can be grown on marginal cropland and provide benefits in terms of soil structure and stability (e.g. reduced soil loss, erosion and runoff), soil quality (e.g. increase in soil fertility, organic matter and nutrient retention) and biodiversity (e.g. cover for native wildlife). Soil erosion, in particular, is one of the biggest environmental threats in the Mediterranean area as it causes pollution of water bodies, critical losses of water, nutrients, soil organic matter and soil biota from the natural ecosystems. The cultivation of perennial grasses has the potential to provide a range of benefits, like surviving over prolonged dry periods, acting as carbon sinks and filter systems for removing agrochemicals from water before these pollutants reach surface and/or groundwater bodies. Perennial grasses are also not seen as competing for agricultural land because they can be grown on marginal or degraded

lands where intensive agricultural practices harm the environment (e.g. promoting soil erosion), and where the economic returns to the farmer's labor and capital are not sustainable. *Miscanthus* spp is one of the most investigated perennial rhizomatous grass for bioenergy. *Miscanthus* is a perennial C_4 plant native to Eastern Asia, which can produce substantial annual yields of dry biomass with limited nutrient input.

For this purpose, three different researches (two field trials and one in controlled environment) were carried out with the aim of studying i) the adaptation and biomass production potential of 18 *Miscanthus* accessions, representing 5 *Miscanthus* species, collected from a wide geographical range (Numata, 1974) for suitability to semi-arid Mediterranean climates; ii) the effect of harvest time (autumn and winter time) on biomass yield, morph-biometric characters, moisture content, cellulose, hemicelluloses and lignin contents for second generation bioethanol production and ash content for combustion purposes in a long term plantation of *Miscanthus x giganteus* in a Mediterranean environment; iii) the effect of heat stress, in controlled-environment, on 5 *Miscanthus* genotypes, coming from the *Miscanthus* germplasm collection at Institute of Biological, Environmental and Rural Sciences (IBERS) of the Aberystwyth University – Wales – UK, to identify how temperatures affect growth, partitioning and physiology of *Miscanthus* plants.

Results suggest that some *Miscanthus* accessions are suitably adapted to maintain high biomass in a semi-arid Mediterranean environment and that the most commonly available commercial *Miscanthus* genotypes (*M. x giganteus* and *Goliath*) are not well adapted to the Mediterranean climate or environments where water is a limiting factor, while other *Miscanthus* accessions produce high biomass yield in semi-arid regions. Long term *Miscanthus* plantations strictly depend by the thermopluviometric trend of the growing season, decreasing biomass yield as rainfall reduces and the biomass for specific end uses presents higher quality (in terms of more hemicellulose and cellulose content and lower ash content), with winter harvest. Relative to temperatures, high temperature decreased the plant height (~48%), above-ground dry biomass (~66%), below-ground dry biomass (~26%) and photosynthetic response to absorbed light (~13%). The most widely available and commonly used variety of *Miscanthus* is sensitive to high temperatures and there are other genotypes that have a higher capacity for carbon assimilation in high temperature environments.

MONOGRAPHIC PART

1 Energy sources

Meeting global energy needs in the future has become a main topic of discussion, for several reasons. The simplest one is the fact that the world population is still growing from the current 6.6 billion people, to a projected stable level of 9 billion in 2050 (Population Institute 2008). That means a 50% increase in the population in just over 40 years. The majority of this population growth is expected to occur in Africa, India and Southeast Asia. Concurrent with this increase in the world population is the increase in the standard of living in several countries with large populations, notably China and India, each with more than 1 billion people. As the standard of living in these countries goes up, so does the need for energy in the form of fuels for automobiles, farm equipment, trucks, and airplanes, and electricity to heat, cool and light houses, offices and factories. Fossil fuels–coal, oil, and natural gas–currently supply 86% of the world's energy, but fossil fuels represent a finite resource that will be used up in the foreseeable future. So the only way the increase in demand for energy can be met, is through the use of alternative energy sources. The question therefore is not if we should develop alternative energy sources, or whether alternative energy is economically competitive with fossil fuels, but *how* we can use alternative energy in a way that is sustainable both economically and ecologically. Conventional energy sources are the fossil fuels (coal, oil and natural gas), but also include hydroelectric and nuclear power, because these two energy sources are each already responsible for providing 6% of the world's energy. Alternative energy, as the name implies, refers to energy that is different from conventional energy and includes a diverse collection of energy sources: solar energy, wind energy, tidal energy, wave energy, geothermal energy, and bioenergy. Renewable energy is energy from sources that are replenished. This includes all the above mentioned alternative energy sources, as well as hydro-electric power. The production of energy crops is expected to benefit the development of new markets, to promote regional economic structures, to provide alternative sources of employment in rural areas, to promote the use of surplus and marginal lands, to reduce CO_2 levels, and to reduce dependence on short-term weather changes experienced by production of other forms of renewable energy (wind, photovoltaic) (Biomass action plan, 2005). Therefore, in the long term, the successful implementation of energy crop systems should seek to ensure income generation, environmental sustainability, energy security, flexibility and replicability (Table 1).

Table 1 – Why to grow energy crops? (Zegada-Lizarazu et al., 2010 based on Sims et al., 2006).

Social benefits	*Environmental benefits*	*Economic benefits*
Energy crops could benefit the production of indigenous renewable energy which in turn increases energy security* and improves trade balances.	The production of dedicated lignocellulosic biomass crops has the potential to provide a range of benefits for water quality improvements, phytoremediation, and treatment of waste water and sludge, carbon emission reductions* and biodiversity.	Production of energy crops will lead to the development of new and profitable markets (biofuels, chemicals, materials, foods and feeds, etc.) that could provide farmers with new sources of income and employment.
Will lead to the creation of alternative sources of employment in rural areas.	Soil degradation problems could also be reduced when perennial herbaceous and short rotation trees are grown.	Will promote the development of regional economic structures
Production of energy crops could lead to the creation of farmers associations/cooperatives.	Will promote the use of marginal lands.	Will create higher value coproducts
Could improve the education, training, and assistance services provided for farmers.	Will provide wildlife and natural habitats. Reduce pressure on finite natural resources	

*EU primary goals are the increase in energy security and GHG emission reduction

In general, all plant species could be used as feedstock for bioenergy generation, but only a limited number of them meet the standard requirements of a good energy feedstock to be used in transport (first- and second-generation biofuels), electricity, and heating. Due to their origin as a cultivated resource, biofuels are closely related to the production of annual crops, while electricity and heating are related to the production of perennial herbaceous and woody crops. Biomass has had a long important use as an energy source for man, and includes that of wood which has been estimated to have been used for hundreds of thousands of years (Goren-Inbar et al, 2004). It is estimated that biomass currently supplies 10-14% of the world's energy (McKendry, 2002). Interest in energy production from crops has resurged in recent years, as evidenced by the recent growth in development of grain based ethanol and biodiesel industries spurred through the interest of replacing transportation fuel and fuel additives with cleaner burning renewable sources.

1.1 Political and economics motivations for using alternative energy

In addition to concerns about the limited supplies of fossil fuels and the effect their use has on the global climate, use of alternative energy has the advantage of reducing the political and economic dependence on imported fossil fuels. In 2007, the United States, with a population of 295 million people, used 542 x 10^9 liter gasoline. This represents approximately 25% of the global oil consumption. The high fuel consumption reflects the large distances traveled by car, truck and airplane due to the large size of the country, the (on average) low population density, the high economic activity, and the limited availability of public transportation. Even though there are active oil fields in primarily Texas, Alaska and the Gulf of Mexico, together accounting for 0.3 x 10^9 liter crude oil in 2006, the vast majority of the oil is imported (587 x 10^9 liter crude). The high fuel consumption combined with the realization that the country depends heavily on imported oil have made energy security a major priority in U.S. politics. The 25-member European Union (EU-25; 460 million people) uses 656 x 10^9 liter crude oil per year. This high level of oil consumption is a reflection of the large and overall affluent population. A considerably amount of oil (17%) is available from EU (primarily Norwegian) oil fields in the North Sea, but the majority of the oil is imported (EC 2005). Japan, another industrialized nation (127 million people) imported 243 x 10^9 l crude oil in 2006 (IEA 2008). China is rapidly increasing its consumption of oil, at an annual rate of more than 10%. This is a reflection of China's large population (1.3 billion people) combined with rapid economic growth. China relies in part on its own oil reserves. In 2007, China imported 142 x 10^9 l crude oil, representing approximately 50% of its total oil consumption. Approximately 16% of the imported oil came from Saudi Arabia. The International Energy Agency (IEA) calculated that in 2006, China and India (1 billion people) consumed 8% and 4% of the world oil, respectively. These countries are projected to increase their use of oil in the future. In 2030 their use of world oil could be as high as 18% and 8%, respectively, but could be several percentage points lower, depending on policies governing population growth, economic development and energy use (IEA 2007).

Without change in energy policy, by 2030 greenhouse gas emissions from China will be as high as the current emission of greenhouse gases from the whole world (Zeng et al. 2008). These data illustrate the dependence on imported oil, which has both economic and political consequences. The oil crisis of the 1970's represents the classic example demonstrating the economic reliance on imported oil is. This crisis resulted from reduced oil exports by oil-producing countries organized in OPEC, and led to a world-wide recession. As global economies have grown since then, and have become even more dependent on international trade, an oil crisis similar to the one in the 1970's would have an even bigger economic impact. The vast majority of oil reserves are in the Middle-East, a region not known for its political stability as illustrated by the ongoing conflicts in that

region. Venezuela and Nigeria, two other major oil-producing countries, generate similar political concerns. As a consequence, being dependent on these countries for energy carries a certain risk.

If liquid transportation fuels would, however, be produced locally, that is, within the country or region where they are needed, and if that involved developing an industry that could produce and process the biological feedstocks and the supporting infrastructure, then the reduced political dependence would have the added benefit of stimulating local economies. The profitability of ethanol production is a function of the price of the feedstock and the price of fossil fuels as well as processing and operation costs (McAloon et al. 2000; Shapouri and Gallagher 2005; Tyner and Taheripour 2007). Fossil fuels play a role in the economics of biofuels, as they supply some of the energy needed to make biofuels, whereas high oil prices (and therefore gasoline prices) make biofuels more competitive as a transportation fuel. As biofuels start to gain economic importance, it may make sense to let market forces determine the price of ethanol. This has the risk that the ethanol industry collapses in case of a sharp decline in oil prices.

1.2 Current legislation on renewable energy

The continued use of fossil fuels to meet the majority of the world's energy demand is threatened by increasing concentrations of CO_2 in the atmosphere and concerns over global warming (Yu et al., 2003; Demirbas et al., 2004). The combustion of fossil fuels is responsible for 73% of the CO_2 production (Wildenborg and Lokhorst, 2005).

In order to meet sustainability goals, in particular the reduction of greenhouse gas emissions agreed under the Kyoto Protocol (and further United Nations Framework Convention on Climate Change), it is therefore essential to find ways of reducing emissions from transportation, heating and industrial processes.

Actions towards this aim have been initiated. In the USA, the Energy Policy Act of 2005 requires blending of 7.5 billion gallons of alternative fuels by 2012 (Gray et al., 2006), and recently the US President, in his state of the union address, set the goal to replace more than 75% of imported oil with alternative fuels by the year 2025 (Herrera, 2006).

The European Commission with the directive 2009/28/CE plans to use 20% renewable energy on the whole energetic consumption in order to reduce 20% CO_2 emission by 2020. In order to reach the ambitious target of a 20% share of energy from renewable sources in the overall energy mix, the EU plans to focus efforts on the electricity, heating and cooling sectors and on biofuels. In transport, which is almost exclusively dependent on oil, the Commission hopes that the share of biofuels in overall fuel consumption will be 10% by 2020.

This Directive establishes a common framework for the production and promotion of energy from renewable sources. EU Member States have to establish national action plans which set the share of energy from renewable sources consumed in transport, as well as in the production of electricity and heating, for 2020. These action plans must take into account the effects of other energy efficiency measures on final energy consumption (the higher the reduction in energy consumption, the less energy from renewable sources will be required to meet the target). These plans will also establish procedures for the reform of planning and pricing schemes and access to electricity networks, promoting energy from renewable sources. Member States are encouraged to exchange an amount of energy from renewable sources using a statistical transfer, and it is also possible to establish cooperation with third countries. However, the following conditions must be met: i) the electricity must be consumed in the Community; ii) the electricity must be produced by a newly constructed installation (after June 2009); and iii) the quantity of electricity produced and exported must not benefit from any other support. Each Member State must be able to guarantee the origin of electricity, heating and cooling produced from renewable energy sources. The information contained in these guarantees of origin is normalised and should be recognised in all Member States. It may also be used to provide consumers with information on the composition of the different electricity sources.

Within June 30 2010, each Member State has to issue its own National Action Plan to implement the EU directive on renewable energy.

In Italy, the EU Directive has been implemented through the "*Decreto Legislativo 3 Marzo 2011, n. 28"*, which set a total energy production from renewable energies of 17% in order to reduce 20% CO_2 emission by 2020 (based on 1990 values), with the use of 10% biofuels in the transport sector. The biofuels share have to follow the obligation of mixing 4% in 2011, 4.5% in 2012, 5% within 2014 to the final 10% in 2020.

For the purposes of demonstrating compliance with national renewable energy obligations and the target for the use of energy from renewable sources in all forms of transport, the contribution made by biofuels produced from wastes, residues, non-food cellulosic material, and ligno-cellulosic material shall be considered to be twice that made by other biofuels (double counting mechanism).

In this context perennial lignocellulosic energy crops may take a leading role in supplying feedstocks for the production of electricity, heating, cooling and biofuels production.

In particular, perennial herbaceous energy crops, well adapted to the climatic and soil conditions of a specific area, could reduce the raw material cost and increase total biomass production. Once established, they do not require annual reseeding. They require lower energy inputs of fertilizer and

pesticide than annual crops. They have a high production of biomass and they can often be grown on marginal cropland (McKendry, 2002; McLaughlin et al., 2002).

However, most herbaceous perennial crops have not been cultivated for biomass production, so their growth yields, compositions, fiber and bioconversion characteristics are not as well-known as traditional agricultural residues (Scordia et al., 2010).

Research and development could further contribute to lower production costs while increasing the biomass yields and efficiencies of the different bioconversion technologies in a sustainable way. In this respect, high priority should be given to the "bio-refinery" concept, finding valuable uses for all parts of the plant, and into second-generation biofuels.

1.3 Reducing global carbon emissions

The atmosphere surrounding Earth consists largely of nitrogen (78%) and oxygen (21%), but contains small amounts of other gases, including argon (Ar), neon (Ne), helium (He), hydrogen (H_2), water vapor, CO_2, CH_4, nitrous oxide (NO_2), ozone (O_3) and man-made halogenated compounds (chlorocarbons, chlorofluorocarbons, hydrofluorocarbons). Among the gases listed above, CO_2, CH_4, NO_2, O_3, and the halogenated compounds are called greenhouse gases, because they retain a portion of the heat from both the incoming solar radiation and from the radiation reflected from Earth's surface. Greenhouse gases absorb and emit energy in the form of infrared (IR) light. The wavelength of IR light is between 800 and 10,000 nm (visible light is in the 400–800 nm range). The energy that is absorbed by the molecule can be emitted in the form of photons, or passed on to other molecules *via* collisions, increasing their kinetic energy, and thus raising the temperature. While the term 'greenhouse gases' generally has a negative connotation, it is important to realize that their presence in Earth's atmosphere has enabled the existence of life as we know it, by raising the temperature 34°C above what would be the normal surface temperature purely based on the position of Earth in the solar system (-19°C). In the discussion about greenhouse gases, CO_2 generally draws most of the attention, because it is the most abundant greenhouse gas (63%), and the greenhouse gas with the fastest rising concentration, even though methane is more effective at trapping heat. The overall effect of the combined effects of population growth and increasing standards of living is that CO_2 emissions have rapidly increased since 1850, raising the CO_2 concentration from 280 ppm prior to the Industrial Revolution to 380 ppm in 2005 (Raupach et al. 2007) (Fig. 1). The potential consequences of global climate include enhanced rates of melting of polar ice in the arctic zone (Arctic Ocean and ice coverage on Greenland) and ice sheets in Antartica, higher sea levels as a result of melting polar ice and expansion of water due to higher temperatures, the potential for more frequent and more severe hurricanes/typhoons across the

Atlantic and Pacific Oceans as a result of warmer seawater, less precipitation in areas that are already arid or semi-arid (the southwestern United States, southern and western Australia, southern Africa, northeastern Brazil, South Europe), less snow on mountain tops and more precipitation in the form of rain, and receding glaciers, resulting in changes in seasonal water levels of snow-fed rivers. In addition, the higher CO_2 levels in the atmosphere will result in higher levels of carbonic acid (H_2CO_3) in the oceans, and therefore in acidification of the oceans and seas. The full consequences of these changes in the ecosystem on human societies are not easy to predict, but scenarios include flooding of low areas, more damage from storms, and shifts in climate zones that will affect agricultural production. This is a result of a combined impact of altered precipitation, changes in temperature, and the occurrence of pests and pathogens that were normally not of concern. Diseases affecting humans may also spread beyond their current boundaries. The acidification of the oceans will impact marine life in ways that are hard to predict because of the still limited understanding of this ecosystem. It is also likely that terrestrial and marine species will become extinct as their habitats change. The expected increase in atmospheric CO_2 concentration will, in part, mitigate these negative effects. It has been proved that C_3 crops positively respond to increased atmospheric CO_2 concentration, increasing net photosynthesis (Ainsworth and Ort, 2010), reducing transpiration and stomatal conductance due to reduced stomatal aperture and density (Drake et al., 1997), reducing the dark transpiration due to a reduction in activity of respiratory enzymes (Ogren, 1984; Bunce, 1994) and increasing water use efficiency, both in C_3 and C_4 species (Olesen and Bindi, 2002). On the other hand, rise in atmospheric CO_2 concentration will lead to higher greenhouse effects and water shortage, especially in southern Europe, which in turn will negatively affect the crop productivity. Recent studies reported that the average crop yield across Europe will change from -3% to 1% due to climate change, from 11% to 32% due to the increase in atmospheric CO_2 concentration, from 25% to 136% due to the advances in technology (Ewert et al., 2005; Rounsevell et al., 2005, 2006). Efficient informatics inclusion and seasonal forecast on agriculture, crop breeding to overcome specific environmental constrains and crop management, including cultivation timing, tillage practices, fertilization practices, new genotypes and varieties introduction, crop protection and assurance will play a key role in strategies of adaption and mitigations to climate change (Olesen et al., 2011). The European Commission has planned to strongly increase the investment on bioenergy crops in short term as important renewable alternatives to replace fossil fuels. Therefore, energy crops in general and perennial no-food ones in particular will enjoy substantial financial support in terms of research, development, crop adaptability and tolerance, as in the case of the Seventh European Framework Programs. As claimed in the European directive 2009/28/EC (European Commission, 2009), European

Commission imposed an obligation to increase the amount of the renewable energy in the EU to 20% of the total energy consumption by 2020, with 10% biofuels in the transport sector in order to reduce the CO_2 emissions by 20% compared to 2005. The EU has estimated that 30.8 million tons of oil equivalent (Mtoe) will be needed to satisfy the obligation of 10% of biofuel sharing in 2020; this would stand at 48 million tons (Mt) of bioethanol or 35 Mt of biodiesel (considering that 1 toe correspond to 1.56 t of bioethanol or 1.14 t of biodiesel) (Eurobserver, 2009). However, the gap of bioethanol or biodiesel production could be reduced through the introduction of high yielding genotypes selected for improved oil or sugar/starch content and/or through the development of high efficient technologies for second generation biofuel (Scordia *et al.*, 2010), as claimed by the European Commission.

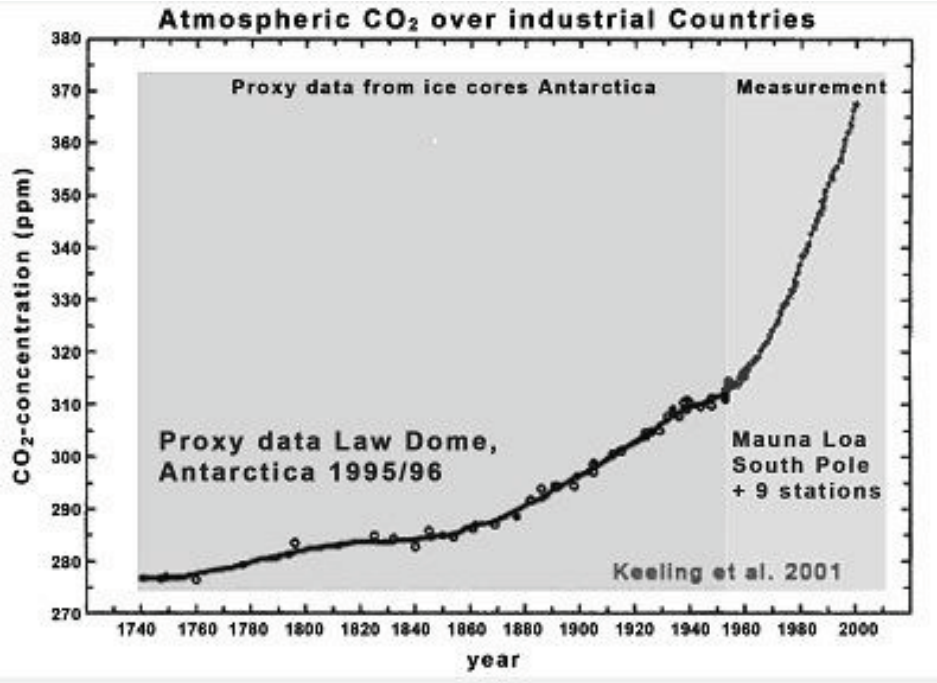

Figure 1 – Atmospheric CO_2 trend from the year 1740 to the year 2000 (based on Keeling et al., 2001 - http://members.shaw.ca/sch25/FOS/IPCC%20CO2.gif)

2 Concerns about bioenergy

Strong global growth and development has increased demand for energy to refine, manufacture and transport products to support the lifestyles of an increasingly developing and globalized world. In recent decades, fossil fuels have become important sources of energy. However, with increasing demand, there has been developing concern over the sustainability of fossil fuels relating to their potential future sources and harmful byproducts of use, specifically large net carbon releases, which has spurred interest towards the use of alternative renewable energy sources. Potential alternatives

are available including wind, solar, hydro, and biomass, all of which are touted to have greater environmental benefits relative to fossil fuels. While the use of bioenergy has many advantages, not everybody agrees that developing bioenergy in general, and ethanol as a transportation fuel in particular, is the best solution for the short or long term. The problems of biofuels have often been discussed in a piecemeal way, but to avoid harm and reap potential benefits, ethical concerns should be part of an integrated analysis that gives a clear policy steer. The Nuffield report identified moral values that are increasingly recognized in debates on global justice, climate change, and environmental ethics: the common good of mitigating climate change, respect for human rights, a commitment to solidarity with vulnerable populations and notions of stewardship, sustainability and intergenerational justice. To apply these values practically to biofuels development, the report authors derived an ethical framework that includes five principles that policy-makers can use to evaluate biofuel technologies and guide policy-making. Biofuels targets have encouraged producers to scale up production rapidly, sometimes developing biofuels in countries with lax regulations (World Bank, Washington, DC, 2010). In some cases, this may have contributed to higher food prices or led to human rights abuses, for example, workers living in near-slavery conditions (Renewable Fuels Agency, 2010; Amnesty International, 2008). Target-based policies need to be more responsive to such unintended consequences and allow for changing the pace of upscaling. The obligation to be responsible stewards of natural resources and ecosystems services for current and future generations is captured in the idea of environmental sustainability (Dobson, 1998). In addition to biodiversity losses caused by land clearing and land-use change (both direct and indirect), biofuel crops can lead to water over-use and pollution through pesticide and fertilizer use.

Climate change is predicted to impose increasing harms, in particular on those most disadvantaged. Thus, climate change mitigation is a vital common good (Nuffield Council on Bioethic). Biofuels are expected to reduce GHG emissions. However, there is considerable uncertainty regarding measurement of emissions, and there are no controls to ensure that imported biofuels offer emissions savings throughout their production life cycle.

Moreover, a global, coordinated response to climate change from the international community should address land-use change directly, with strong international and local measures to prevent destruction of high-carbon stock. Adequate payment for labor has been recognized as a basic human right (Universal Declaration of Human Right) and is a central element of just reward (Nuffield Council on Bioethic). However, producers outside the EU may not abide by these policies, which can lead to overwork and low wage. Policy-makers should consider social impacts of mandated biofuels imports and implement strict requirements with strong audit trails for pay and working conditions that respect vulnerable populations. There may be environmental, political, social, or

economic benefits or burdens that apply only to certain sections of society, leading to questions of distributive justice (Rawls, 1971). For example, investment in biofuels may threaten food security in poor countries, while delivering benefits for climate change and energy security in the developed world. Harms and benefits also depend on crop type and the land it is grown on, as these codetermine GHG emission savings or food versus fuel trade-offs.

2.1 Benefits of using perennial grasses for energy

Perennial grasses have been widely used as fodder crops for centuries, often contributing significantly to energy supply on farms through the use of draft animals. For example, as late as 1920 in the United States, 27 million animals provided traction power on farms and in cities, fuelled by some 35–40 million hectares of grasslands (Vogel, 1996). In the 21st century, perennial grasses may be set for a comeback through a number of different energy conversion pathways. There has been increasing interest in the use of perennial grasses as energy crops in the US and Europe since the mid-1980s. The characteristics which make perennial grasses attractive for biomass production are their high yield potential, the high contents of lignin and cellulose of their biomass and their generally anticipated positive environmental impact. Energy crops are crops which are produced with the express purpose of using their biomass energetically. High contents of lignin and cellulose in their biomass are desirable, especially when they are used as solid biofuels, for two main reasons. First they have a high heating value due to the high content of carbon in lignin (about 64%). Secondly strongly lignified crops can stand upright at low water contents. Therefore their biomass has lower water contents, the biomass can dry 'on the stem' and a late harvest for improved biomass quality is possible (Hartmann, 2001). The biomass of perennial grasses has higher lignin and cellulose contents than the biomass of annual crops. There are many ecological benefits expected from the production and use of perennial grasses. The substitution of fossil fuels or of raw materials based on fossil fuels by biomass is an important contribution to reduce anthropogenic CO_2 emissions. Compared to other biomass sources, like woody crops and other C_3 crops, C_4 grasses may be able to provide more than twice the annual biomass yield in warm and temperate regions because of their more efficient photosynthetic pathway (Clifton-Brown and Jones, 1996). Unlike annual crops, the need for soil tillage in perennial grasses is limited to the year in which the crops are established. The ecological advantages of the long periods without tilling are reduced risk of soil erosion and a likely increase in soil carbon content (Kahle, 2000; Ma et al., 1999). Furthermore, due to the recycling of nutrients by their rhizome systems, perennial grasses have a low demand for nutrient inputs (Christian et al., 1997). Since they have few natural pests, they may also be produced with little or no pesticide use (Lewandowski et al., 2000). Studies of fauna show that due to long-

term lack of soil disturbance, the late harvest of the grasses in winter to early spring and the insecticide-free production, an increase of abundance and activity of different species, especially birds, mammals and insects, occurs in stands of perennial grasses (Jodl et al., 1998; Hoffman et al., 1995). Perennial grasses can therefore contribute to ecological values in agricultural production. They can also function as elements in landscape management and as habitat for different animals. In both the US and in Europe, there are various candidate perennial grasses available which differ considerably in their potential productivity, chemical and physical properties of their biomass, environmental demands and crop management requirements (Tables 2 and 3).

Table 2 – 18 perennial grass species that were screened by the US herbaceous energy crops research program (based on Lewandowski et al., 2003).

English name	Latin name	Photosynthetic pathway
Crested wheatgrass	*Agropyron desertorum* (Fisch ex Link) Schult.	C_3
Redtop	*Agrostis gigantea* Roth	C_3
Big bluestem	*Andropogon gerardii* Vitman	C_4
Smooth bromegrass	*Bromus inermis* Leyss.	C_3
Bermudagrass	*Cynodon dactylon* L.	C_4
Intermediate wheatgrass	*Elytrigia intermedia* [Host] Nevski	C_3
Tall wheatgrass	*Elytrigia pontica* [Podp.] Holub	C_3
Weeping lovegrass	*Eragrostis curvula* (Schrad.) Nees	C_4
Tall Fescue	*Festuca arundinacea* Schreb.	C_3
Switchgrass	*Panicum virgatum* L.	C_4
Western wheatgrass	*Pascopyrum smithii* (Rydb.) A. Love	C_3
Bahiagrass	*Paspalum notatum* Flugge	C_4
Napiergrass (elephant grass)	*Pennisetum purpureum* Schum	C_4
Reed canary grass	*Phalaris arundinacea* L.	C_3
Timothy	*Phleum pratense* L.	C_3
Energy cane	*Saccharum* spp.	C_4
Johnsongrass	*Sorghum halepense* (L.) Pers.	C_4
Eastern gammagrass	*Tripsacum dactyloides* (L.) L.	C_4

Table 3 – Perennial grasses grown or tested as energy crops in Europe (based on Lewandowski et al., 2003).

English name	Latin name	Photosynthetic pathway
Meadow foxtail	*Alopecurus pratensis* L.	C_3
Big bluestem	*Andropogon gerardii* Vitman	C_4
Giant reed	*Arundo donax* L.	C_3
Cypergras, Galingale	*Cyperus longus* L.	C_4
Cocksfoot grass	*Dactylis glomerata* L.	C_3
Tall fescue	*Festuca arundinacea* Schreb.	C_3
Raygras	*Lolium* ssp.	C_3
Miscanthus	*Miscanthus* spp.	C_4
Switchgrass	*Panicum virgatum* L.	C_4
Napier grass	*Pennisetum purpureum* Schum	C_4
Reed canary grass	*Phalaris arundinacea* L.	C_3
Timothy	*Phleum pratense* L.	C_3
Common reed	*Phragmites communis* Trin.	C_3
Energy cane	*Saccharum officinarum* L.	C_4
Giant cordgrass/ Salt reedgrass	*Spartina cynosuroides* L.	C_4
Prairie cordgrass	*Spartina pectinata* Bosc.	C_4

2.2 Why use *Miscanthus*

Miscanthus, like maize, has C_4 photosynthesis. In theory this should increase the efficiency of radiation, nutrient and water utilization (Monteith 1978; Long 1983) above those of C_3 plant species. In practice *M. x giganteus* often exceeded expectations in physiological studies (Beale and Long 1995; 1997; Beale et al. 1999). Naidu et al. (2003) sought the reasons for the high levels of photosynthesis at low temperature. They discovered that *M. x giganteus* could maintain 80% higher photosynthetic quantum yields when grown at 14/11ºC (day/night) than maize. This study showed that the two enzymes involved in photosynthesis, pyruvate orthophosphate dikinase (PPDK) and Rubisco, were unaffected by low temperatures in *M. x giganteus* but were decreased >50% and >30%, respectively, in maize grown under the same conditions. Further *in vivo* experimentation led to the conclusion that maintenance of high photosynthetic rates in *M. x giganteus* at low

temperature, in contrast to *Z. mays*, is most likely the result of different properties of Rubisco and/or PPDK, reduced susceptibility to photoinhibition, and the ability to maintain high levels of leaf absorptance (the ratio of absorbed to incident radiation) during growth at low temperature (Naidu and Long 2004). Recently, an analysis of the temperature effects on fluorescence from *Miscanthus* indicates that *Miscanthus* has an alternative sink to CO_2 assimilation for photosynthetic reducing equivalents. Farage et al. (2006) postulate that oxygen reduction occurs *via* a Mehler reaction, which could act as a mechanism for protection of Photosystem II from photo-inactivation and damage. From the physiological research described above, it would seem that *Miscanthus* productivity would exceed that of maize. This is not always the case, since many agronomic factors impact yield. A recent paper describing side-by-side trials on several candidate energy crop species in Germany showed that a variety of 'energy' maize had higher energy yields per hectare than *M. x giganteus*. These higher yields were achieved, however, at a relatively high input level (Boehmel et al. 2008) and consequently, upon calculation of the energy output:input ratio, *Miscanthus* was far better than maize. Current estimates of the energy ratio for *Miscanthus x giganteus* range between 22 (Lewandowski and Schmidt 2006) and 50 (Lewandowski, pers. comm.), depending on the agronomic methods used. The overall energy ratio is obviously sensitive to the productive lifespan. Current estimates vary between 10 and 30 years (Lewandowski et al. 2000).

To our knowledge there are no continuously monitored trials older than 15 years, making it impossible at present to make complete 'crop life-span' analyses of the energy ratio. In addition to increasing energy ratios, perenniality results in significant environmental benefits. These include erosion control, prevention of leaching (Christian and Riche 1998) and the locking up of more carbon in the rhizosphere (Beuch et al. 2000; Foereid et al. 2004; Hansen et al. 2004). The current limitations with *Miscanthus* are mostly associated with the high establishment costs of sterile triploid genotypes, which must be propagated vegetatively (tissue culture or rhizome division). Considerable progress has been made recently in Europe on reducing the costs of clonal propagation through rhizomes and consequently, costs keep falling each year with increasing planting scales. A further limitation in the eyes of industry is that *Miscanthus* is a grass, and not a tree. Grasses have typically higher ash contents than woody species. In *Miscanthus x giganteus* typical ash contents are 2% depending to some extent on local site conditions and the harvest time. At this level the ash content causes some slagging and fouling of standard wood-burning boilers. Since considerable genetic variation in ash content has been found (Lewandowski et al. 2003), selective breeding will certainly reduce ash content.

2.2.1 Potential contribution of Miscanthus to reducing carbon emissions

Soils are an important sink for the carbon storage in the form of soil organic carbon and reduction of CO_2 in the atmosphere. For example with the sequestration of 1 Mg ha^{-1} of carbon in the soil, the CO_2 emissions could be reduced and ranged from 5% to 15% (Lal 2004). Moreover carbon sequestration could contribute to mitigate drought, salinity stress, and desertification. However it is difficult to quantify the actual amount of carbon added in the soil system by plant roots because the continuous and simultaneous fluxes of carbon compounds between the soil-plant-atmosphere continuum. Clifton-Brown et al. (2004) estimated that the increased production of *Miscanthus* as an energy source could bring about a significant reduction in carbon emissions in the EU. *Miscanthus* could potentially be grown on 10% of agricultural land, resulting in the production of 231 TWh $year^{-1}$ of electricity (9% EU requirement) and a carbon sequestration level of 12 M t C $year^{-1}$. The combination of potential energy production and carbon sequestration from *Miscanthus* growth could realize a carbon mitigation level of 76 M t C/year. In conclusion, Clifton-Brown et al. (2004) proposed that the production of energy crops would contribute substantially to the EU targets for reducing greenhouse gas emissions.

2.2.2 Positive and negative impacts of Miscanthus on the environment

Apart from the use of herbicides in the establishment years (years one to three), *Miscanthus* requires very few agrochemical inputs after establishment. Herbicide application in the establishment years is usually restricted to a single application per year. Once a good crop cover is attained in the second or third year, weed interference is suppressed and there is no need for herbicide application. So far, *Miscanthus* is free from pests, and as such there is no need to use insecticides. Thus, the risk of ground water contamination by agro-chemicals is very low. Of all the crops grown for energy purposes, perennial C_4 grasses such as *Miscanthus* are regarded as the most efficient nitrogen users. This is due to the recycling of nitrogen from year to year through the rhizome system and in the leaf fall. The non-disturbance of the soil, combined with the deep root system results in slow rates of organic nitrogen release and the uptake of nitrogen from deeper soil layers, thereby reducing the risk of nitrogen leaching losses. *Miscanthus* crops do not contribute to phosphorus pollution in water because they are capable of optimum growth in soils with low levels of phosphorus, and also they have low rates of soil erosion. Soil erosion, in particular, is one of the biggest environmental threats in the Mediterranean area as it causes pollution of water bodies, critical losses of water, nutrients, soil organic matter and soil biota from the natural ecosystems. It was estimated that about 10 million hectares a year of cropland is shrinking due to soil erosion. There might be a risk of soil erosion in the first year of planting (e.g. in upland areas) due to wide plant spacing and slow

establishment until complete crop cover. The risk is reduced from the second year onwards. *Miscanthus* has a very long life span (of about 20 years) and can grow up to 4 m in height in some parts of Europe. This may create a visual impact on the rural landscape. Therefore, when selecting sites for *Miscanthus*, one should take account of the landscape aesthetics and public foot path access, as well as local archaeology. There have been some concerns whether *Miscanthus*, as an introduced species, might be an invasive plant. However, this is not a problem because most varieties used for biomass are sterile hybrids and ornamental *Miscanthus* varieties have been around in our gardens for a number of years. In addition, *Miscanthus* is easy to get rid of by harvesting the rhizomes using modified potato harvesters or kill the crop using glyphosate herbicides.

2.2.3 Positive and negative impacts of Miscanthus on ecology

A three-year study on the ecology of perennial grasses has shown that two/three-year-old *Miscanthus* plantations were used as overwintering sites for birds, small-mammals and invertebrates, suggesting immediate benefits to biodiversity (Fig. 2) (Semere and Slater, 2005; 2007). Once established, *Miscanthus* crops compete efficiently with weeds and the closed canopy tends to shade out most vegetation (Semere and Slater, 2005; 2007). The *Miscanthus* crop itself is free from any arthropods; it is the growth of weed flora within the field that encourages the presence of arthropods which in turn leads to an increase in the abundance of small mammals and birds. The most important invertebrate taxa caught using pitfall traps, sweep netting and branch beating in *Miscanthus* fields included *Coleoptera, Hemiptera, Diptera* and *Hymenoptera*. No pest damage to the *Miscanthus* crop was found. Biomass crops are not as biologically diverse as mature woodland or traditional coppice, they do provide suitable habitats for a wide range of birds (skylarks, lapwings, meadow pipits), particularly when situated within intensively farmed land. Tall stands of miscanthus can serve as cover and habitat for small mammals (wood mouse, bank vole, field vole, common shrew, pygmy shrew).

Miscanthus leaves are not palatable to insects, and as such, most of the invertebrate populations are dependent on the weed vegetation within the crop. Therefore, if *Miscanthus* fields are kept weed-free at all times, their effects on invertebrate populations are bound to resemble that of arable crops.

These results were based on newly established *Miscanthus* stands which did not demonstrate any adverse effects to the environment, but the authors concluded that further investigation of more mature plantings would be required to provide a broader view of environmental impact of this energy crop.

Figure 2 – Invertebrate (wasp spider, *Argiope bruennichi*) and chick (stone curlew, *Burhinus oedicnemus*) within *Miscanthus* field (Catania, Italy – August 2013).

3 *Miscanthus*

3.1 Botanical description of *Miscanthus*

The name *Miscanthus* derives from the Greek "*mischos*" (pedicel) referring to its inflorescence that has spikelets borne in pairs with both being pedicellate, and "*anthos*" referring to "flower". *Miscanthus* species are all perennials with erect cane-like stems up to 7 m tall (in *M. lutarioriparius* L. Liu ex Renvoize & S. L. Chen), generally growing from a rhizomatous base, but sometimes tufted. The inflorescence is terminal and consists of a cluster of plumose racemes bearing awned or awnless, paired spikelets. The inflorescence axis may be short and the inflorescence subdigitate with long racemes (as commonly found in *M. sinensis*) or the axis may be long and bear short racemes (as commonly found in *M. floridulus* (Labill.) Warb. Ex K. Schum. & Lauterb.). Taxonomically, *Miscanthus* is classified with several other species of high economic value such maize, sorghum and sugarcane, in the predominantly tropical grass tribe Andropogoneae. Within this tribe it is placed in subtribe Saccharinae (Clayton and Renvoize, 1986; Hodkinson et al., 2002a) which also contains *Eriochrysis* P. Beauv., *Eulalia* Kunth, *Eulaliopsis* Honda, *Homozeugus* Stapf., *Imperata* Cyr., *Lophopogon* Hack., *Microstegium* Nees, *Pogonatherum* P. Beauv., *Polytrias* Hack., *Saccharum* L. (sugarcane) and *Spodiopogon* Trin. *Miscanthus* species are unusual among the Andropogoneae because they possess bisexual paired spikelets (both with hermaphrodite florets). Other Andropogoneae have paired spikelets but, with the exception of a few genera such as *Ischaemum* L. and *Schizachyrium* Ness, one of these is usually male or sterile. *Miscanthus* in a broad sense contains approximately 14-20 species (Hodkinson et al., 1997; Scally et al., 2001), but its genetic limits have been revaluated using molecular phylogenetics (Hodkinson et al., 2002a).

