

Stress-Induced Variation in Leaf Gas Exchange and Chlorophyll a Fluorescence in *Callistemon* Plants

¹Sergio Mugnai, ²Antonio Ferrante, ³Luca Petrognani, ⁴Giovanni Serra and ³Paolo Vernieri

¹Department of Ortoflorofrutticoltura, University of Florence,
viale delle Idee 30, 50019 Sesto F.no (FI), Italy

²Department of Produzione Vegetale, Università degli Studi di Milano,
via Celoria 2, 20100 Milano, Italy

³Department of Biologia delle Piante Agrarie, University of Pisa,
viale delle Piagge 32, 56100 Pisa, Italy

⁴Scuola Superiore Sant'Anna, Piazza Martiri della Libertà 1, 56100 Pisa, Italy

Abstract: The aim of this research was to evaluate the effect of some abiotic stresses commonly present in the Mediterranean environment (drought, salinity and negative physical soil properties) on a native Australian species (*Callistemon citrinus* (Curtis) Stapf), as the introduction of species in a new environment is successful, whenever they are able to overcome peculiar stress conditions. Plants were subjected to salinity stress using tap water added with 200 mM NaCl (23.1 mS cm⁻¹), water stress induced by a reduced irrigation of 450 mL/pot/day and root restriction (1.5 L of pot volume instead of 3 L). Results showed that plant growth and total water potential were significantly reduced with all the stress treatments. Net photosynthesis and the other leaf gas exchange parameters were also reduced by stress conditions. Chlorophyll a fluorescence parameters were lower in salt and root-restricted stress conditions, compared to controls. Results suggest that *C. citrinus* can be used as an ornamental plant in a Mediterranean area, as this species appeared to be particularly resistant to both water stress and root restriction conditions. For this reason, *C. citrinus* could be chosen in compact soil and limited water availability such as urban environments and gardens with low maintenance.

Key words: Australian woody plants, growth, photosynthesis, root restriction, salinity, water stress

INTRODUCTION

Over the past two centuries many woody plants species started to be spread from their native regions as a source of a wide array of basic materials and food but an ever-increasing proportion of them changed their role becoming merely ornamentals (Binggeli, 2001). The transport of a species from a biogeographical region to another has been almost always performed without a preliminary analysis on the impact of the new environment (basically climate and soil characteristics) on the growth and the physiology of the imported species. In fact, they may not perform well in a new location in terms of both growth and production, due to the onset of different environmental stresses compared to those occurring in their native regions. For this reason, their introduction should always be carefully evaluated because of strong morphological, physiological and ecological implications. A good example is Australian woody plants, which adapted and are utilized in Mediterranean regions due to

their tolerance to typical stresses such as drought (especially, during summer), water salinity and mechanical impedance of the soil. Among Australian ornamental plants, one of the most important genus is *Callistemon*, which includes several species showing interesting ornamental features (Mitchem, 1993). In Europe, the most widely used *Callistemon* sp. are *C. citrinus* Skeels and *C. laevis* Anon, the latter characterized by a good tolerance to environmental stresses, such as drought and salinity (Vernieri *et al.*, 2006; Lippi *et al.*, 2003). As the adaptation of *Callistemon* species to a different environment is of importance for their cultivation and use, their physiological responses to stresses, which can normally occur in Mediterranean climates, such as salinity and drought, need to be analyzed in order to predict their responsiveness in terms of product quality and landscape use. Many studies confirm the ability of the Mediterranean woody plants to adjust their physiology to stressful environmental conditions (Mugnai *et al.*, 2005; Bombelli and Gratini, 2003; Gulias *et al.*, 2002;

Larcher, 2000; Filella *et al.*, 1998), but no studies have been previously conducted on *Callistemon* species to investigate the adjustment of their physiology as a response to stress.

