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Research Paper

Indian Horticulture Journal; 8(1): 25-30, January-March (2018)
©Indian Society of Advanced Horticulture
ISSN: 2249-6823

DI: 369-17-IHJ-1810-05

Agronomical, Physiological and Biochemical Characterization of Chinese Mulberry Cultivars under Cuban Tropical Conditions

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Received: 18 October 2017; Revised accepted: 28 February 2018

ABSTRACT

Field experiments were carried out to analyse the response of three promising Chinese mulberry (*Morus alba*) cultivars under Cuban tropical conditions, paying special attention to biomass yield, the principal leaf biochemical characteristics and leaf mechanisms developed to confront water stress. The experiment was carried out in 2014 and 2015 near the city of La Habana (Cuba). The trees were own-rooted GuiSang You 62, Guandong 11 and GuiSang You 12 cultivars**.** High leaf and total biomass yield were obtained each year in the three cultivars, even though Guandong 11 and GuiSang You 12 cultivars showed a more constant production of both, and their leaf composition seemed to be more suitable for rearing silkworms and livestock fodder. The three mulberry cultivars exhibited an anisohydric physiology, exerting low stomatal control which permits a substantial decrease in leaf water potential. These plants were also able to develop active osmotic adjustment to confront water stress, thus maintaining leaf turgor.

Key words: Anisohydric plants, Biomass production, Osmoregulation**,** Water relations, Leaf biochemical contents

Mulberry (*Morus alba* L.)is an economically important multipurpose tree crop which, in addition to its traditional uses for rearing silkworms (*Bombyx mori*), as forage for livestock, providing fruit for human consumption, fuel, wood and in traditional Chinese medicine, is of great interest for its gardening, ethnobotany and agroforestry potential. When mulberry is used for silkworm or livestock the most important characteristic is the yield of foliage, which is chiefly dependent on cultivar and soil moisture and fertility (Madan and Sharma 1999, Ramanjulu and Sudhakar 2000). Mulberry shows great adaptation to various climatic conditions, from arid to humid and from temperate to tropical (Biasiolo *et al*. 2004). In this sense, the selection of new superior mulberry cultivars with better foliage yields in each agrosystem is extremely important because it encourages and optimizes mulberry cultivation in new areas. Mulberry has been grown in Cuba under tropical conditions from the 1930s (Pentón *et al*. 2012) and interest in this crop continues to grow mainly due to the increasing demand as fodder and in the biopharmaceutical industry. So, there is a permanent quest for efficient cultivars, which could be used for producing better foliage yields under tropical conditions even under adverse environmental conditions. For this reason, the main objective of the study described in this paper was to assess the response of three promising Chinese mulberry cultivars, Guandong 11, GuiSang You 12 and GuiSang You 62, to Cuban tropical culture conditions, paying special attention to biomass yield, leaf biochemical characteristics and leaf mechanisms developed to confront water stress.

MATERIALS AND METHODS

Plant material, experimental conditions and treatments

The experiment was carried out in 2014 and 2015 in a mulberry (*Morus alba* L.) orchard located near the city of La Habana (Cuba).The trees were own-rooted4-year old

GuiSang You 62, Guandong 11 and GuiSang You 12 Chinese cultivars at a planting density of 45720 trees/ha. The soil of the orchard is a Lixic Ferralic Nitisol of clayey texture, flat topography and good external and internal drainage. It is slightly acid and has a medium organic matter, low available potassium and very low available phosphorus content. The irrigation water used had an electrical conductivity of between 0.4 and 0.6 dS m^{-1}. Fertilization was that used by local growers applying every year 300 kg/ha N, 120 kg/ha P_2O_5, 300 kg/ha K_2O and 60 t/ha of manure. No weeds were allowed to develop within the orchard. During the growing season, control plants were irrigated above crop water requirements in order to ensure non-limiting soil water conditions. Irrigation was performed daily during the night using a sprinkler-irrigation system. Water stressed plants treatments were irrigated as control plants except for 15 days (DOY 70-85) days when irrigation was withheld.