DNA sequence (Hodkinson et al., 2002a) and fingerprinting (Hodkinson et al., 2002b) data showed that many species included in *Miscanthus sensu lato* are more closely allied to other genera than *Miscanthus*. On the basis of these and other recent taxonomic analyses (Chen and Renvoize 2006; Ibaragi 2003; Ibaragi and Oshashi 2004), *Miscanthus sensu stricto* can be defined as containing approximately 11-12 species:

- *M. floridulus* (Labill.) Warb.
- *M. intermedius* (Honda) Honda
- *M. longiberbis* Nakai
- *M. lutarioparius*
- *M. oligostachyus* Stapf.
- *M. paniculatus* (B. S. Sun) Renvoize & S. L. Chen
- *M. sacchariflorus* (Maxim.) Hack.
- *M. sinensis* Anderss.
- *M. tinctorius* (Steud.) Hack.
- *M. transmorrisonensis* Hayata
- The hybrid *M. x giganteus* Greef & Deuter ex Hodkinson and Renvoize
- *Miscanthus sinensis* ssp. *condensatus* (Hackel) T. Koyama

The latter species is sometimes recognized at specific rank as *M. condensatus*. All these species are characterized by a basic chromosome number of 19. The other species previously included in *Miscanthus* are better placed in several other genera including *Diandranthus* L. Liu, *Miscanthidium* Stapf and *Sclerostachya* A. Camus (Hodkinson et al., 2002a) and have differing basic chromosome numbers (Hodkinson et al. 2002c). Three *Miscanthus* species have been identified as having the highest potential for biomass production (Jones and Walsh 2001). These are *M. x giganteus*, *M. sacchariflorus* and *M. sinensis*. *Miscanthus x giganteus* has been wrongly called *M. sinensis* 'Giganteus', *M. giganteus*, *M. ogiformis* Honda and *M. sacchariflorus* var. *brevibarbis* (Honda) Adati. It is sometimes confused with *M. sacchariflorus* as this species is so variable in morphology. Miscanthus x giganteus Greef et Deuter (Greef and Deuter 1993) is an illegitimate name under the rules of the International Code of Botanical Nomenclature (Greuter et al. 2000) because a type was not specified nor a Latin description given (Hodkinson and Renvoize 2001).

Hodkinson and Renvoize (2001) rectified this by providing a type specimen and Latin description and correctly published the name as *Miscanthus x giganteus* Greef et Deuter ex Hodkinson & Renvoize. They chose to keep the species epithet '*x giganteus*' to prevent confusion in the literature but have updated the authority accordingly. Several subspecies of *M. sacchariflorus* and *M. sinensis* have been described and a high number of varieties and horticultural cultivars of these

two species have also been described (Hodkinson et al. 2002b; IPNI 2007). *Miscanthus sacchariflorus* and *M. sinensis* can hybridize (Adati and Shiotani 1962; Hodkinson et al., 2002c) and form a species complex. This complex is considered to be the source of high yielding plants suitable for biomass production. *Miscanthus floridulus* can also achieve high biomass and may be suitable for more southerly regions of the northern hemisphere. *Miscanthus sensu stricto* is native to eastern or south-eastern Asia and presumably originated somewhere in this broad area. Its natural geographic range extends from northeastern Siberia, 50°N in the temperate zone to Polynesia 22°S, in the tropical zone, westwards to central India and eastwards to Polynesia. It is therefore found in a wide range of climatic zones and biomes. For example, in the Taiwanese islands, *Miscanthus* species are widely distributed from the coast up to high mountain areas above 3,000 m in elevation. They are widely adapted to different habitats from agricultural grasslands, dry grassland and even wet, saline, and polluted land (Chiang 1993). Selection in these habitats has resulted in various ecotypes (Chou et al. 2001).

Some *Miscanthus* species such as *M. floridulus* generally grow best at sea level in tropical conditions but others such *M. paniculatus* can tolerate temperate and/or high altitude conditions up to altitudes of up to 3,100 m on dry mountain slopes in Guizhou, Sichuan and Yunnan of China (Chen and Renvoize 2006). The species *M. sinensis* has a number of well-documented, morphologically distinct intra-specific taxa. *Miscanthus sinensis* var. *sinensis* of the Chinese mainland and Japan, has morphological similarity to the related species *M. sinensis* ssp. *condensatus* of Taiwan. Taxa that are distributed in high mountains (var. *transmorrisonensis*), middle-elevation grasslands (var. *formosanus*) and low elevation wastelands (var. *glaber*) in Taiwan have been described (Hsu, 1978). The morphological distinction of *Miscanthus sinensis* taxa (the *M. sinensis* complex) could have been caused by range expansion during the postglacial recolonization. Using DNA sequencing variations to reconstruct the phylogeny of *Miscanthus* is a powerful tool to identify the species or infrageneric taxa (Chou et al., 2001; Hodkinson et al., 2002a; Chiang et al., 2003). *Miscanthus* is a perennial rhizomatous grass which produces a crop of bamboo-like stems annually. Stands of *M. sacchariflorus* in China which are cut annually have remained productive for 30 years since the establishment of a cellulose industry. Stands of *M. sinensis* in Japan are harvested for forage and for thatching up to the present day. However, consistent annual yield series are not available until biomass trials were established in the late 1980's and early 1990's. The basic chromosome number in *Miscanthus sensu stricto* is 19 (Adati and Shiotani 1962) and its chromosomes, like other members of Andropogoneae, are small (1-2 μm) in comparison to some other grass tribes. Meiotic and mitotic metaphase chromosome photographs of M. floridulus, M. sacchariflorus, M. sinensis, and M. x giganteus can be seen in Linde-Laursen (1993), Hodkinson

and Renvoize (2001), and Hodkinson et al., (2002c). Cytogenetic studies indicate that the basic chromosome number 19 could result from the hybridization of two parental lineages with 10 and 9 chromosomes, respectively (Adati and Shiotani 1962), but this hypothesis remains to be adequately tested. *Miscanthus* species range in ploidy from diploid to hexaploid, but are normally diploid or tetraploid (Hodkinson et al., 2001). *Miscanthus sinensis* is normally diploid (Lafferty and Lelley 1994). However, natural and artificially induced polyploids of *M. sinensis* do exist (Matumura et al., 1985). This has resulted in some polyploidy *M. sinensis* varieties such as the triploid variety known as "*Goliath*". *Miscanthus sacchariflorus* is commonly tetraploid, but there are examples of the whole range of ploidy up to hexaploid in this species (Hodkinson et al., 1997). The highly productive *M. x giganteus* is an allopolyploid hybrid containing genomes from *M. sacchariflorus* (as maternal parent) and *M. sinensis* (Hodkinson et al., 2002c). It could have originated from a cross between an allotetraploid *M. sacchariflorus* and a diploid *M. sinensis* (Greef and Deuter 1993; Linde-Laursen 1993; Hodkinson et al., 2002c).

3.2 Agronomic characteristics and bioprocessing

In the wild, *Miscanthus* reproduces through seeds and spreads through lateral rhizomatous creep. In disturbed environments new clonal plants will result from splitting rhizomes and stems bases. To date, field establishment of *Miscanthus* through direct sowing has been found unreliable in Europe. This may be due to relatively high thermal requirements for seed germination (typical of a tropical species) and very low seed weight, which means the seed has very little reserve carbohydrates to sustain germination in sub-optimal environments. In Europe, most experimental work has concentrated on *M. x giganteus* and therefore the agronomic methodologies are mainly developed for this clone. In brief, the rhizomes are harvested in late winter from mature plants. The rhizomes are divided into pieces using semi-automatic methods to produce propagules between 15 and 50 g fresh weight. Post division, these are kept refrigerated to prevent root growth which would result in the rhizomes clumping together. In the UK efficient machinery has been developed to plant rhizomes at rates above 1 ha h^{-1} at a densities varying between 1 and 2 plants m^{-2} into a well-produced tilth (as is standard for sowing cereals). After planting, weeds are typically controlled by the application of a soil-acting herbicide to prevent the germination of weed seedlings. Depending on the weed burden in the soil, it would be normal to apply a further herbicide application pre-emergence in the second year following establishment. The local site conditions and management have a strong impact on the establishment success. At many locations substantial yields can be harvested after the second growing season. In northern Europe the yield building phase lasts typically 3-4 years as the rhizome biomass increases and growth becomes more vigorous. Current

practice is to harvest in spring time when the plant has had sufficient time to senesce, translocate nutrients and dry out (Jorgensen 1997). Practical moisture contents at harvest vary from 20-50% depending on local climatic conditions, genotype and ripening time before harvest. Where needed, accelerated drying in the swath has been achieved by mowing with a mower that breaks the stems every 10 cm and these break points release trapped water.

3.2.1 Site preparation and planting

To prepare the soil for planting, ploughing to 20-30 cm depth is recommended. Harrowing shortly before planting reduces competition from weeds. The young *miscanthus* plants from micro- or rhizome propagation are frost-sensitive and, therefore, should be planted in spring when no more frost (< -3°C) occurs. Planting densities in various trials have ranged from 1 to 4 plants m^{-2}. Advantages of a higher planting density include a higher yield in the first 2-5 years, but as this yield increase does not compensate for higher planting costs, a density of one plant per square metre is recommended. Mechanical propagation may result in a variable degree of emergence (around 70%), but this does not seem to be a problem since stand density levels out after a few years. In general, irrigation of newly planted *miscanthus* during the first growing season improves establishment rates.

3.2.2 Fertilization

Since *Miscanthus* did not respond to N fertilization on several sites in Europe it was concluded that N fertilization is necessary mainly on soils with low N contents.

At locations with sufficient N mineralization from soil organic matter N fertilization can be avoided or limited to 50-70 kg ha^{-1} yr^{-1}. The overall nutrient requirements for N, phosphorus (P) and calcium (Ca) are about 2-5, 0.3-1.1 and 0.8-1.0 kg per t of dry matter (Clifton-Brown and Lewandowski, 2001) and for K 0.8-1.2 kg (Lewandowski, 2000).

3.2.3 Propagation

The emerging *Miscanthus* industry is currently relying on tissue culture of initial stocks of very high yielding genotypes (usually triploid hybrids) selected from the breeding program. This is followed by rhizomatous propagation to establish near homogenous fields. These methods are expensive, and high yields and high fossil fuel prices are required to make this economically viable.

Establishment from seed is an attractive option, because it has the potential to lower costs considerably. Christian et al. (2005) found that whilst direct seeding (they tested encapsulation, but this did not improve on naked seed) was successful in terms of established plant population, the

methods require further evaluation and refinement before they can be regarded as alternatives to current commercial methods. More effort is needed to identify genotypes and climates where seed propagation is a viable option

3.2.4 Crop protection

In the year of planting, *miscanthus*, competes poorly with weeds, so weed control is needed, either mechanical or chemical. In tests of different herbicides, it was found that those suitable for use on maize or other cereals can be used on *M. x giganteus* (Serafin F., 1995). Once the crop is well established (from year two or three onwards), weed control is no longer necessary (Thiemann R., 1995). To date, there are no reports of plant diseases significantly limiting production, but the crop is known to be susceptible to *Fusarium* (Thinggaard K., 1997), to Barley Yellow Dwarf Luteovirus (Christian DG., 1994) and to *miscanthus* blight (*Leptosphaeria* sp.) (O'Neill and Farr, 1996).

3.2.5 Flowering Time

There is considerable genotypic variation in flowering time in *Miscanthus*. Early flowering shortens the effective length of the growing season, thus reducing the quantity that a particular genotype can produce. At many northern sites *M. x giganteus* does not flower before the onset of the autumn frosts. Where flowering does not occur before the autumn frosts, the onset of senescence and remobilization of nutrients appears less efficient. Which can result in higher ash contents. Higher ash contents are naturally associated with higher offtakes of growth elements such as nitrogen (N), phosphorus (P) and potassium (K). Late flowering has also been associated with higher over-winter losses of plants in the first year following planting.

3.2.6 Harvest frequency and time

Miscanthus can be harvested only once a year since multiple cutting would over-exploit the rhizomes and kill the stands. The harvest window depends on the local conditions and is between November and March/April. The later the harvest can be performed, the more the combustion quality improves since the moisture content and the mineral contents decrease; however, there is a trade-off, since the biomass yield decrease as well. For economic reasons a late harvest at a water content lower than 30% is recommended because the costs for harvesting and drying of the biomass are increasing with the water content (Huisman W., 1998; Van der Heuvel, 1995).

3.2.7 Biomass production

Species currently tested for biomass production include *M. sinensis*, *M. sacchariflorus* and *M. x giganteus*. Because *Miscanthus* is a perennial grass, the first year concerns establishment of the crop, biomass production only being possible during subsequent years. The lifetime of the crop is estimated at between 20 and 25 years (Lewandowski et al., 2003), during which *Miscanthus* biomass is produced during two phases: a yield-building phase, which in *M. giganteus* lasts for two to five years, depending on climate and plant densities, and a plateau phase where the yield is maintained (Clifton-Brown et al., 2000, 2001b; Christian et al., 2008). Yield is very low during the first year (less than 10 t ha^{-1} for *M. giganteus*) but these figures are usually not known as the grass is not harvested. During subsequent years, peak yields are obtained in the autumn, at the full plant flowering phase (Cosentino et al., 2007) and then decline through the winter due to leaf loss. Harvestable yields in the spring are 27% - 50% lower than in the autumn (Clifton-Brown et al., 2001b; Cadoux et al., 2008; Himken et al., 1997; Jorgensen et al., 2003b; Richter et al., 2008). *M. sinensis* is native to eastern Asia, and cited to range from subtropical to subarctic environments (Numata, 1979). It is often classified as a tight clumping grass with little rhizome spreading.

It is a fertile diploid species, and accessions in Europe have demonstrated significant genetic diversity within the species (Hodkinson et al, 2002a). In biomass tests in Europe it has displayed greater winter hardiness than other species, providing more consistent production in cooler climates, but is generally lower yielding than *M. x giganteus* species where both survive (Clifton-Brown et al, 2001a). In central Europe, third year dry matter yields of *M. sinensis* hybrids, defined as crosses between two *M. sinensis* genotypes, or a *M. sacchariflorus* and *M. sinensis* cross, ranged from 6.5-17.7 t ha^{-1} in England to 10.3-20.0 t ha^{-1} in Germany while third year dry matter yields of *M. sinensis* from collections in Denmark and Sweden originally sourced from Japan ranged in yield from 4.6-10.9 t ha^{-1} dry matter in England, and 9.1-12.8 t ha^{-1} dry matter in Germany (Clifton-Brown et al, 2001a). *M. sacchariflorus* commonly exists as a diploid or tetraploid, and is native to warmer regions of east-central Asia (Deuter, 2000). Genotypes used in biomass testing have been characterized by relatively loose, spreading clumps with few, tall culms associated with long, thick rhizomes (Clifton-Brown et al, 2001a). Biomass testing in Europe has been limited to one genotype, making conclusions about *M. sacchariflorus*' biomass potential difficult. For the genotype used, winter hardiness appears to be lower than *M. sinensis* with winter losses of 50% and 67% in Sweden and Denmark respectively, much higher than all *M. sinensis* genotypes tested which ranged from 1-16% across the same locations (Clifton-Brown et al, 2001a). In regions where winter survival was high, third year yields for the *M. sacchariflorus* genotype tested were generally moderate, ranging from 11.1 t ha^{-1} in England to 12.6 t ha^{-1} in Germany; less than *M. x giganteus*

but higher than *M. sinensis* for these same regions. Irrigated yields in Portugal however were very high and comparable to *M. x giganteus*, averaging 35 t ha^{-1} by third year. Similar to *M. sinensis*, *M. sacchariflorus* can pose some invasiveness risk as it has been observed to set fertile seeds even when grown outside of its native region, and has been characterized by having more vigorous rhizome growth relative to other *Miscanthus* species such as *M. x giganteus* which has more bunch-like growth habits (Meyer and Tchida, 1999). While results of the genotype currently published in literature do not appear to make *M. sacchariflorus* a strong candidate for biomass production, interest in this species still exists given its genetic contribution towards *M. x giganteus*. *M. x giganteus* is commonly cited as a strong candidate biomass species, as its sterile nature limits its invasiveness risk when introduced in new regions. It was first introduced in Europe from Japan in the 1930's (Linde-Laursen, 1993). It is believed to have originated from a natural overlapping region of both species in east-central Asia where it displays growth characteristics balanced between *M. sinensis* and *M. sacchariflorus*. A review of autumn yields in Europe showed that the maxima from *M. giganteus* were obtained in France in Lusignan and Grignon under irrigated and fertilised conditions: being, respectively, 49 and 42 t DM ha^{-1} (Clifton-Brown et al., 2004).

These yields ranged from 15 to 25 t ha^{-1} without irrigation. For winter yields of *M. giganteus*, the highest non-irrigated yields were found to be 15-19 t DM ha^{-1} during the trials performed by the Miscanthus Productivity Network that involved 15 European sites. Yields ranged from 7 to 26 t DM ha^{-1} following the third growing season, with some of the trial crops being irrigated (Clifton-Brown et al., 2001b). Outside this network, higher productivity was reported in Central and Southern Europe, but irrigation was always required (Clifton-Brown et al., 2001b; Cadoux et al., 2008).

3.3 End uses of *Miscanthus*

Miscanthus, can be valorized by thermal, chemical or biochemical routes. Thermal conversion processes use heat as the dominant mechanism to convert biomass into another chemical form. The basic alternatives of combustion, torrefaction, pyrolysis, and gasification are separated principally by the extent to which the chemical reactions involved are allowed to proceed (mainly controlled by the availability of oxygen and conversion temperature).

A range of chemical processes may be used to convert biomass into other forms, such as to produce a fuel that is more conveniently used, transported or stored, or to exploit some property of the process itself. Many of these processes are based in large part on similar coal-based processes, such as Fischer-Tropsch synthesis, methanol production, olefins (ethylene and propylene), and similar chemical or fuel feedstocks. In most cases, the first step involves gasification, which step generally is the most expensive and involves the greatest technical risk. Biomass is more difficult to feed into

a pressure vessel than coal or any liquid. Therefore, biomass gasification is frequently done at atmospheric pressure and causes incomplete combustion of biomass to produce a combustible gas consisting of carbon monoxide, hydrogen, and traces of methane. This gas mixture can provide fuel for various vital processes, such as internal combustion engines, as well as substitute for furnace oil in direct heat applications. Conversion of biomass to biofuel can also be achieved via selective conversion of individual components of biomass. For example cellulose can be converted to intermediate platform chemical such a sorbitol, glucose, hydroxymethylfurfural, etc. These chemical are then further reacted to produce hydrogen or hydrocarbon fuels. As biomass is a natural material, many highly efficient biochemical processes have developed in nature to break down the molecules of which biomass is composed, and many of these biochemical conversion processes can be harnessed. Biochemical conversion makes use of the enzymes of bacteria and other microorganisms to break down biomass. In most cases, microorganisms are used to perform the conversion process: anaerobic digestion, fermentation, and composting.

As mentioned above, *Miscanthus,* as a low-moisture-content lignocellulosic biomass, can be valorized by thermochemical routes. The thermochemical routes with the main final products and operating conditions are presented in Fig. 3. They are ranked from left to right as a function of the O_2 supply to the reactor. From left to right the oxygen supply decreases. Figure 4 sums-up a simplified picture of the two main routes for chemicals and fuels production from the thermal conversion of biomass. The first route is gasification followed by Fischer-Tropsch synthesis that needs large scale plants (Larson et al., 2009; Ng KS and Sadhukhan, 2011). Large-scale plants could be not adapted to the biomass supply chain without energetic densification of biomass by pyrolysis before its long distance transport (Raffelt et al., 2006). The second route is biomass fast pyrolysis or liquefaction followed by bio-oils up-grading (HDO) in the actual-modified refineries. Biomass fast pyrolysis could even be conducted in the energy crops or forests by mobile pyrolysis reactors (Badger and Fransham, 2006).

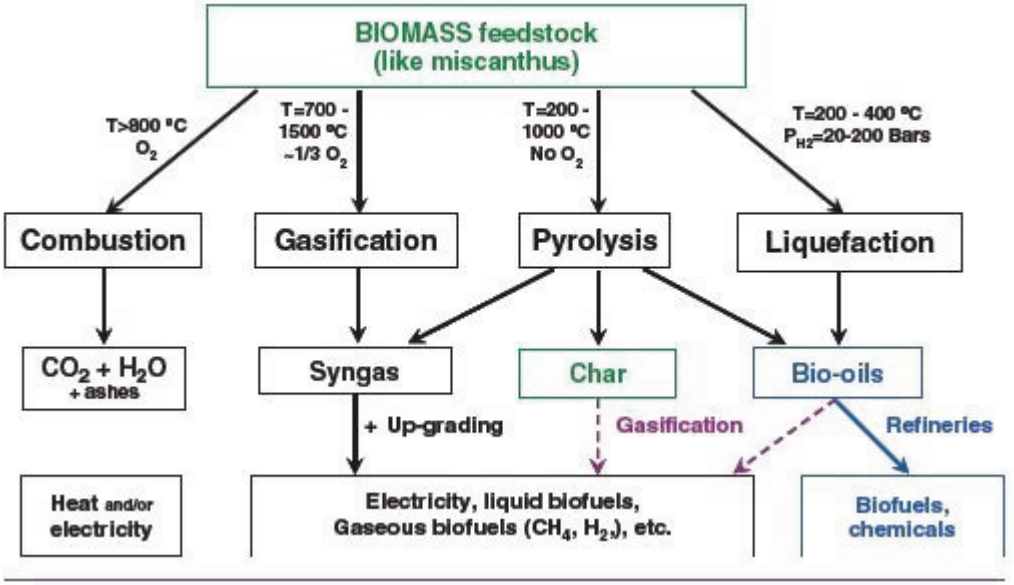

Figure 3 – Main thermo-chemical routes for *Miscanthus* valorization. T refers to the temperature of the reactor but not of the solid biomass decomposition in the reactors (based on Brosse et al., 2012).

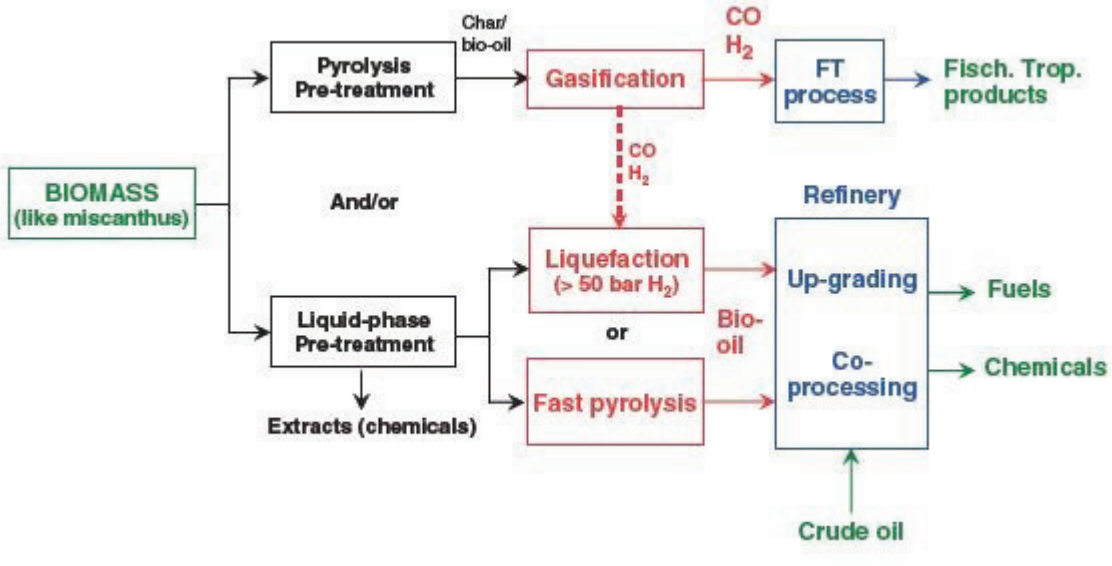

Figure 4 – Simplified scheme of the main routes for fuels and chemicals productions from *Miscanthus* thermal conversion (based on Brosse et al., 2012).

4 Response of *Miscanthus* to stress conditions

Few studies have been devoted to stress conditions, the important one being the European Miscanthus Improvement Project which was designed to broaden the genetic base, test genotypes and develop breeding methods (Lewandowski and Clifton-Brown, 2000). It contributed to developing new screening techniques to determine genotypic variability for traits such as the response to effects of low temperatures, frost tolerance, mineral content and biomass yield (Lewandowski and Clifton-Brown, 2000). Another project, the *Miscanthus* Productivity Network, studied the limitations of low temperatures and other abiotic factors on the growth of *M. giganteus* under European climatic conditions (Walsh, 1998). Studies on the use of environmental resources have focused on the effect of water availability (Christian and Haase, 2001; Clifton-Brown et al., 2002; Cosentino et al., 2007) and nitrogen availability (Lewandowski et al., 1995; Lewandowski and Kicherer, 1997; Lewandowski and Kauter, 2003; Christian et al., 2006; Lewandowski and Schmidt, 2006; Christian et al., 2008).

4.1 Mediterranean environment

The Mediterranean climate is characterized by no freezing temperatures in winter and dry summers. It is the climate typical of the lands in the Mediterranean Basin, and is a particular variety of subtropical climate. The lands around the Mediterranean Sea form the largest area where this climate type is found, but it also prevails in much of California, in parts of Western and South Australia, in southwestern South Africa, sections of Central Asia, and in parts of central coastal Chile (Fig. 5). During summer, regions of Mediterranean climate are dominated by subtropical high pressure cells, with dry sinking air capping a surface marine layer of varying humidity and making rainfall impossible or unlikely except for the occasional thunderstorm, while during winter the polar jet stream and associated periodic storms reach into the lower latitudes of the Mediterranean zones, bringing rain, with snow at higher elevations. As a result, areas with this climate receive almost all of their precipitation during their winter season, and may go anywhere from 4 to 6 months during the summer without having any significant precipitation. The majority of the regions with Mediterranean climates have relatively mild winters and very warm summers. However winter and summer temperatures can vary greatly between different regions with a Mediterranean climate. Because most regions with a Mediterranean climate are near large bodies of water, temperatures are generally moderate with a comparatively small range of temperatures between the winter low and summer high (although the daily range of temperature during the summer is large due to dry and clear conditions, except along the immediate coasts). Temperatures during winter only occasionally fall below the freezing point and snow is generally seldom seen. In the summer, the temperatures

range from mild to very hot, depending on distance from a large body of water, elevation, and latitude.

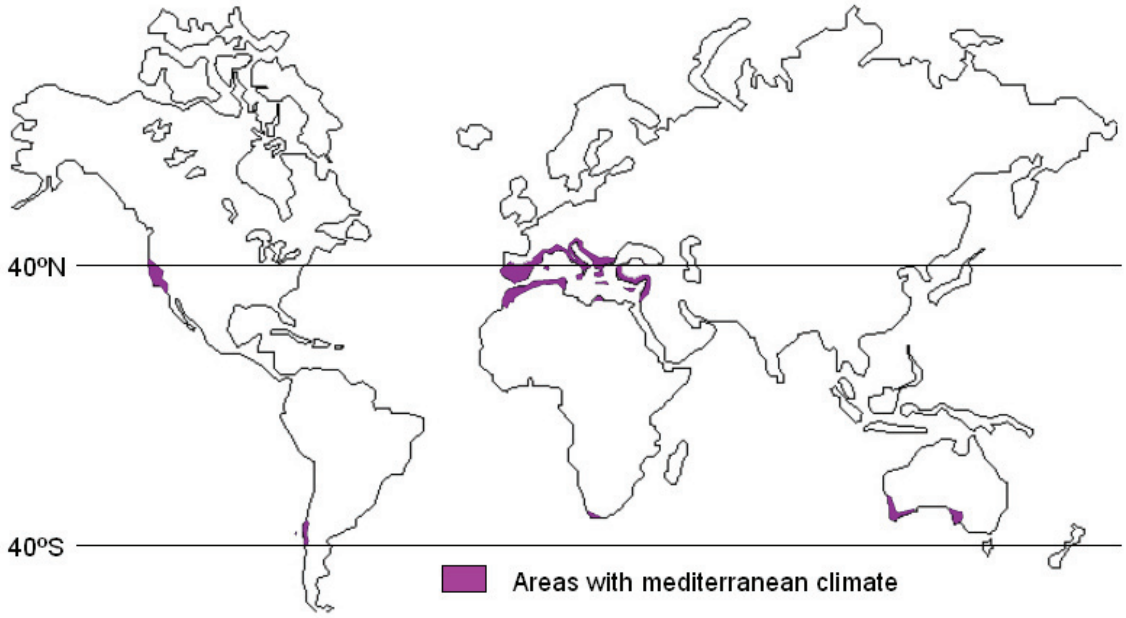

Figure 5 – Areas with Mediterranean climate

4.2 Morphological and physiological adaptations of plants to the Mediterranean environment

Xerophyte (from Greek 'xero' dry, 'phuton' plant) is a group of plant that has adapted to survive in an arid environment. Xerophytic plants may have similar shapes, forms, and structures and look very similar, even if the plants are not very closely related, through a process called convergent evolution. Xerophytic plants can have less overall surface area than other plants, so reducing the area that is exposed to the air and reducing water loss by transpiration. Xerophytes can have smaller leaves or fewer branches than other plants. Other xerophytes may have their leaves compacted at the base, as in a basal rosette, which may be smaller than the plant's flower. Some xerophytes have tiny hairs on their surface to provide a wind break and reduce air flow, thereby reducing the rate of evaporation. The color of a plant, or of the waxes or hairs on its surface, may serve to reflect sunlight and reduce evaporation. Some plants can store water in root structures, trunk structures, stems, and leaves. Tiny pores on the surface of a xerophytic plant called stomata may open only at night, so as to reduce evaporation. Plants may secrete resins and waxes (epicuticular wax) on their surfaces, which reduce evaporation. Plants may drop their leaves in times of dryness (drought deciduous), or modify the leaves produced so that they are smaller. During dry times, xerophytic plants may stop growing and go dormant, change the kind of photosynthesis, or change the allocation of the products of photosynthesis from growing new leaves to the roots. Seeds may be

modified to require an excessive amount of water before germination, so as to ensure a sufficient water supply for the seedling survival.

4.3 Traits of interest in *Miscanthus* in Mediterranean environment

There is big concern for farming systems in the Mediterranean Area. The Mediterranean climate is in fact characterized by hot and dry summers, and most of the global warming models show that the water supply will be much lower and the air temperatures significantly higher in short term, especially during the summertime (Rosenzweig and Tubiello, 1997; Metzger et al., 2005; Black, 2009). This poses serious threats for several conventional crops, particularly in marginal areas. Besides that, there is a growing general awareness and consensus among politicians and farmers on the need to reduce the environmental loads and the energy inputs of farming systems through the introduction of novel species, new insights in plant physiology and biotechnology, optimization of agronomic inputs and natural resources, primarily water. Therefore, alternative crops with high tolerance to hot temperatures and limited water supplies have to be urgently identified, adapted or developed. In general, perennial grasses are drought resistant crops and recently have been attracting growing interest due to their extensive environmental benefits both at global and agricultural community-scale. Compared to traditional row crops, perennial grasses generally require lower energy inputs (fertilizers, pesticides, etc.), can be grown on marginal cropland and provide benefits in terms of soil structure and stability (e.g. reduced soil loss, erosion and runoff), soil quality (e.g. increase in soil fertility, organic matter and nutrient retention) and biodiversity (e.g. cover for native wildlife). The cultivation of perennial grasses has the potential to provide a range of benefits, like surviving over prolonged dry periods, acting as carbon sinks and filter systems for removing agrochemicals from water before these pollutants reach surface and/or groundwater bodies.

4.3.1 Heat stress

Miscathus has been studied for light, drought, nutrient and cold temperature responses, but has never before been studied under realistic increased temperatures. It was noted that some genotypes responded differently to increased temperatures, producing a dwarf phenotype. Most C_4 plants are not able of photosynthesising at temperatures below about 12°C. Controlled environment studies showed that *M. x giganteus* threshold for impairment of the photosynthetic apparatus lies between 8 and 12°C. Leaf photosynthesis in *M. x giganteus* continues down to a temperature of <5°C, while plants can form photosynthetically competent leaves down to 8°C and photosynthetic capacity is unaffected by growth temperatures down to 12°C. This suggests that the threshold for

photosynthesis and the development of the photosynthetic apparatus is 3-5°C below the threshold of other C_4 plants, which is in contrast to other closely related C_4 species utilizing the NADP-malic enzyme (NADP-ME) pathway. When *M. x giganteus* was grown in climates where temperatures are often >30°C, the plant is short stature. C_4 plant species have a higher temperature optimum for photosynthesis than C_3 plants due to the operation of a CO_2-concentrating system that inhibits Rubisco oxygenase activity (Berry and Björkman, 1980; Edwards and Walker, 1983). In C_3 plants, inhibition of net photosynthesis (Pn) at moderately high temperatures has usually been ascribed to an increase in the ratio of Rubisco oxygenase: Rubisco carboxylase activities. As temperature increases, the ratio of dissolved O_2/CO_2 and the specificity of Rubisco for O_2 increase, thus favoring oxygenase activity (Monson et al., 1982; Jordan and Ogren, 1984; Sage and Sharkey, 1987) and resulting in inhibition of Pn. As a consequence, when C_3 plants are exposed to high CO_2 and/or low O_2, i.e. conditions that reduce oxygenase activity, the temperature optimum for Pn is increased (Berry and Björkman, 1980; Edwards and Walker, 1983). For C_3 and C_4 plants, the temperature range for optimum Pn is broad, and at temperatures above this range, Pn decreases (Edwards and Walker, 1983). Temperature-induced decreases in Pn in C_3 species are closely associated with inactivation of Rubisco (Law and Crafts-Brandner, 1999), and when the activation state of Rubisco and gas solubilities are taken into account, the rate of Pn at any given temperature or level of atmospheric CO_2 or O_2 reflects Rubisco kinetics (Crafts-Brandner and Salvucci, 2000). The temperature-induced decrease in Rubisco activation, and the associated inhibition of Pn, in C_3 plants results in large part from the inability of Rubisco activase activity to keep pace with a faster rate of Rubisco inactivation as temperature is increased (Crafts-Brandner and Salvucci, 2000). Activase kinetics and physical denaturation of activase appear to be causative factors contributing to the decrease in Rubisco activation at high temperature (Crafts-Brandner and Salvucci, 2000; Salvucci et al., 2001). Although C_4 plants have a higher temperature optimum than C_3 plants, Pn is usually inhibited when leaf temperatures exceed about 38°C (Berry and Björkman, 1980; Edwards and Walker, 1983). Although the C4 photosynthetic system is more complex than the C3 system, the ultimate limitation to CO_2 fixation for both photosynthetic types is the activity of Rubisco (von Caemmerer et al., 1997; Edwards et al., 2001). Low temperature effects on C_4 photosynthesis have been frequently examined (Labate et al., 1991; Long, 1998). Studies pertaining to the effects of high temperature on C_4 photosynthetic metabolism are less common, and can be hypothesized that high temperature may inactivate Rubisco and limit Pn in a similar manner as for C_3 plants. However, it seemed feasible that heat stress might also impact C_4-specific processes such as fixation of CO_2 by phospho*enol*pyruvate (PEP) carboxylase, shuttling of C_4 acids from mesophyll to bundle sheath cells, or energy balance due to the differential localization of PSII and the Calvin cycle.

4.3.2 Drought stress

Miscanthus being a C_4 plant has higher water use efficiency than plants with C_3 photosynthesis (Beale et al. 1999). However, long periods of drought are serious since they limit the amount of biomass that can be produced and in very extreme cases can lead to plant death when soils are shallow or sandy. The identification of drought-tolerant genotypes that can produce more biomass under water stress conditions remains an essential component in the improvement of *miscanthus* (Clifton-Brown and Lewandowski, 2000a). Irrigation exerts an important influence on yield when *M. giganteus* is grown at sites with a poor water supply (Christian and Haase, 2001). Several studies, carried out throughout Europe, on the use of environmental resources have focused on the effect of water availability and nitrogen availability. Under varying levels of nitrogen inputs (between 60 and 240 kg N ha^{-1}), biomass production increased by between 25% and 84% with irrigation. The difference in yield between well-irrigated plots (100% of evapotranspiration restored) and less irrigated plots (25% of evapotranspiration restored) was higher in autumn than in winter: +84% for an autumn harvest against 26% for a winter harvest (Beale et al., 1999; Ercoli et al., 1999; Cadoux et al., 2008 and Cosentino et al., 2007). These marked differences between rainfed and irrigated yields could be related to the period during which the drought occurred. Richter et al. (2008) determined the main growing season as the period of susceptibility to drought in miscanthus. In terms of biomass components, water availability does not affect shoot production (Christian and Haase, 2001; Cosentino et al., 2007; Cadoux et al., 2008). However, this lack of effect is probably because shoot production takes place during the period of high soil water availability (at the beginning of the growing period). By contrast, the number of stems is more closely dependent on planting densities than on irrigation rate, i.e. water availability. Plant height is not influenced by water availability at the beginning of the growing period. Nevertheless, a reduction in water availability towards the end of the growing period was found to markedly influence plant height (Christian and Haase, 2001; Cosentino et al., 2007; Cadoux et al., 2008).

Irrigated plants were 49% taller than those without irrigation. A similar trend was observed for the leaf area index (Cosentino et al., 2007; Cadoux et al., 2008). Irrigation caused a 77% increase in the leaf area index compared with no irrigation. However, this effect was not reported by Christian and Haase (2001). In *Miscanthus x giganteus*, Cosentino et al. (2007) also observed a one month difference in the flowering date between irrigated and rainfed treatments. During studies to compare *M. giganteus*, *M. sinensis* and *M. sacchariflorus* under controlled conditions, a reduction in leaf area was observed in *M. giganteus* and *M. sacchariflorus* but not in *M. sinensis* under water stress (Clifton-Brown and Lewandowski, 2000a; Clifton-Brown et al., 2002). However, the leaf area of *M. sinensis* was smaller before the water stress was applied.

Furthermore, the water needs of this species may be less than in the other species. The authors concluded that *M. sinensis* might be less sensitive to water stress. In addition, *M. sacchariflorus* and *M. giganteus* presented an increase in the senescence of green leaf area, while *M. sinensis* presented a lack of senescence under all treatments. This experiment suggested that the "stay green" mechanism in *M. sinensis* may be related to stomatal closure with a low soil moisture content. Photosynthetic activity contributes to adapting miscanthus to drought. Weng (1993) showed that both stomatal and non-stomatal photosynthesis factors were affected by water deficit, and suggested that genotypes displaying the highest degree of osmotic adjustment (OA) were the best at maintaining photosynthetic activity under water deficit. Nevertheless, no differences in OA were observed among *M. sinensis*, *M. sacchariflorus* and *M. x giganteus* under severe water deficit, although *M. sinensis* was more tolerant to water stress (Clifton-Brown et al., 2002). Leaf conductance was markedly reduced in *M. sinensis*, even under mild water stress, so that a completely green leaf area was maintained throughout the experiment.