For this reason, the present research aims to investigate the effects of the most important abiotic stresses, which may easily occur during nursery and landscape growth management of *Callistemon* species in the Mediterranean environment (root restriction, salinity and drought) on gas exchange parameters and chlorophyll a fluorescence. In particular, a new procedure for the quantification of the fluorescence transient rise, called JIP-test, was applied (van Heerden *et al.*, 2003). In principle, the polyphasic chlorophyll a fluorescence rise (OJIP) describes photosynthetic rates and therefore, productivity and permits a simultaneous examination of several parameters of photosystem II. Therefore, the JIP-test is being used extensively in stress physiology in a range of plant species (van Heerden *et al.*, 2003).

MATERIALS AND METHODS

Plant material and growing conditions: Experiments were carried out from March to October 2007 at the Department of Crop Biology of the University of Pisa, Pisa, Italy. Rooted cuttings of *Callistemon citrinus* (Curtis) Stapf, grown in 7×7×7 cm pots by a specialized nursery, were transplanted into 3 L polyethylene pots (with a diameter of 18 cm, except for root restriction treatment -1.5 L pots with a diameter of 15 cm), filled with a peat-pumice-sand (60:30:10, v:v:v) substrate and fertilized with 3 kg m⁻³ of a slow release fertilizer (18N-12P-30K; 8 months). Plants were placed inside a glass greenhouse equipped with a separate drip irrigation system to allow different water salinity and regimes. Treatments were chosen on the basis of preliminary results obtained on this species (Lippi *et al.*, 2003). Plants were stressed during the entire experimental period by using the same treatments used by Vernieri *et al.* (2006):

Control (C): About 3 L pots (18 cm of diameter), the substrate moisture was maintained close to container water capacity by daily irrigation (900 mL pot⁻¹; 6 mM NaCl; 1.7 mS cm⁻¹).

Salt Stress (SS): About 3 L pots (18 cm of diameter) using tap water added with 200 mM NaCl (23.1 mS cm⁻¹) at the same quantity as control treatment (900 mL pot⁻¹).

Water Stress (WS): About 3 L pots (18 cm of diameter) irrigated daily with half of the amount of water (450 mL pot⁻¹; 6 mM NaCl; 1.7 mS cm⁻¹) compared to control.

Root Restriction (RR): About 1.5 L pots (15 cm of diameter) by supplying raw water (6 mM NaCl; 1.7 mS cm⁻¹) at the same quantity as control treatment (900 mL pot⁻¹).

Measurements: Plant height, canopy diameter and number of lateral shoots on five plants were measured at the end of the experiment. Five plants in each treatment were collected at the beginning and at the end of the experiment to perform a destructive growth analysis. Data recorded at each harvest included leaf and stem dry weight and leaf area. Dry weight was obtained after drying the samples in an oven at 70°C for 48 h. Leaf area was measured by a leaf area meter (ΔT Area Meter MK2, Delta T-Devices, UK). Growth analysis indices (mean Relative Growth Rate (RGR), Net Assimilation Rate (NAR), Leaf Area Ratio (LAR), Leaf Weight Ratio (LWR) and Specific Leaf Area (SLA)) were calculated according to Hunt (1990). Specifically, mean RGR (g/g/day) was calculated as the rate of increase of total dry weight per unit of plant dry weight, NAR (g/m²/day) as the rate of increase of total dry weight per unit of total leaf area, LAR (m² g⁻¹) as the ratio between the total leaf area and the total plant dry weight, LWR (g g⁻¹) the ratio between the total leaf dry weight and the total plant dry weight and SLA (m² g⁻¹) as the mean area of leaf displayed per unit of leaf weight.