Biomass and leaf characteristics

In both experimental seasons, mulberry biomass was harvested four times per year (every 90 days approximately) pruning the plants with shears 0.60 m above ground. Leaf biomass and total biomass (leaf plus stem) in dry weight basis were calculated according to AOAC (AOAC 1990) procedures. Leaf area was measured using an Image Analysis System (ΔT Devices Ltd., Cambridge, UK) and specific leaf weight was calculated as the dry weight per leaf surface unit.

The chlorophyll (Chl) content was estimated in the field, in 30 leaves per tree, using a SPAD-502 Chl meter (Minolta Camera Co., Ltd, Osaka, Japan). The SPAD meter determines the relative amount of Chl in the leaves by measuring the transmission of light at red (650 nm) and infrared (940 nm) wavelengths, while the difference in transmission at these two wavelengths is an indicator of Chl content per unit leaf area (Markwell *et al*. 1995). Leaves selected for Chl readings were located around the periphery of the tree canopy. Chl measurements were always made both sides of the central vein of the leaves.

To determine contents of each biochemical compound 0.25 g of fresh leaves were used and all measurements were done using a spectrophotometer (Genesys 10 UV, Thermo, Madison, USA). The free amino acids (Aa) were evaluated according to Yemm and Cocking (1955). The reaction mixture contained 1 mL of extract, 0.5 mL sodium citrate buffer 0.2 M pH 6, 0.2 mL of methyl cellosolve-ninhydrin solution 5% (w/v) and 1 mL of potassium cyanide (0.01 M)-methyl cellosolve solution 2% (v/v), which was incubated at 90°C for 15 min and stopped at 4°C for 5 min. Samples were read at 570 nm.

Leaf protein content (Prot) was assessed by the Micro-Lowry method using bovine serum albumin as standard (Lowry *et al*. 1951). Leaf material was ground with liquid nitrogen and homogenized in 100 mM sodium phosphate buffer (pH 7.8) with 0.1 mM EDTA, 0.1% Tritón X-100 and 1.5% polyvinylpirrolidone (w/v). Then, it was centrifuged at 10 000 g 20 min at 4°C.

The total soluble carbohydrates (CH) were extracted grounding leaf samples with liquid nitrogen and 3 mL of ethanol (80% in water, v/v) and incubating the mixture at 85°C for 30 min and then centrifuged 15 min at 8000 rpm. These steps were repeated 3 times and the supernatants were collected in the same tube. The quantification of CH was achieved by the procedure of anthrone method (Withman *et al*.1971), using glucose as the standard.

The total phenols (Ph) were assessed by the protocol described by Slinkard and Singleton (27), with some modifications. The extracts were obtained similar to CH (ethanol 80%), but incubated at room temperature and centrifuged at 3000 rpm. The reaction mixture contained 500 μL of diluted samples, 2.5 mL Folin-Ciocalteu reagent and 2 mL $Na_2CO_3$7%, which was shaking up and incubated at 50°C 5 min, and then it was keep at room temperature for 5 min. The absorbance was measured at 765 nm using gallic acid as standard.

Plant water status

Leaf conductance (g_{leaf}) and leaf (Ψ_{leaf}) and stem (Ψ_{stem}) water potential were measured on fully developed leaves from the south-facing side and the middle third of the trees of four trees per treatment. The g_{leaf} was measured with a porometer (Delta T AP4, Delta-T Devices, Cambridge, UK) on the abaxial surface of two leaves per tree. Ψ_{leaf} and Ψ_{stem} were measured in two leaves similar to those used for g_{leaf} using a pressure chamber (PMS 600-EXP, PMS Instruments Company, 209 Albany, USA). Leaves for Ψ_{stem} were enclosed in small black plastic bags covered with aluminium foil for at least 2 h before the measurements were made.