4.3.3 Composition

Miscathus is classified as lignocellulosic species. Cellulose, hemicellulose, and lignin are the three main components in *Miscanthus* lignocellulosic feedstock (Fig. 6), with the relative proportions of the three dependent on the material source (Reddy and Yang, 2005). Lignocellulosic cell wall in mainly represented by cellulose (30-50%), hemicellulose (20-40%) and lignin (10-30%), with pectin, resin lipids and ashes closing the balance (Wyman et al., 1994). Cellulose, the main structural component of plant cell walls, is a long chain of glucose molecules, linked to one another primarily by glycosidic bonds (van Wyk, 2001). Hemicellulose, the second most abundant constituent of lignocellulosic biomass, is not a chemically well-defined compound but rather a family of polysaccharides, composed of different 5- and 6-carbon monosaccharide units, that links cellulose fibres into microfibrils and cross-links with lignin, creating a complex network of bonds that provide structural strength (van Wyk, 2001). Finally lignin, a three-dimensional polymer of phenylpropanoid units, can be considered as the cellular glue providing the plant tissue and the individual fibres with compressive strength and the cell wall with stiffness (Del Rio et al., 2007), in addition to providing resistance to insects and pathogens. The composition of cellulose, hemicellulose and lignin in *Miscanthus* plays a crucial role in optimizing strategies for biochemicals, biopower, and biofuels. Harvesting *Miscanthus* in February generally leads to higher cellulose, hemicelluloses and lignin contents and lower ash content for most *Miscanthus* species. The major elemental composition based on dry matter in *Miscanthus* includes 47.1 to 49.7 % C, 5.38 to 5.92 % H, and 41.4 to 44.6 % O, which reflects the variation of three major lignocellulosic

components to some extent (Lewandowski and Kicherer, 1997; Hodgson et al., 2011; Lygin et al., 2011). Mineral content including K, Cl, N, and S plays an important role in affecting biomass combustion quality. K and Cl enrichment can reduce ash melting point and cause corrosion issue. High concentrations of N and S can result in emissions of NO_x and SO_2. Mineral content varies significantly depending on different genotypes, harvest time, locations, and even fertilization. Delayed spring harvest time benefits the *Miscanthus* combustion quality due to relatively lower K, Cl and N elemental level (Lewandowski et al., 2003). Ash concentration of *Miscanthus* can affect combustion quality especially heating value. Ash consisting of 20 to 40% SiO_2, 20 to 25% K_2O, 5% P_2O_5, 5% CaO and 5% MgO is closely related to silt and clay content of the soil, its lower melting point brings about slag and causes agglomeration during thermal process thereby lowers combustion efficiency. The optimum composition of harvested biomass depends on the application to which the biomass is to be put. For combustion it is essential to minimize the moisture, ash and mineral contents because these reduce boiler efficiency (Lewandowski and Kicherer 1997). For fermentation the organic composition (e.g. lignin, cellulose, hemicellulose) will change the total efficiency of conversion from solid to other fuel formats. The composition of the harvested biomass is influenced by the harvest time, the genotype, the fertilizer inputs and weather in the months preceding harvest. Harvest time is probably the most important factor, since ripening ensures the death and detachment of leaves (which contain much ash) and allows translocation of the nutrients to the overwintering rhizome. Ripening has the benefit of increasing the overall nutrient use efficiency by retaining nutrients within the rhizosphere. Fertilization with a chloride-rich fertilizer (such as potassium chloride) had the highly undesirable effect of raising the Cl content (Clifton-Brown 2007), which upon combustion would lead to acidification and reduced boiler life.

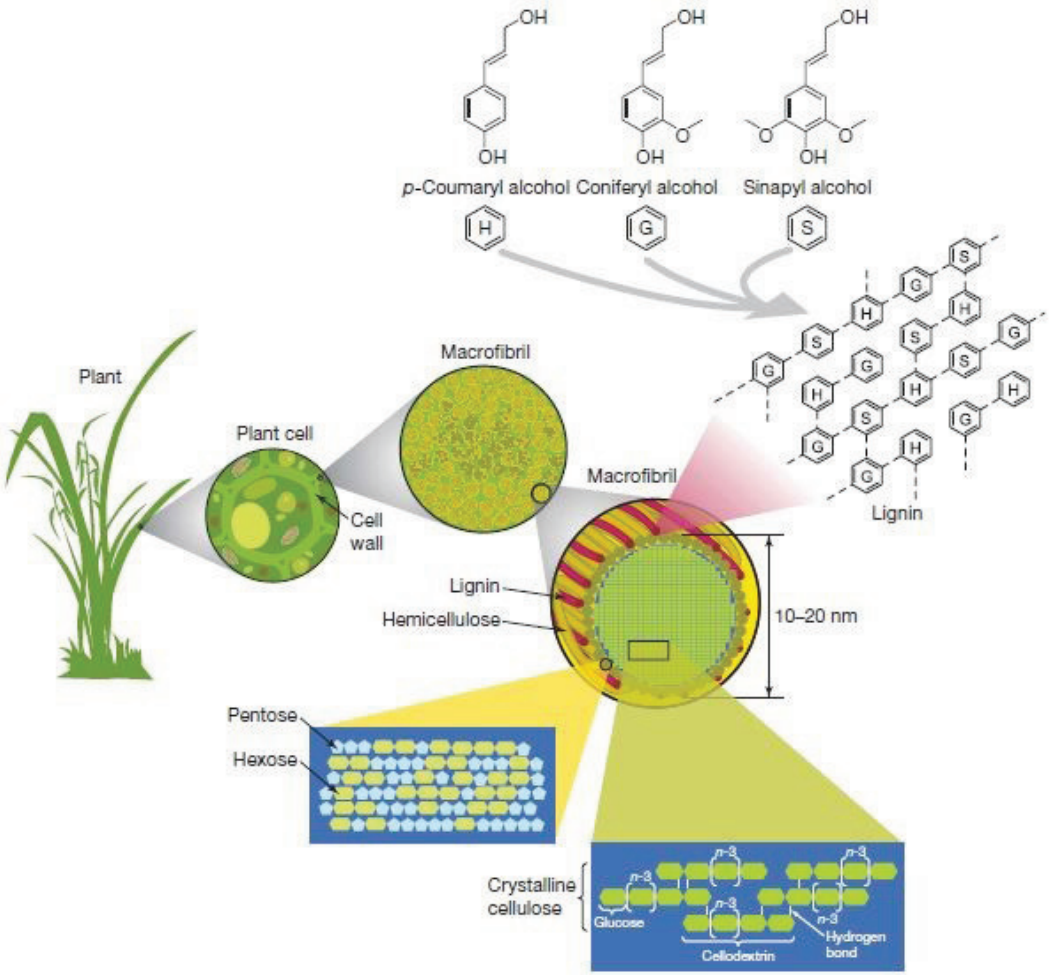

Figure 6 – Structure of lignocellulose cell wall (based on Rubin 2008).

EXPERIMENTAL PART

1 Purpose of the work

Two major events that impacted significantly on the development of human-kind involved the use of plants: our ability to make fire and our change from being hunter-gatherers to food-producers. Estimates suggest that agriculture arose some 10 000 years ago, whilst the control of fire may date back some 790 000 years (Goren-Inbar et al., 2004). As food production became more efficient, it became possible for larger numbers of people to live together. Human populations expanded and civilisations were born (Heiser CB Jr., 1973). During this expansion, the requirement for plants to provide fuel was not in conflict with food production. Rather, this requirement diminished as alternative sources of energy were developed. As a result, twenty-five years ago, although plants were still being used for fuel in underdeveloped regions of the world, it was oil, coal, natural gas and nuclear power that together fulfilled most of the world's energy needs (Sims et al., 2006). Interest in energy production from crops has resurged in recent years, as evidenced by the recent growth in development of grain based ethanol and biodiesel industries spurred through the interest of replacing transportation fuel and fuel additives with cleaner burning renewable sources. While grain derived biofuels may provide environmental benefits when compared to traditional fossil fuels, there are concerns about the sustainability of their adoption due to their limited improvements in net carbon release and net energy yields relative to fossil fuels and the limitations in total achievable grain production which will limit their expansion and potential for offsetting fossil fuels (Hill et al, 2006). Grain based biofuels have also faced scrutiny through the development of a market which may compete for grains which would otherwise have been primarily grown for food purposes. Given the limitations of grain-based bioenergy production, biomass has received attention as an alternative bioenergy feedstock due to its ability to overcome many of the challenges associated with grain-based bioenergy. Biomass has had a long important use as an energy source for man, and includes that of wood which has been estimated to have been used for hundreds of thousands of years (Goren-Inbar et al, 2004). It is estimated that biomass currently supplies 10-14% of the world's energy (McKendry, 2002). There is a growing general awareness and consensus among politicians and farmers on the need to reduce the environmental loads and the energy inputs of farming systems through the introduction of novel species, new insights in plant physiology and biotechnology, optimization of agronomic inputs (fertilizers, etc.) and natural resources, primarily water. Perennial grasses are an excellent fit for biomass systems through their perennial establishment, ability to recycle nutrients, and their potentially significant yields of high quality combustible biomass (Lewandowski et al, 2003). In general, perennial grasses are drought resistant crops and recently have been attracting growing interest due to their extensive environmental

benefits both at global and agricultural community-scale. Compared to traditional row crops, perennial grasses generally require lower energy inputs (fertilizers, pesticides etc.), can be grown on marginal cropland and provide benefits in terms of soil structure and stability (e.g. reduced soil loss, erosion and runoff), soil quality (e.g. increase in soil fertility, organic matter and nutrient retention) and biodiversity (e.g. cover for native wildlife). Perennial grasses are also not seen as competing for agricultural land because they can be grown on marginal or degraded lands where intensive agricultural practices harm the environment (e.g. promoting soil erosion), and where the economic returns to the farmer's labour and capital are not sustainable (OPTIMA project, Grant Agreement 289642). In terms of perennial grasses, C_4 photosynthetic types present an advantage to conventional C_3 types through their greater nutrient and water use efficiencies (Brown, 1978), suggesting the potential for higher biomass yields relative to C_3 types. Beyond yield, *Miscanthus* has demonstrated many key elements of efficiency: high nutrient use efficiency including some reports of no nitrogen response at sites (Christian et al, 2008); positive environmental benefits including less nitrogen leaching (Christian et al, 1998); greater potential for wildlife biodiversity (Smeets et al, 2009); and higher levels and improved quality of organic matter (Kahle et al, 2001). *Miscanthus* is a C_4 perennial grass native to eastern Asia. Workers, primarily in Europe (Schwarz, 1993; Lewandowski et al., 2000; Jones & Walsh, 2001; Lewandowski & Schmidt, 2006) and the United States (Heaton et al., 2008; Khanna et al., 2008; Villamil et al., 2008), but also in Asia (Yoshida et al., 2008), have evaluated the potential of several members of the *Miscanthus* genus as bioenergy crops, particularly high-yielding taxa such as *Miscanthus sinensis*, *Miscanthus sacchariflorus*, and their hybrids (Clifton-Brown et al., 2001; Jones & Walsh, 2001). Owing to its C_4 photosynthesis (Naidu et al., 2003), low-nutrient requirements (Lewandowski et al., 2003), high water-use efficiency (Clifton-Brown et al., 2002), capability of C mitigation (Clifton-Brown et al., 2007), and high yields in various climates and environments (Clifton-Brown et al., 2001), *Miscanthus x giganteus* has been the primary hybrid of choice as a potential bioenergy crop. Species currently studied for their biomass potential include *Miscanthus sinensis* (*M. sinensis*) and *Miscanthus sacchariflorus* (*M. sacchariflorus*) also.

For this purpose, two field researches were carried out with the aim of studying i) the adaptation and biomass production potential of 18 *Miscanthus* accessions, representing 5 *Miscanthus* species, collected from a wide geographical range (Numata, 1974) for suitability to semi-arid Mediterranean climates and ii) the effect of harvest time (autumn and winter time) on biomass yield, morph-biometric characters, moisture content, cellulose, hemicelluloses and lignin contents for second generation bioethanol production and ash content for combustion purposes in a long term plantation of *Miscanthus x giganteus* in a Mediterranean environment; iii) a third experiment, in controlled-

environment, was carried out to investigate the effect of heat stress, on 5 *Miscanthus* genotypes, coming from the *Miscanthus* germplasm collection at Institute of Biological, Environmental and Rural Sciences (IBERS) of the Aberystwyth University – Wales – UK, to identify how temperatures affect growth, partitioning and physiology of *Miscanthus* plants.

2 Materials and Methods

2.1 Evaluating wild *Miscanthus* germplasm for biomass potential in Southern Europe

The research was carried out between 2010 and 2011 at Catania (Sicily,10 m a.s.l., 37°25'N Lat., 15°30'E Long.,) on a Typic and/or Vertic Xerofluvents - Typic and/or Vertic Xerochrepts soil association (Fierotti et al., 1988) whose characteristics are reported in Table 1.

18 different *Miscanthus* accessions, representing 5 *Miscanthus* species (Table 2) were compared in a randomized complete block design, planted on 10th June, 2009, at 2 plants m^{-2} each containing a 5 x 5 m plot with 3 replicates per accession. A second transplanting was carried out on 20th October, 2009, for replacing the dead plants/rhizomes.

After transplanting and until the autumnal vegetative quiescence, the soil water content was kept at a good level in order to allow a good plant establishment (200 mm of water has been applied during this period). Weeds were controlled mechanically. In 2010, the plots were partly irrigated (from June to August), while in 2011 the plots were rainfed (not irrigated). The water was distributed by means of a drip irrigation system. The irrigation was determined on the basis of the maximum available water content in the first 0.6 m of soil, where most of the root is expected to grow, calculated by means of the following formula (Doorembos and Pruitt, 1977):

$$V = 0.66\ (FC - WP)\ \Phi\ D$$

where V = water amount; 0.66 = fraction of readily available soil water permitting unrestricted evapotranspiration; FC = soil water at field capacity, equal to 27% of dry soil weight; WP = soil water at wilting point, equal to 11% of dry soil weight; Φ = apparent volumetric mass, equal to 1.2 kg m^{-3}; D = equal to 0.6 m, which is the soil depth were the bulk of roots is expected to develop (Beale et al., 1999).

The irrigation was carried out when the sum of daily ETm (maximum evapotranspiration), calculated as follows, corresponded to V:

$$ETm = E_0\ K_p\ K_c$$

where ETm = daily maximum evapotraspiration (mm); E_0 = evaporation of class 'A' pan (mm); K_p = pan coefficient, equal to 0.80 in semi-arid environment (Doorembos and Pruitt, 1977); K_c = crop

coefficient, ranging between 0.4 and 0.7 from plant emergence to beginning of jointing, between 0.7 and 1.1 from beginning to end jointing, equal to 1.1 from end jointing to flowering and between 1.1 and 0.7 from flowering until October. The crop coefficient adopted refers to those of C_4 plants (e. g. sorghum and maize) for the Mediterranean environment (Doorembos and Pruitt, 1977).

In 2010, some >280mm of water has been applied during the summer season from June to August. Air temperature and rainfall were recorded using a meteorological station (CR10, Campbell-USA). Yields were estimated from plot interior quadrat to minimise edge effects. During the growing seasons (and before the final harvests), the date of flowering (when at least 50% of plants in the plots were flowered), canopy and panicle height, canopy width and width at base, senescence score and lodging resistance were recorded.

Flowering observations were recorded one time per week in whole plants. Four flowering stages (FS) were scored in accordance with Jensen et al. (2011a):

- FS1, pre-exertion of the panicle, is recorded as the day of year (DOY) when the first flag leaf of the plant emerge but the panicle is still within the leaf sheath. This is the first observable indication that floral transition has occurred.
- FS2, start of flowering, is recorded as the DOY when 1 cm of panicle is showing on at least one stem, either above the flag leaf ligule or from a split in the side of the sheath.
- FS3, mid-point of the flowering process, is recorded as the DOY when approximately 50% of all the stems contributing to the canopy height have exerted more than 1 cm of panicle.
- FS4, flowering completion, is recorded as the DOY when more than 80% of the stems contributing to the canopy have exerted more than 1 cm of panicle.

Development of senescence was scored by observation of the whole visible aerial parts of the plant, primarily leaf, and was scored on a scale of 0 to 10. A value of zero represented no visible leaf senescence; a value of 1 represented approximately 10% loss of green leaf and so on up to a value of 10 which represented a fully senesced plant with 100% loss of green leaf. Development of lodging resistance was scored on scale of 1 to 9. A value of 1 represented a low strong plant (soft and floppy); a value of 5 represented a medium plant and so on up to a value of 9 which represented a very strong plant.

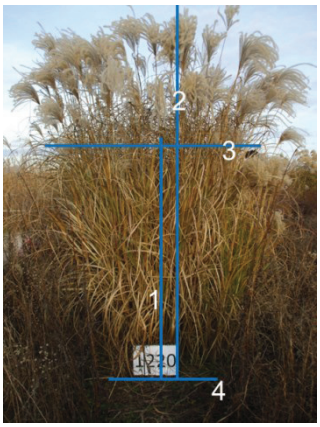

Figure 1 – Basic morphological measurements on the studied *Miscanthus* accessions.

The basic morphological measurements were taken as shown in figure 1, using a ruler: 1-height to top of canopy; 2-height to top of panicle (when present); 3-width of plant canopy; 4-width at base of plant.

Harvests were carried out in spring in 2011 and 2012 (for growing seasons 2010 and 2011 respectively). Harvestable biomass was calculated from quadrat fresh weight and subsample moisture content. For above-ground dry biomass estimation, plant samples were oven dried at 65°C to constant weight.

For both harvests (2011 and 2012) the crop water use efficiency (WUE), expressed as ratio between dry biomass production at final harvest and water used by the crop, was calculated, adopting the following formula:

$$WUE = \frac{P}{I + R}$$

where WUE = water use efficiency (g l^{-1}); P = above-ground dry biomass (g ha^{-1}); I = water supplied by means of irrigation (l ha^{-1}) and R = precipitation (l ha^{-1}).

The data of productive characteristics were analyzed by a two-way ANOVA (Snedecor and Cochran, 1989), using CoStat Version 6.003 (CoHort Software) related to the randomized complete block design in field. With significant difference, the Student-Newman-Keuls (SNK) method for the means separation was applied.

Table 1 – Soil characteristics of the field site in the top 0-50 cm

Soil characteristic	*Value*
Sand (%) (Gattorta method; Lotti and Galoppini, 1980)	49.27
Loam (%) (Gattorta method; Lotti and Galoppini, 1980)	22.43
Clay (%) (Gattorta method; Lotti and Galoppini, 1980)	28.30
pH (in water solution)	8.6
Total calcareous (%) (gas-volumetric method)	15.24
Organic matter (%) (Walkley and Black method)	1.40
Total N (‰) (Kjeldahl method)	1.00
P_2O_5 availability (ppm) (Ferrari method)	5
K_2O availability (ppm) (Dirks and Sheffer method)	244.8
Field capacity at -0.03 MPa (%)	27
Wilting point at -1.5 MPa (%)	11

Table 2 – The *Miscanthus* species used within the trial and associated accession numbers

M. floridulus	*M. sinensis*	*M. condensatus*	*M. sacchariflorus*	*M. x giganteus*
M2	M5	M13	M9	M19
M3	M18		M1	
M11	M20		M6	
M12			M7	
			M8	
			M10	
			M15	
			M16	
			M14	

2.2 *Miscanthus* biomass yield and biomass quality as affected by harvesting dates

The experimental field of *Miscanthus* x *giganteus* was established at the experimental farm of Catania University (Catania plain, 10 m a.s.l., 37° 24' N, 15° 03' E) on a vertic xerochrepts soil (Soil taxonomy, USDA), whose characteristics are similar to those reported above (Table 1).

Micro-propagated plantlets of *M. x giganteus* provided by Piccoplant (Oldenburg, Germany) were transplanted in the field on June 10th 1993, adopting a 4 plants m^{-2} density.

A split-plot experimental design with three replicates was applied and the following factors were studied until 1996:

• three levels of maximum evapotranspiration (ETm) restoration: 25%, 50% and 100%;

• three levels of nitrogen fertilization: 0 kg ha^{-1}, 60 kg ha^{-1} and 120 kg ha^{-1} of nitrogen.

After transplanting and until the autumnal vegetative quiescence, the soil water content was kept at a good level in order to allow a good plant establishment. The irrigation treatments were differentiated from the second year. The nitrogen fertilization was carried out supplying 60 kg ha^{-1} of N, as ammonium sulphate, at the spring crop re-growing (in both fertilized treatments) and the further 60 kg ha^{-1} at the beginning of jointing (only in 120 kg ha^{-1} treatment).

After 1996/1997 growing season, the plantation was managed without agronomical input supply and biomass was harvested annually in winter time.

Starting from February 2012, the following factors were studied:

- Harvest time: winter and autumn;
- Nitrogen fertilization: no fertilization (N0) and 80 kg N ha^{-1} (N80);
- Biomass quality (Van Soest et al., 1991).

The plantation consists of two blocks, 9 plots in each block for a total of 18 plots. Fertilization has been applied in one block after harvest and compared to the other one without fertilization. In the post-autumn harvest 80 Kg N ha^{-1} have been supplied as ammonium sulphate, while in the post-winter harvest 80 Kg N ha^{-1} as ammonium nitrate. The experimental layout is shown in figure 2.

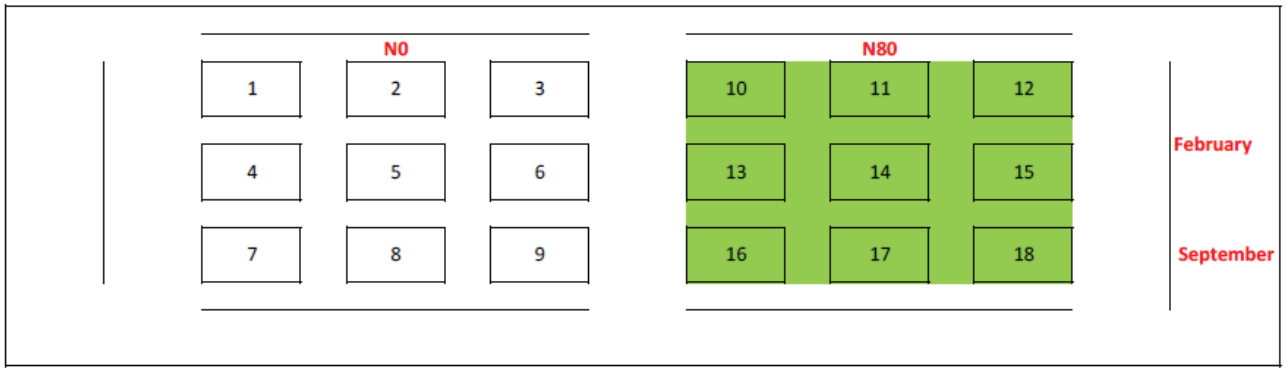

Figure 2 - Experimental layout of the long term plantation at Experimental farm of UNICT (Catania plain, 10 m a.s.l., 37°27'N, 15° 03' E). In green the nitrogen fertilized blocks, while in white the unfertilized one.

At harvest the following morphological and biometric measurements were carried out in ten random plants: stem density (plant on square meter), weight of one stem (g), node number, basal stem diameter (mm), plant height (from the base of the cut up to the panicle), stems and leaves fresh weight (g), moisture content (% w/w) and biomass partitioning (% stems, leaves and panicle where present).

Fresh and dry matter yield were obtained harvesting a sub-plot inside the large plot after removing 2 linear meter plants in each plot edge to minimize the edge effect. To determine the dry matter yield, sub-samples were placed in a ventilated oven dry at 60 ± 5 °C until constant weight and biomass yield for unit surface was calculated (t ha^{-1}).

Biomass fiber composition of autumn (September 2012) and winter (February 2013) harvests have been performed in order to compare the best harvest time for specific end-uses.

Determinations of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were made in accordance with the Van Soest fiber analysis method (1991). Analysis were conducted in triplicate.

Procedure for NDF determination (Neutral detergent fiber)

1. Grind the air dried sample to pass 1 mm screen.
2. Weigh in a crucible 1 g of grinded sample with 1 mg approximation.
3. Add 100 ml of neutral detergent solution at room temperature into crucible with 0.5 g of sodium sulfite and some drops of n-octanol.
4. Heat to boiling and reflux 60 minutes from onset of boiling.
5. Filter and wash 3 times with boiling water, then twice with cold acetone.
6. Dry 8 hours at 105 °C and let cool in a desiccator.
7. Weigh.
8. Calculate neutral detergent fiber: NDF % = (weight of crucible + weight of residue) - weight of crucible / weight of sample x 100. Neutral detergent solubles: NDS % = 100 - NDF %.
9. Ash in a muffle at 550 °C 2 hours and let cool in a desiccator.
10. Weigh.
11. Calculate ash insoluble in neutral detergent: loss on ashing / weight of sample x 100.

Procedure for ADF determination (Acid detergent fiber)

1. Grind the air dried sample to pass 1 mm screen.
2. Weigh in a crucible 1 g of grinded sample with 1 mg approximation.
3. Add 100 ml of acid detergent solution at room temperature and some drops of n-octanol.
4. Heat to boiling and reflux 60 minutes from onset of boiling.
5. Filter and wash 3 times with boiling water, then twice with cold acetone.
6. Dry 8 hours at 105 °C and let cool in a desiccator.
7. Weigh.
8. Calculate acid detergent fiber: ADF % = (weight of crucible + weight of residue) - weight of crucible / weight of sample x 100.
9. Ash in a muffle at 550 °C 2 hours and let cool in a desiccator.
10. Weigh.
11. Calculate ash insoluble in acid detergent: loss on ashing / weight of sample x 100.

ADL (acid detergent lignin) was determined by treating the sample with 72% H_2SO_4 for about 4 hours and repeatedly washed with deionized water and vacuum filtered until the liquid fraction did not present any solubilized substance (as clear water). Subsequently the sample was posed in a muffle furnace at 550 ± 50 °C for 8 hours. The weight difference between the treated samples and ashes estimated the ADL content.

The data of biological and productive characteristics were analyzed by a two-way ANOVA (Snedecor and Cochran, 1989), using CoStat Version 6.003 (CoHort Software) according to the experimental design. With significant difference, the Student–Newman–Keuls (SNK) method for the means separation was applied.

2.3 Effect of heat stress on the biomass production and physiology in *Miscanthus* genotypes

The heat experiment was conducted in a controlled glasshouse at the Institute of Biological, Environmental and Rural Sciences (IBERS) of the Aberystwyth University, Wales (UK) (52°24′50″N - 04°04′54″W).

The experiment was performed on five *Miscanthus* types sourced from the *Miscanthus* germplasm collection at IBERS (Table 3). Plants were grown in two different growth cabinet at different temperatures arranged in a completely randomized block design and were randomized twice weekly throughout the course of the experiment to avoid shadowing effects due to faster growing of some genotypes. The high temperature treatment (T_{high}) followed the climatic conditions of Taipei (Taiwan) and the low temperature treatment (T_{low}) followed the typical climate conditions for Braunschweig (Central Germany) (Fig. 3). T_{high} averaged 28.5°C, with a max and min of 30.4 and 23.6°C over the experimental period and T_{low} averaged 19.4°C, with a max and min of 22.1 and 12.4°C over the experimental period.

Rhizomes were trimmed to be of similar size and weight then planted in 9L plastic pots in John Innes No. 3 compost. Rhizome pieces had a fresh weight of 26.9 ± 2.4 g. Water was applied automatically to achieve an identical water content between the treatments. Irradiance was controlled at an average of 600 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), at the top of the leaves, with a 14:10-h day:night cycle.

To determine the dry biomass, the final yield plants were harvested at the end of the experiment and the plants were separated into above-ground, roots and rhizomes. These were then oven dried at 65°C to constant weight. Dry matter yield was estimated by calculating the moisture content in stems, leaves and inflorescences, for above-ground biomass and, in rhizomes and roots, for below-ground biomass, dried as above described.

During the experiment morphological and physiological measurements were made in order to investigate the metabolic characteristics and changes of the *Miscanthus* genotypes in both low and high heat controlled conditions.

Morphological measurements:

- Main stem height (to the 1st meristem).
- Main stem diameter.
- Stem and leaf number in each plant.
- Leaf surface area on main stem (by the width and length leaves).
- Fresh and dry weight of the above-ground (plant) and below-ground biomass (rhizome and roots).

Physiological measurements: (on the youngest fully expanded leaf, defined by ligule emergence)

- A/Ci curve: response of the light saturated CO_2 assimilation rate (A_{sat}) to leaf internal CO_2 mole fraction (Ci).
- A/Q Curve: response of CO_2 assimilation rate (A) to absorbed light (Q) and pulse-modulated fluorescence.
- Relative chlorophyll *a* estimation.
- Leaf Absorbance.
- Continuous chlorophyll fluorescence.
- Stomatal density and cell size.

Leaf CO_2 uptake per unit leaf area of the youngest fully expanded leaves (defined by ligule emergence) was measured with a portable gas exchange fluorescence system (GFS-3000, Heinz Walz GmbH, Germany) with an open system design (Fig. 4). With the GFS-3000 all environmental parameters relevant for plant photosynthesis (CO_2, H_2O, temperature, light, ventilation and flow) are controlled automatically and over the full physiological range. The response of photosynthetic CO_2 uptake rate (A) to photosynthetic photon flux density (Q) and temperature was assayed by enclosing the leaf in a cuvette under a light source. For light response measurement, the Q was adjusted from darkness to 2000 μmol m^{-2} s^{-1} (while maintaining all other conditions the same). Steady-state rates of A were recorded after equilibration at each successive light level. The reference CO_2 was set to 390 ppm for ambient CO_2 and all the measurements were performed between 09:00 and 14:00 h on all dates, to reduce any effect of sucrose feedback photoinhibition.

Each *Miscanthus* A/Q response curve was modelled by a non-rectangular hyperbola where the initial slope is the apparent quantum efficiency (ϕ_{CO2}), the light compensation point (LCP) and apparent respiration (Rd) are estimated from axis intercepts and light saturated maximum (A_{max}) is the upper asymptote.

The fluorometer parameters (flash intensity and duration) were adjusted such that the fluorescence emissions were maximal and steady for the duration of the saturating pulse. Leaves were dark-

adapted within the chamber until respiration and minimal fluorescence (F_0) were at steady-state (average 20 minutes). Dark-adapted variable fluorescence (F_v=F_m-F_0) over maximal fluorescence (F_m) was then determined. Subsequently the actinic light was switched to 500 μmol m^{-2} s^{-1} and the leaf light-adapted until steady-state was achieved. Photosynthetic and light-adapted fluorescence parameters were then measured at 10 actinic light levels ranging from the equivalent of full sunlight (2000 μmol m^{-2} s^{-1}) to low (25 μmol m^{-2} s^{-1}). All gas-exchange and fluorescence data are graphed and/or calculated on an absorbed-light basis.

The response of photosynthetic CO_2 uptake rate (A) to intercellular CO_2 mole fraction (C_i) curve was measured with Q = 1500 μmol m^{-2} s^{-1} at a series of ambient CO_2 concentrations (C_a), while maintaining all other conditions the same. For each *Miscanthus* A/C_i response curve, carboxylation efficiency (CE) of PEPc was calculated as the slope of the initial linear portion of the curve (C_i<100 μl l^{-1}). The CO_2-saturated photosynthetic rate (V_{pr}) was estimated from the horizontal asymptote of each individual A/C_i curve. Photosynthesis in this region of the A/C_i curve is controlled by PEP regeneration and/or carboxylation limitation within the bundle sheath. Measurements were corrected to 25°C using the temperature responses of Bernacchi et al. (2001) and Long and Bernacchi (2003) for the Rubisco and RuBP-limited portions of the A vs C_i curves, respectively (Long and Bernacchi, 2003). The operating point of photosynthesis ($C_{i,390}$) was calculated as the C_i that corresponds to a given C_a of 390 μl^{-1}, fit using a linear regression of C_i vs. C_a for each individual leaf (Farquhar and Sharkey, 1982; Naidu and Long, 2004). The photosynthetic rate where C_i = C_a (390 μl l^{-1}) represents the hypothetical scenario in which there is no stomatal limitation to photosynthesis. The percent reduction in photosynthesis due to stomatal limitation (l_s) was calculated from each replicate A/C_i curve (Long and Bernacchi, 2003).

Measurements of continuous chlorophyll fluorescence were made on the youngest fully expanded leaves on the adaxial leaf surface using a continuous fluorescence portable fluorimeter (Handy PEA, Hansatech, UK), with an excitation radiation of 3000 μmol m^{-2} s^{-1} for 1 second (Fig. 5). The leaves were enclosed in a small plastic leafclips and dark-adapted for 20 minutes.

The relative amount of chlorophyll (R_{chl}) in the leaves were estimated using a SPAD-502 meter (Konica Minolta, Inc.) (Fig. 6). Measurements were made on the youngest fully expanded leaves (the same leaves used for the gas exchange measurements) and a single average recorded for five readings taken uniformly along the length of the leaf. The adaxial side of the leaves was always placed toward the emitting window of the instrument and major veins were avoided.

After gas exchange measurements were made, leaf peels were taken by the addition of Perspex dissolved in chloroform to the leaf and a microscope slide firmly pressed on to the leaf surface. Once the Perspex had solidified the microscope slide was carefully removed. This was repeated on

the abaxial and adaxial leaf surfaces. Slides were analysed using a Leica micro-dissection microscope (LMD6000 with LMD software version 6.5, Leica Microsystems [UK] Ltd) (Fig. 7). For each individual leaf peel an area of 0.04 mm^2 was selected and the number and size of the stomata recorded, this was repeated a minimum of 10 times per leaf peel.

A dual channel spectrometer (Ocean Optics SD2000) (Fig. 8) was used to determine the peak emitting wavelengths of red (640 nm) and blue (470 nm) light from the Walz GFS-3000FL light source. The same portion of the leaf that was within the portable gas exchange fluorescence cuvette was inserted in a tungsten halogen light source (LS1-LL, Ocean Optics). Transmittance (T) and reflectance (R) were measured with the spectrometer and used to calculate the leaf absorbance: $A_{leaf} = 1 - (T + R)$, at these peak wavelengths. Transmittance (T) was determined by placing a leaf sample between the light source and the inlet port of the integrating sphere. Reflectance (R) was determined by placing the leaf sample at the sample port with the upper surface facing into the sphere while the sphere was illuminated via the opposite inlet port. Total absorbed light (Q_{abs}) at each incident light level (Q) was then calculated by combining the percentage of actinic light emitted by the blue and red LEDs with the leaf absorbance for the peak wavelength of each LED. All gas exchange and fluorescence data are graphed and/or calculated on an absorbed light basis.

Table 3 - Plant material used in experiments

Species	Other name	Abbreviation	Ploidy
M. x giganteus	---	M. x gig	3
M. floridulus	---	M. flo	2
M. sinensis	Goliath	Gol	3
M. sinensis	EMI-6	M. sin	3
M. sacchariflorus	EMI-5	M. sac	4

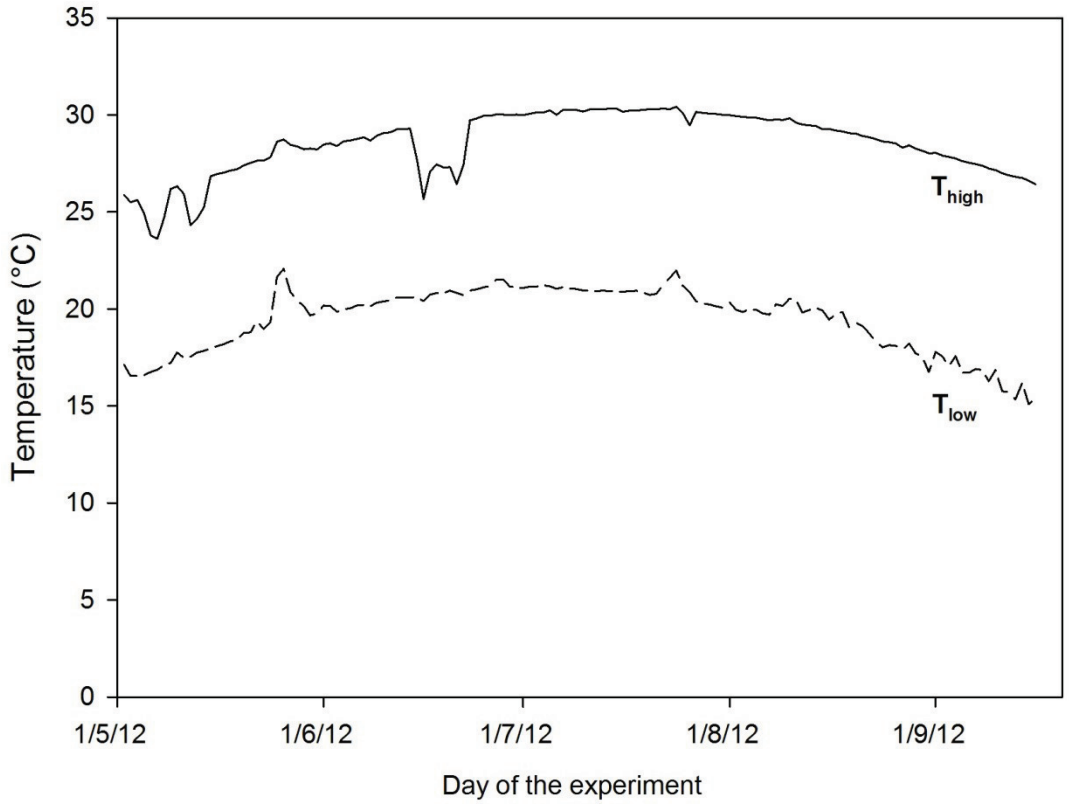

Figure 3 - Growth temperatures for the two treatments over the duration of the experiment

Figure 4 – The gas exchange fluorescence system used on the *Miscanthus* leaves during the heat stress for the leaf gas exchange measurements.

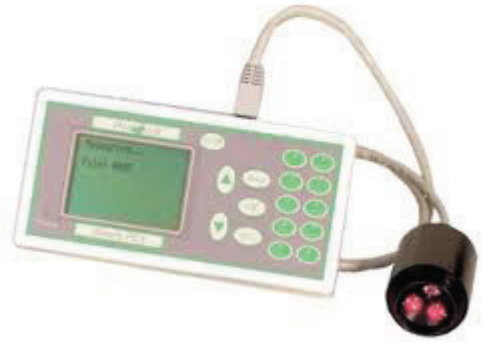

Figure 5 – The continuous excitation chlorophyll fluorimeter used to determine the chlorophyll fluorescence on the *Miscanthus* leaves during the heat stress experiment.

Figure 6 – The SPAD meter used to estimate the relative amount of chlorophyll *a* on the *Miscanthus* leaves during the heat stress experiment.

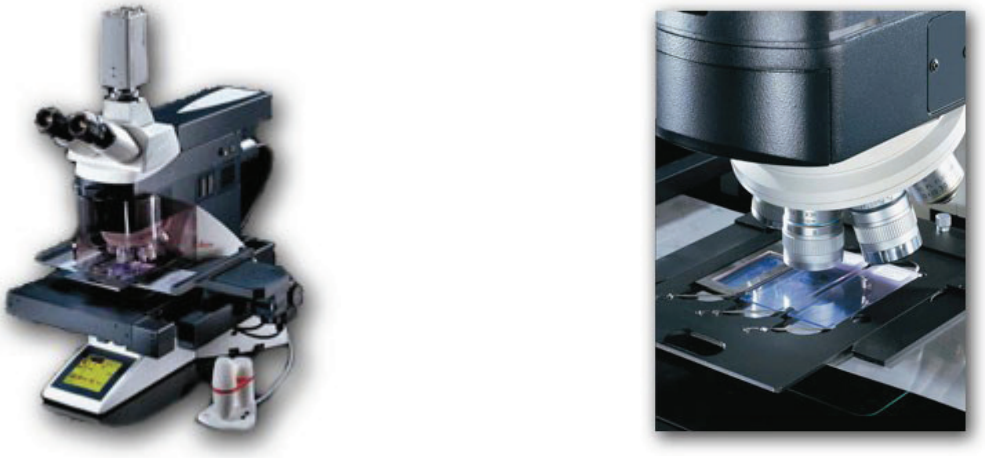

Figure 7 – The Leica micro-dissection microscope used to count and analyze the stomata on the adaxial and abaxial *Miscanthus* leaves grown and measured at different growth temperature.

Figure 8 – The spectroradiometer used to determine the transmittance and reflectance on the studied *Miscanthus* genotypes at different growth temperature.

3 Results and discussions

3.1 Evaluating wild *Miscanthus* germplasm for biomass potential in Southern Europe

3.1.1 Meteorological data

Meteorological data were those of a typically Mediterranean environment: rain almost absent during the summer period and high intensity rain in the autumn-winter time (Fig. 1).