Chlorophyll a fluorescence was determined around noon after illumination with a light intensity of 3000 μmol/m²/s (Ferrante and Maggiore, 2007) on randomly selected dark-adapted leaves in mid-June and early September using leafclips and a portable Handy Plant Efficiency Analyzer (PEA, Hansatech, UK). Fluorescence parameters were automatically calculated: Fv/Fm or (Fm-Fo)/Fm. The JIP-test was performed for the determination of the following indexes (Force *et al.*, 2003) Performance Index (PI), Dissipation of energy/CS (DIO/CS) and density of reaction Centers at P stage (RC/CSm). Net CO₂ Assimilation (A), transpiration (E) and stomatal conductance (g) were determined by a CIRAS-1 portable system (differential CO₂/H₂O infrared gas analyzer; PP systems, USA) around noon at ambient temperature and illumination on at least five apical and fully-expanded healthy young leaves for each plant during a clear sunny day in mid-June and early September. Measurements were replicated on five plants for each treatment. Leaf water potential (Ψ_w, MPa) was measured at noon by a pressure chamber, according to Pardossi *et al.* (1991).

Statistical analysis: The experiment consisted in a randomized block design, a single block referred to a single treatment. Growth data were subjected to one-way ANOVA, whereas leaf water potential, leaf gas exchange

and chlorophyll a fluorescence data were subjected to two-ways ANOVA. Means were separated by Tukey's test (one-way ANOVA, $p < 0.05$) or Bonferroni's test (two-ways ANOVA, $p < 0.05$). Each treatment was composed at least by 5 replicate samples ($n \geq 5$). All statistical analysis was carried out using GraphPad® 4.0 software.

RESULTS

Leaf water potential: Stressed plants showed significantly lower values of Ψ_w compared to control after three months, except for plants subjected to a reduced pot volume (Table 1). At the end of the experiment (early September), all stressed plants showed a marked decrease in Ψ_w values compared to the initial measurements (June) with control plants maintaining comparable higher Ψ_w values during all the experimental period.

Growth analysis: Abiotic stresses greatly affected size and growth of *C. citrinus* plants, with a significant decrease in stem height (Fig. 1a), total dry weight (Fig. 1b) and leaf area (Fig. 1c) at the end of the experiment in all the three treatments, but canopy diameter was comparable (Fig. 1d) to control. SS, followed by RR, reduced plant size the most in terms of both height and biomass production (total dry weight and leaf area). No differences among the

stress treatments were found in RGR (Table 2); however WS, SS and RR significantly decreased NAR and increased LAR, LWR and SLA (Table 2).

Table 1: Leaf water potential (MPa) measured on *Callistemon* plants subjected to abiotic stresses in two different moments of the experimental season

Treatments	Total water potential	
	June	September
Control	-0.77Ab	-0.88Ab
Water stress	-1.68Aa	-1.98Ba
Salt stress	-1.34Aa	-1.67Ba
Root restriction	-1.03Aab	-1.24Bb

Data are reported as mean values ($n = 5$) and were subjected to a two-way ANOVA, with means separated by Bonferroni's test. Different capital letters represent a statistical difference among different months for the same treatments ($p < 0.05$). Lower case letters represent a statistical difference among treatments within the same month ($p < 0.05$).

Table 2: Growth indices (Relative Growth Rate (RGR, g/g/d), Net Assimilation Rate (NAR, g/m²/d), Leaf Area Ratio (LAR, m² g⁻¹), Leaf Weight Ratio (LWR, g g⁻¹), specific leaf area (SLA, g m⁻²)) measured at the end of the experiment on *Callistemon* plants subjected to abiotic stresses

Treatments	RGR (g/g/d)	NAR (g/m ² /d)	LAR (m ² g ⁻¹)	LWR (g g ⁻¹)	SLA (m ⁻² g)
Control	0.012a	0.046a	258.92b	0.38b	607.32b
Water stress	0.011a	0.032b	352.51a	0.47a	635.68a
Salt stress	0.011a	0.034b	317.63a	0.51a	663.46a
Root restriction	0.010a	0.029b	336.43a	0.47a	639.33a

Values are reported as means ($n = 5$). Data were subjected to one-way ANOVA. Different letters represent statistical difference for $p < 0.05$, when means were separated by Tukey's test