Leaf osmotic potentials were determined in the same leaves used for Ψ_{leaf}. Leaves were frozen in liquid nitrogen and the osmotic potential was measured using a vapour pressure osmometer (Wescor 5600, Logan, USA) after thawing the samples and expressing the sap. Leaf turgor potentials (Ψ_p) were derived as the difference between osmotic and water potentials. To estimate leaf osmotic potential at full turgor (Ψ_{os}), excised leaves were sealed in plastic bags immediately after excision and subjected to a rehydration treatment by dipping their petioles in distilled water for 24 h at 4°C. Rehydrated leaves were frozen in liquid nitrogen before measuring the osmotic potential after thawing the samples and expressing sap, in an osmometer as indicated above (Cruz 2012).

The diurnal patterns of g_{leaf}, Ψ_{leaf} and Ψ_p in response to both irrigation treatments were performed on DOY 85 (2015). Measurements were performed from sunrise to sunset on two leaves per tree and four trees per treatment, using the above described procedures.

Statistical design and analysis

The experimental design was completely randomized with four replications, each replication consisting of three adjacent tree rows, each with fifteen trees. Measurements were taken on the inner tree of the central row of each replicate, which were very similar in appearance (leaf area, trunk cross sectional area, height, ground shaded area, etc.), while the other trees served as border trees. Data were analysed using SPSS (2002) software. Analysis of variance was performed and mean values were compared by $LSD_{0.05}$ test. Values for each replicate were averaged before the mean and the standard error of each treatment were calculated.

RESULTS AND DISCUSSION

Very similar leaf and total biomass production values were observed in the three mulberry cultivars in 2014 and 2015, with the characteristic that these values were also nearly constant between seasons, except for GuiSang You 62 cultivar, which showed a slight decrease in total biomass yield in 2015 with respect to 2014 (Table 1). This cultivar was also characterized both seasons by lower leaf surface per plant and higher specific leaf weight than the other mulberry cultivars (Table 2).

Table 1 Average leaf and total biomass from the four harvests a year for three different mulberry cultivars (2014-2015). Means within a column for each factor and season followed by different small letter, and within a row for each factor and cultivar followed by different capital letter are significantly different by $LSD_{0.05}$ test.

Cultivar	2014	2015
Leaf biomass (t/ha/yr)		
GuiSang You 62	10.39aA	9,23bA
Guandong 11	10.57aA	12,13aA
GuiSang You 12	12.36aA	11,33abA
Total biomass (t/ha/yr)		
GuiSang You 62	19.20aA	16,96bB
Guandong 11	18.04aA	20,08aA
GuiSang You 12	20.75aA	18,93abA

Table 2 Average leaf surface and specific leaf weight from the four harvests a year for three different mulberry cultivars (2014-2015). Means within a column for each factor and season followed by different small letter, and within a row for each factor and cultivar followed by different capital letter are significantly different by $LSD_{0.05}$ test.

Cultivar	2014	2015
Leaf surface (m^2/plant)		
GuiSang You 62	0.95bA	0.90bA
Guandong 11	1.59aA	1.76aA
GuiSang You 12	1.88aA	1.60aA
Specific leaf weight (g/cm^2)		
GuiSang You 62	5.97aA	5.69aA
Guandong 11	3.58bA	3.71bA
GuiSang You 12	3.64bA	3.85bA

The harvested leaves from GuiSang You 62 had a higher free Aa content than the leaves from the other cultivars (Table 3). However, this cultivar exhibited lower total soluble CH, total Ph, Chl and Prot content than the other cultivars (Table 3). In both seasons, the content of all the studied biochemical compounds was very similar in Guandong 11 and GuiSang You 12 leaves, except in 2014 when the Prot content was higher in Guandong 11 (Table 3).