During the first transplanting time (June 2009) the average minimum air temperature was 16.9 °C and the maximum was 28.6 °C and during the second transplanting time (October 2009) the average minimum was 14.1 °C and the maximum was 24.3 °C. The average temperatures, from June 2009 to March 2012, reached the maximum value (41.4 - 36.2 °C) in July and August. The daily evaporation in these months was 8 – 10 mm. Subsequently, temperatures decreased gradually and reached the minimum values in January 2010 (6.5 °C) and February 2011 (5.3 °C). Mean air temperatures, recorded during the growing season (June – September), were similar for the two years. The average minimum air temperature for the period was 19.2°C and the maximum was 30.0°C. Rainfall recorded was 622.4 and 846.4 mm during the first (2010) and second (2011) growing season, respectively amounted at 2,140.60 mm from June 2009 to March 2012 (establishment- last harvest time), mm well distributed during the years of experiment (from September to March each year). In 2010 growing season (June – September) the total rainfall was 65.2 mm, while 109.2 mm in the second growing season (2011), with the highest rainfall consistently in September.

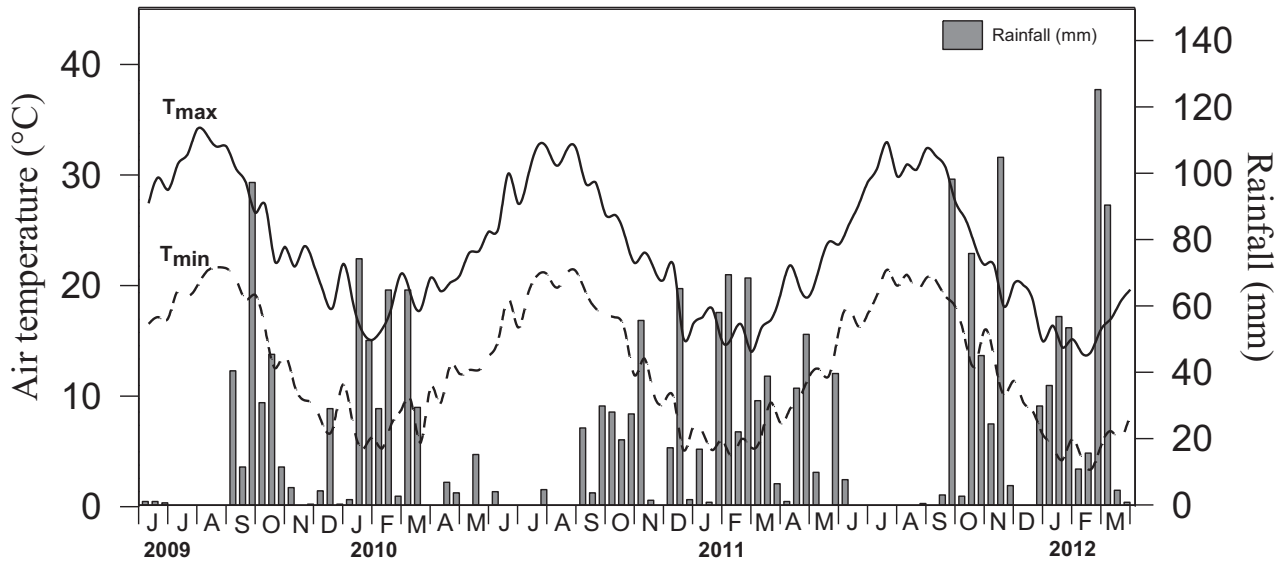

Figure 1 – Changes in maximum and minimum air temperature (mean 10-day values) and rainfall (total 10-day values).

3.1.2 Phenology

On the average of the two years, the *Miscanthus* crop attained the flowering stage between the beginning of August and the beginning of November. (Table 1). In literature it has reported that *Miscanthus* has a broad natural geographical range that provides extensive and currently unexploited genetic diversity. The optimization of flowering time is considered important for the improvement of yield and quality of biomass: early flowering associates with reduced yield and is therefore undesirable, whilst flowering per se associates with senescence and the subsequent remobilization of nutrients to the underground rhizome. Flowering therefore contributes towards crop sustainability and reduced moisture content, whilst improving biomass quality through removing residual nutrients from the harvested biomass.

Premature genotypes were those that showed the lowest above-ground biomass quantity, so looking at the flowering stages, showed on the below table, they were M1 and M5 (*M. sacchariflorus* and *M. sinensis*, respectively). They showed the flag leaf stage (FS1) at the beginning of August and the flowering completion (FS4) at the beginning of the second half of September (Table 1). For M13 (*M. condensatus* - the most yielding *Miscanthus* genotype, on the average of the two years), the flag leaf stage was reached at the beginning of the second half of September, while the flowering completion at the beginning of November (Table 1). For M2, M3 and M12 (*Miscanthus* accessions belonging to the *M. floridulus* species), the flag leaf stage was reached at the beginning of

September, while the flowering completion at the end of October (Table 1). The other genotypes varied the flowering stage between the middle of August to the end of September (Table 1).

In the current study was found variations in flowering date among genotypes. The induction of the flowering is dependent on day length and variations can be related to the geographical origin of genotypes (Lewandowski et al., 2003). Experiments under artificial light conditions have been carried out to induce flowering. A short-day light period may be necessary to induce the flowering of *M. sacchariflorus* (Deuter, 2000). By contrast, day length may be less important than cumulative degree-days in *M. sinensis*. Cosentino et al. (2007) showed that in the Mediterranean environment *M. x giganteus* attained the flowering between the end of August and the beginning of October. High temperature and low photoperiod conditions probably regulate the flowering of the crop. The same species not always flowered in the colder regions of Central and North Europe (Beale et al., 1999; Clifton-Brown and Lewandowski, 2002). This may probably allow the crop to grow more in height and being more productive. In this research *M. x giganteus* attained the flowering (FS3) on the beginning of September and attained the flowering completion (FS4) at the end of the same month. *M. x giganteus* showed the flag leaf stage (FS1) at the beginning of August.

Jensen et al. (2012) indicated that *M. sacchariflorus*, irrespective of origin, is a quantitative short-day plant. *M. sacchariflorus* flowering phenology closely resembles that of *Sorghum* and *Saccharum* (sugar cane), indicating potentially similar floral pathways and suggesting that determination of the underlying genetic mechanism will be facilitated by the syntenic relationships existing between these important C_4 grasses. Within the *Miscanthus* genus, *M. sacchariflorus* flower less readily than *M. sinensis*, both in North European (Clifton-Brown et al., 2001; Jensen et al., 2011a) and in diverse Chinese (Yan et al., 2011) field conditions, and reports have indicated that some *M. sacchariflorus* lines require short-day treatments for floral induction (Deuter, 2000). Field observations in Aberystwyth (Wales, UK) have shown that photoperiod alone does not explain flowering time in a number of *M. sacchariflorus* lines (Jensen et al., 2011a). Temperature is also known to have a role in determining maturity and can interact with photoperiod to regulate flowering, including in *Sorghum* (Hammer et al., 1989; Ellis et al., 1997; Craufurd and Qi, 2001). Flowering time prediction is further complicated by water availability. For example, in *Sorghum* (Craufurd et al., 1993), panicle initiation and flowering are delayed in response to drought, and similar effect has been observed in *Miscanthus*, where water deficits appear to impose a delay on flowering (Jensen et al., 2011b). In general, flowering terminates the production of leaves at the stem apex. This has the potential to reduce the length of the growing season, thereby reducing the time over which radiation is intercepted and hence reducing potential biomass accumulation. Flowering is likely to be at least partially coupled to senescence (Wingler *et al*., 2009), with

associated impact on nutrient remobilization to the underground rhizome, thereby promoting crop sustainability and improving combustion quality by removing undesirable elements (i.e. N, S, and Cl) from the harvested biomass.

Table 1 – Flowering stages of the studied *Miscanthus* genotypes during the two years (on the average of the two years)

Miscanthus accessions	Flowering process stages			
	FS1	FS2	FS3	FS4
M1	10/8	20/8	5/9	20/9
M2	10/9	20/9	5/10	30/10
M3	5/9	15/9	30/9	20/10
M5	10/8	20/8	5/9	20/9
M6	15/8	25/8	10/9	30/9
M7	15/8	25/8	10/9	30/9
M8	15/8	25/8	10/9	30/9
M9	15/8	25/8	10/9	30/9
M10	15/8	25/8	10/9	30/9
M11	15/8	25/8	10/9	30/9
M12	5/9	15/9	30/9	20/10
M13	20/9	5/10	20/10	5/11
M14	15/8	25/8	10/9	30/9
M15	15/8	25/8	10/9	30/9
M16	15/8	25/8	10/9	30/9
Goliath	15/8	25/8	10/9	30/9
M. x gig	15/8	25/8	10/9	30/9
M20	15/8	25/8	10/9	30/9

FS1=1st flag leaf; FS2=1st panicle > 1 cm; FS3=visible anthers on first panicles; FS4=flowering end (no live anthers)

3.1.3 Morphological traits

The highest panicle height was recorded for M10 (*M. sacchariflorus*), it reached the maximum value equal to 356.7 cm. The lowest panicle height was recorded for M5 (*M. sinensis*), it reached the minimum value equal to 120.0 cm. The other studied *Miscanthus* accessions ranged between 202.5 cm (M9 – *M. sacchariflorus*) and 351.7 cm (M16 – *M. sacchariflorus*). For *M. x giganteus* and *Goliath*, the panicle heights were equal to 305.0 and 210.0 cm, respectively (Table 2).

Canopy width values ranged between 35.0 cm (M1 – *M. sacchariflorus*) and 130.0 cm (M13 – *M. condensatus*). For *M. x giganteus* and *Goliath*, the canopy width was equal to 68.0 and 62.0 cm, respectively (Table 2). The lowest canopy width at base value was recorded for M8 (*M. sacchariflorus*), it reached a value equal to 18.0 cm, followed by M5 (*M. sinensis*) with a value equal to 20.0 cm. The highest canopy width at base values were recorded for M11 (*M. floridulus*), M16 (*M. sacchariflorus*) and M13 (*M. condensatus*), they reached values equal to 50.0 cm,

respectively (Table 2). At harvests, the *Miscanthus* accessions belonging to *M. floridulus* species (M2, M3, M11, M12) and M13 (*M. condensatus*) showed few loss of green leaf, reaching the lowest values in accordance with the senescence score scale. *M. x giganteus* and *Goliath* showed a fully senesced plant with 80-90% loss of green leaf (Table 2). Regarding the lodging resistance score, the best genotypes were M3 and M12 (*M. floridulus*), M7 and M16 (*M. sacchariflorus*) and M19 (*M. x giganteus*) (Table 2).

Table 2 – Morphological measurements (panicle height ±SE, canopy width and canopy width at base [cm] and senescence and lodging observations, carried out on the studied *Miscanthus* accessions before the two spring harvests (2011 and 2012).

Genotype	Panicle height (cm)	Canopy width (cm)	Width at base (cm)	Senescence score (1 to 10)	Lodging Resistance (1 to 9)
M1	210.0±5.8	35	22	9	5
M2	280.0±5.8	85	40	2	7
M3	315.0±2.9	98	38	1	8
M5	120.0±8.7	37	20	8	4
M6	345.0±2.9	85	45	8	7
M7	342.5±1.4	82	48	10	8
M8	205.0±8.7	37	18	10	4
M9	202.5±21.6	48	25	10	5
M10	356.7±18.8	97	47	10	6
M11	210.0±5.8	72	50	2	6
M12	297.5±10.1	103	45	1	8
M13	321.7±4.4	130	50	1	7
M14	270.0±5.8	95	38	8	5
M15	326.7±8.8	98	48	8	7
M16	351.7±4.4	103	50	8	8
Goliath	210.0±2.9	62	40	10	7
M. x gig	305.0±2.9	68	48	10	8
M20	213.3±10.1	73	45	10	7

3.1.4 Productive traits

The stem height, on the average of the studied *Miscanthus* genotypes, was consistent: 181.0 cm in 2011 and 176.2 cm in 2012, but there was not any significant difference (Fig 2). On the average of the two years, the tallest genotype were M6, M16, M7and M10: they reached a final height equal to 276.7, 272.5, 268.3 and 248.3 cm, respectively, (Fig. 2). All these *Miscanthus* accessions belong to the *M. sacchariflorus* species. The smallest genotype, on the average of the two years, was M5. It

reached a final height equal to 80.0 cm (Fig. 2). This *Miscanthus* accession belong to the *M. sinensis* species. The other *Miscanthus* accessions varied their final height between 111.7 cm (M9 – *M. sacchariflorus*) to 237.5 cm (M15 – *M. sacchariflorus*) (Fig. 2). For *M. x giganteus* (M19), the height on the average of the two years was equal to 207.5 cm, while for *Goliath* (M18), it was equal to 130.0 cm (Fig. 2). In the first year the tallest genotype was M16 (300.0 cm) and the smallest was M5 (60.0 cm). The other *Miscanthus* accessions varied the final height between 113.3 cm (M1) to 293.3 cm (M6) (Fig. 2). In the second year the tallest genotype was M6 (260.0 cm) while the smallest was M5 (100.0 cm). The other *Miscanthus* accessions varied the final height between 106.9 cm (M9) to 246.7 cm (M7) (Fig. 2).

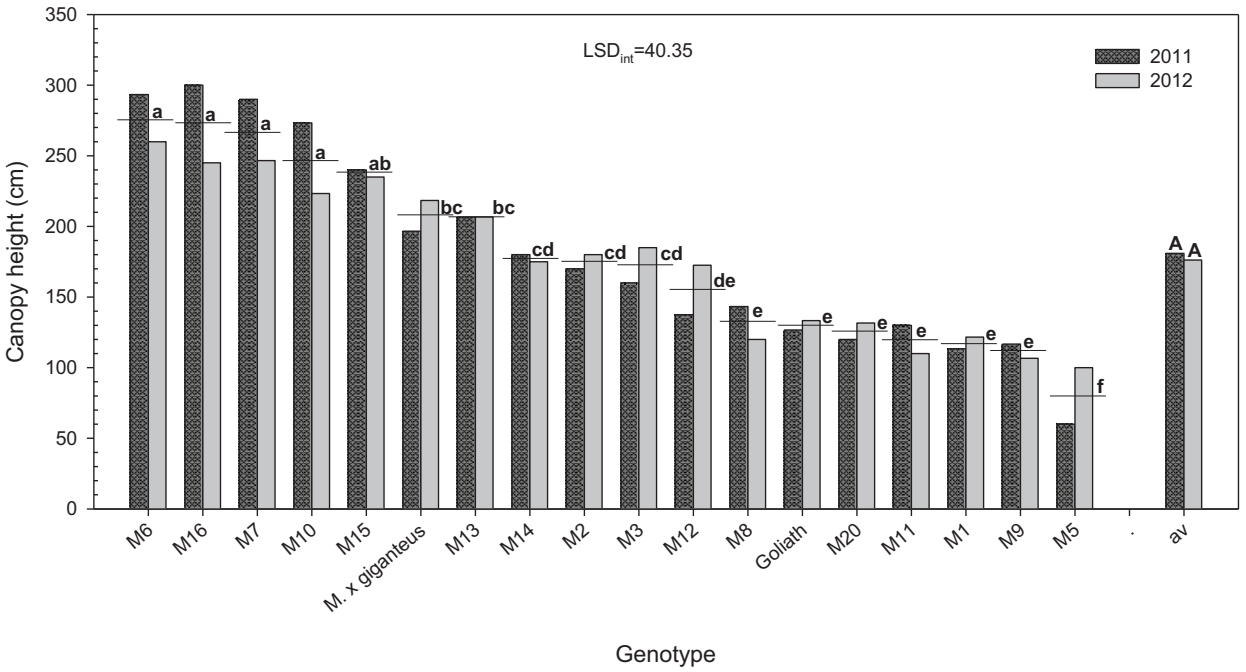

Figure 2 – Canopy height (cm) of the studied *Miscanthus* accessions after the first (2011) and second (2012) spring harvest. Bars represent the average value of 3 measurements. Horizontal lines within each bar represent the average value of two years. Small letters, for averaged values of years within each genotype; capital letters for averaged values of all genotypes within each year. Different letters indicate significant differences at P≤0.05 by SNK Test.

The above-ground fresh biomass on the average of the studied *Miscanthus* genotypes was equal to 19.0 t ha^{-1} and 18.2 t ha^{-1}, in the first and second year, respectively, and there was not any significant difference (Fig. 3).

On the average of the two years, the most yielding genotype was M13 (*M. condensatus*): it reached a total above-ground fresh biomass equal to 66.5 t ha^{-1}, followed by the three *Miscanthus* accessions belonging to the *M. floridulus* species: M2, M3 and M12. They reached a value of fresh biomass

equal to 54.9, 51.8 and 42.8 t ha^{-1}, respectively (Fig. 3). The other *Miscanthus* genotypes ranged their above-ground fresh biomass between 3.0 t ha^{-1} (M5 – *M. sinensis*) to 13.5 t ha^{-1} (M7 – *M. sacchariflorus*) (Fig. 3).

In the first year, among genotype, the highest yielding were M2 (*M. floridulus*) and M13 (*M. condensatus*), reaching a fresh biomass quantity equal to 65.6 and 64.7 t ha^{-1}, respectively. The lowest yielding genotype was M20 (*M. sinensis*), whose fresh biomass attained a minimum value of 1.9 t ha^{-1}. The other genotypes varied their total fresh biomass quantity between 2.0 t ha^{-1} (M9 – *M. sacchariflorus*) to 50.4 t ha^{-1} (M3 – *M. floridulus*) (Fig. 3).

In the second year, among genotype, the highest yielding was M13 (*M. condensatus*), reaching a fresh biomass quantity equal to 68.4 t ha^{-1}, while M2 (*M. floridulus*) decreased its final fresh biomass (-33%). The lowest yielding genotype was M5 (*M. sinensis*), whose fresh biomass attained a minimum value of 3.7 t ha^{-1}. The other genotypes varied their total fresh biomass quantity between 4.7 t ha^{-1} (M1 – *M. sacchariflorus*) to 53.2 t ha^{-1} (M3 – *M. floridulus*) (Fig. 3).

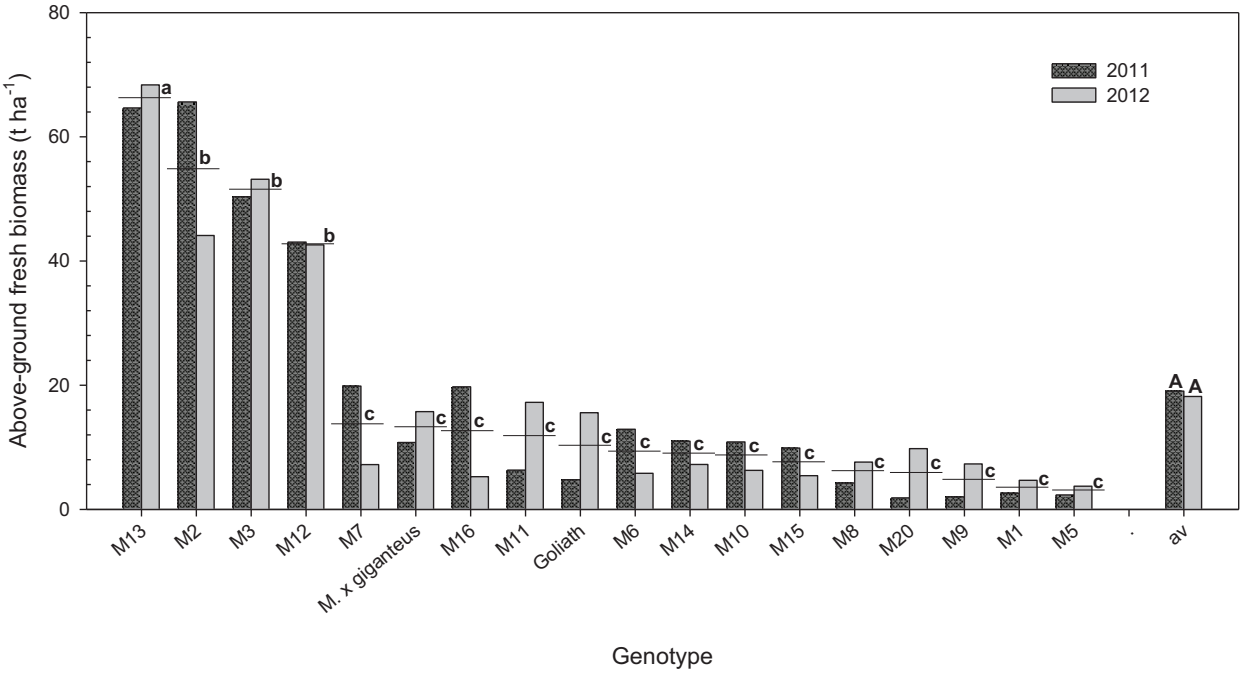

Figure 3 – Above-ground fresh biomass (t ha^{-1}) of the studied *Miscanthus* accessions after the first (2011) and second (2012) spring harvest. Bars represent the average value of 3 measurements. Horizontal lines within each bar represent the average value of two years. Small letters, for averaged values of years within each genotype; capital letters for averaged values of all genotypes within each year. Different letters indicate significant differences at P≤0.05 by SNK Test.

The biomass moisture content at the first harvest (2011) was equal to 30.2% and 36.8% at the second harvest (2012). The same trend was recorded for the above-ground dry biomass. The above-ground dry biomass on the average of the studied *Miscanthus* genotypes was equal to 10.8 t ha^{-1} and 10.5 t ha^{-1}, in the first and second year, respectively, and there was not any significant difference (Fig. 4).

On the average of the two years, the most yielding genotype was M13 (*M. condensatus*): it reached a total above-ground dry biomass equal to 36.0 t ha^{-1}, followed by the three *Miscanthus* accessions belonging to the *M. floridulus* species: M3, M2, and M12. They reached a value of dry biomass equal to 23.2, 23.1 and 19.8 t ha^{-1}, respectively (Fig. 4). The other *Miscanthus* genotypes ranged their above-ground dry biomass between 2.3 t ha^{-1} (M5 – *M. sinensis*) to 11.3 t ha^{-1} (M19 – *M. x giganteus*) (Fig. 4).

In the first year, among genotype, the most yielding was M13 (*M. condensatus*), reaching a dry biomass quantity equal to 35.0 t ha^{-1}. The lowest yielding genotype was M20 (*M. sinensis*), whose dry biomass attained a minimum value of 1.5 t ha^{-1}. The other genotypes varied their total dry biomass quantity between 1.8 t ha^{-1} (M5 – *M. sinensis* and M9 – *M. sacchariflorus*) to 27.7 t ha^{-1} (M2 – *M. floridulus*) (Fig. 4).

In the second year, among genotype, the most yielding was M13 (*M. condensatus*), reaching a dry biomass quantity equal to 36.9 t ha^{-1}. The lowest yielding genotype was M5 (*M. sinensis*), whose dry biomass attained a minimum value of 2.8 t ha^{-1}. The other genotypes varied their total dry biomass quantity between 3.9 t ha^{-1} (M1 – *M. sacchariflorus*) to 23.9 t ha^{-1} (M3 – *M. floridulus*) (Fig. 4).

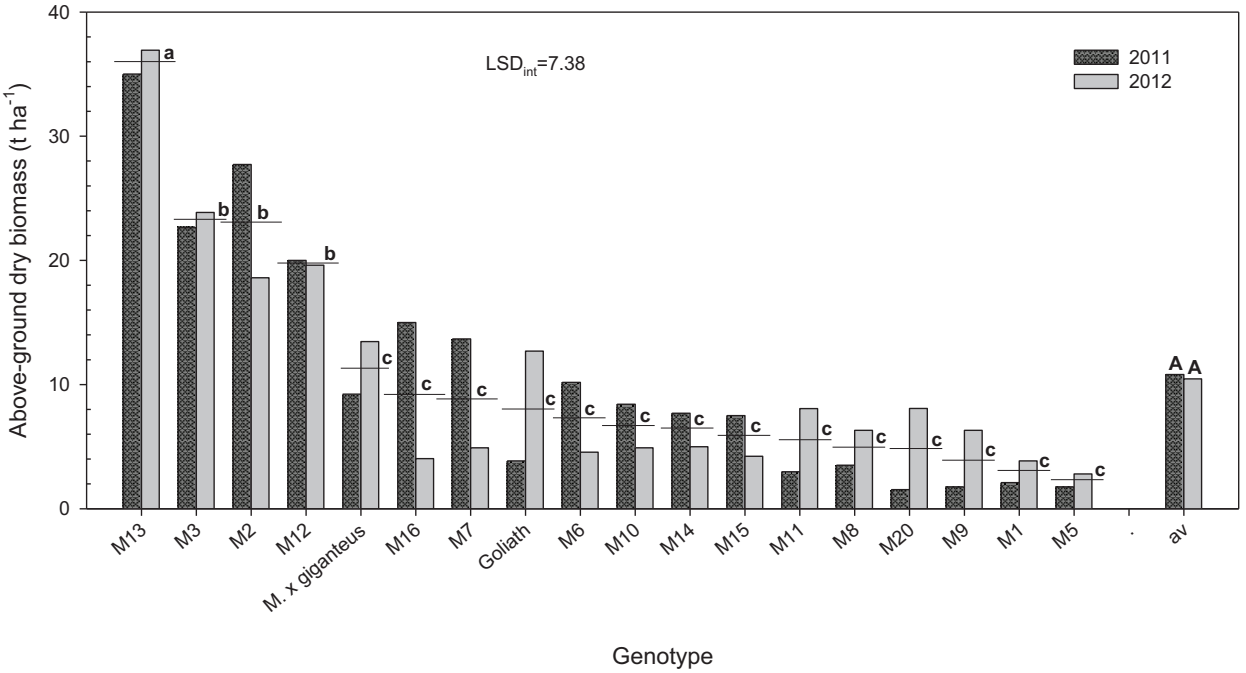

Figure 4 – Above-ground dry biomass (t ha^{-1}) of the studied *Miscanthus* accessions after the first (2011) and second (2012) spring harvest. Bars represent the average value of 3 measurements. Horizontal lines within each bar represent the average value of two years. Small letters, for averaged values of years within each genotype; capital letters for averaged values of all genotypes within each year. Different letters indicate significant differences at P≤0.05 by SNK Test.

In central Europe, third year dry matter yields of *M. sinensis* hybrids, defined as crosses between two *M. sinensis* genotypes, or a *M. sacchariflorus* and *M. sinensis* cross, ranged from 6.5-17.7 t ha^{-1} in England to 10.3-20.0 t ha^{-1} in Germany while third year dry matter yields of *M. sinensis* from collections in Denmark and Sweden originally sourced from Japan ranged in yield from 4.6-10.9 t ha^{-1} dry matter in England, and 9.1-12.8 t ha^{-1} dry matter in Germany (Clifton-Brown et al, 2001a). In this research the *Miscanthus* accessions belonging to *M. sinensis* species reached a dry matter yield equal to 2.3 t ha^{-1} (M5), 8.3 t ha^{-1} (M18) and 4.8 t ha^{-1} (M20).

In biomass tests in Europe *M. sinensis* has displayed greater winter hardiness than other species, providing more consistent production in cooler climates, but is generally lower yielding than *M. x giganteus* species where both survive (Clifton-Brown et al, 2001a). In this research, for *M. x giganteus*, the dry matter biomass was equal to 11.3 t ha^{-1}, while for *M. sinensis* was equal to 5.1 t ha^{-1}, on average of the three *M. sinensis* accessions.

In regions where winter survival was high, third year yields for the *M. sacchariflorus* genotype tested were generally moderate, ranging from 11.1 t DM ha^{-1} in England to 12.6 t DM ha^{-1} in Germany; less than *M. x giganteus* but higher than *M. sinensis* for these same regions. In this

research, the average value of the dry matter yield, for all *Miscanthus* accessions belonging to *M. sacchariflorus* species, was equal to 6.3 t ha^{-1}, with a maximum value for M16 (9.5 t ha^{-1}) and a minimum value for M1 (3.0 t ha^{-1}).

A review of autumn yields in Europe showed that the maxima from *M. x giganteus* were obtained in France, under irrigated and fertilized conditions: being, respectively, 49 and 42 t DM ha^{-1} (Clifton-Brown et al., 2004). These yields ranged from 15 to 25 t ha^{-1} without irrigation. For winter yields of *M. x giganteus*, the highest non-irrigated yields were found to be 15-19 t DM ha^{-1} during the trials performed by the Miscanthus Productivity Network. Cosentino et al. (2007), demonstrated that *M. x giganteus*, in Mediterranean environment showed a high yield potential even in very limited water availability conditions (more than 14 t DM ha^{-1} with a 25% ETm restoration). In this research, the above-ground dry biomass for *M. x giganteus* was equal to 9.23 and 13.46 t ha^{-1} for the growing seasons 2010 (when some >280mm of water has been applied during the summer season from June to August) and 2011 (when the plots were not irrigated during the summer season), respectively.

3.1.5 Water Use Efficiency (WUE)

Water Use efficiency, expressed as ratio between dry biomass production (g ha^{-1}) at final harvest and water (l ha^{-1}) used by the crop (irrigation + rainfall), showed on the average of the studied treatments (genotype and growing season) significant differences, to be attributed to the different ability of the *Miscanthus* genotypes to exploit the water resource (Table 3). During the growing seasons (from April to November), the water soil content (irrigation + rainfall) reached a value equal to 502.8 mm and 504.6 mm, for 2010 and 2011 growing season, respectively. The highest WUE value was recorded for M13 (*M. condensatus*), it reached a maximum value equal to 7.1 g l^{-1}. The lowest WUE values ranged between 0.5 g l^{-1} (M5 – *M. sinensis*) and 2.3 g l^{-1} (M19 – *M. x giganteus*) (Table 3). The water use efficiency, on the average of the studied *Miscanthus* accessions, was equal to 2.1 g l^{-1}. There was not any significant difference between the WUE values of the first and second year (Table 3). The most yielding genotypes, in terms of dry biomass, (M13 – *M. condensatus*, M3-M2-M12 – *M. floridulus*) reached the highest WUE values, on the average of the studied treatments: they reached WUE values equal to 7.1, 4.6, 4.6 and 3.9 g l^{-1}, respectively (Table 3).

Table 3 – Water Use Efficiency (WUE) of the studied *Miscanthus* accessions in relation to the above-ground dry biomass. Small letters, for averaged values of years within each genotype**.** Capital letters, for averaged values of all genotypes within each year. Different letters indicate significant differences at P≤0.05 by SNK Test.

Genotype	Dry biomass (t ha^{-1})			WUE (g l^{-1})		
	2011	2012	av	2011	2012	av
M13	35.00	36.92	**35.96a**	7.0	7.3	***7.1a***
M3	22.63	23.86	**23.25b**	4.5	4.7	***4.6b***
M2	27.72	18.60	**23.16b**	5.5	3.7	***4.6b***
M12	20.00	19.62	**19.81b**	4.0	3.9	***3.9b***
M. x giganteus	9.23	13.46	**11.35c**	1.8	2.7	***2.3c***
M16	15.00	4.04	**9.52c**	3.0	0.8	***1.9c***
M7	13.68	4.91	**9.30c**	2.7	1.0	***1.8c***
Goliath	3.85	12.69	**8.27c**	0.8	2.5	***1.6c***
M6	10.18	4.56	**7.37c**	2.0	0.9	***1.5c***
M10	8.42	4.91	**6.67c**	1.7	1.0	***1.3c***
M14	7.69	5.00	**6.35c**	1.5	1.0	***1.3c***
M15	7.50	4.23	**5.87c**	1.5	0.8	***1.2c***
M11	2.98	8.07	**5.53c**	0.6	1.6	***1.1c***
M8	3.51	6.32	**4.92c**	0.7	1.3	***1.0c***
M20	1.54	8.08	**4.81c**	0.3	1.6	***1.0c***
M9	1.75	6.32	**4.04c**	0.3	1.3	***0.8c***
M1	2.11	3.86	**2.99c**	0.4	0.8	***0.6c***
M5	1.75	2.81	**2.28c**	0.3	0.6	***0.5c***
av	**10.8A**	**10.5A**	**10.6**	**2.1A**	**2.1A**	**2.1**

Because of its C_4 photosynthetic pathway and perennial rhizome, *Miscanthus* displays quite good combination of water-use efficiency for biomass production (Lewandowski et al., 2000; Heaton et al., 2004; Lewandowski and Schmidt, 2006). Water limitation is relevant especially in Southern Europe where, due to high temperature and irradiation, there are potentially high productive sites for C_4 crops. Since irrigation of biomass crops is unlikely to be economic, it is important to identify genotypes that optimize the use of water in different climatic regions, and those which are tolerant of water stress. Under water and nitrogen supplies, the water use efficiency studies showed that an adult *M. x giganteus* stand reached between 9.1 and 9.5 g DM l^{-1} in the UK (Beale et al., 1999) and between 6 and 10 g DM l^{-1} in France (Cadoux et al., 2008). Lower values were found in a Mediterranean environment, with a negative correlation of -0.87 between water availability and WUE (Cosentino et al., 2007). Beale et al. (1999) showed that irrigation during dry periods reduced the WUE of the crop by 15%, with a higher water consumption of 45%. Clifton-Brown and Lewandowski (2000), demonstrated that differences in biomass partitioning were observed among

Miscanthus genotypes (*M. x giganteus*, *M. sacchariflorus* and a *M. sinensis* hybrid), in a pot experiment, which was conducted to measure the influence of reduced water supply on the water use efficiency and biomass partitioning, in a controlled environment. This does not agree with observations made on other species (e.g. maize, soybean, cotton and squash) where water stress led to stimulation of root growth and the suppression of shoot growth (Spollen et al., 1993).

Cosentino et al. (2007) demonstrated that WUE for *M. x giganteus*, in Mediterranean environment, achieved the highest values in limited soil water availability (between 4.51 and 4.83 g l^{-1}), whilst in non-limiting water conditions it decreased down to 2.56 and 3.49 g l^{-1}. These values are comparable to those obtained in the present experiment, where WUE for *M. x giganteus* was equal to 1.8 and 2.7 g l^{-1}, for the 2011 and 2012 harvests, respectively (Table 3). These values are comparable to those obtained in other field experiments carried out in the same environment, with other C_4 crops such as sweet sorghum (from 4.52 to 6.10 g l^{-1}) (Cosentino, 1996). Previous experiments carried out with *M. x giganteus* gave similar results (from 2.88 to 3.57 g l^{-1}) (Foti et al., 1996).

Genotypic variability has been studied with respect to WUE. Under controlled conditions, Clifton-Brown et al. (2000) demonstrated similar WUE values for young shoots of *M. x giganteus* and *M. sinensis* (2 g DM l^{-1}), but the highest value was seen in *M. sacchariflorus* (3.8 g DM l^{-1}). Several studies comparing the WUE (Beale et al., 1999) of *Miscanthus* with other energy crops concluded as to the superiority of *Miscanthus* efficiencies over other species.

3.2 *Miscanthus* biomass yield and biomass quality as affected by harvesting dates

3.2.1 Harvest time effect on above-ground dry biomass yield

The harvest time effect is shown in figure 5. Above-ground biomass yield was determined by hand harvest of small sub-plots (3x3m) inside the large fields. The dry biomass yield obtained in February 2012 (15.4 t DM ha^{-1}), after 16 years from transplant, was similar to those obtained from Cosentino et al. (2007) in absence of external input (e.g. fertilization and irrigation). The subsequent winter harvest (February 2013), lower biomass DM yield was observed probably due to less rainfall recorded during 2012/2013 growing season as compared to the previous one. Indeed, only 378.1 mm of rainfall were recorded than the 550 mm typical of the experimental area and of the 2011/2012 growing season. In these conditions *Miscanthus* produced 9.1 ± 1.6 t DM ha^{-1}. In both autumn harvests (September 2012 and 2013) a very low dry biomass production was recorded (2.5 t DM ha^{-1} and 2.1 t DM ha^{-1}, respectively). While the first autumn harvest was performed after seven month growing season (from February 2012 to September 2012) and therefore with no

optimal biomass accumulation, the second autumn harvest (September 2012 to September 2013) was characterized by extreme drought conditions, with only 342.7 mm of rainfall registered (Fig. 6).

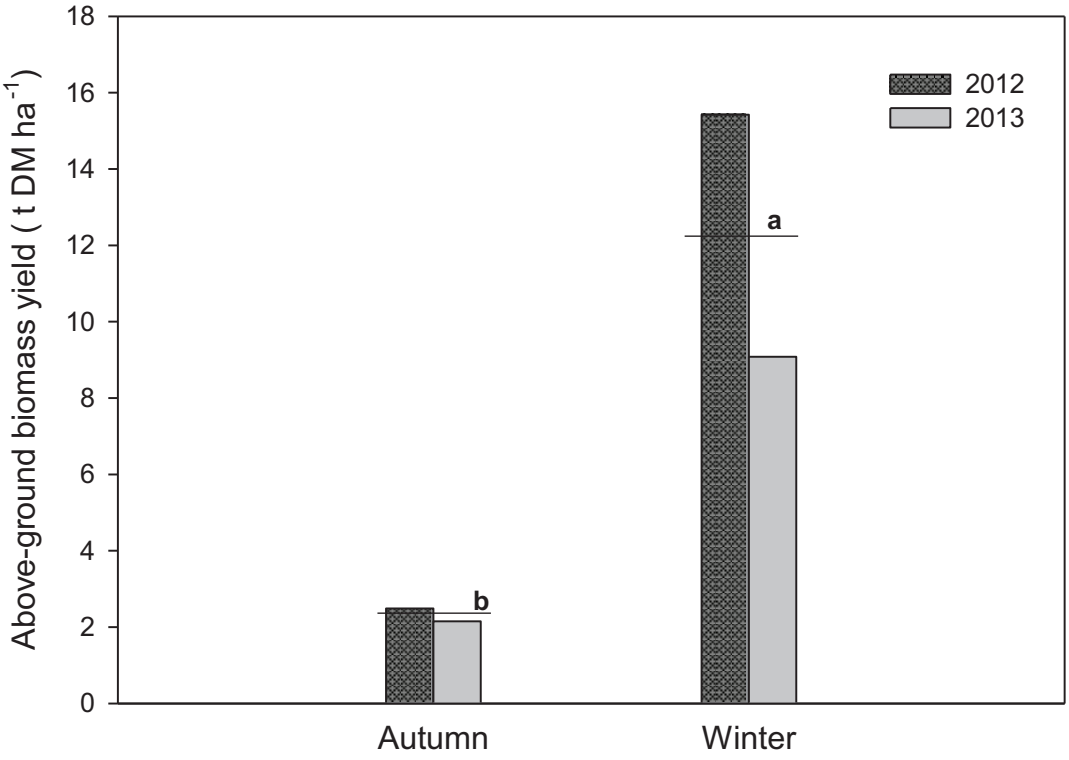

Figure 5 - Above-ground dry matter yield (t ha^{-1}) at different harvest dates and year of harvest (autumn and winter 2012 and 2013). Horizontal lines represent average value of two years harvest. Different letters indicate significant differences at $P \leq 0.05$ by SNK Test.

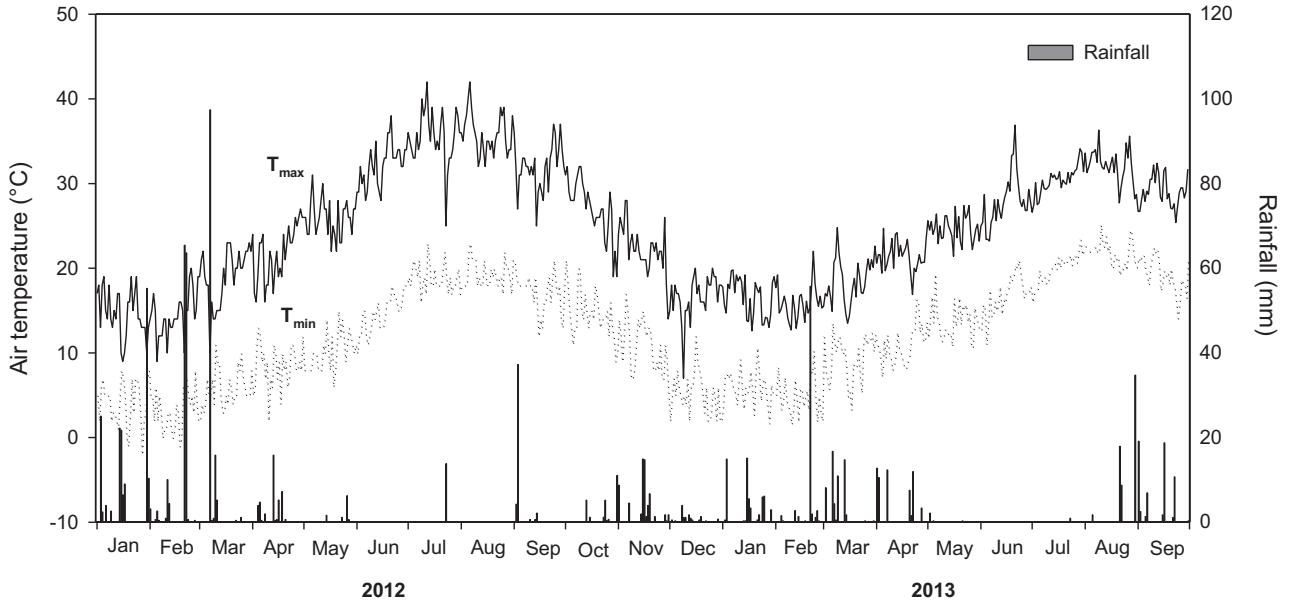

Figure 6 - Meteorological trend during 2012 and 2013 growing season at the experimental site of Catania University (Catania plain, 10 m a.s.l., 37°27'N, 15° 03' E).