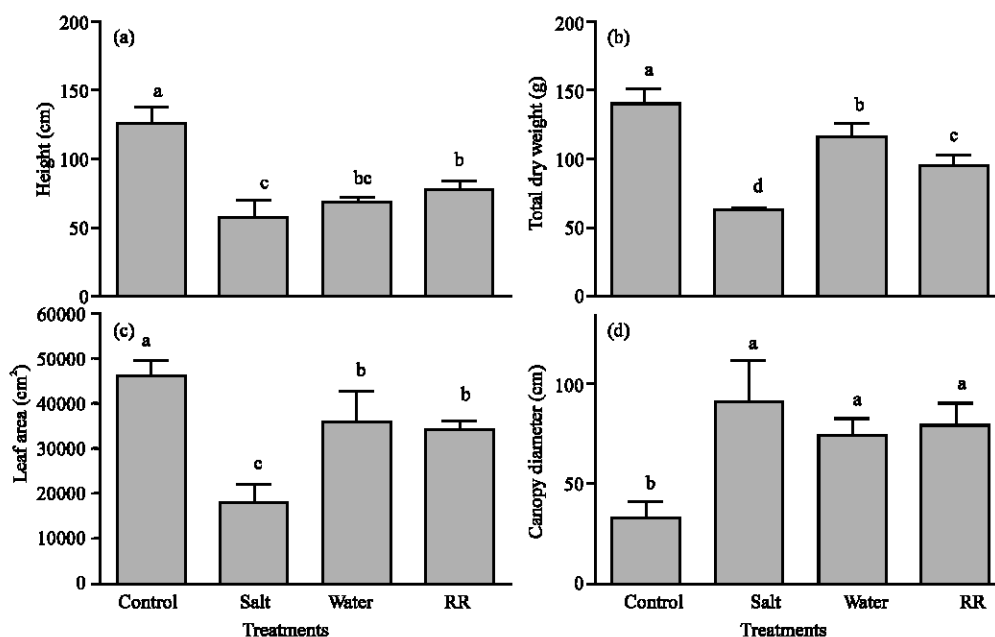


Fig. 1: Growth parameters, a): Height; b): Total dry weight; c): Leaf area; d): Canopy diameter, measured at the end of the experiment on *Callistemon* plants subjected to abiotic stresses. Values are reported as means with bars indicating standard errors ($n = 5$). Data were subjected to one-way ANOVA. Different letters represent statistical difference for $p < 0.05$ when means were separated by Tukey's test

Table 3: Chlorophyll a fluorescence parameters measured in *Callistemon* subjected to abiotic stresses

Parameters	Fv/Fm		Fm		Fo		Dio/CS		RC/CSm		PI	
	June	Sept.	June	Sept.	June	Sept.	June	Sept.	June	Sept.	June	Sept.
Control	0.80ns	0.78a	2928ns	2479a	584ns	629ns	117ns	120b	1327ns	965a	3.47a	1.85a
Water stress	0.81ns	0.74ab	2726ns	2514a	568ns	641ns	102ns	166ab	1243ns	933a	3.32a	1.45a
Salt stress	0.79ns	0.71b	2721ns	2252b	583ns	543ns	120ns	183a	1147ns	786b	2.21b	0.90b
Root restriction	0.78ns	0.71b	2644ns	2223b	523ns	644ns	130ns	182a	1193ns	758b	3.06a	1.00b

Values are reported as means (n = 5). Data were subjected to two-ways ANOVA. Different letters represent statistical difference inside the seasonal period for each parameter, when means were separated by Tukey's test (p<0.05). Dio/CS = Dissipation of energy per cross section, RC/CSm = Density of active reaction centers at P stage, PI = Performance Index

Table 4: Gas exchange parameters (net assimilation rate, A; stomatal conductance, g; transpiration, E) measured on *Callistemon* plants subjected to abiotic stresses in two different periods of the experimental season