Withholding irrigation water from the three mulberry cultivars induced a significant decrease in Ψ_{stem}, Ψ_{os} and leaf Chl levels (Table 4). The circadian rhythm of the g_{leaf} values in control mulberry plants was characterized by a gradual increase in stomata opening from sunrise to midday (12:00 h solar time) and a progressive decrease thereafter, although g_{leaf} values stabilised at around 14:00 – 16:00 h in GuiSang You 12 (Fig 1). Theg_{leaf} values were similar in Guandong 11 and GuiSang You 62 plants but significantly higher in GuiSang You 12 plants (Fig 1). The g_{leaf} values in water stressed plants decreased with respect to control plants in the three mulberry cultivars, even though the values reached can still be considered as substantial. Differences between water stressed treatments were higher between 10:00 and 16:00 h, when a g_{leaf} values almost stabilised in stressed plants took place (Fig 1).

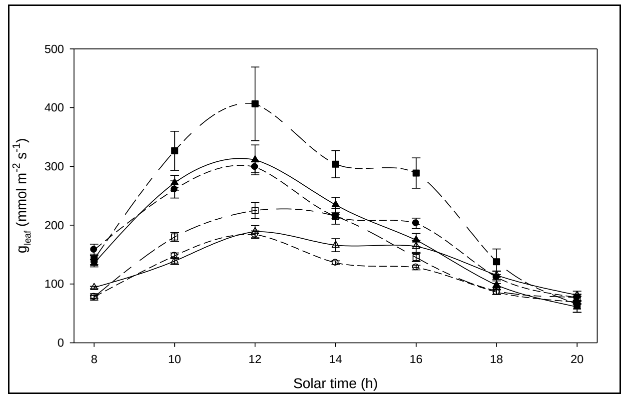

Fig 1 Diurnal course of leaf conductance (g_{leaf}) values (mean ± S.E., not shown when smaller than symbols) for GuiSang You 62 (triangles), Guandong 11 (circles) and GuiSang You 12 (squares) mulberry cultivars in control (closed symbols) and water withholding (open symbols) treatments

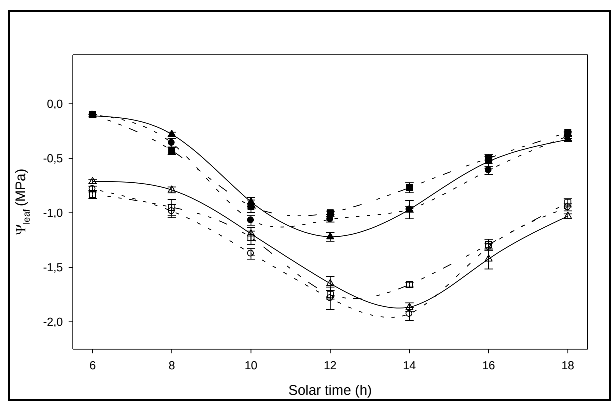

Fig 2 Diurnal course of leaf water potential (Ψ_{leaf}) values for GuiSang You 62, Guandong 11 and GuiSang You 12 mulberry cultivars in control and water withholding treatments. Symbols as in Fig 1.

Daily Ψ_{leaf} values in control and water stressed plants showed a similar qualitative diurnal pattern, reaching maximum values at predawn, decreasing early in the morning, regardless of the treatment, and attaining minimum values at around 10.00–12.00 h in control plants and at around 12:00-14:00 h in water stressed plants and then recovering in the afternoon(Fig 2).Water stress induced a significant decrease in Ψ_{leaf} values in treated plants throughout the day, with the characteristic that no differences between cultivars were observed (Fig 2).

