



**Full Length Article**

## Divergent Gas-exchange, Physiological, Isotopic and Compositional Responses of Two Wood-crop Species to Water Deficit: *Ziziphus nummularia* and *Corymbia citriodora*

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### Abstract

We assessed the response to drought in *Corymbia citriodora*, a fast growing wood-crop with high levels of water-loss, and *Ziziphus nummularia*, a tree species that occurs in arid areas of Asia and a potential alternative wood-crop plantation species in drought-prone regions. *Z. nummularia* was able to sustain higher levels of stomatal conductance at lower levels of soil water availability than *C. citriodora*. The leaves of *Z. nummularia* also contained higher levels of the antioxidant peroxidase, offering enhanced protection from drought induced oxidative stress. The carbon isotopic and nitrogen concentration of *C. citriodora* foliage was strongly affected by decreasing soil water availability, but a compositional effect was only apparent in *Z. nummularia* leaves at the lowest level of soil water. The higher leaf levels of stomatal conductance and nitrogen are indicative of relatively high assimilation rates in *Z. nummularia*, suggesting that this species is capable of fully exploiting brief periods where conditions are limited for growth. These attributes, in combination with its inherent tolerance to drought, may make *Z. nummularia* a suitable wood crop species for rain-fed plantations in drought-prone areas. © 2015 Friends Science Publishers

**Keywords:** Carbon isotope composition; Drought; Lemon-scented eucalypts; Peroxidase; Stomatal conductance; Wood crop

### Introduction

Water availability is a major constraint to vegetation growth in many parts of the Earth, frequently limiting production of both food and biofuel crops (Chaves *et al.*, 2003; Centritto *et al.*, 2011b; Pinheiro and Chaves, 2011). Global population growth will necessitate improvements in agricultural practises, irrigation techniques and the development/identification of drought tolerant species to exploit previously unproductive lands (Grierson *et al.*, 2011). Furthermore, future climatic changes are likely to result in more frequent drought events of greater duration in many regions. Prolonged water shortages are not only expected to occur more frequently in the future, but the extent of land affected by drought is expected to rise by 50% (IPCC, 2007). This is particularly relevant for fuel and hard-wood tree plantations that are often grown on marginal lands without supplementary irrigation (Thomas, 2008; Searchinger, 2010; Sedjo *et al.*, 2013). The identification of plant species likely to be vulnerable to water stress, and/or the traits that confer drought tolerance, are vital to the mitigation of the impacts of climate change to ensure the future security of food and fuel crops (Ren *et al.*, 2007;

Centritto *et al.*, 2009; Chaves *et al.*, 2009). Analysis of the photosynthetic and water-use responses of plants to drought may permit the elucidation of mechanisms that underpin plant responses and adaptation to water-deficits (Centritto *et al.*, 2011b).

Water-deficit induces a number of responses in plants depending upon the species, duration and severity of drought, occurrence of co-existing biotic stresses and environmental factors such as temperature and light intensity. The primary negative effect of drought is diminished rates of photosynthesis caused by reductions in the diffusive uptake of CO<sub>2</sub> and metabolic limitations (Loreto and Centritto, 2008; Pinheiro and Chaves, 2011). As water availability becomes limited plant stress hydraulic (Centritto *et al.*, 2011b) and chemical signals, such as abscisic acid (ABA) (Wilkinson and Davies, 2002) and pH (Wilkinson *et al.*, 1998; Tahiri *et al.*, 2007), cause stomata to close and stomatal conductance ( $G_s$ ) to decrease (Haworth *et al.*, 2013). This reduced  $G_s$  is often accompanied by reduced mesophyll conductance to CO<sub>2</sub>, resulting in lower availability of CO<sub>2</sub> at the sites of carboxylation within the chloroplast envelope (Centritto *et al.*, 2003; Centritto *et al.*, 2011a; Flexas *et al.*, 2013). These reductions in  $G_s$  also

result in changes to the carbon isotopic composition of leaves, as the discrimination of the lighter  $^{12}\text{C}$  in favour of the heavier  $^{13}\text{C}$  isotope becomes less pronounced (Farquhar *et al.*, 1989). Metabolic limitations to  $\text{CO}_2$ -uptake such as diminished regeneration of RuBP caused by impaired ATP production (Lawlor and Tezara, 2009) tend to occur under conditions of severe water-deficit following an extended duration of drought (Centritto *et al.*, 2003; Centritto *et al.*, 2009; Centritto *et al.*, 2011a). To protect the photosynthetic physiology from the effects of reduced photochemical energy use, a corresponding increase in the non-photochemical dissipation of light energy as heat occurs (Harbinson *et al.*, 1990). During episodes of extreme or prolonged water-deficit these protective mechanisms may become impaired, resulting in inhibition of the photosynthetic physiology (Pinheiro and Chaves, 2011). As photosynthesis becomes increasingly diminished by the reduced uptake of  $\text{CO}_2$  during drought stress, this results in increased generation rates of harmful reactive oxygen species (ROS) within the leaf (Reddy *et al.*, 2004). These ROS are 'mopped-up' by anti-oxidants such as peroxidase (POX) as part of the cell's protective metabolism. The capacity to 'mop-up' these harmful ROS via anti-oxidant cycling may influence the resistance to drought of a particular plant species or variety (Fu and Huang, 2001; Türkan *et al.*, 2005).