Parameters	A ($\mu\text{mol}/\text{m}^2/\text{s}$)		g ($\text{mmol}/\text{m}^2/\text{s}$)		E ($\text{mmol}/\text{m}^2/\text{s}$)	
	June	Sept.	June	Sept.	June	Sept.
Control	8.79a	8.99a	221.00a	148.78a**	4.45a	2.71a***
Water stress	5.30b	6.70b	96.69b	109.14b	2.73b	2.14b
Salt stress	5.35b	5.71bc	85.62b	69.43c	2.52b	1.85b*
Root restriction	4.30b	5.23c	88.77b	79.86c	2.28b	2.02b

Values are reported as means (n = 5). Data were subjected to two-way ANOVA. Different letters represent statistical difference among treatments inside a month for p<0.05 when means were separated by Tukey's test. Asterisks represent statistical difference between months for the same treatments for p<0.05 (*), p<0.01 (**) and p<0.001 (***) when means were separated by Bonferroni's test

Chlorophyll a fluorescence: Chlorophyll a fluorescence parameters such as Fo, Fm and Fv/Fm were not affected by treatments in June. On the contrary, late summer (early September) measurements showed a significant decrease in Fm and Fv/Fm values in RR and SS plants compared to control (Table 3). In details, Fv/Fm ranged from 0.80-0.78 in control plants, while RR and SS plants showed the lowest value (0.71) (Table 3). Fm showed the same trend of Fv/Fm: also in this case, the lower values were found in RR and SS plants. On the contrary, Fo did not significantly change among treatments in both the months. Chlorophyll a fluorescence data were then subjected to the JIP-test. Among the derived indices, the most interesting results were shown by RC/CSm, Dio/CS and PI. RC/CSm showed the same trend of Fm and Fv/Fm ratio. In fact, leaves of RR and SS plants showed lower numbers of active reaction centers (21 and 19%, respectively) than control leaves (Table 3). The Dissipation energy (Dio/CS) maintained the same values in control plants in both the measurements, while the values reported by all treatments increased over the summer. Contrary to all the other indices, PI was able to show up stress conditions also in both months. In June, PI indicated significant differences in salt stressed plants, but progressively decreased by increasing environmental temperature (Table 3).

Gas exchange parameters: Abiotic stress treatments affected stomatal functionality. In fact, a significant reduction in gas exchange parameters was detected in both months compared to control (Table 4). Interestingly, no significant differences were noticed among treatments in the same month, meaning that abiotic stresses affected

plant photosynthetic physiology with a same order of magnitude inside the same season. The only exception was given by stomatal conductance measured in September, when g values were significant higher in WS plants than both SS and RR ones. Stress treatments already led to a strong decrease in gas exchange values in June compared to the controls: A values showed a 40% (WS and SS plants) and 52% (RR plants) reduction, g decreased of 56.3% (WS), 61.3% (SS) and 59.84% (RR), while E values decreased of 49.7% (WS), 43.4% (SS) and 49% (RR). Measurements performed in September reported similar trends with a slight difference. In fact, SS and RR plants continued to show comparable values, while a significant reduction was noticed in the transpiration values of WS plants despite similar A and g values.

DISCUSSION

Plant growth is plastically influenced by the surrounding environment. The effects of salinity, drought and decreased pot volume on plant growth and dry matter accumulation have been described in other ornamental species (Shao *et al.*, 2008; Vernieri *et al.*, 2006; Mugnai *et al.*, 2005) and are known to affect also woody plant physiology, especially photosynthetic parameters (Lawlor and Cornic, 2002). Our experiment reported that the application of stresses to plant substrate led to smaller plants and to a significant constraint in both growth parameters (lower NAR, i.e., reduced capability in carbon assimilation and allocation and higher LAR, i.e., a tendency to maximize leaf surface) and water status. This situation seems to be due, in order of significance: to a