Yield is very low during the first year (less than 10 t ha^{-1} for *M. giganteus*) but these figures are usually not known as the grass is not harvested. During subsequent years, peak yields are obtained in the autumn, at the full plant flowering phase (Cosentino et al., 2007) and then decline through the winter due to leaf loss. Harvestable yields in the spring are 27% - 50% lower than in the autumn (Clifton-Brown et al., 2001b; Cadoux et al., 2008; Himken et al., 1997; Jorgensen et al., 2003b; Richter et al., 2008). However, there are no consistent information in autumn or winter long term yields. Our results point out that satisfactory yields, in south Mediterranean area, can be sustained only by well rainfall distribution during the growing stages of the crop.

3.2.2 Fertilization effect on the above-ground dry biomass yield

The fertilization effect on the above-ground dry biomass yield in autumn 2013 harvest after differentiation of nitrogen fertilization is shown in figure 7. Plots #7, 8 and 9 were not fertilized, while in #16, 17 and 18 (see Figure 2 in materials and methods) ammonium sulphate was distributed after September 2012 harvest.

Plots, fertilized with ammonium sulphate, increased of 0.94 t DM ha^{-1} in *Miscanthus* than unfertilized plots. However, above-ground biomass yield was slightly higher as compared to the harvest of September 2012 which was not fertilized since several year probably due to the thermopluviometric trend.

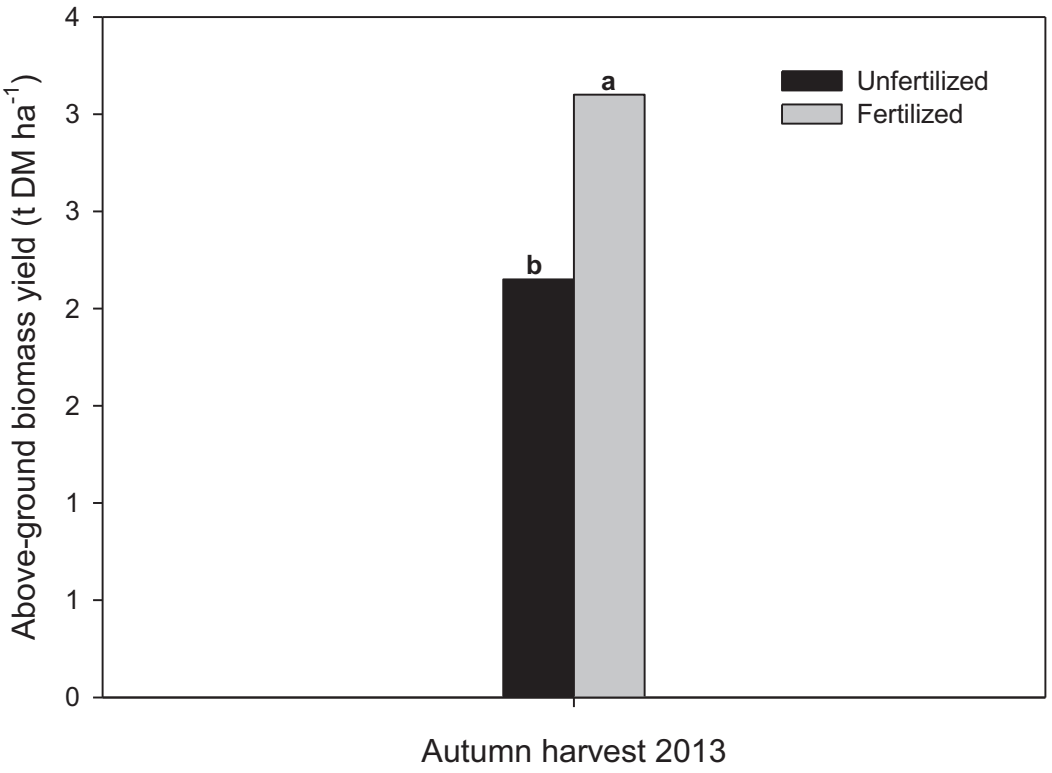

Figure 7 - Nitrogen fertilization effect on above-ground dry biomass yield in *Miscanthus* x *giganteus* (plots harvested in September 2013). Different letters indicate significant differences at P≤0.05 by SNK Test.

Biomass production by *M. x giganteus* has been described as being dependent on soil water availability, air temperature and precipitation (Richter et al., 2008), but there is no consensus yet in terms of this crop's nitrogen fertilization requirement. Indeed, many authors suggest that N fertilization has no effect on biomass production (Christian et al., 2008; Clifton-Brown et al., 2007; Danalatos et al., 2007; Himken et al., 1997) whereas others report that nitrogen fertilization is needed to achieve maximum biomass production (Boehmel et al., 2006; Cosentino et al., 2007; Ercoli et al., 1999). However, a consensus view is that the nitrogen requirement of *M. x giganteus* to achieve maximum biomass yields is low compared with that of other crops (Lewandowski and Schmidt, 2006).

3.2.3 Biometric measurements at different harvest dates and year of harvest

Biometric measurements have been performed in both harvests (autumn and winter) and compared in terms of moisture content, basal stem diameter, stem height, stem node number, fresh weight of stems and leaves, fresh biomass yield and biomass partitioning (table 4).

Basically, autumn harvest lead to higher leaves to stems ratio, weight of one stem and higher moisture content of both stems and leaves, while winter harvest lead to higher stem density, stem node number, plant height and fresh biomass yield.

The highest stem number was observed in winter 2012 (125.2 stem m^{-2}), the lowest in autumn 2013 (38.0 stem m^{-2}). Weight of one stem was highest in autumn 2012 (27.0 g), while quite similar values were recorded in autumn and winter 2013 (15.0 g and 16.6g , respectively). Plant height and node number were highest in February 2012 and 2013 (169 and 149 cm with 10.2 and 14.9 nodes, respectively), while plant height measured only 58.9 and 41.9 cm in autumn 2012 and 2013, respectively, with 5.4 and 5.7 node number. Basal stem diameter was highest in winter 2012 (7.2 mm) than autumn 2012 (5.7); in the subsequent growing season basal stem diameter was quite similar in both autumn 6.6 mm and winter harvest (6.4 mm).

Stem fresh weight was higher in 2012 harvests (16 g and 14.5 g in winter and autumn) than 2013 harvest (9.7 g and 10.9 g in winter and autumn), while leaves were completely lost in winter 2012 and amounted at 12.4 g in autumn 2012. Winter harvest in 2013 had lower leaves weight than autumn harvest (4.9 g and 6.1 g, respectively).

Biomass was partitioned as 100% stems in 2012 due to leaves senescence and lost, while in winter 2013 34 and 66% of leaves and stems were recorded. Autumn harvest had higher leaves than winter harvest, with a biomass partitioned as 52.4% and 46.6% stem and leaves in 2012 and 57.2% and 41.2% in 2013.

Moisture content was extremely lower in winter harvests as compared to autumn harvests. It was only 14.2% in the stems in winter 2012, while 9.2% and 8.2% in stems and leaves in winter 2013. In both autumn harvests moisture content was higher than 50% in both leaves and stems, except in leaves harvested in autumn 2013 (41.2%).

As discussed previously for dry matter yield, fresh biomass yield was highest in winter 2012 (17.9 t ha^{-1}), followed by winter 2013 (10.5 t ha^{-1}), while only 6.6 and 5.4 t ha^{-1} were recorded in autumn 2012 and 2013 harvests, respectively.

Table 4 - Biometrical measurements and at different harvest dates and year of harvest (February 2012 and September 2012, February 2013 and September 2013).

	Miscanthus x *giganteus*			
	Feb 2012	*Sep 2012*	*Feb 2013*	*Sep 2013*
Stem numbers (n.)	125.5±3.6	39.9±0.6	65.9±24.87	38.0±6.1
Weigth of 1 stem (g)	16.1±0.6	27.0±1.7	15.04±5.6	16.61±1.4
Node number (n.)	10.2±0.5	5.4±0.2	14.89±1.59	5.67±0.3
Basal stem diameter (mm)	7.2±0.4	5.7±0.1	6.4±0.6	6.59±0.9
Plant height (cm)	169.3±1.5	58.9±2.4	149±41.1	41.92±4.6
Stem fresh weigth (g)	16.0±0.7	14.5±1.2	9.72±3.6	10.91±1.6
Leaf fresh weigth (g)	0.0±0.0	12.4±0.7	4.9±1.7	6.11±0.7
Stem %	100±0.0	52.4±0.8	65.93±3.9	57.20±2.6
Leaf %	0.0±0.0	46.6±0.3	34.07±3.5	41.21±3.6
H_2O stems %	14.2±0.6	58.6±0.5	9.2±1.5	57.20±2.6
H_2O leaves %	0.0±0.0	55.0±0.4	8.6±1.8	41.21±3.5
Fresh biomass yield (t ha^{-1})	17.9±2.1	6.6±0.5	10.5±4.4	5.41±1.1

3.2.4 Raw material composition of Miscanthus x giganteus

Raw material composition has been analysed taking into account two specific end uses: second generation bioethanol production and energy/heat production through biomass combustion. In the former case fiber quality has been determined, while in the latter ash content, among others, is a good indicator of the biomass quality for combustion purposes.

Lignocellulosic cell wall, as *Miscanthus* cell wall, is made-up by structural and no-structural compounds. The latter are represented by NDS (Neutral Detergent Soluble), namely the non-cell wall soluble substances easily removed from the biomass when reacts with a neutral detergent, as sucrose, pectin, starch and non-starch compounds.

The cell wall structure (hemicellulose, cellulose and lignin), on the other hand, is identified with NDF (Neutral Detergent Fiber), ADF (Acid Detergent Fiber) and ADL (Acid Detergent Lignin), according to Van Soest method (1991). In detail the NDF contain hemicellulose, cellulose and lignin, ADF cellulose and lignin and ADL lignin only. By calculation of the difference between NDF and ADF it is possible to estimate the hemicellulose, while the cellulose comes from the difference between ADF and ADL. Burning the residues in a muffle furnace at 550 ± 50 °C and obtaining the ash we can calculate the lignin as difference between ADL and ash.

Raw material composition of autumn (September 2013 unfertilized) and winter (February 2013) harvests have been performed in order to compare the best harvest time for specific end-uses. Fiber composition has been carried out separately on stems and leaves in order to ascertain the contribution of each biomass part on quality. Basically, it was noted that the winter harvest lead to a decrease of NDS and ash content and increase of NDF, ADF and ADL content than the autumn harvest in both leaves and stems (figure 8).

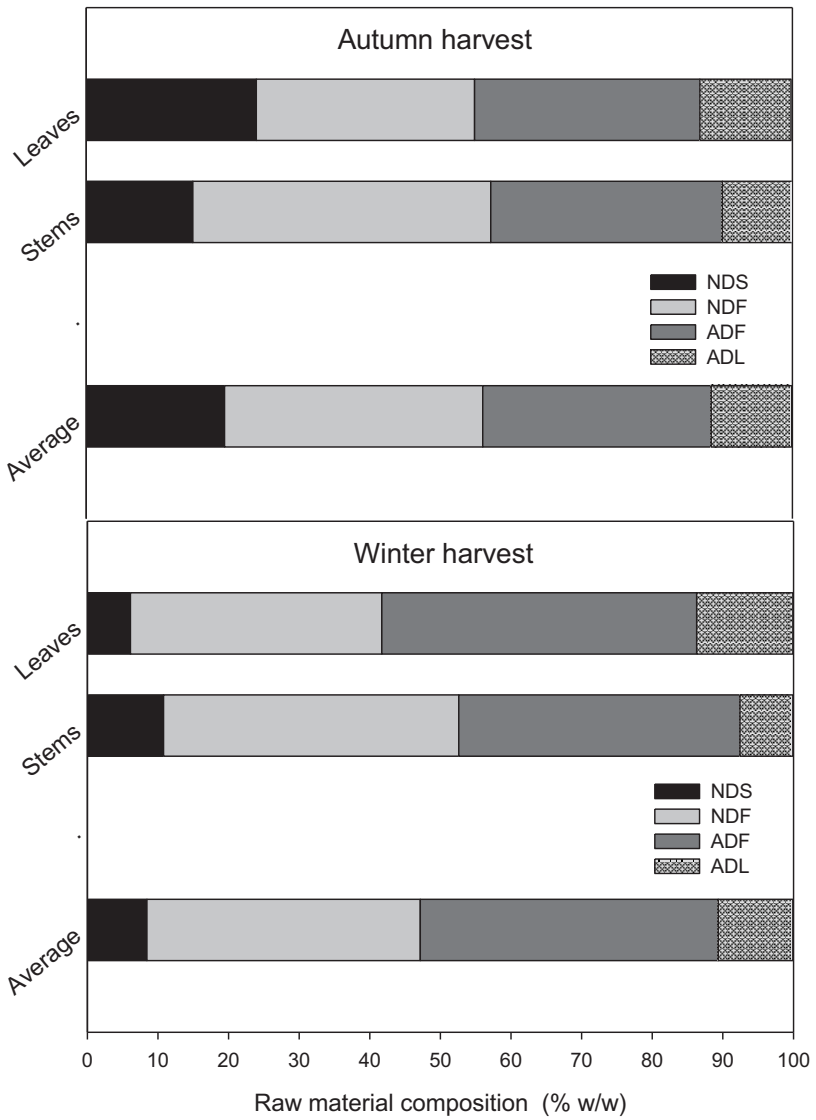

Figure 8 – Raw material composition (leaves and stems) of *Miscanthus* x *giganteus* harvested in autumn and winter 2013. Average value of three determinations.

In the average of stem and leaves, NDS reduced from 19.4 to 8.4% moving from autumn to winter harvest, while ADL was quite similar in both harvest (11.4% in autumn and 10.6 in winter). NDF and ADF which represent the hemicellulose and cellulose, primary substrate for ethanol production, increased, in the average of stems and leaves, from 36.5% to 38.7% (NDF) and from 32.3% to 42.2% (ADF) in autumn and winter harvest, respectively.

In autumn harvest leaves were richer in NDS and ADL content than stems (24.1% vs. 15.1% and 12.9% vs. 9.9%), while stems had higher content of NDF than leaves (42.2% vs. 30.9%) and quite similar ADF values (32.8% in stems and 31.9% in leaves). In winter harvest the opposite trend was observed for NDS content (10.8% in stems and 6.1% in leaves), while NDF was again higher in stems than leaves (41.8 vs. 35.6%). ADF and lignin content were higher in leaves than stems (44.6% vs. 39.8% and 13.6% vs. 7.5%).

Taking into account biomass partitioning (stems to leaves) and biomass composition, winter harvest lead to higher biomass quality for second generation bioethanol, since higher structural polysaccharides, lower lignin content and lower leaves to stem ratio is achieved than autumn harvest.

Scordia et al. (2013) has reported that structural polysaccharides of *M.* x *giganteus* raw material is mainly constituted by cellulose fraction, with hemicelluloses mostly represented by xylan and arabinan, and galactan, mannan and rhamnan making up a little part (less than 1%).

A total polysaccharide content of 67.4% was found in *M. x giganteus* grown in Belgium (Vanderghem et al., 2012), while lower values (54.8%) were reported with *M. x giganteus* cultivated in Korea (Kim et al., 2012).

The present results agree with Scordia et al. (2013) and Vanderghem et al. (2012) if considered autumn harvest, while higher values than the literature have been obtained in polysaccharide content in winter harvest.

It is worth to note, however, that cell wall composition may differ due to method of analysis, climate and harvesting date, crop age, cultivation practices and part of the plant analysed (stem and leaves ratio).

3.2.5 Ash content in leaves and stems of Miscanthus x giganteus

Ash content has been determined separately on stems and leaves and harvesting date (autumn and winter), as shown in figure 9. In the average of stems and leaves, about two-fold higher ash content was found in autumn than winter harvest (4.8% vs 2.6%). According to the plant part, leaves contained more ash than stems both in autumn (6.1% vs 3.5%) and winter harvest (3.1% vs 2.1%).

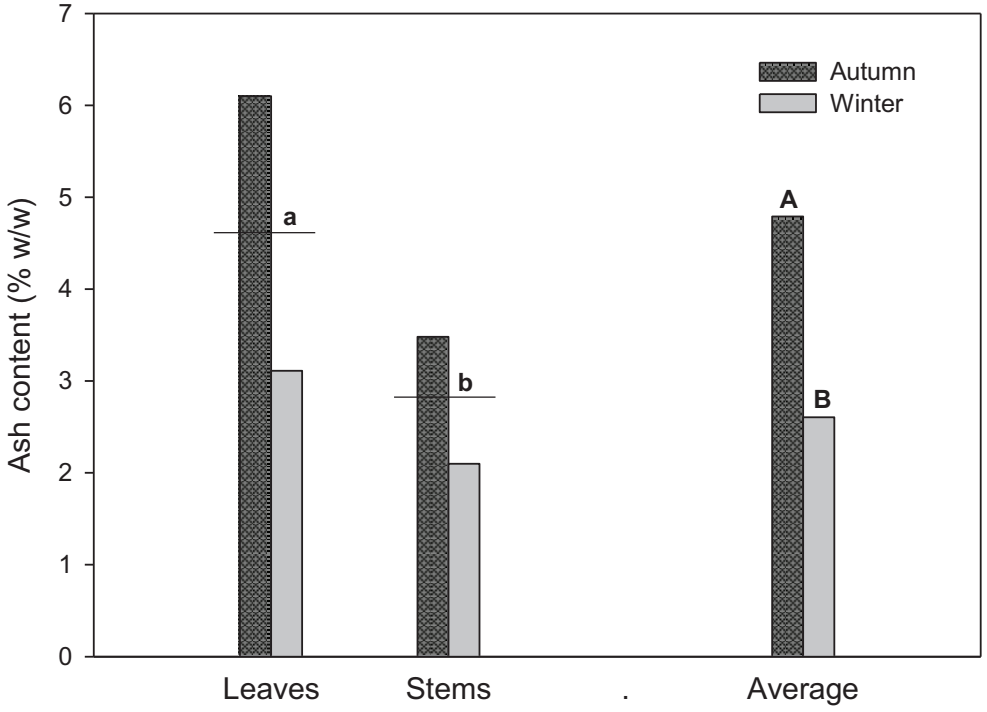

Figure 9 - Ash content in leaves and stems of *Miscanthus* x *giganteus* harvested in winter and autumn 2013. Horizontal lines represent average value of two dates harvest. Small letters, for averaged values of dates harvest within leaves and stems; capital letters for averaged values of all leaves and stems within each dates harvest. Different letters indicate significant differences at P≤0.05 by SNK Test.

This is mainly due to N cycling within the crop. In spring, part of the rhizome nitrogen stocks are remobilized from belowground to aboveground organs (hereafter referred to as spring remobilization). Part of the nitrogen accumulated in aboveground parts is subsequently remobilized from aboveground to belowground organs (hereafter referred to as autumn remobilization) during autumn and winter (Beale and Long, 1997; Christian et al., 2006; Himken et al., 1997).

M. x giganteus is currently used in combustion to produce heat and electricity, and is thus harvested in late winter to benefit from improved quality with regard to combustion processes, i.e. low mineral and moisture content (Lewandowski and Heinz, 2003).

The development of an industrial process for converting cellulose to ethanol is likely to make early harvest of green material interesting, since the quality criteria for this type of conversion relate to

lignocellulose content and recalcitrance (Karp and Shield, 2008). In a recent study, Le Ngoc Huyen et al. (2010) showed that saccharification yields of early harvested biomass were higher than those of late harvested plants. However, early harvest could increase the crop nitrogen requirement due to preventing or limiting leaf losses and autumn remobilization, which in turn could prevent or limit nitrogen recycling in the soil-crop system.

3.3 Effect of heat stress on the biomass production and physiology in *Miscanthus* genotypes

a) Morphological traits of the studied *Miscanthus* accessions

3.3.1 Stem height

The height of the plants at final harvest was significantly affected by the different growth temperature. On the average of all studied genotypes it was 112.1 cm for the plants grown and measured at low temperature and 58.4 cm for plants grown and measured at high temperature (Fig. 10). Relative to low temperature, high temperature decreased plant height (~48%).

On the average of the two different growth temperature, *M. x giganteus* was significantly affected by the different growth temperature showing the highest final height (137.2 cm) while *M. sinensis* was the lowest genotype (48.3 cm). The height of the other genotypes ranged between 67.5 cm (*M. floridulus*), 72.6 cm (*Goliath*) and 100.7 cm (*M. Sacchariflorus*) (Fig. 10).

In T_{low} the highest genotype was *Miscanthus x giganteus* (171.3 cm) while the lowest one was *M. floridulus* (44.8 cm). The height of the other genotypes ranged between 84.4 cm (*M. sinensis*), 127.2 cm (*Goliath*) and 132.9 cm (*M. sacchariflorus*) (Fig. 10).

In T_{high} all the studied *Miscanthus* genotypes showed a lower final height than in T_{low}. Only *M. floridulus* showed a different response to T_{high}, it doubled its final height from 44.8 cm in T_{low} to 90.1 cm in T_{high}, *M. x giganteus* was again the tallest genotype (103.1 cm), showing a percentage decrease equal to 40%. The height of the other genotypes ranged between 12.2 cm (*M. sinensis*), 18.0 cm (*Goliath*) and 68.5 cm (*M. Sacchariflorus*) (Fig. 10).

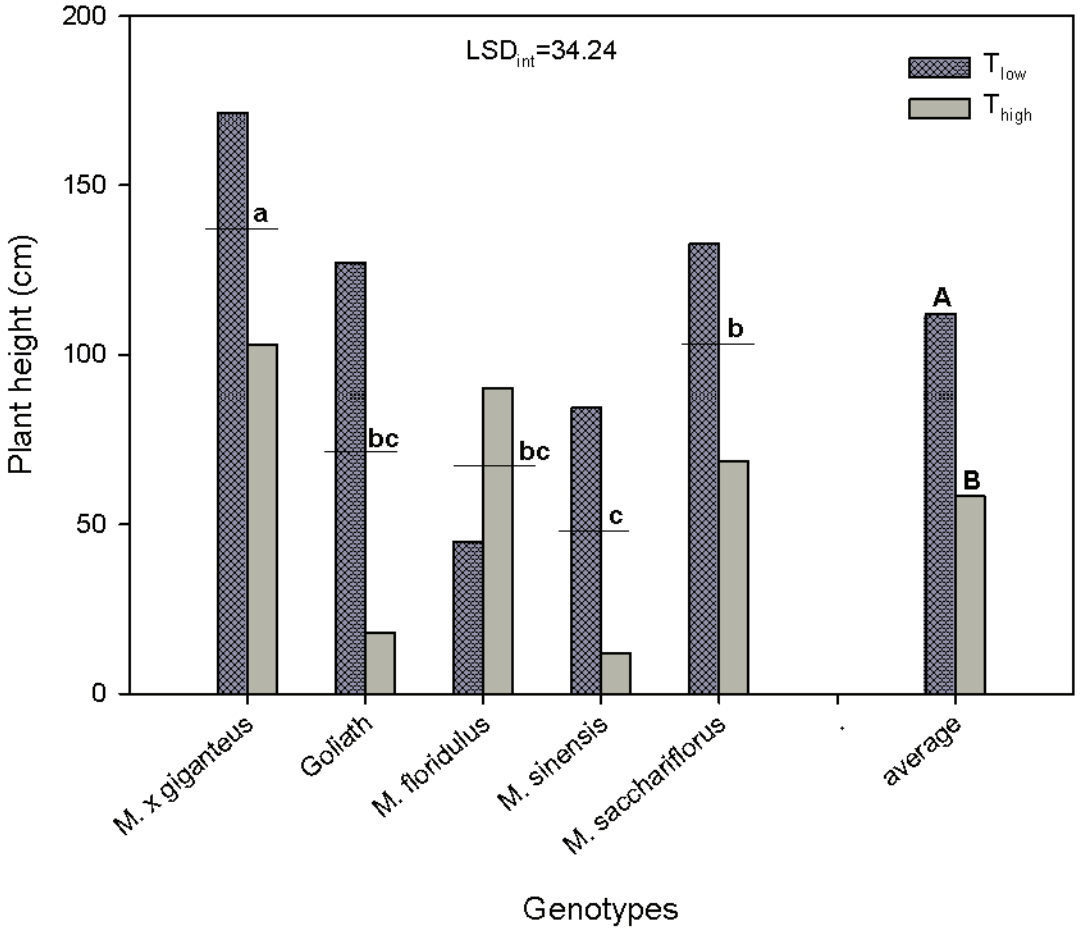

Figure 10 – Height plants (cm) of *Miscanthus* genotypes grown and measured at low and high temperature at the end of the heat stress experiment. Bars represent the average value of 5 measurements. Horizontal lines represent average value of the two growth temperature. Small letters, for averaged values of temperatures within each genotype; capital letters for averaged values of all genotypes within each temperature. Different letters indicate significant differences at P≤0.05 by Tukey's HSD Test.

3.3.2 Stem and leaf number per plant

The growth temperature, to which *Miscanthus* genotypes were exposed to, affected significantly the stem and leave number per plant (Figs. 11 and 12). On the average of all studied genotypes the stem number per plant was 6.9, for the plants grown and measured at low temperature, and 9.0, for plants grown and measured at high temperature (Fig. 11). Relative to high temperature, low temperature decreased the stems number per plant (~23%). On the average of the two different growth temperature, *M. sinensis* was the genotype that showed the highest stems number (18.8 stems/plant), while the genotype that showed the lowest value was *M. sacchariflorus* (4.7 stems/plant) (Fig. 11). *Goliath* and *M. sinensis* showed the highest stems number per plant in T_{high} (13.0 and 18.8 stems/plant, respectively), than in T_{low} (7.2 and 9.2 stems/plant, respectively). By contrast, *M. x giganteus*, *M. floridulus* and *M. sacchariflorus* showed the highest stems number per

plant in T_{low} (5.4, 7.6 and 5.2 stems/plant, respectively) than in T_{high} (4.2, 5.0 and 4.2 stems/plant, respectively).

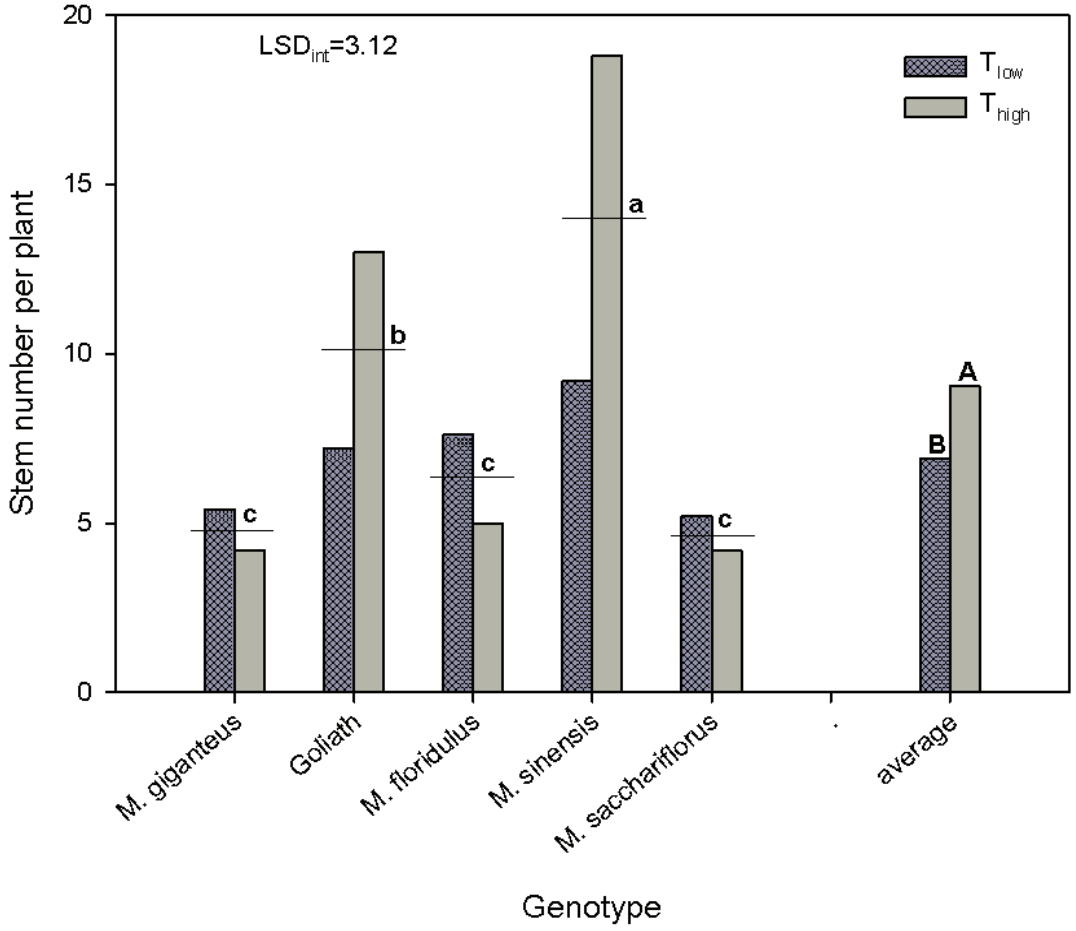

Figure 11 – Stem number of the studied *Miscanthus* genotypes grown and measured at low and high temperature at the end of the heat stress experiment. Bars represent the average value of 5 measurements. Horizontal lines represent average value of the two growth temperature. Small letters, for averaged values of temperatures within each genotype; capital letters for averaged values of all genotypes within each temperature. Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD Test.

Regarding the leaf number per plant, most of the genotypes showed more leaves at high temperature but *M. floridulus* showed a different trend, producing more leaves at low temperature (Fig. 12). On the average of all studied genotypes the leaf number per plant was 59.1, for the plants grown and measured at low temperature, and 91.2, for plants grown and measured at high temperature (Fig. 12). Relative to high temperature, low temperature decreased the number of stem per plant (~35%). On the average of the two different growth temperatures, *M. sinensis* showed the greater leaf number per plant (112.8), followed by *Goliath* (84.8), while the other genotypes varied between 45.8 (*M. floridulus*) and 68.7 leaves/plant (*M. x giganteus*) (Fig. 12). At high temperature the genotype that produced the highest leaf number was *M. sinensis* (145.0 leaves/plant), followed by *Goliath* (121.8 leaves/plant). All the other genotypes varied between 71.6 (*M. x giganteus*) and 75.2

leaves/plant (*M. sacchariflorus*) (Fig. 12). At low temperature, as mentioned above, *M. floridulus* showed greater leaf number per plant than in T_{high} (49.4 and 42.2 leaves/plant, respectively in T_{low} and in T_{high}). *M. sinensis* showed the greater leaf number per plant (80.6 leaves/plant), followed by *M. x giganteus* (65.8 leaves/plant), while the other genotypes varied between 47.8 (*Goliath*) and 52.0 leaves/plant (*M. sacchariflorus*) (Fig. 12).

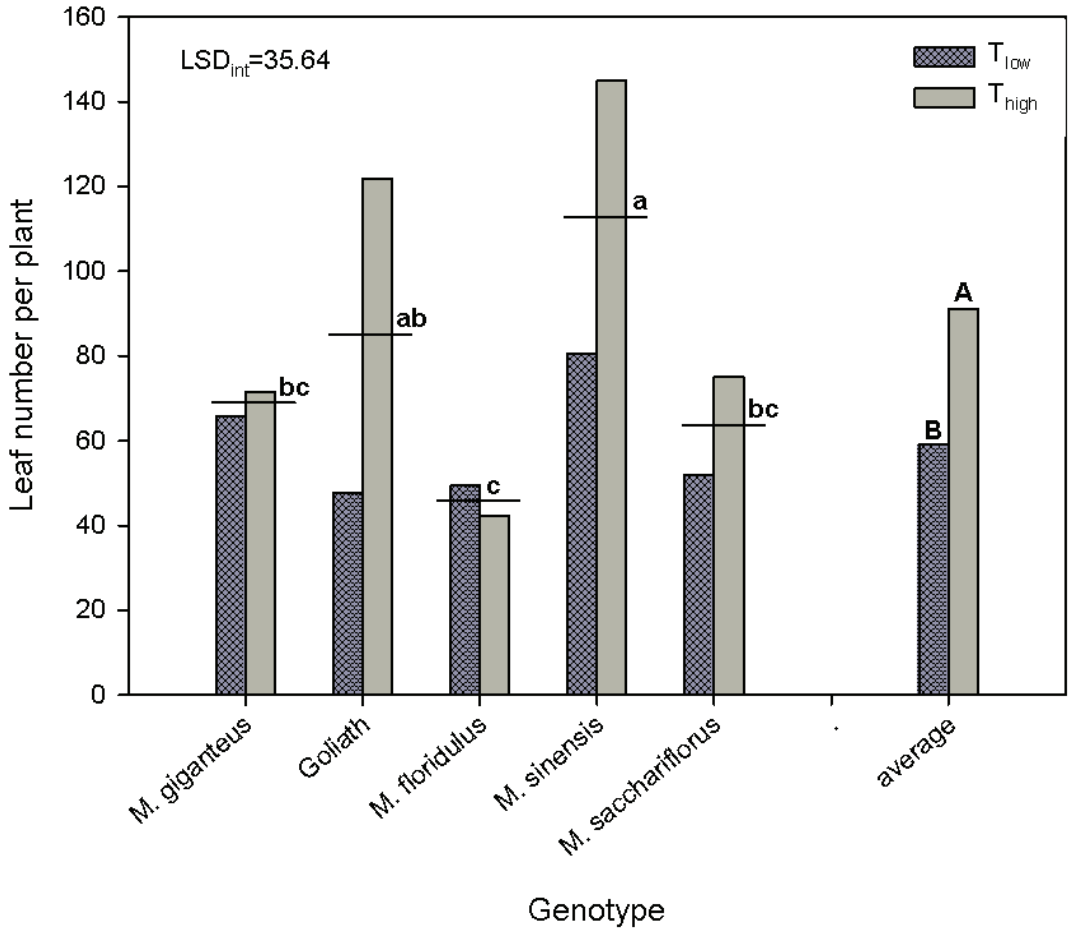

Figure 12 - Leaf number of the studied *Miscanthus* genotypes grown and measured at low and high temperature at the end of the heat stress experiment. Bars represent the average value of 5 measurements. Horizontal lines represent average value of the two growth temperature. Small letters, for averaged values of temperatures within each genotype; capital letters for averaged values of all genotypes within each temperature. Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD Test.

3.3.3 Leaf surface area

During the heat stress experiment, the leaf surface area was calculated. To determine it, leaf length and width were determined on a regular basis throughout the experiment using a ruler and green lamina area was calculated from the empirically derived relationship as described by Clifton-Brown, 1997:

Area (cm^2): length (cm) x width (cm) x 0.74

As shown in the figure 13, at the beginning of the heat stress experiment, when the plants were immature, the values of the leaf surface area were lower than that of the same values measured after twenty-three days, since the experiment started. The value of the leaf surface area was equal to 15 cm^2, on the average of all studied *Miscanthus* genotypes at low temperature (Fig 13), after twenty-three days after the experiment started. The higher value was recorded for *M. x giganteus* (20.9 cm^2), while the lower value was recorded for *Goliath* (8.6 cm^2). The value of the leaf surface area was equal to 17 cm^2 (Fig. 13), on the average of all studied *Miscanthus* genotype grown and measured at high temperature, after twenty-three days after the experiment started. The higher value was recorded for *M. floridulus* (23.3 cm^2), while the lower value was recorded for *M. sinensis* (13.7 cm^2). The values of the leaf surface area increased and acclimated gradually, towards the end of the experiment. In general the high growth temperature resulted in the reduction of the leaf area surface in the *Miscanthus* genotypes (Fig. 13). At the end of the experiment, for *M. x giganteus*, the leaf area surface was equal to 169.0 and 49.0 cm^2, for plants grown and measured at low and high temperature, respectively; for *M. floridulus*, the leaf area surface was equal to 115.2 and 68.6 cm^2, for plants grown and measured at low and high temperature, respectively; for *Goliath*, the leaf area surface was equal to 103.4 and 23.8 cm^2, for plants grown and measured at low and high temperature, respectively; for *M. sinensis*, the leaf area surface was equal to 52.4 and 6.6 cm^2, for plants grown and measured at low and high temperature, respectively; for *M. x sacchariflorus*, the leaf area surface was equal to 106.1 and 34.0 cm^2, for plants grown and measured at low and high temperature, respectively (Fig. 13).

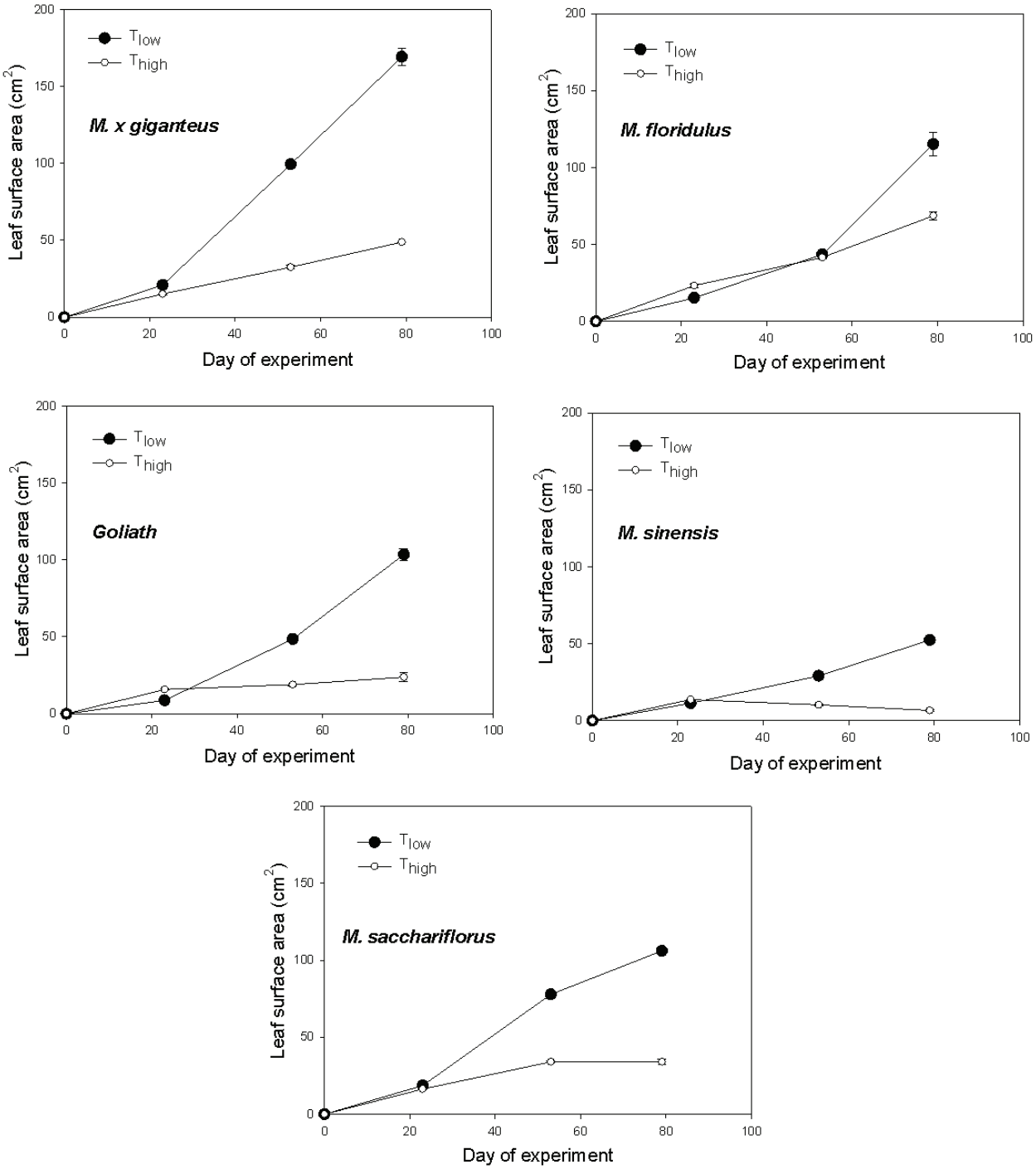

Figure 13 – The response of the leaf surface area to the different growth temperature for the studied *Miscanthus* genotypes grown and measured at low and high temperature. The bars on each curve represent the standard error of the difference of the means (n=5).