The sustainable production of biofuel crops is of increasing importance in the developed world as a carbon neutral energy source and in the developing world as a source of fuel for domestic uses such as cooking and heating. The establishment of commercial stands of fast growing tree species as a hard and fuel-wood biomass crop is increasing in many countries, providing economic benefits and reducing the clearance of slower growing mature mixed species forests that provide important ecosystem services and enhance biodiversity (Searchinger, 2010). Many of these tree crops have rapid growth; however, this accumulation of biomass is often accompanied by high water consumption. Wood-fuel crops are often grown under rain-fed conditions the relative high water requirements frequently restrict their cultivation in marginal land types. This, in turn, may lead to either higher pressure and eventually loss of native forests to provide fuel wood, or the conversion of productive agricultural land from food to biofuel crop production, that would result in exacerbating food security concerns (Grierson *et al.*, 2011). Furthermore, the high water requirements of these fast growing biofuel species frequently makes them vulnerable to drought, thus constraining their potential cultivation in the provision of fuel-wood (Thomas, 2008; Orikiriza *et al.*, 2009; Agaba *et al.*, 2010).

The Australian Eucalypt, *Corymbia citriodora*, exhibits rapid growth and has been planted extensively as a hard and fuel-wood species in Australia, Central and South East Asia, Africa and South America. To be economically productive, plantations of *C. citriodora* require consistent

water availability to maintain growth and prevent loss of plants (Thomas, 2008; Agaba *et al.*, 2010). Drought results in a pronounced decline in rates of photosynthesis and  $G_s$  in *C. citriodora*. Additionally, under severe water-deficit the emission of the biogenic volatile organic compound isoprene declines, indicating that the efficacy of any protective role against thermal and oxidative damage is diminished (Brilli *et al.*, 2013). To fully exploit marginal land types for wood production it may be necessary to identify alternative tree species that may be more adapted to growth and survival under water-deficit conditions (Allen *et al.*, 2010). The genus *Ziziphus* grows in arid areas of Africa and Asia and is composed of shrubs and trees that possess structural and physiological adaptations to prevent water-loss (Clifford *et al.*, 1998; Arndt *et al.*, 2001). *Z. nummularia* has been suggested as possible fruit and wood-crop species for growth in water-limited areas (Pandey *et al.*, 2010). The ability of *Z. nummularia* to tolerate water-stress may permit its growth in commercial hard and fuel-wood plantations in marginal land-types more prone to drought than those required by *C. citriodora*. Furthermore, the afforestation of low-grade upland areas and marginal lands by a drought tolerant species may assist in the prevention of desertification and soil degradation (Zuazo and Pleguezuelo, 2009).

The gas-exchange and physiological responses of *C. citriodora* and *Z. nummularia* to water-deficit were investigated to assess the suitability of both trees as hard and fuel-wood species in areas likely to be affected by drought events. This study aimed to: (i) assess the  $G_s$  response of *C. citriodora* and *Z. nummularia* relative to the content of soil water available for transpiration, to characterise the ability of the respective species to sustain gas-exchange and hence  $\text{CO}_2$  uptake during water-deficit; (ii) investigate changes in leaf water potential and leaf area as possible adaptations to drought; (iii) analyse shifts in the carbon isotopic and nitrogen composition of leaves as potential indicators of drought stress, and; (iv) study alterations in peroxidase activity as an indicator of the efficiency of physiological protective mechanisms in mopping-up harmful ROS generated during drought stress.

## Materials and Methods

### Plant Material and Experimental Design

Two-year-old saplings of *C. citriodora* and *Z. nummularia* were grown in  $5 \text{ dm}^3$  pots containing a mixture of commercial soil, sand and manure (1:1:1) in the glasshouse at Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi. The seedlings of both species were collected from areas adjacent to the Chenab River in Muzaffargarh District, south-western Punjab, Pakistan. All the saplings were regularly watered and fertilized with Hoagland solution once a week to supply mineral nutrients at free access rates. On the afternoon prior to the beginning of the