significant decrease in plant water potential and stomatal conductance, affecting net CO₂ assimilation rate and leading to a lower plant biomass production and to a reduction in photochemical efficiency during summer period. Regarding the behavior of the other *Callistemon* species, Aihong *et al.* (2006) reported a bimodal photosynthetic curve with an obvious midday depression phenomenon in *Callistemon rigidus* R. Br. under optimal conditions. In a previous research (Vernieri *et al.*, 2006), the researchers performed net CO₂ assimilation rate and stomatal conductance measurements under stress on *C. laevis*, where different plant responses to stress compared to control were evident, as root restriction had no significant effects on net assimilation, whereas salinity and drought greatly reduced both parameters (net CO₂ assimilation rate and stomatal conductance). In the study, however, only salinity appeared to greatest impact growth with drought and root impediment showing similar levels of effectiveness. In these experiments, 200 mM NaCl treatment was applied since this concentration represents the threshold level for distinguishing the two groups of tolerant plants to salinity (Greenways and Munns, 1980) plants with rapid growth and which can survive at high salinity with NaCl concentration ranging between 200-500 mM or plants that reduce growth by increasing salt concentration up to a maximum of 200 mM NaCl.

Even though, several *Callistemon* species are characterized by a high degree of tolerance to salinity, others are salt-sensitive (Lippi *et al.*, 2003). The severe reduction of *C. citrinus* growth in a saline environment suggests that this species can be classified as tolerant with a reduction of growth. Moreover, this species also showed a significantly altered water status, stomatal behavior and photosynthetic efficiency. Growth inhibition is one of the most common effects of salinity (White and Broadley, 2001), with Na⁺ being the primary cause of ion-specific damage (such as a reduction in K⁺ channel activity). In cells, the accumulation of Na⁺ in the cytoplasm inhibits the enzyme activities with dramatic effect on the whole plant metabolism. Halophyte plants are able to limit Na⁺ concentration and regulate the substrate and enzymes concentration in cells for allowing the normal physiological processes (Flowers *et al.*, 1977).

The impact of salinity on photosynthetic parameters in woody plants has been already reported (Tattini and Traversi, 2008; Tabatabaei, 2006). Reduced net CO₂ assimilation rate with increasing salinity has been attributed: to a stomatal closure, which leads to a reduction in intracellular CO₂ partial pressure, to concurrent non-stomatal factors (i.e., the reduction in protein concentration), to a decline in photosynthetic pigments and to changes in ion concentrations (Demetriou *et al.*, 2007). Photosynthetic efficiency in Mediterranean plants progressively decreases also during

water shortage (Mugnai *et al.*, 2005; Flexas *et al.*, 2001; Abril and Hanano, 1998) and it is strongly influenced by atmospheric conditions and daytime (Martinez-Ferri *et al.*, 2000; Beyschlag *et al.*, 1992; Tenhunen *et al.*, 1990). During summer, the maximum rate of carbon fixation occurs during relatively low light intensity and temperature, at the beginning and at the end of the day (Noguès *et al.*, 2001; Beyschlag *et al.*, 1992). In the case, both salinity and drought treatments led to a lower capacity in leaf gas exchange in *C. citrinus* plants compared to control (a and g concurrently decreased during stress treatments), with no seasonal effects regarding photosynthetic behavior, as we detected an earlier onset in gas exchange constraint than photochemical efficiency decrease, which happened only during the summer. Photosystem II (PSII) activity plays a key role in the response of photosynthesis to environmental perturbations (Martinez-Ferri *et al.*, 2000). Photochemistry of PSII has been extensively studied using chlorophyll a fluorescence, a non-destructive method for assessing stress conditions in plants grown in different environmental conditions. Stressed plants usually show lower efficiency in leaf photosynthetic light harvesting and energy transfer (Strasser and Srivastava, 1995). Many studies use a sustained decrease in maximum efficiency of PSII in dark-adapted leaves (Fv/Fm) as reliable diagnostic indicators of photoinhibition in response to stresses, as well as Fo and Fm. It has been suggested that Fv/Fm below 0.83 can be usually referred to stressed plants (Maxwell and Johnson, 2000). However, other researchers suggested that evergreen trees are not stressed until Fv/Fm values decrease below 0.76 (Percival, 2004). Considering this last hypothesis, the results showed that plants grown under reduced pot volume and salt treatments became stressed later in the summer. Chlorophyll a fluorescence has been also used for studying a wide range of stresses (Force *et al.*, 2003). The most interesting studies for understanding the results were those that utilized temperature, drought and saline water as treatments or variables. For example, high temperatures strongly affected chlorophyll a fluorescence (Petkova *et al.*, 2007). Moreover, salinity decreases the PSII activity and efficiency (Lu and Vonshak, 2002) and inhibits the quantum yield of PSII electron transport (Xia *et al.*, 2004). Our results showed a decrease in Fm and Fv/Fm values under salt stress when environmental temperature increased, while drought slightly reduced the Fv/Fm values in September, showing a minor effect on photochemical efficiency. The Fv/Fm ratio was the best indicator of salt stress in sweet almond (Ranjbarfordoei *et al.*, 2006). Analogous experiments carried out in *Fraxinus* genotypes under drought showed a reduction of chlorophyll a parameters, with a drastic reduction in Fv/Fm ratio, Fo and net CO₂ assimilation rate