3.3.4 Stem diameter

Stem diameter was significantly affected by the different growth temperatures (Table 5). The three parts of the stem, basal, median and apical, were, on average, larger in plants grown at low temperature (T_{low}) compared to plants at high temperature (T_{high}). On the average of all genotypes, in T_{low}, the basal diameter was approximately 6.9 mm, the median one 5.8 mm and finally the apical one 3.9 mm (Table 5). In T_{high}, by contrast, the basal diameter was equal to 5.5 mm, the median one 4.1 and the apical one 2.0 mm (Table 5). On the average of the two different growth temperature, there was not any statistical different regarding the apical diameter, it ranged between 2.5 mm (*M. sacchariflorus*) and 3.2 mm (*M. x giganteus*, *Goliath* and *M. floridulus*), but there were statistical differences regarding the median and basal diameter (Table 5). About the median diameter, *M. x giganteus* and *M. floridulus* showed the highest value, 5.9 and 5.8 mm respectively, while no differences were recorded regarding the other genotypes (Table 5). About the basal diameter, *M. floridulus* showed the highest value (7.1 mm) (Table 5). In general, the high growth temperature resulted in the formation of thinner stems in the *Miscanthus* genotypes.

Table 5 – Values of the stem diameter (apical, median and basal) of all studied *Miscanthus* genotypes grown and measured at low and high temperature. Capital letters, for averaged values of all genotypes within each temperature; small letters, for averaged values of temperatures within each *Miscanthus* genotype. Different letters indicate significant differences at P≤0.05 by Tukey's HSD Test

	Apical (mm)			Median (mm)			Basal (mm)		
Genotype	T_{low}	T_{high}	***av.***	T_{low}	T_{high}	***av.***	T_{low}	T_{high}	***av.***
M. x giganteus	4.5	1.8	***3.2ns***	7.8	3.9	***5.9a***	8.9	4.9	***6.9ab***
M. floridulus	5.1	1.4	***3.2ns***	6.4	5.3	***5.8a***	7.6	6.6	***7.1a***
Goliath	3.6	2.8	***3.2ns***	4.8	4.7	***4.8b***	5.6	6.5	***6.1ab***
M. sinensis	2.7	2.5	***2.6ns***	4.8	3.9	***4.4b***	5.8	5.8	***5.8bc***
M. sacchariflorus	3.5	1.5	***2.5ns***	5.3	2.6	***4.0b***	6.4	3.4	***4.9c***
av.	***3.9A***	***2.0B***	***2.9***	***5.8A***	***4.1B***	***5.0***	***6.9A***	***5.5B***	***6.2***

b) Productive traits of the studied *Miscanthus* accessions

3.3.5 Fresh and dry above-ground biomass

The different plant growth temperature affected significantly the total production of fresh and dry above-ground biomass (Figs 14 and 15) . On the average of all studied genotypes fresh above-ground biomass was equal to 185.0 and 69.1 g for plants grown and measured at low and high temperature, respectively (Fig. 14). Relative to low temperature, high temperature decreased fresh biomass production (~63%). On the average of the two different growth temperature, *M. x giganteus* was the most yielding genotype, it reached a final fresh above-ground biomass equal to 213.5 g; the low yielding genotype was *Goliath*, it reached a final fresh above-ground biomass equal to 85.0 g. The other genotypes varied their final fresh biomass between 93.0 g (*M. sinensis*) and 135.0 g (*M. floridulus*) (Fig. 14).

At low temperature the most yielding genotype was *Miscanthus x giganteus*, it reached a final fresh biomass of 349.3 g, while the low yielding genotype was *Goliath*, 106.0 g. The fresh biomass of the other genotypes varied between 132.5 g (*M. sinensis*) and 172.8 g (*M. sacchariflorus*) (Fig. 14).

At high temperature the most yielding genotype was *M. floridulus*, it reached a final fresh production equal to 105.4 g (Fig. 14), even though its final production decreased of 36% at high temperature, it was the best genotype at high temperature, followed by *M. x giganteus* (high temperature decreased its final fresh biomass production of 78%) (Fig. 14). All the other genotypes ranged between 45.1 g (*M. sacchariflorus*) and 64.1 g (*Goliath*) (Fig. 14).

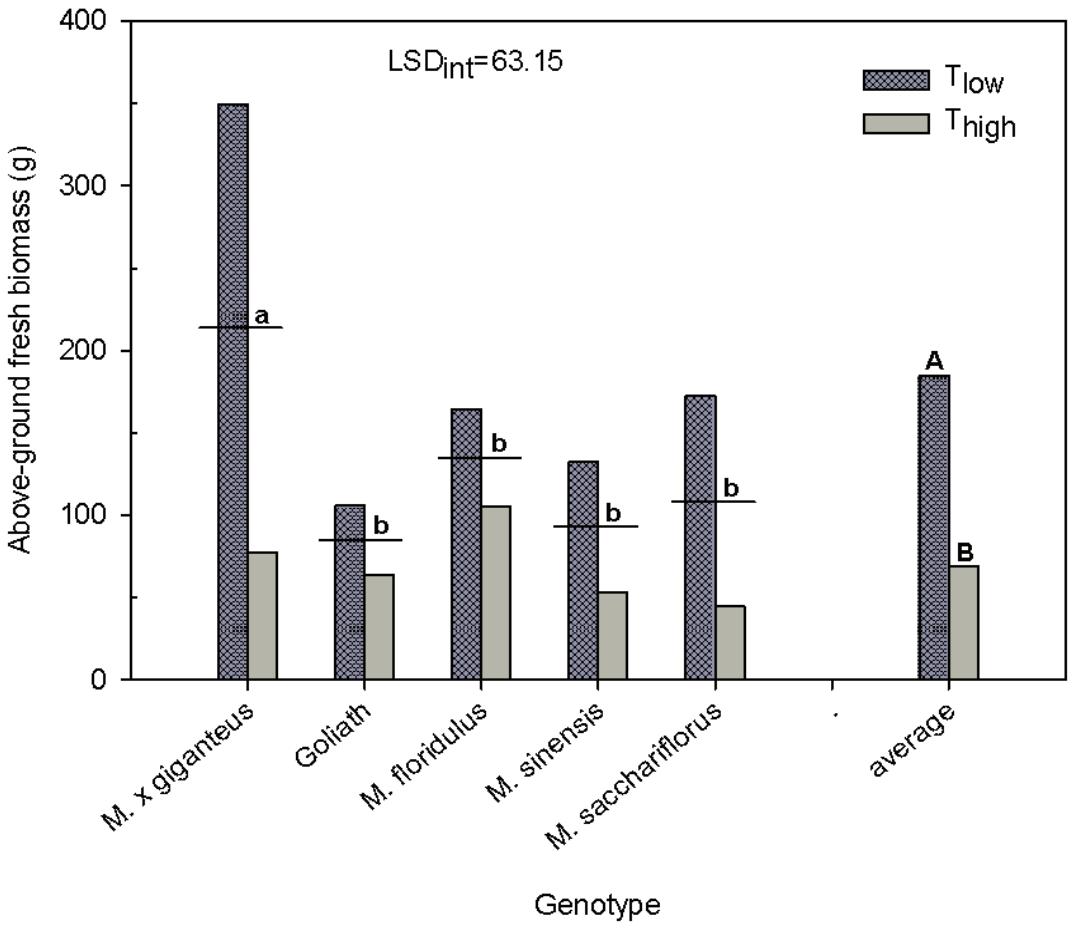

Figure 14 – Above-ground fresh biomass (g) of the studied *Miscanthus* genotypes grown and measured at different temperature. Bars represent the average value of 5 measurements. Horizontal lines represent average value of the two growth temperature. Small letters, for averaged values of temperatures within each genotype; capital letters for averaged values of all genotypes within each temperature. Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD Test.

The same trend was recorded regarding to the total dry above-ground biomass (Fig. 15). On the average of all studied *Miscanthus* genotypes, the dry biomass was equal to 59.0 g and 19.8 g, for plants grown and measured at low and high temperature, respectively (Fig. 15). Relative to low temperature, high temperature decreased the dry biomass (~66%).

On the average of the two different growth temperature, *M. x giganteus* was the best yielding genotype than the other studied genotypes, in fact *M. x giganteus* reached a final dry biomass production equal to 70.3 g, while the other genotypes ranged from 25.0 g (*Goliath*) to 39.8 g (*M. sacchariflorus*) (Fig. 15).

At low temperature the most yielding genotype was *M. x giganteus* (117.2 g), while the low yielding genotype was *Goliath* (33.4 g). The final dry biomass of the other genotypes ranged between 38.1 g (*M. floridulus*) and 64.0 g (*M. sacchariflorus*) (Fig. 15).

At high temperature the most yielding genotype was *M. floridulus* (28.8 g) while the low yielding genotype was *M. sinensis* (14.5 g) . The final dry biomass of all other genotypes ranged from 15.6 g (*M. sacchariflorus*) and 23.4 g (*M. x giganteus*) (Fig. 15).

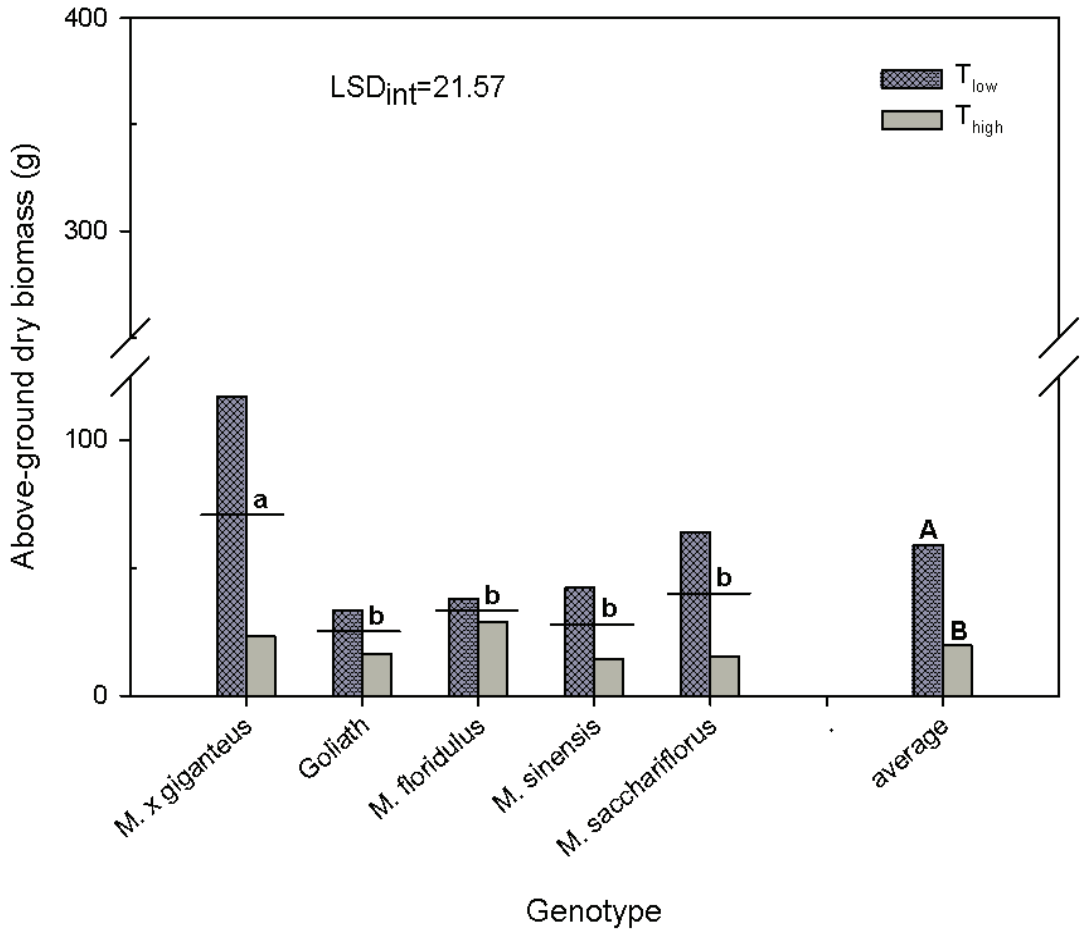

Figure 15 - Above-ground dry biomass (g) of the studied *Miscanthus* genotypes grown and measured at different temperature. Bars represent the average value of 5 measurements. Horizontal lines represent average value of the two growth temperature. Small letters, for averaged values of temperatures within each genotype; capital letters for averaged values of all genotypes within each temperature. Different letters indicate significant differences at P≤0.05 by Tukey's HSD Test.

M. x giganteus is closely related to sugarcane (*Saccharum* ssp.) and related to maize (*Zea mays* L.), but it contrasts to these in its superior ability to develop leaves and maintain photosynthesis at chilling temperatures (<14°C) (Farage et al., 2006; Wang et al., 2008). *M. x giganteus* produced 60% more above-ground biomass than a modern maize cultivar in a side-by-side trial. This could be related to its ability to form photosynthetically active leaves earlier in the growing season and maintain them later, thereby extending the growing season and allowing greater interception of the available annual solar radiation (Dohleman and Long, 2009).

3.3.6 Dry above-ground biomass partitioning

On the average of all studied *Miscanthus* genotypes, at low temperature, the above-ground dry matter biomass partitioning was equal to 57.9%, 37.5% and 4.5% for stems, leaves and inflorescences, respectively; while at high temperature, it was equal to 44.8%, 54.8% and 0.4% for stems, leaves and inflorescences, respectively. Relative to low temperature, high temperature decreased the percentage of dry matter stem yield (~23%) and the percentage of dry matter inflorescence yield (~91%), while relative to high temperature, low temperature decreased the percentage of dry matter leaf yield (~32%) (Fig. 16).

At high temperature, only *M. floridulus* showed a higher percentage stem weight than that at low temperature. It reached a stem weight equal to 16.4 and 18.2 g, for plants grown and measured at low and high temperature, respectively; relative to low temperature, high temperature increased the stem weight (~11%) (Fig. 16). *Goliath* showed a higher percentage leaf weight than that at low temperature. It reached a leaf weight equal to 9.5 and 10.7 g, for plants grown and measured at low and high temperature, respectively; relative to low temperature, high temperature increased the leaf weight (~13%) (Fig. 16).

At high temperature, the other studied *Miscanthus* genotypes decreased their percentage stem and leaf weight than that at low temperature values (Fig. 16). For *M. x giganteus*, high temperature decreased the dry matter stem and leaf yield, 81.7 and 11.0 g, for stems (-86%), and 32.7 and 12.0 g for leaves (-63%), for plants grown and measured at low and high temperature, respectively; for *M. sacchariflorus*, high temperature decreased the dry matter stem and leaf yield, 44.0 and 7.1 g, for stems (-84%), and 18.8 and 8.4 g for leaves (-55%), for plants grown and measured at low and high temperature, respectively; for *M. sinensis*, high temperature decreased the dry matter stem and leaf yield, 23.2 and 5.1g for stems (-78%), and 18.0 and 9.4 g for leaves (-48%), for plants grown and measured at low and high temperature, respectively.

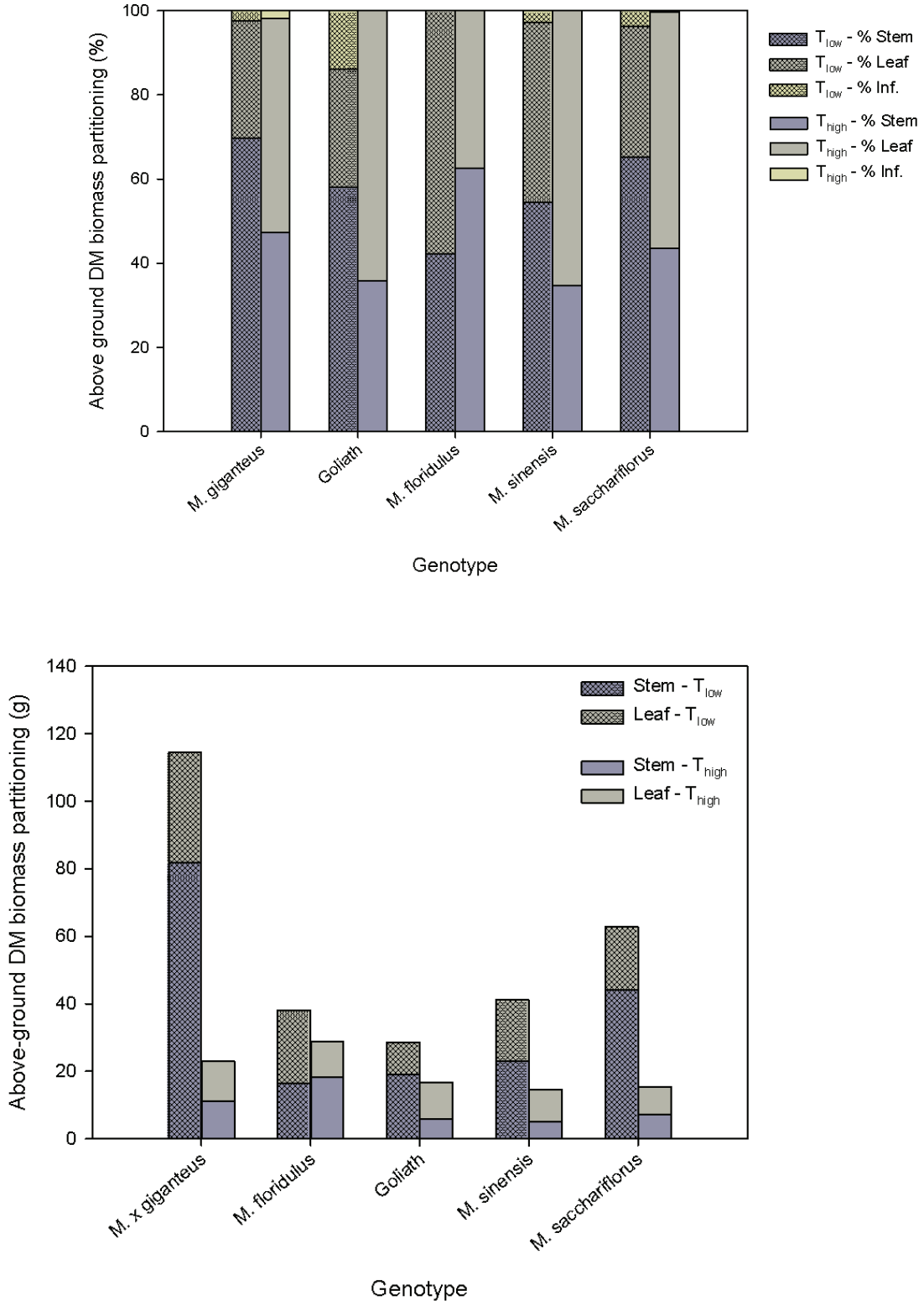

Figure 16- Above-ground dry matter biomass partitioning, in terms of weight (g) and percentage (%) of the studied *Miscanthus* genotypes, at the end of the heat stress experiment. Average value of five determinations.

3.3.7 Fresh and dry below-ground biomass

At the end of the heat stress experiment the below-ground biomass was harvested. On the average of all studied *Miscanthus* genotypes, the total below-ground fresh biomass (rhizomes and roots) was equal to 156.0 g for the plants grown and measured at low temperature and 107.2 g for the plants grown and measured at high temperature (Fig. 17). Relative to low temperature, high temperature decreased (31%) the total below-ground fresh biomass.

On the average of the two different growth temperature, *M. x giganteus* was the most yielding genotype reaching a final below-ground fresh biomass equal to 250.4 g, followed by *M. sacchariflorus* with 158.0 g fresh matter, while the other genotypes ranged from 70.5 g (*M. floridulus*) to 98.1 g (*M. sinensis*) (Fig. 17).

At low temperature all the studied *Miscanthus* genotypes produced more below-ground fresh biomass quantity than the plants grown at high temperature, and *M. x giganteus* was the most yielding genotype reaching 304.8 g fresh matter, followed by *M. sacchariflorus* with 181.6 g fresh matter, while the other genotypes varied their final fresh below-ground biomass from 85.8 g (*M. floridulus*) to 119.5 g (*M. sinensis*) (Fig. 17). At high temperature was recorded the same trend, the most yielding genotype was *M. x giganteus* (196.0 g fresh matter), followed by *M. sacchariflorus* (134.4 g fresh matter), while the other genotypes ranged between 55.2 g (*M. floridulus*) and 76.7 g (*M. sinensis*) (Fig. 17).

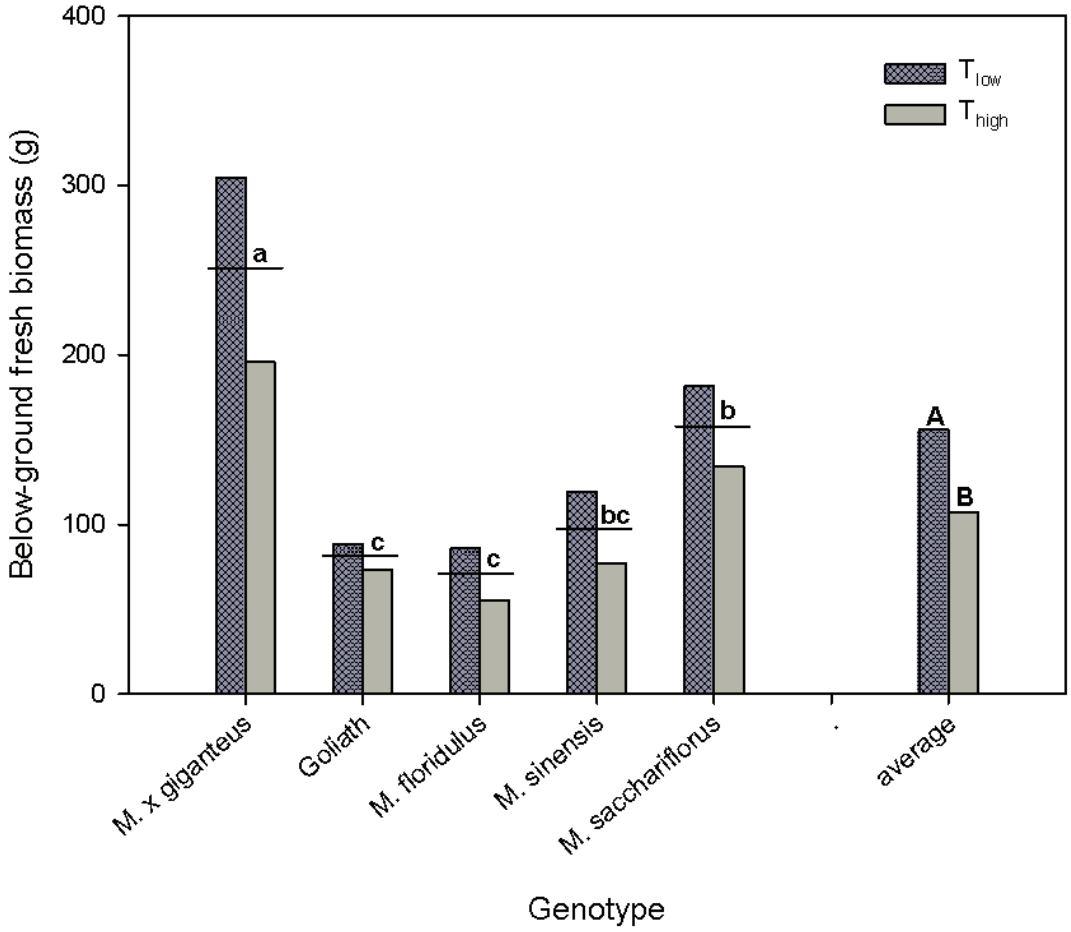

Figure 17 – Total below-ground (rhizomes + roots) fresh biomass (g) of the studied *Miscanthus* genotypes grown and measured at different temperature. Bars represent the average value of 5 measurements. Horizontal lines represent average value of the two growth temperature. Small letters, for averaged values of temperatures within each genotype; capital letters for averaged values of all genotypes within each temperature. Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD Test.

The same trend was recorded regarding to the total below-ground dry biomass: it was equal to 35.5 g dry matter for the plants grown at low temperature and 26.2 g dry matter for the plants grown at high temperature (Fig. 18). Relative to low temperature, high temperature decreased the total below-ground dry biomass (~26%).

On the average of the two different growth temperature, *M. x giganteus* was the most yielding genotype (54.1 g dry matter) followed by *M. sacchariflorus* (45.0 g dry matter), while the other genotypes ranged from 15.5 g dry matter (*M. floridulus*) to 21.5 g dry matter (*M. sinensis*) (Fig. 18). At low temperature all the studied *Miscanthus* genotypes produced more below-ground dry biomass quantity than the plants grown at high temperature, and *M. x giganteus* was the most yielding genotype reaching 61.8 g dry matter, followed by *M. sacchariflorus* with 52.5 g dry matter, while the other genotypes varied their final dry below-ground biomass from 18.4 g (*M. floridulus*) to 25.2 g (*M. sinensis*) (Fig. 18). At high temperature was recorded the same trend, the most yielding

genotype was *M. x giganteus* (46.5 g dry matter), followed by *M. sacchariflorus* (37.5 g dry matter), while the other genotypes ranged between 12.7 g (*M. floridulus*) and 17.8 g (*M. sinensis*) (Fig. 18).

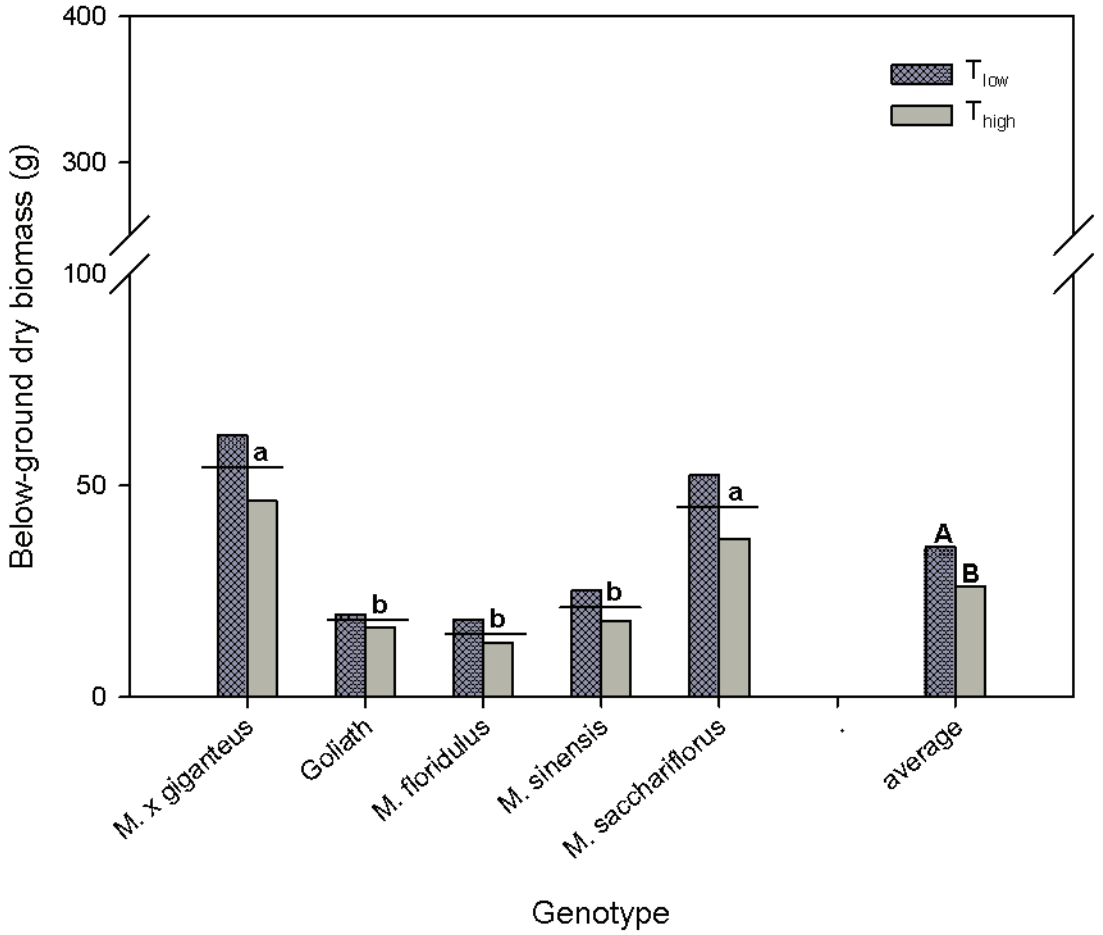

Figure 18 – Total below-ground (rhizomes + roots) dry biomass (g) of the studied *Miscanthus* genotypes grown and measured at different temperature. Bars represent the average value of 5 measurements. Horizontal lines represent average value of the two growth temperature. Small letters, for averaged values of temperatures within each genotype; capital letters for averaged values of all genotypes within each temperature. Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD Test

3.3.8 Fresh and dry below-ground biomass partitioning

The total below-ground biomass was divided into rhizomes and roots at the end of the experiment. Regarding the fresh matter rhizome yield, on the average of all studied genotypes, there was not a significant difference: at low temperature the fresh rhizome yield was equal to 78.8 g, while at high temperature it was 63.3 g (Fig. 19). On the average of the two different growth temperature, *M. x giganteus* and *M. sacchariflorus* were the most yielding genotypes reaching 146.6 and 104.5 g, respectively, while the other studied *Miscanthus* genotypes ranged between 29.3 g (*M. floridulus*) and 37.3 g (*Goliath*) (Fig. 19). *M. x giganteus* and *M. sacchariflorus* were the most yielding genotypes in both temperatures: 166.9 and 113.7 g, respectively, at low temperature and 126.9 and 96.1 g, respectively, at high temperature (Fig. 19). For *Goliath*, the fresh rhizome yield was almost the same in both temperature: 36.0 g for plants grown and measured at low temperature and 38.5 g for plants grown and measured at high temperature (Fig. 19).

The same trend was recorded regarding the dry matter rhizome yield: it was equal to 23.1 g at low temperature and 18.7 g at high temperature, on the average of all studied *Miscanthus* genotype. As shown in the fresh matter rhizome yield, there was not any significant difference in relation to the studied genotype for the dry matter rhizome yield (Fig. 19).

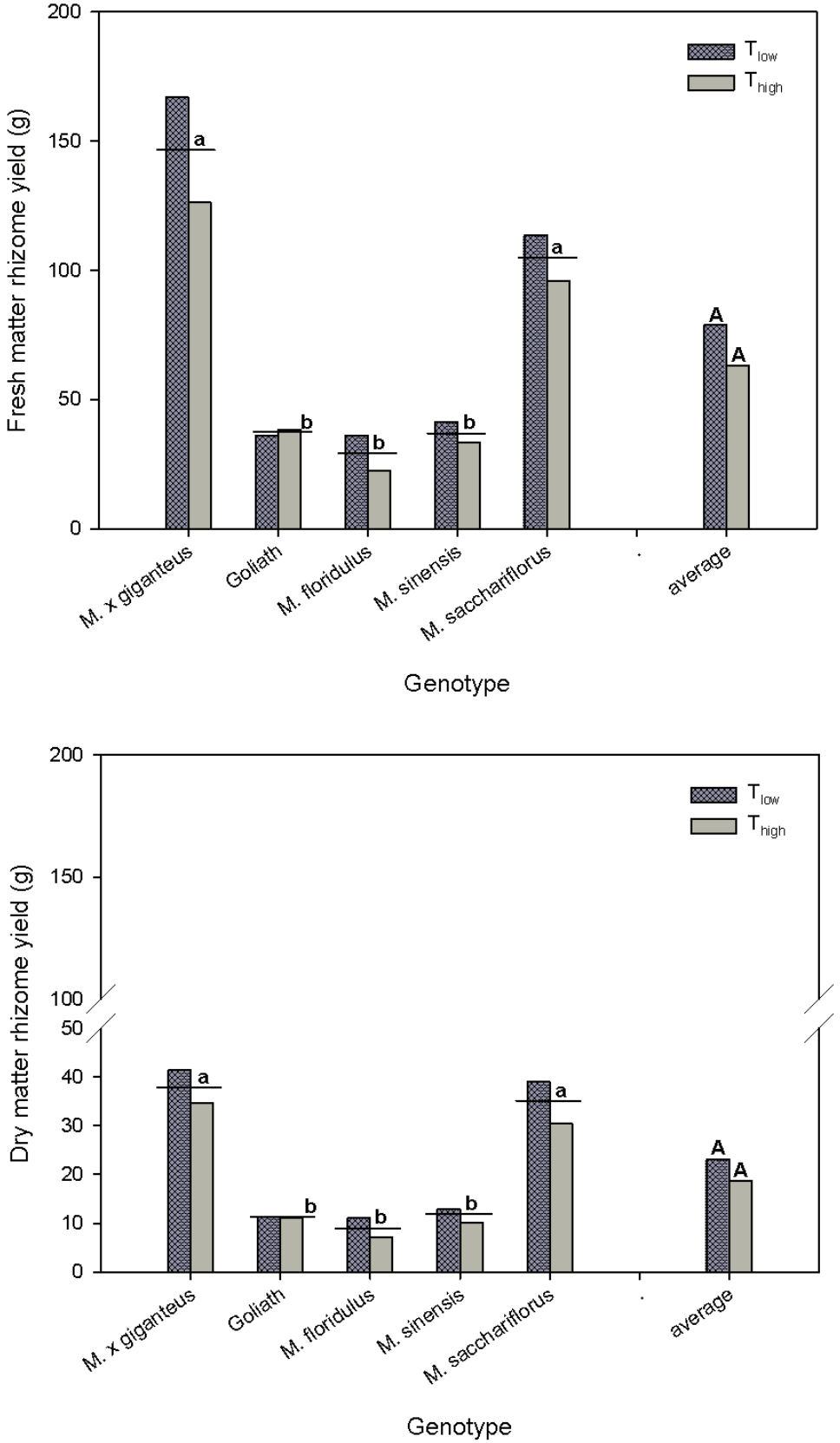

Figure 19 – Fresh and dry matter rhizome yield (g) of the studied *Miscanthus* genotypes grown and measured at different temperature. Bars represent the average value of 5 measurements. Horizontal lines represent average value of the two growth temperature. Small letters, for averaged values of temperatures within each genotype; capital letters for averaged values of all genotypes within each temperature. Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD Test.

The final fresh and dry root yield was affected by the different growth temperature (Fig. 20). On the average of all studied *Miscanthus* genotypes the total fresh roots biomass was equal to 77.3 g at low temperature and 43.8 g at high temperature (Fig. 20).

On the average of the two different growth temperature, *M. x giganteus* was the most yielding genotype. It reached a total fresh roots biomass equal to 103.8 g, while the other genotypes ranged from 41.2 g (*M. floridulus*) to 60.9 g (*M. sinensis*) (Fig. 20). At low temperature the most yielding genotype was *M. x gigantesus*, (137.9 g fresh matter), followed by *M. sinensis* (78.3 g fresh matter), while the other genotypes ranged from 49.8 g fresh matter (*M. floridulus*) to 68.0 g fresh matter (*M. sacchariflorus*) (Fig. 20). At high temperature the most yielding genotype was *M. x gigantesus*, (69.8 g fresh matter), followed by *M. sinensis* (43.5 g fresh matter), while the other genotypes ranged from 32.6 g fresh matter (*M. floridulus*) to 38.4 g fresh matter (*M. sacchariflorus*) (Fig. 20).

The same trend was recorded regarding to the dry matter root yield. On the average of all studied *Miscanthus* genotypes, it was equal to equal to 12.3 g at low temperature and 7.5 g at high temperature (Fig. 20). On the average of the two different growth temperatures *M. x giganteus* showed the highest value, equal to 16.1 g dry matter, while the other *Miscanthus* genotypes did not differ statistically and showed values between 6.4 g dry matter (*M. floridulus*) and 10.3 g dry matter (*M. sacchariflorus*).

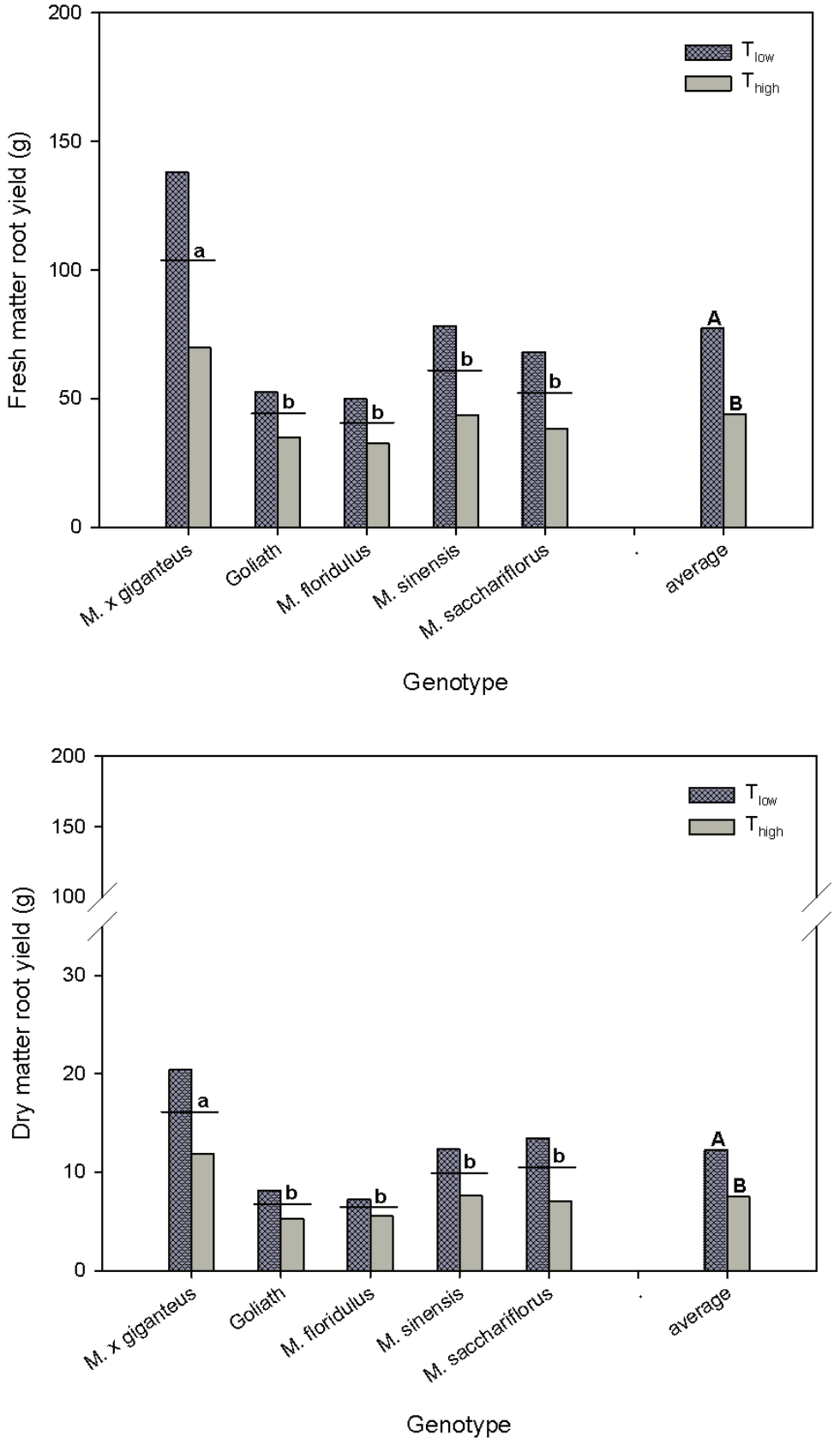

Figure 20 – Fresh and dry matter root yield (g) of the studied *Miscanthus* genotypes grown and measured at different temperature. Bars represent the average value of 5 measurements. Horizontal lines represent average value of the two growth temperature. Small letters, for averaged values of temperatures within each

genotype; capital letters for averaged values of all genotypes within each temperature. Different letters indicate significant differences at P≤0.05 by Tukey's HSD Test.

c) Physiological traits of the studied *Miscanthus* accessions

3.3.9 Response of CO_2 assimilation rate (A) to photon flux density (Q): A/Q curves

The radiation light level plays a key factor for determining the photosynthetic rate assimilation. During the heat stress experiment carried out at Institute of Biological, Environmental and Rural Sciences (IBERS) at Aberystwyth University, photosynthesis rates from several measurements were plotted against light intensity, getting a photosynthesis light response curve. Studied plants showed differences in the shape of their light response curves, which revealed characteristics of the underlying photosynthesis processes including the light-dependent and light-independent reactions, the efficiency at which light is utilized by photosynthesis, and even the rate of O_2 uptake (Fig. 21). The response of A to photon flux (Q) describes a curve or curvilinear progression and can be divided into two sections. The first section is under low-light levels (*light limited* section) the rate of photosynthesis increases as the irradiance level is increased and is limited by the concentration of chlorophyll and the efficiency of the light-dependent reactions. The initial slope of the light curve is called apparent quantum efficiency of photosynthesis (Φ_{CO2}), which represents the ratio of the absorbed quanta utilized in photochemical conversions to the total quanta absorbed. The second section is under high-light levels (*carboxylation limited* section) the rate of photosynthesis decreases progressively due to the carboxylation efficiency, which is influenced by both the CO_2 availability into the leaves tissues and by the efficiency of RuBisCo. X-axis point of intersection is called 'light compensation point' (LCP), which is the amount of light intensity where the rate of photosynthesis exactly matches the rate of respiration. At this point, the uptake of CO_2 through photosynthetic pathways is exactly matched to the respiratory release of carbon dioxide, and the uptake of O_2 by respiration is exactly matched to the photosynthetic release of oxygen. Y-axis point of intersection is called 'dark respiration' (Rd), which is the CO_2 quantity released without the aid of sun light (photosynthesis). Photochemical activity is limited by the rate of electron transport under these conditions. Changes in quantum yield are thus caused by changes in the partitioning between carboxylation and oxygenation reactions of Rubisco.