experiment, all of the plants were fully irrigated and the excess water was allowed to drain overnight. After draining, the pots were weighed to 1-g precision on a digital balance (model ACS Electronic Scale, Meezan Ltd., Rawalpindi, Pakistan) to determine the weight at pot water capacity (Initial<sub>pot weight</sub>). Each pot was then enclosed in a plastic bag that was tied around the stem of the sapling to prevent evaporation from the soil. Twelve (*C. citriodora*) and (*Z. nummularia*) plants were then water-stressed by withholding water, while an equal number of control plants were watered to pot capacity each day. The development of water-deficit stress was characterised by recording the daily pot weight relative to  $G_s$  values at the start and end of the drought treatment 20 days later, and then expressed as the fraction of transpirable soil water (FTSW). The mean daily weight of twelve pots of each species and treatment was used to calculate FTSW (Sinclair and Ludlow, 1986; Brill *et al.*, 2013). The physiological lower limit of available soil water was defined as the FTSW at which stomatal conductance approached zero (i.e., soil water decreased to a level where there was no longer water available to support transpiration) (Sinclair and Ludlow, 1986; Centritto *et al.*, 2011a). Once this level was achieved, the water-stressed pots were weighed to determine the final pot weight (Final<sub>pot weight</sub>). Thereafter, every morning the plastic bags were unwrapped to weigh the water-stressed saplings (Daily<sub>pot weight</sub>) and to water the control plants. Then, the FTSW was calculated for individual pots as follows:

$$\text{FTSW} = (\text{Daily}_{\text{pot weight}} - \text{Final}_{\text{pot weight}}) / (\text{Initial}_{\text{pot weight}} - \text{Final}_{\text{pot weight}})$$

### Estimation of Physiological and Chemical Parameters

Stomatal conductance ( $G_s$ ) was measured on sunny days between 08:00 a.m. and 10:00 a.m. with a steady-state porometer (AP4-UM-3, Delta-T Devices Ltd., Cambridge, England) by enclosing a portion of a single fully expanded leaf in the porometer head whilst avoiding the midrib. The youngest fully expanded leaf was selected from the uppermost canopy of each plant, this leaf was then marked and its levels of  $G_s$  recorded throughout the experiment. Three measurements of  $G_s$  were recorded for each leaf per day; these were then averaged to produce a mean value for each plant, and the average of 12 plants taken for each point in Figure 1. Leaf area was measured by using planimetric techniques on leaves developed after the onset of the water-stress treatment. Paper replicas of the planar leaf surface were made and then measured using a leaf area meter (LI 3100, LI-COR Inc., Lincoln, NE, USA). The pre-dawn leaf water potential of *C. citriodora* was measured using a pressure bomb/pressure chamber. Unfortunately due to the relatively small leaf size of *Z. nummularia* it was not possible to measure leaf water potential without collecting a large number of leaves and causing significant injury to the plant that would likely have biased other physiological measurements. Non-destructive measurements such as  $G_s$

and recording of pot weight for the calculation of FTSW was undertaken each day on all twelve plants. Destructive samples of leaves were collected at FTSW levels of 100, 45, 15 and 5% on three replicates.

To determine total leaf nitrogen, three leaf samples of three replicates were oven dried at 65°C and then ground into powder. Nitrogen concentration of the dried leaf powder (0.2 g) was determined calorimetrically, measuring absorbance at 655 nm, as described by Anderson and Ingram (1993). The carbon isotopic composition ( $\delta^{13}\text{C}$ ) of 1 mg of ground freeze-dried ground leaf tissue was measured using an elemental analyser (NA 1500, Carlo Erba, Milan, Italy) as described by (Farquhar and Richards, 1984).

To assess peroxidase (POX) activity, newly developed fully expanded leaves (three plants per water treatment) were immediately frozen in liquid nitrogen, lyophilized (Cryodos-50, Telstar, Spain), and then stored at -80°C until analysed. The frozen leaves were weighed and then 1 mg ground with 1.5 ml of 0.1 M sodium phosphate (pH 6.5) buffer containing 5% (w/v) polyvinylpyrrolidone (PVP). The Peroxidase activity was assayed using the Guaiacol test (Chance and Maehley, 1955).

### Statistical Analyses

Data were analysed using a factorial ANOVA (two-way maximum interactions) to determine the main effects of water treatment and species on all dependent variables measured using Sigma Plot 10.0. A chi-square goodness of fit was used to test whether all variables were normally distributed. No interaction was observed between variables.

### Results

*C. citriodora* and *Z. nummularia* exhibited pronounced reductions in  $G_s$  as water stress increased. However, these  $G_s$  reductions occurred at different stages of the drought treatment, possibly reflecting differential responses to reductions in soil water availability between *C. citriodora* and *Z. nummularia* (Fig. 1a). *Z. nummularia* ( $0.5 \text{ mol m}^{-2} \text{ s}^{-1}$  at 100% FTSW) exhibited significantly higher rates of  $G_s$  than *C. citriodora* ( $0.2 \text{ mol m}^{-2} \text{ s}^{-1}$  at 100% FTSW) throughout the experiment as the soils dried (Table 1). Nonetheless, differences in the kinetics of drought were apparent between the two species as  $G_s$  of *Z. nummularia* began to reduce as the fraction of transpirable soil water (FTSW) declined below 80%, while *C. citriodora* maintained full rates of  $G_s$  until the FTSW reached 50%. The reduction in  $G_s$  at higher FTSW values in *Z. nummularia* was accompanied by a more gradual decline in  $G_s$  at lower FTSW values than was apparent in *C. citriodora*.