in different genotypes (Percival *et al.*, 2006). The absence of significant differences in the experiment suggests that *C. citrinus* appeared to be a drought-tolerant species. PI is an overall index that combines different fluorescence parameters. It was the only parameter that showed the effect of salinity stress in early summer (June). PI has been used for monitoring a wide range of stresses (Ferrante and Maggiore, 2007). For example, PI is sensitive to temperature, so measurements must be carefully taken under the same environmental conditions. The results also suggest that *C. citrinus* is particularly resistant to water stress. Löscher *et al.* (1981) found that midday stomatal closure, normally occurring in Mediterranean woody plants during the dry season, was progressively reduced during fall and completely disappeared during winter months. As confirmed in the experiment, the direct and cumulative effects of both treatments and an environmental stress induced by summer high temperatures depressed net CO₂ assimilation rate and transpiration. Generally, optimal water availability permits a less water conservation due to a higher stomatal conductance (Castell and Terradas, 1995). Some researchers (Flexas *et al.*, 2001; Abril and Hanano, 1998; Penuelas *et al.*, 1998) asserted that an increasing dryness (i.e., decreased total water potential) causes a reduction in net CO₂ assimilation rate and stomatal conductance in Mediterranean species, as confirmed in the conditions. There are contradictory reports on how plant water relations and photosynthetic rate play a significant role in the response of aboveground organs to the restricted root growth (Ferree and Schupp, 1992). In the study, the reduction in plant growth and stomatal opening caused by root restriction was not related to changes in plant water status, as observed in plants subjected to drought and salinity (Table 1). Root zone restriction and water stress have different mechanisms of action (Ismail and Noor, 1996) and the impairment of growth might involve different physiological processes. Tschaplinski and Blake (1985) reported a reduction in the stomatal opening of alder (*Alnus glutinosa* L.) seedlings grown under root restriction, suggesting a stomatal factor to explain the reduced photosynthetic rate. In the case, chlorophyll a fluorescence parameters significantly decreased under root restriction treatment, suggesting a lower photochemical efficiency of PSII and a downward regulation of photosynthesis as a result of root restriction, leading to the reduced growth. Downward regulation of A due to root restriction has also been observed in other woody species, attributed to carbohydrate build up in the leaves as a result of limiting the strength of root sink (feedback mechanism) (Schaffer *et al.*, 1997).

CONCLUSION

Results suggest that *C. citrinus* can be successfully used as ornamental plants in Mediterranean environment, because the species appeared to be particularly resistant to both water stress and root restriction conditions. In fact, plants reacted with a physiological adaptation with a slight reduction in total dry weight and leaf area, though significant compared to control, due to modification of leaf gas exchange and chlorophyll fluorescence.

We could conclude that *C. citrinus* can be chosen in compact soil and limited water availability such as urban environments and gardens with low maintenance cares.

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