M. sinensis and *Goliath* demonstrated significant reduction in photosynthetic rate across all light levels in response to high temperature (Fig. 21). Analysis of light curves for *Goliath* and *M. sinensis* indicated large significant decrease in A_{max}, 39% and 57%, respectively for *Goliath* and *M. sinensis* for leaves grown and measured at high temperature compared to low temperature grown plants

(Table 6). Decreased photosynthesis was also evident in *M. x giganteus* leaves grown and measured at high temperature, but the magnitude of the decrease was much less than in *M. sinensis* and *Goliath* (Table 6). For *M. x giganteus*, A_{max} was 9% lower in high temperature leaves compared to the plants grown at low temperature (Table 6). For *M. sacchariflorus*, photosynthetic rate was not too much affected by different growth temperature: the values of A_{max} were 18.8 and 17.6 μmol m^{-2} s^{-1} for plants grown and measured at high and low temperature, respectively (Table 6). For *M. sacchariflorus*, the maximum rate of photosynthesis (A_{max}) was 6% lower in low temperature leaves compared to the plants grown at high temperature. Decreased photosynthesis was also evident in *M. floridulus* leaves grown and measured at low temperature (Table 6). For *M. floridulus*, A_{max} was 23% lower in low temperature compared to the plants grown at high temperature.

Regarding the initial slope of the light response curve ($\Phi_{CO2,max}$) can be seen that all the *Miscanthus* genotypes were not affected by different growth temperature (Table 6). For *M. giganteus* and *Goliath*, $\Phi_{CO2,max}$ was not affected by different growth temperature: the values of $\Phi_{CO2,max}$ were 0.05 and 0.03 for *M. giganteus* and *Goliath* plants grown and measured at high and low temperature, respectively (Table 6). Analysis of $\Phi_{CO2,max}$ for *M. sinensis* and *M. sacchariflorus* indicated very small decreases. For *M. sinensis*, $\Phi_{CO2,max}$ was equal to 0.03 and 0.02 for plants grown and measured at low and high temperature, respectively; for *M. sacchariflorus*, $\Phi_{CO2,max}$ was equal to 0.03 and 0.04 for plants grown and measured at low and high temperature, respectively (Table 6). For *M. floridulus*, $\Phi_{CO2,max}$ was equal to 0.03 and 0.06 for plants grown and measured at low and high temperature, respectively (Table 6). All the *Miscanthus* genotypes demonstrated increases in dark respiration (Rd) rate in response to high temperature (Table 6). Increased dark respiration was evident in *M. giganteus*, *M. floridulus* and *M. sacchariflorus*, but the magnitude of the variation was much less than in *M. Sinensis* and *Goliath* (Table 6). For *M. giganteus*, dark respiration was equal to -0.51 and -0.85 μmol m^{-2} s^{-1} for plants grown and measured at low and high temperature, respectively; for *M. floridulus*, dark respiration was equal to -0.34 and -0.64 μmol m^{-2} s^{-1} for plants grown and measured at low and high temperature, respectively; for *M. sacchariflorus*, dark respiration was equal to -0.31 and -0.87 μmol m^{-2} s^{-1} for plants grown and measured at low and high temperature, respectively (Table 6). For *M. Sinensis*, dark respiration was equal to -0.19 and -0.22 μmol m^{-2} s^{-1} for plants grown and measured at low and high temperature, respectively; while for Goliath, dark respiration was equal to -0.15and -0.31 μmol m^{-2} s^{-1} for plants grown and measured at low and high temperature, respectively (Table 6). All the *Miscanthus* genotypes demonstrated increases in light compensation point (LCP) rate in response to high temperature (Table 6). Increased light compensation point was evident in *M. floridulus* and *M. sacchariflorus*, but the magnitude of the variation was much less than in *M. giganteus*, *Goliath* and *M. Sinensis* (Table 6).

For *M. floridulus*, LCP was 61% lower in low temperature leaves compared to the plants grown at high temperature, while for M. sacchariflorus, LCP was 68% lower in low temperature leaves compared to the plants grown at high temperature (Table 6). For *M. giganteus*, LCP was equal to 7.28 and 9.40 μmol m^{-2} s^{-1} for plants grown and measured at low and high temperature, respectively; for *Goliath*, LCP was equal to 4.52 and 8.59 μmol m^{-2} s^{-1} for plants grown and measured at low and high temperature, respectively; for *Sinensis*, LCP was equal to 8.79 and 10.79 μmol m^{-2} s^{-1} for plants grown and measured at low and high temperature, respectively (Table 6). *Miscanthus x giganteus* demonstrates a remarkable tolerance of C_4 photosynthesis to low growth temperatures in contrast to other C_4 NADH-ME-type species such as *Zea mays* (Beale and Long 1995; Bullard et al. 1995; Beale et al. 1996). There are several potential mechanisms by which this may occur. Naidu and Long (2004) found that inferred enzyme function, particularly that associated with photosynthesis under high light and CO_2, as well as the maintenance of relatively high levels of leaf absorptance and quantum yield, all contributed to high conversion efficiencies of radiation into biomass at low temperature in this species. Leaves of *M. x giganteus* grown under both warm and low temperatures exhibited a similar response of photosynthesis to measurement temperature, in according to Naidu et al. (2003), but in this case, with a temperature optimum of about 35°C. Most C_4 plants are not able of photosynthesising at temperatures below about 12°C. Controlled environment studies showed that *M. x giganteus* threshold for impairment of the photosynthetic apparatus lies between 8 and 12°C. Leaf photosynthesis in *M. x giganteus* continues down to a temperature of <5°C, while plants can form photosynthetically competent leaves down to 8°C and photosynthetic capacity is unaffected by growth temperatures down to 12°C. This suggests that the threshold for photosynthesis and the development of the photosynthetic apparatus is 3-5°C below the threshold of other C_4 plants, which is in contrast to other closely related C_4 species utilizing the NADP-malic enzyme (NADP-ME) pathway. When *M. x giganteus* was grown in climates where temperatures are often >30°C, the plant is short stature. C_4 plant species have a higher temperature optimum for photosynthesis than C_3 plants due to the operation of a CO_2-concentrating system that inhibits Rubisco oxygenase activity (Berry and Björkman, 1980; Edwards and Walker, 1983). In C_3 plants, inhibition of net photosynthesis (Pn) at moderately high temperatures has usually been ascribed to an increase in the ratio of Rubisco oxygenase: Rubisco carboxylase activities. As temperature increases, the ratio of dissolved O_2/CO_2 and the specificity of Rubisco for O_2 increase, thus favoring oxygenase activity (Monson et al., 1982; Jordan and Ogren, 1984; Sage and Sharkey, 1987) and resulting in inhibition of Pn. As a consequence, when C_3 plants are exposed to high CO_2 and/or low O_2, i.e. conditions that reduce oxygenase activity, the temperature optimum for Pn is increased (Berry and Björkman, 1980; Edwards and Walker, 1983). For C_3 and C_4 plants, the

temperature range for optimum Pn is broad, and at temperatures above this range, Pn decreases (Edwards and Walker, 1983). Temperature-induced decreases in Pn in C_3 species are closely associated with inactivation of Rubisco (Law and Crafts-Brandner, 1999), and when the activation state of Rubisco and gas solubilities are taken into account, the rate of Pn at any given temperature or level of atmospheric CO_2 or O_2 reflects Rubisco kinetics (Crafts-Brandner and Salvucci, 2000). The temperature-induced decrease in Rubisco activation, and the associated inhibition of Pn, in C_3 plants results in large part from the inability of Rubisco activase activity to keep pace with a faster rate of Rubisco inactivation as temperature is increased (Crafts-Brandner and Salvucci, 2000). Activase kinetics and physical denaturation of activase appear to be causative factors contributing to the decrease in Rubisco activation at high temperature (Crafts-Brandner and Salvucci, 2000; Salvucci et al., 2001). Although C_4 plants have a higher temperature optimum than C_3 plants, Pn is usually inhibited when leaf temperatures exceed about 38°C (Berry and Björkman, 1980; Edwards and Walker, 1983). Although the C_4 photosynthetic system is more complex than the C_3 system, the ultimate limitation to CO_2 fixation for both photosynthetic types is the activity of Rubisco (von Caemmerer et al., 1997; Edwards et al., 2001). Low temperature effects on C_4 photosynthesis have been frequently examined (Labate et al., 1991; Long, 1998). Studies pertaining to the effects of high temperature on C_4 photosynthetic metabolism are less common, and can be hypothesized that high temperature may inactivate Rubisco and limit Pn in a similar manner as for C_3 plants. However, it seemed feasible that heat stress might also impact C_4-specific processes such as fixation of CO_2 by phospho*enol*pyruvate (PEP) carboxylase, shuttling of C_4 acids from mesophyll to bundle sheath cells, or energy balance due to the differential localization of PSII and the Calvin cycle.

In this experiment, light response curves were performed on all species and were corrected for absorbed quanta (Naidu and Long, 2004). Light saturated assimilation rate decrease in response to increased temperature for *Goliath* and *M. sinensis* and however *M. x giganteus* and *M. sacchariflorus* are unchanged and *M. floridulus* assimilation rate is increased with the higher temperature. Similarly the initial slope, were lower in the higher temperature for *Goliath* and *M. sinensis*, increased in *M. floridulus*, and unaffected in *M. x giganteus* and *M. sacchariflorus*. This confirms that the photosynthetic mechanism is perturbed by the increased temperature.

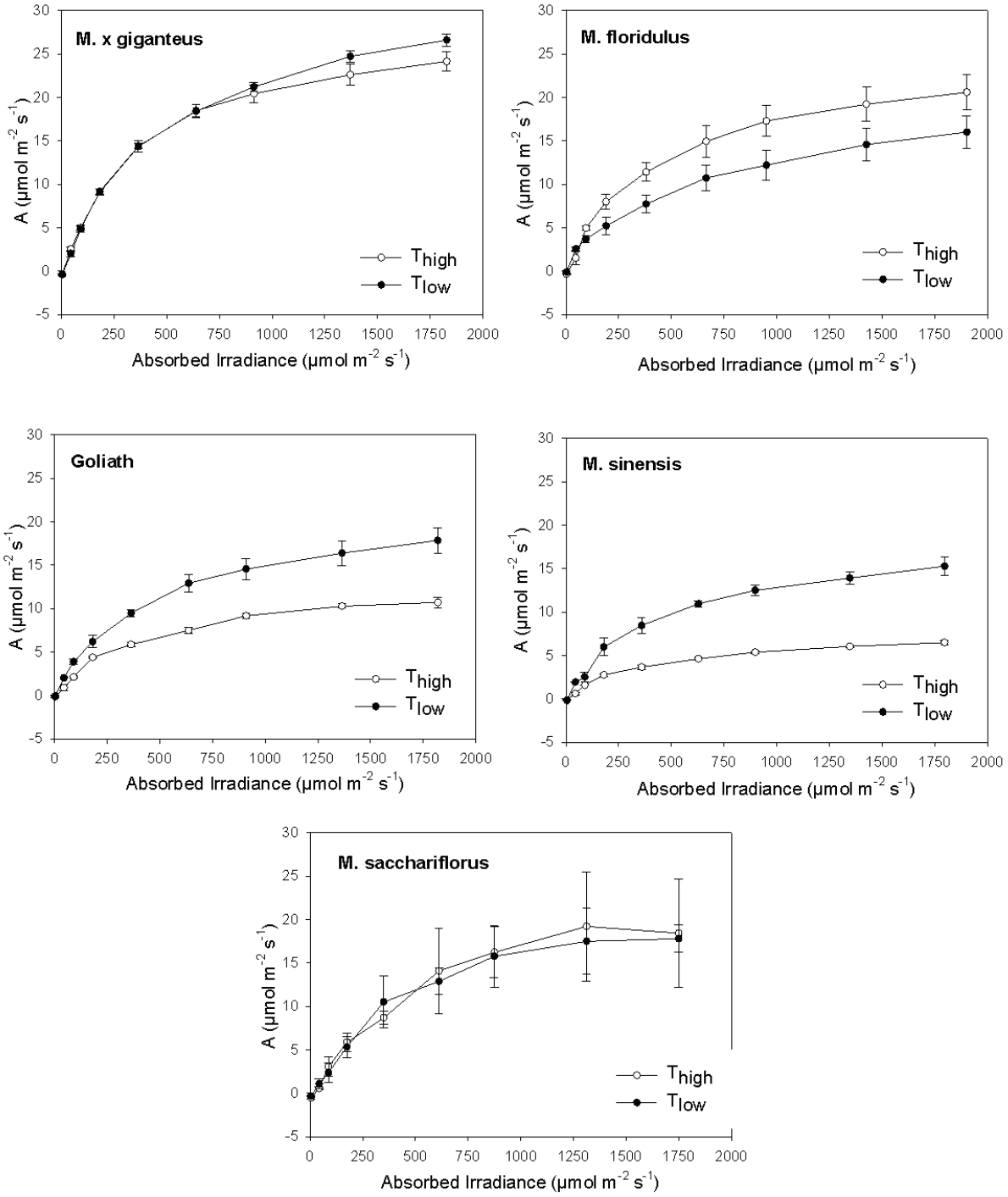

Figure 21 – Response of CO_2 assimilation rate (A) to absorbed light (Q) of the studied *Miscanthus* genotypes grown and measured at different temperature. Values are means, ± standard error of the mean.

Table 6 – Analysis of A/Q response curves for the studied *Miscanthus* genotypes. $\mathbf{A_{max}}$ **–** light saturated maximum rate of photosynthesis. $\boldsymbol{\phi_{CO2}}$ **-** apparent quantum efficiency of photosynthesis. **Rd** - dark respiration. **LCP** - light compensation point

Genotype	Treatment	A_{max} (μmol m^{-2} s^{-1})	ϕ_{CO2}	Rd (μmol m^{-2} s^{-1})	LCP (μmol m^{-2} s^{-1})
M. gig	T_{low}	25.7 (0.94)	0.05 (0.001)	-0.51 (0.06)	7.28 (0.66)
	T_{high}	23.4 (0.77)	0.05 (0.001)	-0.85 (0.15)	9.40 (0.39)
M. flo	T_{low}	15.3 (0.71)	0.03 (0.003)	-0.34 (0.10)	5.10 (0.96)
	T_{high}	20.0 (0.69)	0.06 (0.003)	-0.64 (0.13)	13.00 (2.74)
Gol	T_{low}	17.1 (0.74)	0.03 (0.004)	-0.15 (0.05)	4.52 (0.36)
	T_{high}	10.5 (0.35)	0.03 (0.001)	-0.31 (0.13)	8.59 (1.62)
M. sin	T_{low}	14.6 (0.68)	0.03 (0.005)	-0.19 (0.02)	8.79 (1.59)
	T_{high}	6.3 (0.22)	0.02 (0.000)	-0.22 (0.05)	10.79 (1.36)
M. sac	T_{low}	17.6 (1.56)	0.03 (0.007)	-0.31 (0.06)	6.82 (1.59)
	T_{high}	18.8 (0.39)	0.04 (0.007)	-0.87 (0.15)	21.34 (0.58)

(Values are means, +/- standard error of the mean).

3.3.10 Response of CO_2 assimilation rate (A) to leaf internal CO_2 mole fraction (Ci): A/Ci curves

CO_2 diffuses from the atmosphere into leaves, first through stomata, then through the intercellular air spaces, and ultimately into cells and chloroplasts. In the presence of adequate amounts of light, higher CO_2 concentrations support higher photosynthetic rates and the reverse is also true: low CO_2 concentrations can limit the amount of photosynthesis. Expressing photosynthetic rate as a function of the partial pressure of CO_2 in the intercellular air space (ci) within the leaf makes it possible to evaluate limitations to photosynthesis imposed by CO_2 supply. At very low intercellular CO_2 concentrations, photosynthesis is strongly limited by the low CO_2. Increasing intercellular CO_2 to the concentration at which these two processes balance each other defines the *CO_2* compensation point, at which the net efflux of CO_2 from the leaf is zero. At low to intermediate CO_2 concentrations, photosynthesis is limited by the carboxylation capacity of Rubisco. At high CO_2 concentrations, photosynthesis becomes limited by the capacity of the Calvin cycle to regenerate the acceptor molecule ribulose-1,5-bisphosphate, which depends on electron transport rates. However, photosynthesis continues to increase with CO_2 because carboxylation replace oxygenation on rubisco. By regulating stomatal conductance, most leaves appear to regulate their ci (internal partial pressure for CO_2) such that it is at an intermediate concentration between limitations imposed by carboxylation capacity and limits in the capacity to regenerate ribulose-1,5-bisphosphate.

During the heat stress experiment carried out at Institute of Biological, Environmental and Rural Sciences (IBERS) at Aberystwyth University, *Miscanthus* accessions' analysis of the A/Ci response curves indicated that carboxylation efficiency of PEPc (CE), which represents the initial slope of the A/Ci response, was affected by different growth temperature. For *M. x giganteus*, CE was not significantly affected by temperature: it was equal to 0.20 and 0.21 for leaves grown and measured at low temperature and high grown plants, respectively (Table 7). In contrast, there was a large decrease of the carboxylation efficiency of PEPc in the other studied genotypes in response to high temperature. For *Goliath*, *M. sinensis*, *M. floridulus* and *M. sacchariflorus*, CE was 56%, 45%, 32% and 20%, respectively, lower in high temperature leaves (Table 7). For *Goliath*, CE was equal to 0.18 and 0.08 for plants grown and measured at low and high temperature, respectively; for *M. sinensis*, CE was equal to 0.11 and 0.06 for plants grown and measured at low and high temperature, respectively; for *M. floridulus*, CE was equal to 0.19 and 0.13 for plants grown and measured at low and high temperature, respectively; for *M. sacchariflorus*, CE was equal to 0.20 and 0.16 for plants grown and measured at low and high temperature, respectively (Table 7).

On the average of all studied genotypes, the values of CO_2-saturated photosynthetic rate (V_{pr}) was not affected by different growth temperature: it was equal to 19.8 and 20.5 μmol m^{-2} s^{-1} for plants grown at low temperature and high grown plants, respectively. Regarding V_{pr} there were differences

among genotypes (Table 7). For *M. x giganteus*, *M. floridulus* and *M. sacchariflorus*, V_{pr} was 32%, 15% and 6%, respectively, higher in high temperature leaves, while for *Goliath* and *M. sinensis*, the values of V_{pr} were 37% and 6%, respectively, lower in high-temperature leaves (Table 7).

The operating point of photosynthesis ($C_{i,390}$), on the average of all studied genotypes, was equal to 176 and 216 μl l^{-1}, for plants grown and measured at low and high temperature, respectively. For *Sinensis*, $C_{i,390}$ was not significantly affected by temperature: it was equal to 194 and 195 μl l^{-1}, for leaves grown and measured at low temperature and high grown plants, respectively (Table 7). In contrast, there was a large increase of $C_{i,390}$ in the other studied genotypes, above all in *Floridulus*, in high-temperature plants (Table 7). For *Goliath*, *M. sacchariflorus*, *M. x giganteus* and *M. floridulus*, $C_{i,390}$ was 15%, 18%, 23% and 69%, respectively, higher in high temperature leaves compared to low temperature grown plants (Table 7).

The percent reduction in photosynthesis due to stomatal limitation (l_s), on the average of all studied genotypes, was equal to 0.14 and 0.12%, for plants grown and measured at low and high temperature, respectively. For *M. floridulus*, *M. x giganteus* and *M. sacchariflorus*, l_s was 41%, 25% and 69% higher in low temperature leaves compared to high temperature grown plants, while in *Goliath* and *M. sinensis* was 63% and 62% higher in high temperature leaves compared to low temperature grown plants (Table 7).

Atmospheric CO_2 concentration [CO_2] is forecast to increase from today's concentration of 394 to 550 μmol mol^{-1} by 2050. While it is broadly accepted that this increase in [CO_2] will boost the yield of C_3 plants, there is much less certainty about C_4 plants (Ainsworth and Long, 2005). The photosynthetic C4 cycle serves as a light-energy driven mechanism that maintains [CO_2] at around 10-20 times that of the current atmosphere at Rubisco in the bundle sheath cells (Furbank et al., 1989; Jenkins et al., 1989). In theory, therefore, there should be no direct effect of an increase in atmospheric [CO2] on photosynthetic rate. However, this has proved less certain in practice. Some studies have seen no response (Hocking and Meyer, 1991; Ziska et al., 1991) and others have seen significant increases in photosynthesis and growth at elevated [CO_2] (Knapp et al., 1993; Amthor et al., 1994; Poorter et al., 1996; Wand et al., 1999; Anderson et al., 2001; de Souza et al., 2008). As reviewed by Leakey et al. (2004) greater photosynthesis of C_4 plants at elevated [CO_2] has been suggested to result from a range of processes. These include: i) direct effects on Rubisco CO_2 saturation; ii) insufficient PEP carboxylase activity to gain CO_2-saturation of the primary carboxylase, as could occur during nitrogen deficiency; iii) C_3-like photosynthesis in immature C_4 leaves and iv) indirectly due to lower stomatal conductance. Lower stomatal conductance leads to conservation of water resources and improved plant water status (Leakey et al., 2004) and appears to be the basis of the majority of enhancements in photosynthesis and growth in C_4 plants.

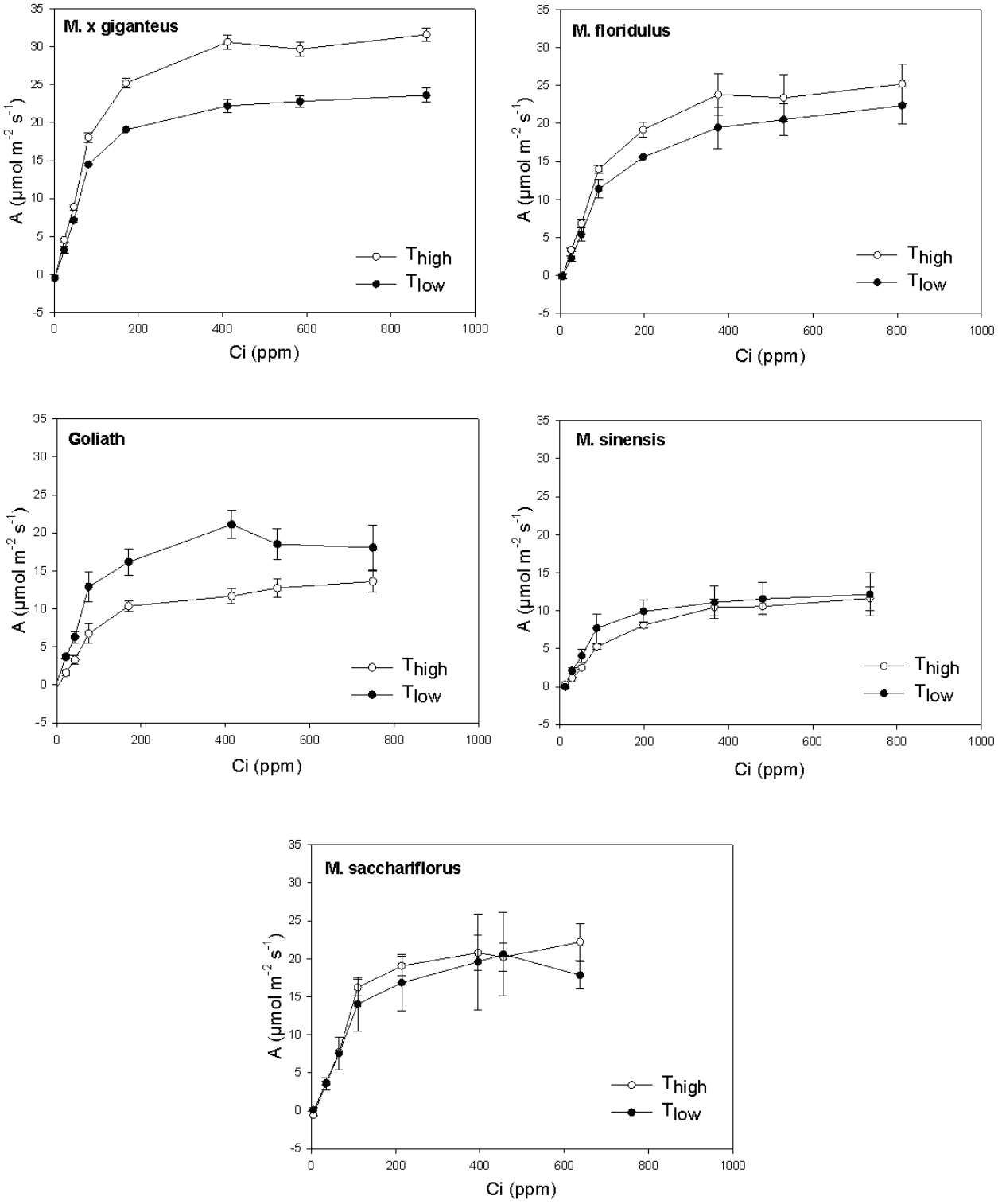

Figure 22 - Response of CO_2 assimilation rate (A) to leaf internal CO_2 mole fraction (Ci) of the studied *Miscanthus* genotypes grown and measured at different growth temperature. Values are means, +/- standard error of the mean.

Table 7 – Analysis of A/Ci response curves for the studied *Miscanthus* genotypes. **CE** - carboxylation efficiency of PEPc. V_{pr} - CO_2-saturated photosynthetic rate. C_i **390** - operating point of photosynthesis. l_s - percent reduction in photosynthesis due to stomatal limitation

Genotype	Treatment	*CE*	V_{pr} (µmol m^{-2} s^{-1})	C_i, 390 (µl l^{-1})	l_s *(%)*
M. x gig	T_{low}	0.20 (0.004)	23.2 (0.8)	176 (13.0)	0.16 (0.017)
	T_{high}	0.21 (0.008)	30.6 (0.9)	217 (11.0)	0.12 (0.018)
M. flo	T_{low}	0.19 (0.032)	21.7 (2.1)	143 (4.0)	0.17 (0.049)
	T_{high}	0.13 (0.007)	24.9 (2.3)	241 (32.0)	0.10 (0.002)
Gol	T_{low}	0.18 (0.012)	20.9 (1.9)	184 (8.0)	0.08 (0.031)
	T_{high}	0.08 (0.015)	13.2 (1.3)	211 (8.0)	0.13 (0.013)
M. sin	T_{low}	0.11 (0.029)	11.7 (2.1)	194 (11.0)	0.13 (0.049)
	T_{high}	0.06 (0.002)	11.0 (1.1)	195 (4.0)	0.21 (0.016)
M. sac	T_{low}	0.20 (0.020)	21.4 (5.2)	182 (6.0)	0.16 (0.002)
	T_{high}	0.16 (0.015)	22.7 (2.5)	214 (5.0)	0.05 (0.002)

(Values are means, +/- standard error of the mean).

The response to CO_2 (A/Ci) provides more information about the CO_2 concentrating mechanism and the functioning efficiency of PEPc. Additionally there can be feedback effects where Calvin Cycle processes become limited through either activation state, substrate limitation or enzyme content amongst other factors. In this work, the response to Ci shows that there is little effect of temperature on the velocity of PEPc regeneration in *M. sacchariflorus*, *M. sinensis* or *M. floridulus* species, however *Goliath* is reduced and there is a marked increase in the rate of PEPc efficiency in *M. x giganteus* at the higher temperature treatment. The carboxylation efficiency of carbon assimilation is significantly reduced in *Goliath*, *M. sinensis*, *M. floridulus* and *M. sacchariflorus*, and however unaffected by increased temperature in *M. x giganteus*. This could indicate that there is either a reduction in PEPc carboxylation efficiency, or PEPc content. The stomatal limitation to photosynthesis (ls) and the intracellular CO_2 concentration at 390ppm extracellular CO_2 concentration (Ci390) are intrinsically linked to the stomata number and conductance, and the rate at which CO_2 is fixed. There appears to be contradictory results. In response to increased temperature, *M. sinensis* net photosynthesis is reduced because of a lower CE, which is in turn affected by an increased stomata limitation (ls). Although dark respiration (Rd) does not change the relationship of Φ_{PSII} and Φ_{CO2} clearly shows that there is a large treatment effect on the number of photons required to fix CO_2 (e^-/CO_2) (Fig. 23). Therefore *M. sinensis* photosynthesis is primarily reduced in increased temperature mainly by reduced capacity for electron transport. *M. x giganteus*

net photosynthesis is unchanged with increased temperature however there is a marked increase in Rd, and V_{pr} is limited at low temperature. The relationship of Φ_{PSII} and Φ_{CO2} is unchanged by temperature. So although there is no change to carbon fixation, there is a marked change in biomass and morphology, maybe because of to the high Rd.

3.3.11 Chlorophyll Fluorescence

The capacity of a plant to carry out photochemistry is limited and will depend upon a range of factors including stresses caused by environmental conditions. Absorbed light energy in excess of that used for photochemistry must be effectively dissipated by non-photochemical processes. Such processes include the emission of heat and re-emission of small but diagnostically significant amounts of the absorbed radiation as longer wavelength red/far-red light energy. This re-emission of light is termed chlorophyll fluorescence. Although chlorophyll fluorescence emission from whole leaf systems is too weak to be viewed with the naked eye, it can be observed from illuminated extracts of a chlorophyll solution. Peak chlorophyll fluorescence occurs in the red region of the spectrum (685 nm) and extends into the infra-red region to around 800 nm. Each of these processes operate in direct competition for a finite pool of absorbed energy, any change in energy utilisation by one process produces a complementary change in the others. This fact enables chlorophyll fluorescence to be used as a rapid and reliable non-invasive probe of photochemistry. Specialist equipment is required for the analysis of the chlorophyll fluorescence signature. A chlorophyll fluorimeter is designed specifically to detect the chlorophyll fluorescence emission from a sample. There are several different types of chlorophyll fluorimeter available:

- pulse modulated chlorophyll fluorometers: they use sophisticated electronics to separate chlorophyll fluorescence from ambient light. The systems achieve this using a rapid pulsing excitation light in order to induce a corresponding pulsed fluorescence emission. The fluorometer uses a highly sensitive photodiode to detect and record the pulsed fluorescence signal and to ignore any non-pulsed signal;
- continuous excitation chlorophyll fluorimeters: they measure fast chlorophyll fluorescence induction (Kautsky and Hirsch, 1931).

Modulated chlorophyll fluorescence in relation to photosynthesis

During the heat stress, simultaneous measurements of CO_2 uptake and fluorescence were made using a portable gas exchange fluorescence system (GFS-3000, Heinz Walz GmbH, Germany). The electrons produced by the photochemical process are not necessarily used for carbon fixation. In conditions where carbon fixation is limited (e.g. low temperature, shortage of CO_2 due to stomatal

closure, etc.), alternative sinks for electrons might be enhanced, namely i) photorespiration and ii) reduction of molecular oxygen (part of Mehler reaction). Whilst the first can be considered as a waste of energy, the latter may lead to a dangerous oxidative stress. The allocation of electrons produced by the oxygen evolving complex can be studied by simultaneous measurement of the quantum yield of electron transfer (Φ_{PSII}) at photosystem II measured by chlorophyll fluorescence and the quantum yield of CO_2 fixation (Φ_{CO2}) measured by gas exchange. If more electrons are used for photorespiration or for the Mehler reaction, the ratio Φ_{PSII}/Φ_{CO2} will increase. During the heat stress experiment, carried out at Institute of Biological, Environmental and Rural Sciences (IBERS) at Aberystwyth University, fluorescence and gas-exchange parameters were used to calculate the operating quantum efficiency of whole-chain electron transport through photosystem II (ϕ_{PSII}) versus the efficiency of CO_2 assimilation (ϕ_{CO2}) (Fig. 23). There is a strong linear relationship between these parameters, however, a discrepancy between these two parameters may occur under certain stress conditions, due to changes in the rate of photorespiration or pseudocyclic electron transport. The slope and Y-intercept for the portion of the relationship between ϕ_{PSII} and ϕ_{CO2} that is linear (and measured at $Q > 40$ μmol m^{-2} s^{-1}) were calculated for each leaf. One-half the value of the slope of this line represents the number of electrons used per molecule of CO_2 fixed. The linear relationship between ϕ_{PSII} and ϕ_{CO2} suggests that the proportion of electron transport used for photosynthesis remained constant, regardless of growth temperature and heat treatment.

As shown in the graph number 23, there was no evidence of alternative energy sinks in *M. x giganteus*, *M. floridulus* and *M. sacchariflorus* at low and high temperature. Analysis of linear trendline of *M. x giganteus* scatter graph suggests that the Y-intercept of the relationship between ϕ_{PSII} and ϕ_{CO2} was not significantly greater than zero (Table 8). There was no significant difference in the slopes with temperature in either *M. x giganteus* plants. The values of the slope of these curves were 13.8 for low temperature *M. x giganteus* and 12.7 for high temperature *M. x giganteus* leaves (Table 8). Thus, the average number of electrons used per CO_2 evolved is approximately 7 for leaves grown and measured at low temperature and 6 for leaves measured at high temperature (Table 8). For *M. floridulus*, the values of the Y-intercept was equal to -0.0006 and -0.0479, for leaves grown and measured at low temperature and high grown plants, respectively; the values of the slope were 14.0 for low temperature and 12.8 for high temperature leaves and the average number of electrons used per CO_2 evolved is approximately 7 and 6 for leaves grown and measured at low temperature and high grown plants, respectively (Table 8). For *M. sacchariflorus*, the values of the Y-intercept was equal to 0.0966 and 0.0873, for leaves grown and measured at low temperature and high grown plants, respectively; the values of the slope were 10.5 for low temperature and 13.4 for high temperature leaves and the average number of electrons used per CO_2

evolved is approximately 5 and 7 for leaves grown and measured at low temperature and high grown plants, respectively (Table 8). There was important difference of alternative energy sinks in *Goliath* and *M. sinensis* at low and high temperature (Fig. 23). Both genotypes were more efficient at low temperature, as they needed less light for each CO_2 molecule fixed than the plants grown and measured at high temperature. At high temperature these plants required more light for each CO_2 molecule fixed (Fig. 23). For *Goliath*, the values of the Y-intercept was equal to -0.0784 and 0.0416, for leaves grown and measured at low temperature and high grown plants, respectively; the values of the slope were 16.6 for low temperature and 22.6 for high temperature leaves and the average number of electrons used per CO_2 evolved is approximately 8 and 11 for leaves grown and measured at low temperature and high grown plants, respectively (Table 8). For *M. sinensis*, the values of the Y-intercept was equal to -0.0553 and 0.1103, for leaves grown and measured at low temperature and high grown plants, respectively; the values of the slope were 16.9 for low temperature and 31.2 for high temperature leaves and the average number of electrons used per CO_2 evolved is approximately 9 and 16 for leaves grown and measured at low temperature and high grown plants, respectively (Table 8).

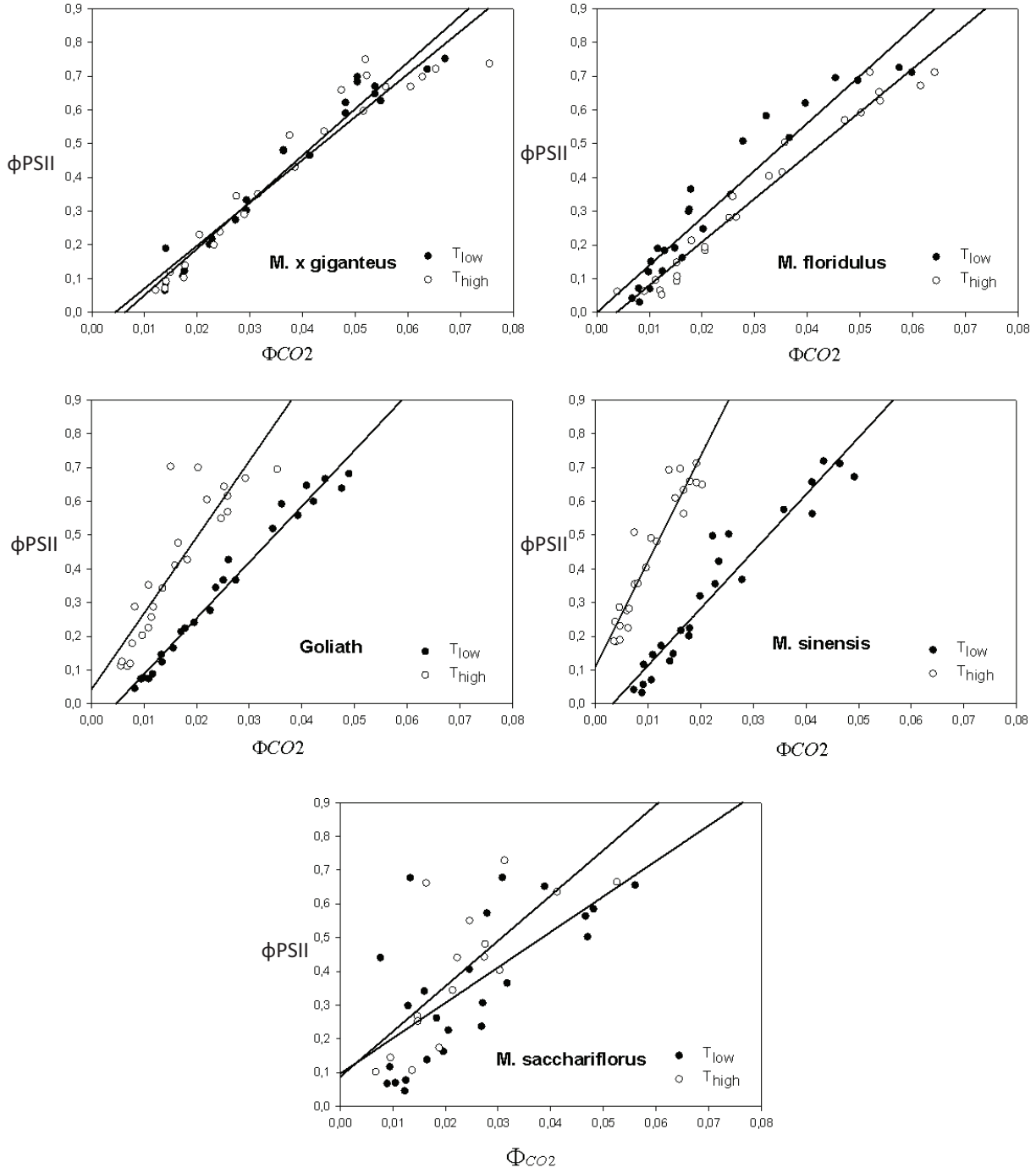

Figure 23 – Relationship between the operating quantum efficiency of whole-chain electron transport through PSII (ϕ_{PSII}), measured by modulated chlorophyll fluorescence, versus the efficiency of CO_2 assimilation (ϕ_{CO2}), measured by gas exchange.