As drought progressed the leaf-water potential of drought-treated *C. citriodora* plants declined (Fig. 2) following a similar pattern to that observed in  $G_s$  (Fig. 1a). The leaf-water potential of control plants remained constant throughout the experiment, while the drought stressed plants

**Table 1:** ANOVA of stomatal conductance ( $g_s$ ) values of *C. citriodora* and *Z. nummularia* following drought"

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1771657	3	590552.5	86.316	5.24E-25	2.718785
Within Groups	547340	80	6841.75			
Total	2318997	83				

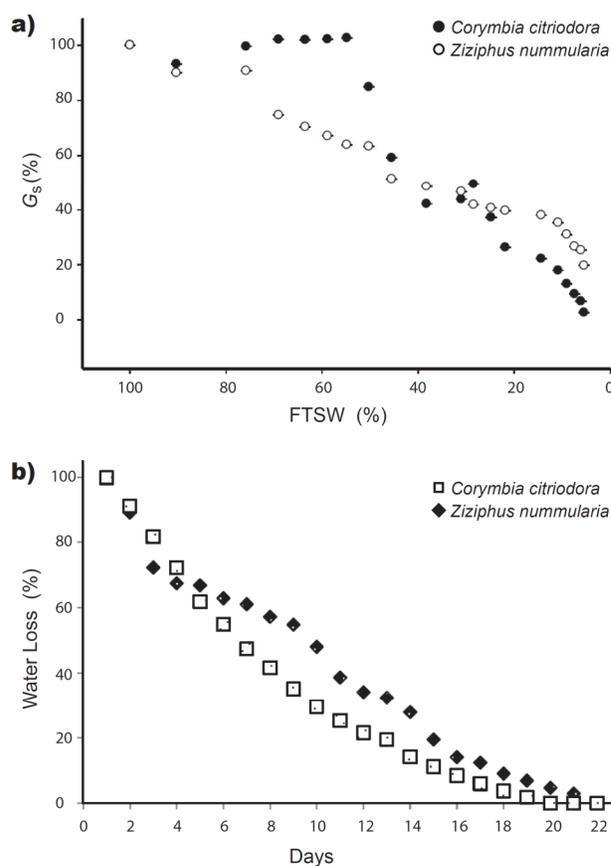
**Table 2:** ANOVA of leaf area values of *C. citriodora* and *Z. nummularia* following drought"

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1075.8849	3	358.6283	39.35758	4.5E-07	3.343889
Within Groups	127.56872	14	9.1120516			
Total	1203.4536	17				

exhibited a 20% decline at 45% FTSW (co-incident with the level of soil water availability at which  $G_s$  values fell), and pronounced reductions of 60% and 125% at FTSW levels of 15 and 5%, respectively (Fig. 2).

*C. citriodora* possessed significantly greater leaf area than *Z. nummularia* (Table 2), accounting for the observation that while leaf-level  $G_s$  was higher in *Z. nummularia*, whole plant transpiration was greater in *C. citriodora*. The leaf area of *Z. nummularia* under both control and water-deficit conditions was constant throughout the experimental period. In contrast, well-watered control *C. citriodora* plants exhibited a significant ~100% increase in leaf area over the 20-day experimental period, reflecting the rapid growth of the species under optimal conditions. Leaf area in drought-stressed *C. citriodora*, despite exhibiting a 21.8% increase by day-10 (45% FTSW) was not significantly altered over the course of the study (Fig. 3). This suggests that neither *C. citriodora* or *Z. nummularia* reduced available leaf area as an acclimation response for coping with severe drought over a 20-day period.

The carbon isotopic composition ( $\delta^{13}C$ ) of the leaves of *C. citriodora* and *Z. nummularia* became enriched in the heavier  $^{13}C$  isotope as drought progressed (Fig. 4). The  $\delta^{13}C$  of *C. citriodora* leaves was more sensitive to the early stages of drought as a significant difference between the carbon isotope values of control and drought treatment leaves was apparent at 45%, and this upturn in  $\delta^{13}C$  was increasingly evident as FTSW declined further; whereas, the leaves of *Z. nummularia* only showed a significant increase in  $\delta^{13}C$  values at the lowest 5% FTSW level. The divergence between the bulk carbon isotopic composition of leaves of *C. citriodora* and *Z. nummularia* (Table 3) exposed to drought may reflect differences in the growth rate of new foliage between the leaves (Fig. 3) and leaf-lifespan, in addition to compositional alterations of metabolites within the leaves. A similar pattern of shifts in composition was evident in the total nitrogen concentration of the leaves of the two species. The leaves of *Z. nummularia* showed a progressive decline in total nitrogen as the drought progressed (Table 4). In contrast, the leaves of *C. citriodora* exhibited a 22% increase in total nitrogen as the FTSW fell to 45%, before total nitrogen fell by 38%



**Fig. 1a:** Stomatal conductance ( $G_s$ ) response of *Ziziphus nummularia* and *Corymbia citriodora* to soil drying expressed as the fraction of transpirable soil water (FTSW), and; **(b)** water-loss *Z. nummularia* and *C. citriodora* over the 20-day period of drought treatment (S.E 0.0001 - 0.0005)

relative to control 100% FTSW values (Fig. 5).