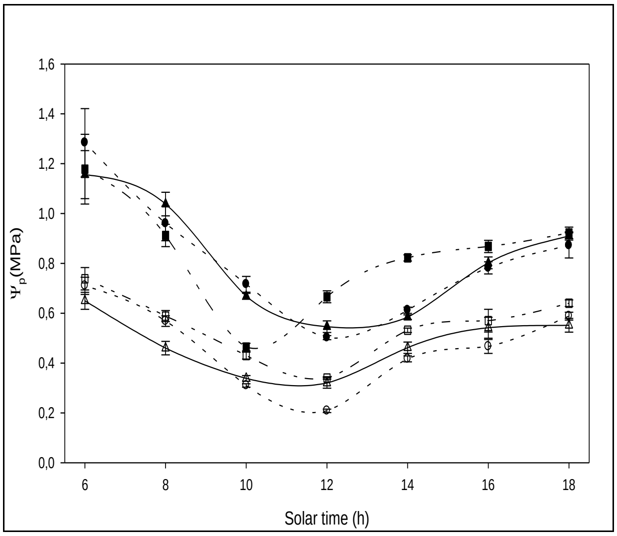

Fig 3 Diurnal course of leaf turgor potential (Ψ_p) values for GuiSang You 62, Guandong 11 and GuiSang You 12 mulberry cultivars in control and water withholding treatments. Symbols as in Fig 1.

Ψ_p values showed maximum values at predawn, before gradually decreasing to reaching minimum values at midday, and then recovering in the afternoon (Fig 3). Regardless of the water stress induced in all three mulberry cultivars, leaf turgor was maintained above zero in the three mulberry cultivars (Fig 3).

Mulberry biomass production is the consequence of the interaction of a multiplicity of factors, mainly those associated with environmental conditions as such climate, soil characteristics, soil water availability (Cordoví *et al*. 2013, Madan and Sharma 1999) and culture practices including planting distance, pruning height and cutting frequency (Boschini *et al*. 1998, Noda *et al*. 2007ab). However, leaf and total biomass harvested per year in the GuiSang You 62, Guandong 11 and GuiSang You 12 cultivars under the experimental conditions (Table 1) could be considered adequate because of the high values obtained (Cordoví *et al*. 2013, Noda *et al*. 2007ab). Moreover, these results showed that these Chinese cultivars constitute an excellent option for mulberry culture under Cuban tropic conditions.

Considering each of the cultivars studied, it is important to emphasize that Guandong 11 and GuiSang You 12 cultivars were more constant as regard leaf and total biomass production than GuiSang You 62 (Table 1), which presented lower leaf surface area but higher specific leaf weight than the other cultivars (Table 2), even though this behaviour compensated leaf and total biomass yield only in 2014 (Table 1).

Table 3 Average leaf content in total chlorophyll (Chl), free aminoacids (Aa), proteins (Prot), total soluble carbohydrates (CH) and total phenols (Ph) from the four harvests a year for the three different mulberry cultivars (2014-2015). Means within a row for each factor and season that do not have a common letter are significantly different by $LSD_{0.05}$ test

	2014			2015		
	GuiSang You 62	Guandong 11	GuiSang You 12	GuiSang You 62	Guandong 11	GuiSang You 12
Aa (mg / g FW)	29.24a	21.07b	21.04b	32.32a	21.19b	21.26b
Prot (mg / g FW)	3,69b	4,97a	4,11b	3,87b	4,31a	4.06ab
CH (g / g FW)	165.45b	276.16a	300.93a	149.78b	267.89a	306.66a
Ph (µg gallic acid / g FW)	157.90b	280.72a	257.54a	154.34b	256.85a	262.46a
Chl (SPAD-502 units)	34.13b	38.08a	36.82a	38.27b	39.28ab	41.43a

Probably, the higher Chl content in Guandong 11 and GuiSang You 12 than in GuiSang You 62 leaves (Table 3) could be related with a more efficient assimilation of the carbon allocated to the synthesis of primary (total soluble carbohydrates) and secondary (total phenols) metabolites (Buchanan *et al*. 2000). In addition, the fact that Guandong 11 and GuiSang You 12 leaves presented the highest levels of total soluble CH, Prot and total Ph indicated that the leaves of these cultivars can be considered more suitable to rear silkworms and as livestock forage than the other cultivar (Pescio *et al*. 2009, Rodríguez *et al*. 2013).