Table 8 – Analysis of linear regression of all studied *Miscanthus* genotypes grown and measured at different temperature and number of electrons used per molecule of CO_2 fixed.

Genotype	Treatment	Slope	Intercept	R^2	e^-/CO_2
M. x gig	T_{low}	13.8	-0.0869	0.96	6.9
	T_{high}	12.7	-0.0562	0.93	6.4
M. flo	T_{low}	14.0	-0.0006	0.92	7.0
	T_{high}	12.8	-0.0479	0.96	6.4
Gol	T_{low}	16.6	-0.0784	0.98	8.3
	T_{high}	22.6	0.0416	0.79	11.3
M. sin	T_{low}	16.9	-0.0553	0.92	8.5
	T_{high}	31.2	0.1103	0.89	15.6
M. sac	T_{low}	10.5	0.0966	0.47	5.3
	T_{high}	13.4	0.0873	0.57	6.7

Continuous excitation fluorescence

During the heat stress experiment, measurements of chlorophyll fluorescence were made using a continuous fluorescence portable fluorimeter (Handy PEA, Hansatech, UK), and the light-adapted photosystem II (PSII) maximum quantum efficiency (Fv'/Fm') and the dark-adapted photosystem II (PSII) maximum quantum efficiency (Fv/Fm) have been calculated. The light-adapted PSII maximum quantum efficiency (Fv'/Fm') was not significantly altered by the different growth temperature (Fig. 24). The means for light-adapted Fv'/Fm' were 0.725 and 0.733 for low and high temperature genotypes leaves, respectively. At low temperature the highest value of Fv'/Fm' was recorded for *Miscanthus x giganteus* (0.749) while the lowest one was for *M. sinensis* (0.708). At high temperature the highest value of Fv'/Fm' was recorded for *Miscanthus x giganteus* (0.754) while the lowest value was for *M. sinensis* (0.716).

Fv/Fm is a parameter widely considered to be a sensitive indication of plant photosynthetic performance with healthy samples typically achieving a maximum Fv/Fm value of approx. 0.85. Values lower than this will be observed if a sample has been exposed to some type of biotic or abiotic stress factor which has reduced the capacity for photochemical quenching of energy within photosystem II. Fv/Fm is presented as a ratio of variable fluorescence (Fv) over the maximum

fluorescence value (Fm). During the dark adaptation, all the reaction centers are fully oxidised and available for photochemestry and any fluorescence yield is quenched.

The dark-adapted PSII maximum quantum efficiency (Fv/Fm) was not significantly altered by the different growth temperature, on any of the measurement dates. Only the *M. Sacchariflorus* showed an increasing trend at high growth temperature (Fig. 25). The means for dark-adapted Fv/Fm were 0.784 and 0.791 for low and high temperature genotypes leaves, respectively. Relative to low-temperature, high-temperature increased Fv/Fm (~1%). In low-temperature the highest value of Fv/Fm was recorded for *Miscanthus x giganteus* (0.796) while the lowest one was for *Goliath* (0.776). The value of Fv/Fm of the other genotypes ranged between 0.777 (*M. sinensis*), 0.783 (*M. sacchariflorus*) and 0.787 (*M. floridulus*). In high-temperature the highest value of Fv/Fm was recorded for *Miscanthus x giganteus* (0.803) while the lowest value was for *Goliath* (0.781). The value of Fv/Fm of the other genotypes ranged between 0.782 (*M. sinensis*), 0.792 (*M. floridulus*) and 0.798 (*M. sacchariflorus*) (Fig. 25).

Where photosystem II (PSII) maximum efficiency is the efficiency with which light absorbed by the pigment matrix associated with PSII is used to drive stable photochemistry when all PSII centres are in the open state. Fv/Fm is reduced in the lower temperature for *M. floridulus*, *Goliath*, *M. sinensis* and *M. sacchariflorus*. Considering the relationship between relative chlorophyll content (RCC) and light harvesting complexes (LHC), and subsequent availability of reductant for PSII there is a good correlation between the higher RCC and fluorescence parameters, except in the instance of *M. x giganteus*. Those species that have the higher RCC in the higher temperature treatment also have a higher PSII efficiency in the light and dark.

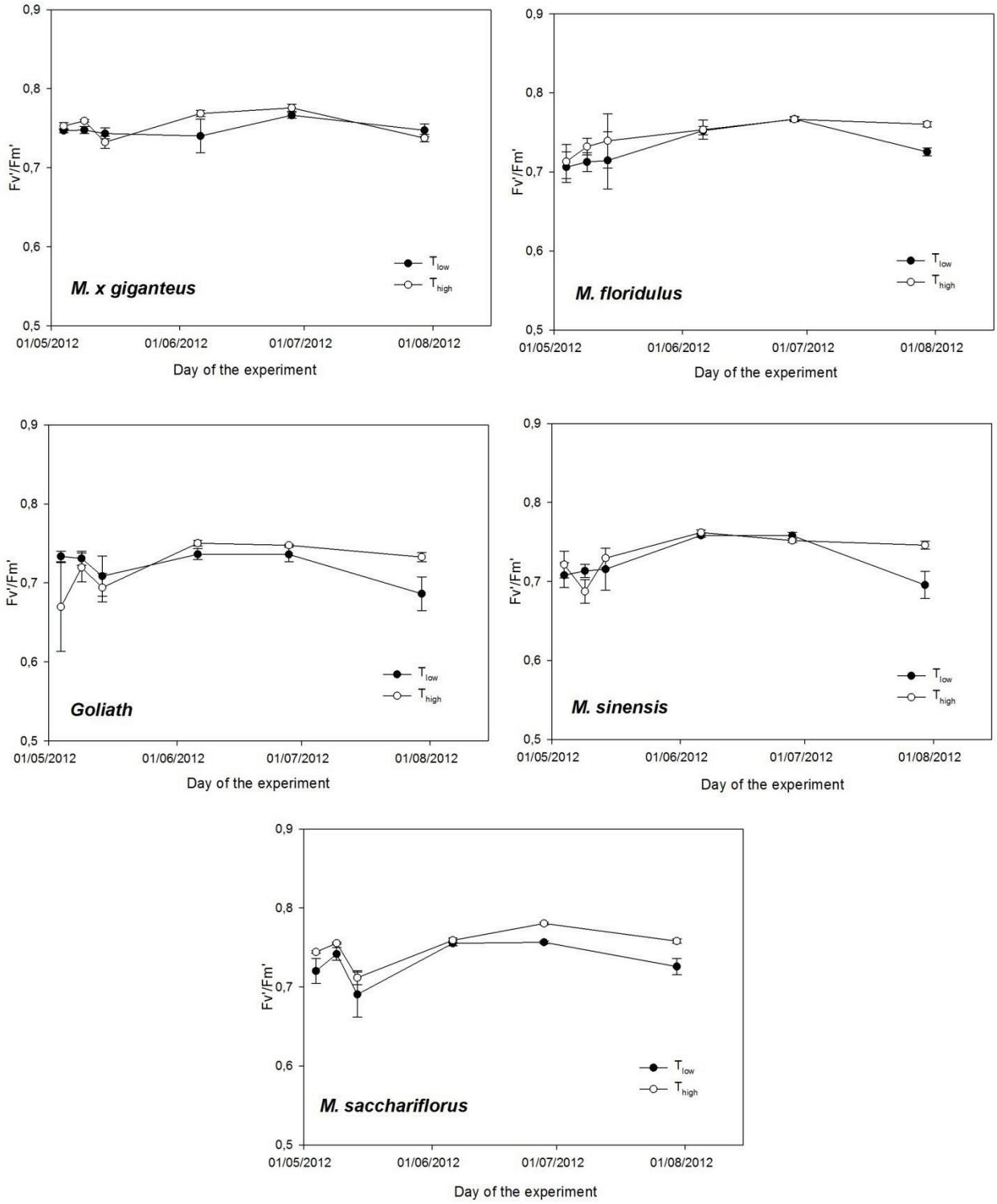

Figure 24 – Light-adapted photosystem II maximum quantum efficiency (Fv'/Fm'), for all measured leaves of the *Miscanthus* genotypes grown and measured at low- and high-temperature. Values are means, +/- standard error of the mean.

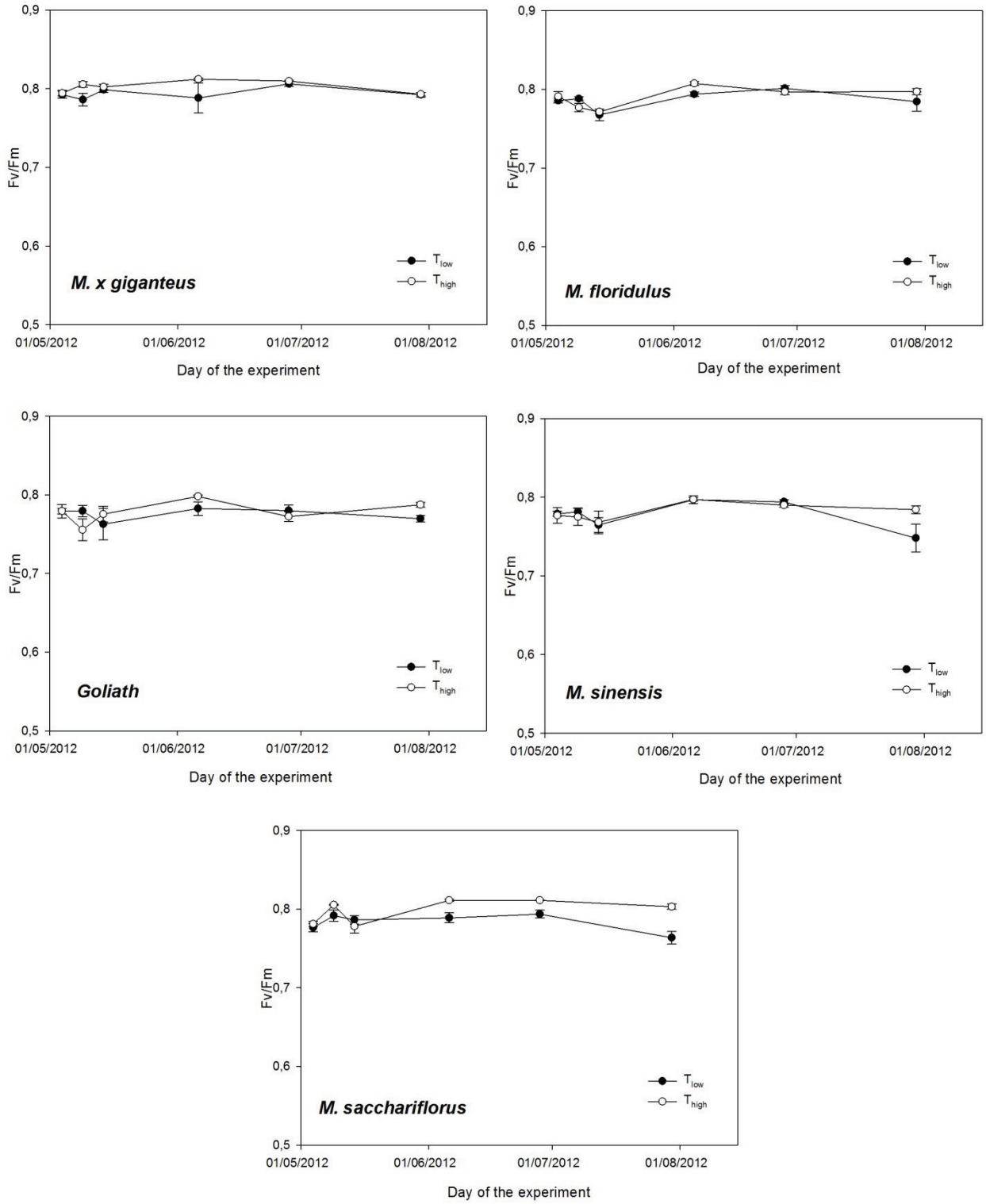

Figure 25 – Dark-adapted photosystem II maximum quantum efficiency (Fv/Fm), for all measured leaves of the *Miscanthus* genotypes grown and measured at low- and high-temperature. Values are means, +/- standard error of the mean.

3.3.12 Stomata number and size

Stomata number was calculated both on the adaxial and abaxial leaf surface of all studied *Miscanthus* plants. On the average of all studied genotypes, the stomata number on the adaxial leaf surface was not affected by the different growth temperature, whilst the number of stomata was affected by the growth temperature on the abaxial leaf surface (Fig. 26). The stomata number was higher on the abaxial leaf surface compared to the stomata number on the adaxial leaf surface (Fig. 26). On the adaxial part of the leaf, on average of all studied genotypes, the stomata number was equal to 68.3 and 65.8 per mm^2 for leaves grown and measured at low and high temperature, respectively (Fig. 26), while the stomata number was equal to 293.2 and 268.8 for leaves grown and measured at low and high temperature, respectively, on the abaxial leaf surface (Fig. 26).

Relative to low temperature, high temperature decreased the stomata number on the adaxial leaf surface (-4%) and on the abaxial leaf surface (-8%).

On the adaxial leaf surface, on average of the two different growth temperature, *M. x giganteus* and *M. Sacchariflorus* showed the highest stomata number per mm^2, 110.8 and 98.8, respectively, but *M. sacchariflorus* showed the highest stomata number on the adaxial leaf surface at high temperature (122.5) than that *M. x guganteus* (96.7). The other *Miscanthus* genotypes ranged between 32.5 (*M. sinensis*) and 47.5 stomata per mm^2 (*M. floridulus*) (Fig. 26). At low temperature, the highest number of stomata per mm^2 was recorded for *M. x giganteus* (125.0), while the lowest number of stomata for *M. sinensis* (33.3). At high temperature, the lowest number of stomata per mm^2 was recorded for *M. sinensis* (31.7) (Fig. 26). On the abaxial leaf surface, on average of the two different growth temperature, *M. floridulus* showed the highest stomata number per mm^2, reaching a value equal to 430.0, but the stomata number was higher at high temperature (455.8) than that at low temperature (404.2). The other *Miscanthus* genotypes ranged between 230.4 (*Goliath*) and 260.0 (*M. x giganteus*), on the average of the two growth temperature (Fig. 26).

Regarding the stomatal size, the studied *Miscanthus* genotypes responded differently to increased temperature (Fig. 27). *M. x giganteus*, *M. floridulus* and *Goliath* showed higher length and width stomata values on the adaxial and abaxial leaf surface at low temperature than that at high temperature. For *M. x giganteus*, on the adaxial leaf surface, the stomata size were 34.8x14.2 μm and 28.3x12.5 μm, for plants grown and measured at low and high temperature, respectively; for *M. floridulus*, the stomata size were 28.2x16.0 μm and 25.4x14.3 μm, for plants grown and measured at low and high temperature, respectively; for *Goliath*, the stomata size were 34.5x17.5 μm and 29.1x15.1 μm, for plants grown and measured at low and high temperature (Fig. 27). *M. sacchariflorus* showed higher stomata size at low temperature on the adaxial leaf surface, reaching a value equal to 30.9x15.3 μm compared to 28.7x14.9 μm recorded at high temperature, while at

high temperature the stomata size was higher on the abaxial leaf surface, reaching a value equal to 29.2x14.5 μm compared to 28.7x12.5 μm recorded at low temperature (Fig. 27). *M. sinensis* showed higher length stomata at high temperature on the adaxial and abaxial leaf surface, while the width stomata value was higher at low temperature on the adaxial leaf surface but none difference was recorded on the abaxial leaf surface. For *M. sinensis*, the stomata size were equal to 30.6x17.6 μm and 31.7x15.1 μm for plants grown and measured at low and high temperature on the adaxial leaf surface, while the stomata size were equal to 27.7x14.8 μm and 30.5x14.8 μm for plants grown and measured at low and high temperature on the abaxial leaf surface.

Stomatal number and density has been shown to have plasticity in response to abiotic drivers such as water, CO_2 and temperature (Fraser et al., 2009). There is a common response within the species studied, that stomatal density, the number of stomata within a defined leaf area, are reduced in the higher temperature treatment. This is occurring on both the upper and lower leaf surfaces except in *M. sacchariflorus* (upper surface) and *M. floridulus* (lower surface). The change in stomata size and distribution is often associated with reduced water. It has been shown that in some instances stomata distribution changes in favour of the upper leaf surface in species where leaf rolling response can occur, the rolling thus protecting the integrity of the boundary layer around the stomata (Xu and Zhou, 2008). In this experiment, there appears in general to be a reduction in both stomata size and density, which could suggest that the increased temperature is effecting the same response as water stress and as such the leaf cellular morphology is changing to protect the leaves from water loss.

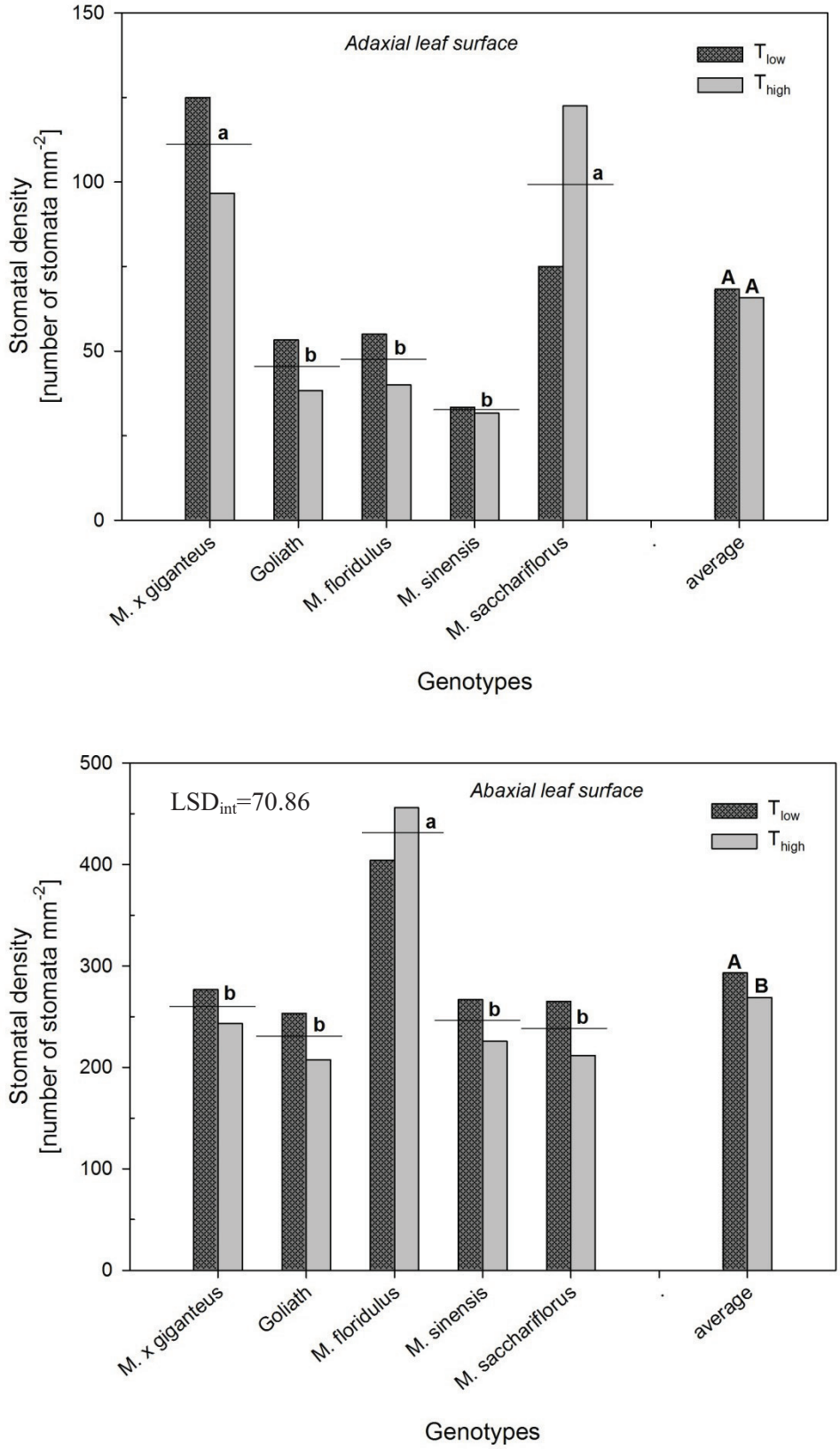

Figure 26 – Stomatal density (number/mm^2) on the adaxial and abaxial leaf surface in the *Miscanthus* genotypes grown and measured at different growth temperature. Bars represent the average value of 6 measurements. Horizontal lines represent average value of the two growth temperature. Capital letters, for averaged values of all Miscanthus genotypes with each temperature; small letters, for averaged values of temperatures within each Miscanthus genotypes. Different letters indicate significant differences at P≤0.05 by Tukey's HSD Test.

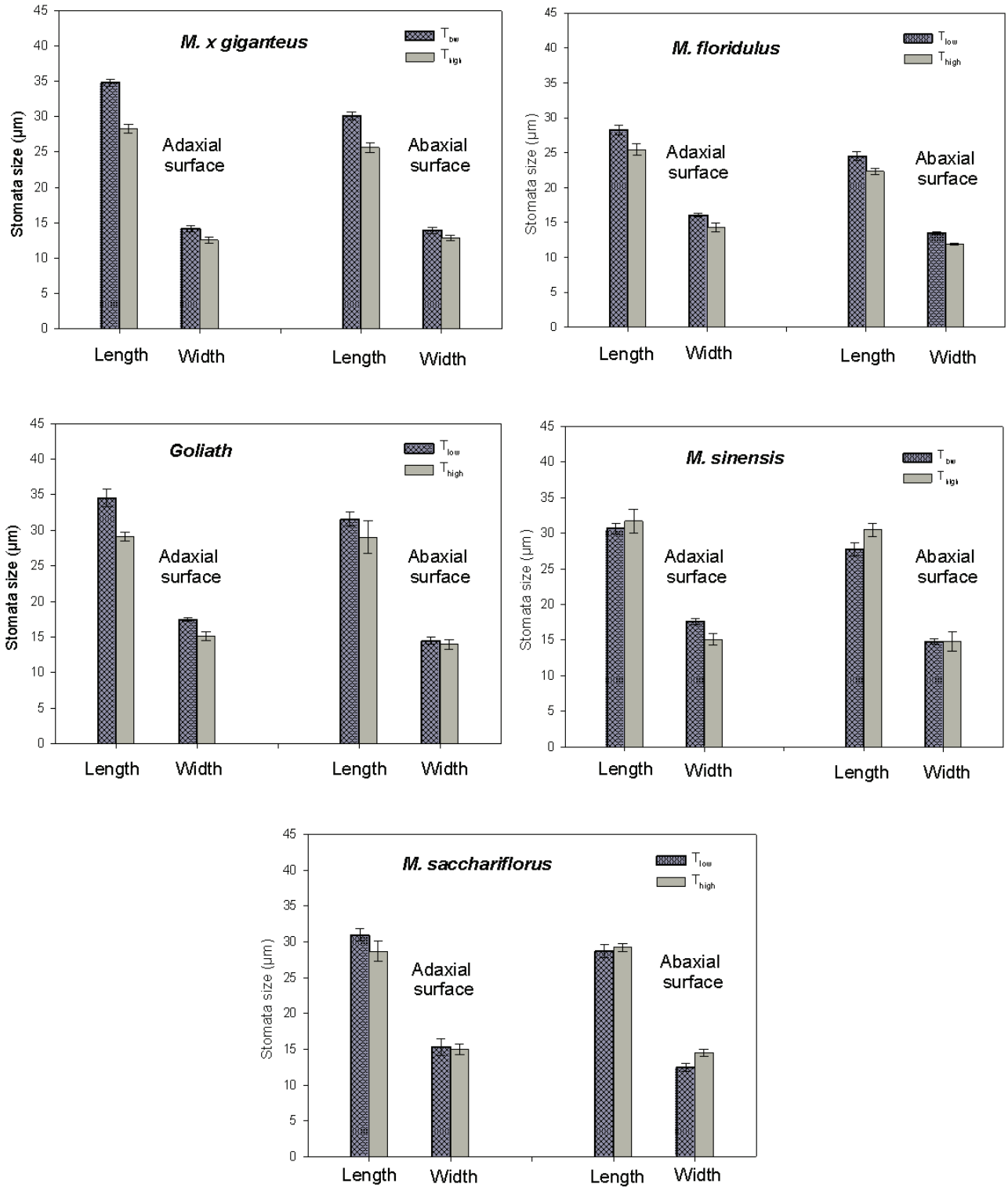

Figure 27 – Stomata size (um) of the studied Miscanthus genotypes grown and measured at different temperature. Bars represent the average value of 6 measurements, +/- errors standard of the mean.

3.3.13 Leaf absorbance

Photosynthesis depends upon the absorption of light by pigments in the leaves of plants. The most important of these is chlorophyll-a, but there are several accessory pigments that also contribute. When the sunlight shines on a leaf, a proportion is reflected from the leaf surface, another proportion is transmitted through the leaf, the remaining light is absorbed by the leaf and can be used in photochemistry. Leaf absorbance was determined essentially for two reasons: i) determine if there is a difference in the leaf morphology and ii) to correct the photosynthesis measurements for the actual absorbed irradiance. The different growth temperature had an effect on the amount of light absorbed, but the effect varied between the species. At high growth temperature leaf absorbance reduction increased in all *Miscanthus* accessions (Table 9), however the *M. sacchariflorus* showed a different trend: it had a higher absorbance at high-temperature (Table 9). The *Miscanthus* accessions all have had reduced absorbance of PAR at 470 and 640 nm, particularly the *Goliath* and *M. sinensis*. For *Goliath*, the values of the reduced absorbance were equal to -6.4 and -11.7 at 600 and 1800 PAR, respectively, for leaves grown and measured at low- and high temperature (Table 9); for *M. sinensis*, it was equal to -8.8 and -11.5% at 600 and 1800 PAR, respectively, for leaves grown and measured at low- and high temperatures (Table 9). *M. x giganteus* had the same light reduction at both growth temperatures: it was equal to -8.6 and -8.8% at 600 and 1800 PAR, respectively, for leaves grown and measured at low- and high temperature (Table 9). For *M. floridulus*, the value of the absorbed light reduction was equal to -3.4 and -6.3% for leaves grown and measured at low- and high-temperature at 600 and 1800 PAR, respectively (Table 9). However, the *M. sacchariflorus* was the opposite and has had higher absorbance in the higher temperature than that of all other studied genotypes. For *M. sacchariflorus*, the light reduction was equal to -14.8 and -7.8% at 600 and 1800 PAR, respectively, for plants grown and measured at low- and high temperatures (Table 9).

Table 9 – Amount of light absorbed by the *Miscanthus* leaves at 600 and 1800 µmol PAR

				Absorbed Q at 600 and 1800 PAR			
Genotype	Treat	A_{470}	A_{640}	600	1800	% at 600	% at 1800
M. x gig	T_{low}	0.9255	0.9130	549	1646	-8.6	-8.6
	T_{high}	0.9290	0.9096	547	1641	-8.8	-8.8
M. flo	T_{low}	0.9677	0.9654	579	1738	-3.4	-3.4
	T_{high}	0.9518	0.9349	562	1686	-6.3	-6.3
Goliath	T_{low}	0.9400	0.9357	562	1685	-6.4	-6.4
	T_{high}	0.8971	0.8819	530	1590	-11.7	-11.7
M. sin	T_{low}	0.9332	0.9092	547	1641	-8.8	-8.8
	T_{high}	0.8953	0.8841	531	1593	-11.5	-11.5
M. sac	T_{low}	0.8763	0.8491	511	1533	-14.8	-14.8
	T_{high}	0.9355	0.9200	553	1659	-7.8	-7.8

3.3.14 Relative chlorophyll a estimation (SPAD values)

The relative amount of chlorophyll *a* in the leaves were estimated during the heat stress experiment. The chlorophyll content (or SPAD meter) is a simple, portable diagnostic tool that measures the greenness or relative chlorophyll content (RCC) of leaves (Percival *et al.*, 2008). Compared with the traditional destructive methods of chlorophyll extraction, the use of this equipment saves time, space, and resources. Chlorophyll *a* is a specific form of chlorophyll used in oxygenic photosynthesis. This photosynthetic pigment is essential for photosynthesis because of its role as primary electron donor in the electron transport chain. Chlorophyll *a* also transfers resonance energy in the antenna complex, ending in the reaction center where specific chlorophylls P680 and P700 are located. The molecular structure of chlorophyll *a* consists of a N-ring with a Mg center, side chains, and a hydrocarbon tail. Chlorophyll *a* contains a central magnesium ion encased in a 4-ion nitrogen ring known as a chlorin ring. The chlorin ring is a heterocyclic compound derived from a pyrrole that encases a metal. The Mg within the center uniquely defines the structure as a chlorophyll molecule. The side chains are attached to the porphyrin ring of chlorophyll *a*. Different side chains characterize each type of chlorophyll molecules, and alters the absorption spectrum of light. Chlorophyll *a* contains only methyl groups (CH_3) as side chains. A tail attached to the porphyrin ring (of the chlorophyll *a* molecule) is a long hydrocarbon tail. This long hydrophobic extension anchors the chlorophyll *a* molecule to other hydrophobic proteins in the thylakoid membrane of the chloroplast. In all studied *Miscanthus* accessions, the estimation of chlorophyll *a*

content, SPAD values, plotted against time, shows an increasing trend at both growth temperatures, and then levels off and/or decreases slightly from the second half of June until the end of the experiment (Fig. 28). At the beginning of the heat stress experiment, when the plants were immature, the values of the SPAD were lower than that of the same values measured after two months, since the experiment started (Fig. 28). From the end of June until the end of August, the values of the SPAD increased and acclimated gradually, towards the end of the experiment (Fig. 28). Heat treatment reduced chlorophyll *a* in *M. x giganteus*, *M. sacchariflorus* and *M. Floridulus*, but increased in *M. sinensis* and *Goliath* (Fig. 28). For *M. x giganteus*, the values of the SPAD were not affected by the different growth temperature from the beginning of the experiment until the beginning of June: they were equal to 46.4 and 46.9 for plants grown and measured at low- and high temperature. The difference increased subsequently, and the SPAD value was equal to 56.9 and 46.6 for plants grown and measured at low and high temperature (Fig. 28). The same observations have been seen for *M. floridulus* and *M. sacchariflorus*. For *M. floridulus*, the SPAD values were equal to 46.4 and 45.1 initially, for plants grown and measured at low- and high temperature then they were equal to 56.2 and 50.2 at low- and high-temperature, respectively. For *Sacchariflorus*, the SPAD values were equal to 43.0 and 40.3 at the beginning, for plants grown and measured at low- and high temperature, then they were equal to 53.4 and 45.5 at low- and high-temperature, respectively (Fig. 28). As mentioned above, high growth temperature increased chlorophyll *a* content for *Goliath* and *M. sinensis*. For *Goliath*, the SPAD values were equal to 42.5 and 46.5 at the beginning, for plants grown and measured at low- and high temperature, then they were equal to 51.4 and 53.3 at low- and high-temperature, respectively. For *M. sinensis*, the SPAD values were equal to 41.8 and 44.2 at the beginning, for plants grown and measured at low- and high-temperature, then they were equal to 49.7 and 52.1 at low- and high-temperature, respectively (Fig. 28). The relative chlorophyll content can relate to the pigment matrix of light harvesting complexes (LHC) associated with photosystem II (PSII). The increased temperature treatment caused a reduction in relative chlorophyll content in *M. x giganteus*, *M. floridulus* and to a lesser extent in *M. sinensis*. However *Goliath* relative chlorophyll content was not affected and *M. sacchariflorus* was affected but with little statistical confidence.

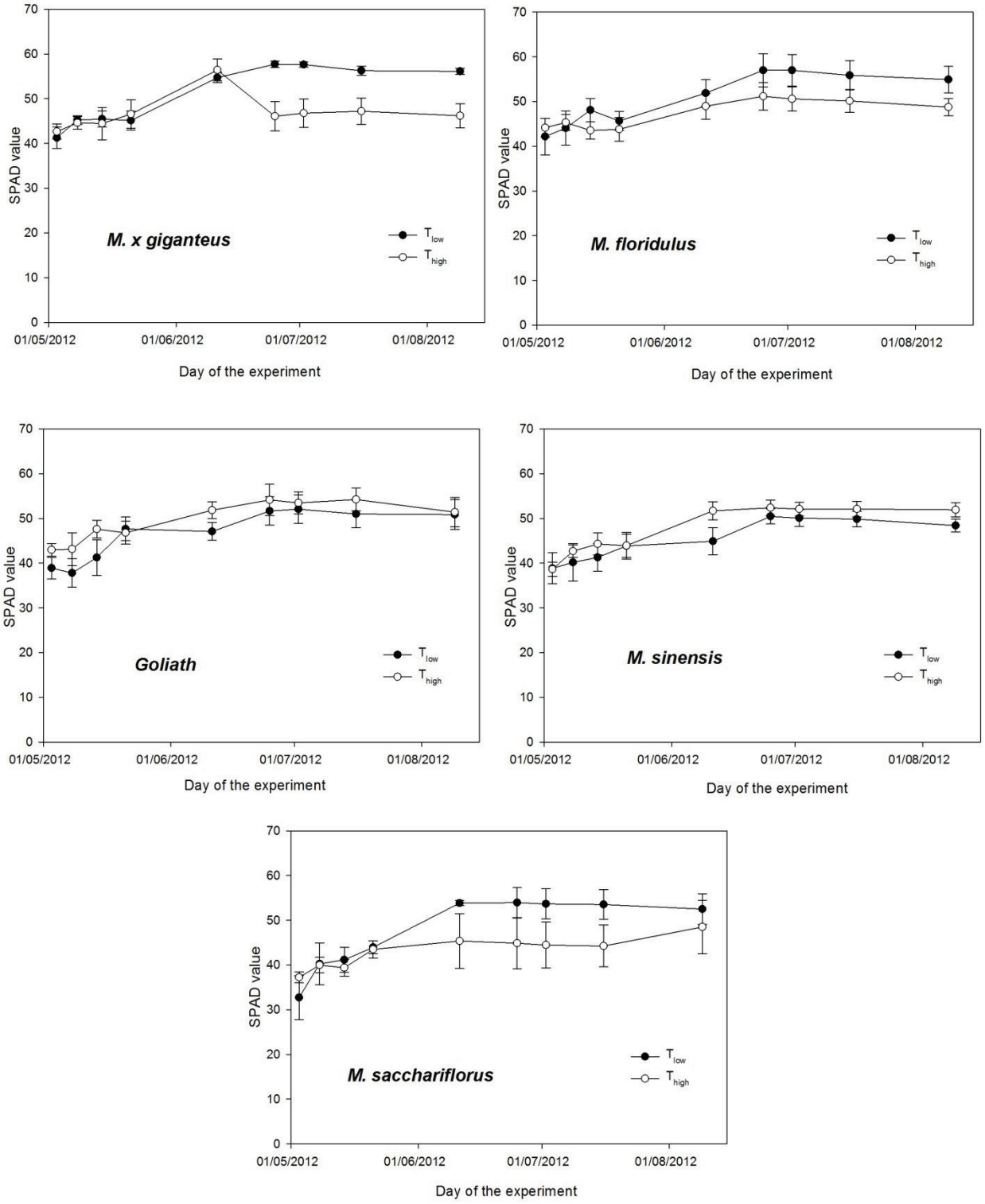

Figure 28 – Trend of the relative chlorophyll *a* estimation of leaves in the studied *Miscanthus* genotypes grown and measured at low and high temperature. Values are means, +/- standard error of the mean.

4 Conclusions

4.1 Evaluating wild *Miscanthus* germplasm for biomass potential in Southern Europe

This study showed that some *Miscanthus* accessions are suitably adapted to maintain high biomass in a semi-arid Mediterranean environment. *Miscanthus x giganteus* (M19) and *Goliath* (M18) have previously been studied for drought sensitivity and water use efficiency. *Goliath* types, in contrast to *M. x giganteus,* actively down regulate stomatal water loss during drought. In this experiment it has been found that yield *Goliath* is consistent with the relative change seen between these species in the two harvest years. It can be surmised from this that accessions from *M. sacchariflorus* are generally drought sensitive, as the yield is reduced in the water limited 2011 growing season, whereas those *M. condensatus* and *M. floridulus* species maintained or increased their yield without irrigation, having a higher water use efficiency and drought tolerance. This study confirmed that the most commonly available commercial *Miscanthus* genotypes are not well adapted to the Mediterranean climate or environments where water is a limiting factor, and there are other *Miscanthus* accessions that produce high biomass in water limited semi-arid regions.
The induction of the flowering is dependent on day length and variations observed among the accessions can be related to the geographical origin of genotypes.

4.2 *Miscanthus* biomass yield and biomass quality as affected by harvesting dates

Long term *Miscanthus* plantations strictly depend by the thermopluviometric trend of the growing season, decreasing biomass yield as rainfall reduces. Basically, autumn harvest lead to higher leaves to stems ratio, weight of one stem and higher moisture content of both stems and leaves, while winter harvest lead to higher stem density, stem node number, plant height and fresh and dry biomass yield. Biomass for specific end uses presents higher quality with winter harvest; indeed, a decrease of NDS and ash content and an increase of NDF and ADF content than the autumn harvest in both leaves and stems was noticed. NDF and ADF (hemicellulose and cellulose, respectively) are the most important polymers for second generation bioethanol production, while ash content for biomass combustion purposes. An increase in polymers, mainly cellulose and hemicellulose corresponds to higher bioconversion yield, while the higher the ash and mineral content the higher the combustion boiler troubles due to slagging, fouling and corrosion tendencies. Furthermore, the higher ash content was found in the leaves, therefore strategies aiming at reducing the leaf component (e.g. winter harvest) may considerably improve the biomass quality for current combustion plants.

4.3 Effect of heat stress on the biomass production and physiology in *Miscanthus* genotypes

Miscanthus has been studied for light, drought, nutrient and cold temperature responses, but has never before been studied under realistic increased temperatures. In this experiment it was noted that the *Miscanthus* genotypes responded differently to increased temperatures, producing a dwarf phenotype. As a C_4 grass, there is an assumption that it would be more sensitive to cold temperatures and high temperatures would benefit growth. However this experiment has identified that the most widely available and commonly used variety of *Miscanthus* is sensitive to high temperatures and has demonstrated that the heat stress result in a reduction of *Miscanthus x giganteus* and *Goliath* growth and that there are other genotypes that have a higher capacity for carbon assimilation in high temperature environments. Morphological and physiological reductions *Miscanthus floridulus* were less than in *Miscanthus x giganteus*, reflecting probably their climatic origins. The ability of *M. x giganteus* to maintain relatively high levels of leaf absorptance, quantum yield and light saturated photosynthesis are all characteristics that contribute to high conversion efficiencies of radiation into biomass at low temperature in this species. Rather, the maintenance of high photosynthetic rates at low temperature in *M. x giganteus* is dependent on the properties of Ribulose bisphosphate carboxylase/oxygenase (RuBisCO) and/or Pyruvate orthophosphate dikinase (PPDK), reduced susceptibility to photoinhibition and the ability to maintain high levels of leaf absorptance.

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