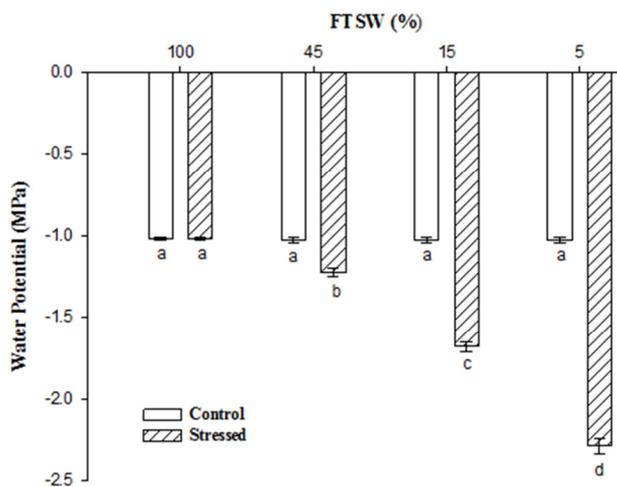
Levels of the protective antioxidant peroxidase (POX) within the leaves of the two species examined in this study showed divergent responses to drought stress. *Z. nummularia* exhibited a decline in POX activity as the FTSW reached 5%. In contrast, POX activity in *C.*

**Table 3:** ANOVA of carbon isotopes values of *C. citriodora* and *Z. nummularia* following drought"

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	11.08675052	3	3.695583506	16.5167	0.000147	3.490295
Within Groups	2.68498019	12	0.223748349			
Total	13.77173071	15				

**Table 4:** ANOVA of total leaf nitrogen values of *C. citriodora* and *Z. nummularia* following drought"

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1.395065234	3	0.465021745	21.6125	1.61E-05	3.343889
Within Groups	0.30122864	14	0.021516331			
Total	1.696293874	17				

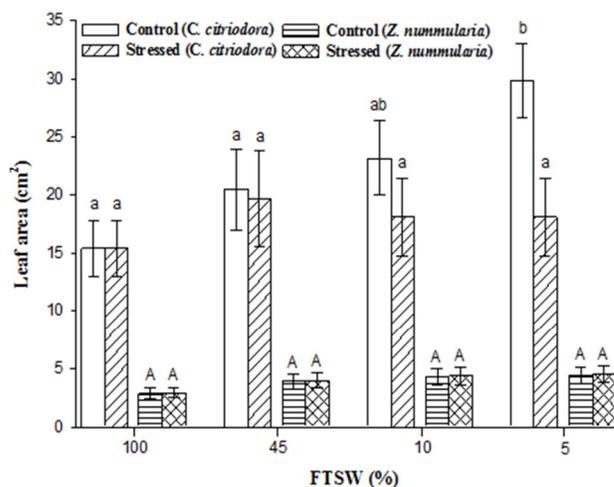


**Fig. 2:** Change in leaf water potential of *Corymbia citriodora* plants following drought treatment expressed as a function of the fraction of transpirable soil water (FTSW). Values represent the mean of three individuals. Error bars indicate one standard error either side of the mean. Letters indicate significant difference between measurements using a one-way ANOVA

*citriodora* progressively increased with the severity of drought (Fig. 6). However, it is noteworthy that while the relative changes in POX levels during drought differ between the two species, absolute levels of POX are generally higher in drought stressed *Z. nummularia* than drought stressed *C. citriodora*, with the exception of those recorded at 5% FTSW (Table 5). This may be indicative of a greater antioxidant protective capacity in leaves of *Z. nummularia* than *C. citriodora* in the event of water deficit occurring, or the use of different antioxidant systems between the two species.

### Discussion

This study has shown contrasting physiological, morphological and compositional responses to drought stress between *Z. nummularia* and *C. citriodora*. *C. citriodora* is characterised by high rates of water-use to



**Fig. 3:** Leaf area response of *Corymbia citriodora* and *Ziziphus nummularia* to soil drying expressed as a function of the fraction of transpirable soil water (FTSW). Values represent the mean of three individuals. Error bars indicate one standard error either side of the mean. Letters indicate significant difference between measurements of *C. citriodora* (lower case) and *Z. nummularia* (upper case) using a one-way ANOVA

sustain photosynthesis and rapid growth that has led to its extensive use in fuel and hard-wood plantations. However, the results of this and previous investigations (eg. Thomas, 2008; Agaba *et al.*, 2010; Brilli *et al.*, 2013) indicate that seedlings of *C. citriodora* are relatively incapable of tolerating extended periods of water deficit. In contrast, *Z. nummularia* was able to maintain  $G_s$  at lower levels of soil water availability than *C. citriodora* (Fig. 1a). This suggests that the physiological and morphological adaptations of *Z. nummularia* that permit it to survive in arid regions (Clifford *et al.*, 1998; Arndt *et al.*, 2001) may also make it a suitable species for plantations in drought prone marginal lands (Pandey *et al.*, 2010). The ability of *Z. nummularia* to sustain higher rates of  $G_s$  at lower levels of soil water availability than *C. citriodora* may be related to differences in the water-uptake and transport systems between the two species (Fig. 1b). *Z. nummularia* possesses