The inhibitory effect of drought on leaf Chl content (Table 4) has been observed previously in mulberry by other authors (Barathi *et al*. 2001, Guha *et al*. 2010 ab) and may be due to enhanced chlorophyllase activity (Dražkiewicz 1994) or decreased synthesis. In woody crops, osmo regulation or active osmotic adjustment is a plant tolerance mechanism which take place at cellular level and is developed mainly when water stress is imposed gradually, the capacity for osmotic adjustment being a function of species as well as cultivar (Lakso 1990, Torrecillas *et al*. 1996). In this sense, it has been demonstrated that mulberry genotypes exposed to drought have a lower leaf content of starch and sucrose but higher total sugar content than control plants. Moreover, the leaf soluble Prot content decreased under water stress, increasing the free amino acid content, especially the proline content (Barathi *et al*. 2001, Ramanjulu and Sudhakar 1997, Ramanjulu *et al*. 1998ab). Nevertheless, this increase in leaf osmolyte levels does not necessarily imply the development of osmo regulation because osmolytes increase takes place through an active accumulation of osmolytes but not as a result of concentration following dehydration. So, our results demonstrate that the three mulberry cultivars are able to develop osmo regulation through the effect of water stress to maintain leaf turgor (Ψ_p above zero) (Table 4, Fig 3).

Table 4 Total leaf chlorophyll (Chl), leaf osmotic potential at full turgor (Ψ_{os}) and stem water potential (Ψ_{stem}) at the end of water withholding period for the three different mulberry cultivars (2015). Means within a column for each factor and irrigation treatment followed by different small letter, and within a row for each factor and cultivar followed by different capital letter are significantly different by $LSD_{0.05}$ test

Cultivar	Control plants	Water-stressed plants
	Ψ_{stem} (MPa)	
GuiSang You 62	-0.69bA	-1.15aB
Guandong 11	-0.64abA	-1.31bB
GuiSang You 12	-0.57aA	-1.25abB
	Ψ_{os} (MPa)	
GuiSang You 62	-1.40aA	-1.97aB
Guandong 11	-1.46aA	-1.91aB
GuiSang You 12	-1.38aA	-1.87aB
	Chl (SPAD-502 units)	
GuiSang You 62	41.05bA	35.92bB
Guandong 11	44.08aA	38.60abB
GuiSang You 12	45.57aA	39.75aB

The trend in the circadian Ψ_{leaf} values recorded in water stressed plants was characterized by significantly more negative values in midday Ψ_{leaf} values compared with the morning values (Fig 2). This behaviour could be due to the absence of strong stomatal control of water loss via transpiration (Fig 1).

Midday leaf water stress achieved at the end of the water-withholding period for the three different mulberry cultivars can be regarded as not having been too severe, because Ψ_{stem} and Ψ_{leaf} values in the control plants were around - 0.65 and - 1.0 MPa, respectively, whereas in water stressed plants these values were around - 1.25 and above - 2.0 MPa, respectively (Table 4, Fig 2). Nevertheless, the leaf water status and g_{leaf} data recorded in drought-stressed mulberry plants agree with an anisohydric control of water use where by plants exert only moderate stomatal control and Ψ_{leaf} decreases strongly with the evaporative demand (Chaves *et al*. 2010, Schultz 2003). In this sense, other authors showed a similar anisohydric control of water use in *Morus indica* (Guha and Reddy 2014).

The main conclusions are that the GuiSang You 62, Guandong 11 and GuiSang You 12 Chinese mulberry cultivars constitute an excellent option for mulberry culture under Cuban tropical conditions. Nevertheless, Guandong 11 and GuiSang You 12 cultivars are more constant as regard leaf and total biomass production and their leaf composition seems to be more adequate for use to rear silkworms and as livestock forage. Moreover, our data show some of the leaf-level mechanisms adopted by these mulberry cultivars to tolerate water stress. In this sense, mulberry cultivars exhibited an anisohydric physiology, exerting low stomatal control which permits a substantial decrease in Ψ_{leaf}. These plants were also able to develop osmoregulation or active osmotic adjustment to confront water stress, maintaining leaf turgor.

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