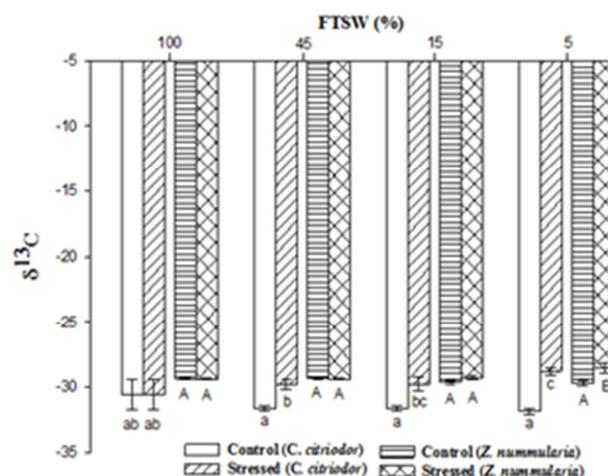
**Table 5:** ANOVA of POX values of *C. citriodora* and *Z. nummularia* following drought"

ANOVA among POX activity of both plant species						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.053148869	3	0.01771629	14.72428	0.000131	3.343889
Within Groups	0.016844831	14	0.001203202			
Total	0.0699937	17				

a large deep and extensive root system that allows it to access water in rocky arid soils (Pandey *et al.*, 2010). This effective water-uptake system permits *Z. nummularia* to sustain photosynthesis during episodes of water deficit. Furthermore, drought tolerance in tree species is often associated with resistance to xylem embolism (Brodrribb *et al.*, 2003). As a tree species occupying arid water-limited environments with a high vapour pressure deficit it may be expected that *Z. nummularia* exhibits a high level of resistance to xylem cavitation that allows it to maintain transport of water to the photosynthetic organs during episodes of low water availability and high transpirative demand (Sun *et al.*, 2011).

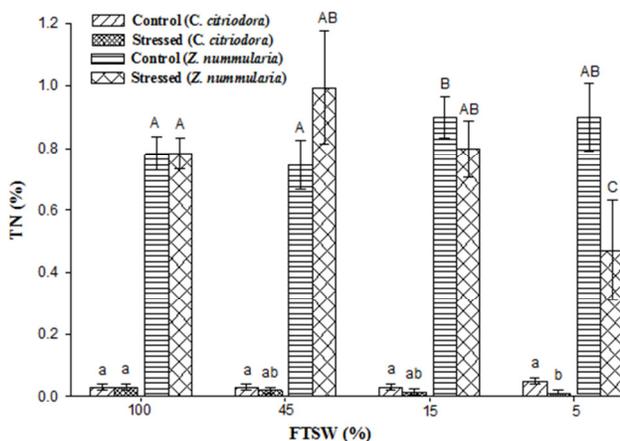
*Z. nummularia* reduces from full  $G_s$  at higher levels of soil water availability (80% FTSW) than *C. citriodora*, which maintains full  $G_s$  until soil water levels are lower (45% FTSW). This may represent different ‘water-use behaviours or acclimation responses between the two species, with the more drought tolerant *Z. nummularia* exhibiting more conservative water-use (isohydric species) and the earlier modification of leaf physiology than *C. citriodora* (Figs. 1 and 5) (anisohydric species) (see Maseda and Fernandez, 2006; Centritto *et al.*, 2011b). The native habitat of *C. citriodora* is sub-tropical and tropical summer dry forests of north-eastern Australia that receive 500–800 mm of precipitation annually, with most falling during the winter. During the dry period *C. citriodora* frequently loses a high proportion of older leaves and branches to reduce water loss (Pook, 1985; Prior *et al.*, 1997). Relatively few episodes of drought occur during the winter-wet period in the native habitat of *C. citriodora*. *Z. nummularia* occurs in arid drought prone areas that frequently experience a large range in temperatures and levels of annual precipitation as low as 100 mm (Gupta *et al.*, 2002; Orwa *et al.*, 2009; Pandey *et al.*, 2010); potentially inducing selective pressures favouring the ability to tolerate persistent water-deficit stress (Kolb and Sperry, 1999). The adaptation of *Z. nummularia* to drought is apparent in early stages of soil drying (at 80% FTSW), the resultant plant water-uptake behaviour and the capacity of the leaf physiology obstruct the negative effects of potential drought that were employed by the plants in this study (Figs. 1 and 6).

Enhanced antioxidant levels are associated with increased tolerance to drought induced oxidative stress in *Olea europea* (Sofa *et al.*, 2005; Aganchich *et al.*, 2009). Levels of the protective antioxidant POX were 875% higher in unstressed (100% FTSW) leaves of *Z. nummularia* than *C. citriodora* (Fig. 6). *Z. nummularia* maintained these



**Fig. 4:** Carbon isotopic composition ( $\delta^{13}C$ ) of *Corymbia citriodora* and *Ziziphus nummularia* leaves following the onset of drought treatment gauged relative to the fraction of transpirable soil water. Values represent the mean of three individuals. Error bars indicate one standard error either side of the mean. Letters indicate significant difference between measurements of *C. citriodora* (lower case) and *Z. nummularia* (upper case) using a one-way ANOVA

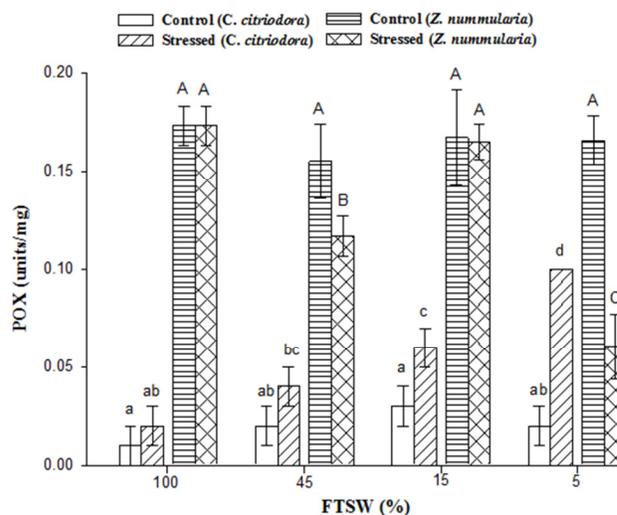
comparatively higher levels of POX activity under drought stress until the FTSW declined below 15%, whereupon POX activity fell by 65.8% as FTSW reached 5%. In contrast, levels of POX activity rose with increasing levels of drought stress in *C. citriodora*. This may suggest that leaves of *Z. nummularia* possess greater capacity against oxidative stress, or that *C. citriodora* utilises alternative antioxidant systems such as the glutathione and ascorbate systems (Noctor and Foyer, 1998). This divergence in the activity of POX between the two species may reflect differing degrees of drought tolerance and investment in foliage. *C. citriodora* produces large numbers of leaves with high photosynthetic rates and relatively short leaf lifespans of less than one-year (Poorter and Bongers, 2006; Laclau *et al.*, 2009). A common response to drought marked by the onset of the dry season in Eucalypts is to rapidly lose older leaves, thus reducing photosynthetic area from which transpirative water-loss can occur (Pook, 1985; Prior *et al.*, 1997). The leaves of *Z. nummularia* are generally more robust with a longer leaf lifespan reflecting a greater investment in structural material such as lignin (Niinemets, 1999; Haworth and Raschi, 2014). In response to water



**Fig. 5:** Total nitrogen concentration of *Corymbia citriodora* and *Ziziphus nummularia* leaves following soil drying expressed as the fraction of transpirable soil water (FTSW). Values represent the mean of three individuals. Error bars indicate one standard error either side of the mean. Letters indicate significant difference between measurements of *C. citriodora* (lower case) and *Z. nummularia* (upper case) using a one-way ANOVA

deficit, *Z. nummularia* generally does not shed leaves to reduce total leaf area unless the drought event is particularly prolonged or severe (Pandey *et al.*, 2010). This would be indicative of a relatively lower level of investment in the foliage of *C. citriodora* (Niinemets, 2001; Poorter *et al.*, 2009), and the selective pressures exerted by the environments of the two species may have shaped their respective responses to drought (Kane and Rieseberg, 2007). In this study the total leaf area of stressed *C. citriodora* and *Z. nummularia* remained constant throughout the drought period (Fig. 3). This may be due to the 20-day duration of the experiment, if water-deficit had been allowed to progress over a longer time period reductions in leaf area may have occurred. However, well-watered *C. citriodora* exhibited a rapid doubling of total leaf area during the experimental period, while the canopy size of *Z. nummularia* did not significantly increase. This corroborates the high growth rate in Eucalypts observed in other studies (Mooney *et al.*, 1978; Poorter *et al.*, 1990), and suggests that under growth conditions where the supply of water is unimpeded, a species such as *C. citriodora* is an effective hard and fuel-wood crop species due to the rapid accumulation of biomass. Nonetheless, such ideal conditions of uninterrupted water supply are unlikely to occur in real world situations, particularly in rain-fed forest plantations on marginal lands where the death of seedlings due to drought constitutes a significant economic loss (McDowell *et al.*, 2008; Agaba *et al.*, 2010).

The effect of the different leaf economic strategies of the species analysed in this study may also be evident in the compositional changes of the leaves following drought.



**Fig. 6:** Activity of the protective anti-oxidant peroxidase (POX) of *Corymbia citriodora* and *Ziziphus nummularia* leaves during drought treatment measured as the fraction of transpirable soil water (FTSW). Values represent the mean of three individuals. Error bars indicate one standard error either side of the mean. Letters indicate significant difference between measurements of *C. citriodora* (lower case) and *Z. nummularia* (upper case) using a one-way ANOVA

Carbon isotopic analysis of the leaves of *C. citriodora* indicated progressive increases in  $\delta^{13}\text{C}$  as soil water availability declined (Fig. 4); indicative of diminished  $G_s$  resulting in increased uptake of  $^{13}\text{C}$  within the leaves (Farquhar *et al.*, 1989; Feng *et al.*, 2013). In contrast, *Z. nummularia*, despite showing a decline in  $G_s$  at higher FTSW values than *C. citriodora*, did not exhibit any significant increases in leaf carbon isotopic composition until the level of soil water available for transpiration reached its lowest (5% FTSW). As the carbon isotopic composition of the bulk leaf was analysed in both cases, the lack of a detectable isotopic shift in the leaves of *Z. nummularia* at FTSW values above 5% may reflect the greater structural content invested in the leaves. The absence of alteration evident over the experimental period in the total leaf area of *Z. nummularia* (Fig. 3) is indicative that the leaves grew prior to the stress treatment and therefore the carbon isotopic composition of much of this structural material will likely reflect the more favourable growth conditions. The faster growth and lower structural content in the foliage of *C. citriodora* was more likely to show the carbon isotopic effect of drought in the analysis of bulk leaf material. The lack of any increase in the carbon isotopic ratio of the leaves of *Z. nummularia* may also be indicative of the importance of  $G_s$  in determining the degree of *Z. nummularia* carbon isotope discrimination. Leaf-level  $G_s$  of *Z. nummularia* was consistently higher than that of *C. citriodora*. An increase in  $\delta^{13}\text{C}$  values of *C. citriodora*

coincided with a reduction of  $G_s$  values from  $\sim 0.2$  to  $0.1 \text{ mol m}^{-2} \text{ s}^{-1}$  at 45% FTSW. Stomatal conductance values of *Z. nummularia* only declined below  $0.1 \text{ mol m}^{-2} \text{ s}^{-1}$  at the lowest level of available soil water recorded of 5% FTSW (Fig. 1); raising the possibility that the apparent lack of leaf carbon isotopic response to drought stress is due to the relatively high rates of  $G_s$  sustained by *Z. nummularia*. However, the carbon isotopic composition of a leaf is generally associated with the long-term WUE of a plant, and often determined by  $G_s$  (Farquhar and Richards, 1984; Farquhar *et al.*, 1989). The  $\delta^{13}\text{C}$  of *C. citriodora* would suggest a lower WUE than *Z. nummularia*, yet the  $G_s$  values of *Z. nummularia* were consistently greater than those of *C. citriodora*, possibly suggesting more efficient  $\text{CO}_2$ -uptake in *Z. nummularia*; potentially associated with the observations of higher leaf nitrogen concentration in this study (Fig. 5). Nonetheless, to constrain these potential sources of variation, future studies of the effect of drought-stress on the isotopic composition of wood-crop species should utilise the analysis of recently synthesised photosynthates (Scartazza *et al.*, 1998) or compound specific analysis of wax compounds on newly developed leaves (Tipple *et al.*, 2013).

Similar patterns of the effect of drought on foliage composition are evident in the nitrogen concentration of the leaves of the two species. The total nitrogen concentration of *Z. nummularia* leaves was unaffected as the available soil water declined from 100 to 15% FTSW. A significant reduction in leaf nitrogen was only apparent at the lowest level of water availability (5% FTSW). In contrast, the leaves of *C. citriodora* showed progressive reductions in nitrogen concentration as FTSW declined from 45 to 5% (Fig. 5). These divergent compositional responses may reflect different physiological adaptations to water-stress and/or the differential degrees of investment in the leaves between the two species. It is however noteworthy that the longer lived foliage of *Z. nummularia* possessed significantly higher levels of leaf nitrogen than the foliage of *C. citriodora*. This pattern is not consistent with numerous observations of leaf economic strategies, where those leaves representing a greater proportional investment with longer leaf lifespans generally possess lower levels leaf nitrogen relative to leaf mass (Poorter *et al.*, 1990; Poorter *et al.*, 2009). Leaf-level photosynthetic rates are often positively associated with the concentration of nitrogen (Evans, 1989), and rates of stomatal conductance (Haworth *et al.*, 2011). Therefore, the higher levels of leaf nitrogen in the foliage of *Z. nummularia* are consistent with observations of relatively higher rates of  $G_s$  than were found in *C. citriodora*. This may suggest that despite being adapted to relatively arid conditions, *Z. nummularia* is capable of high rates of leaf-level photosynthesis during periods of water availability to fully exploit favourable growth conditions (Attiwill and Clayton-Greene, 1984; Hetherington and Woodward, 2003). During drought-stress *Z. nummularia* can then reduce photosynthetic rates and  $G_s$  to conserve water (isohydric behaviour), and in these periods the comparatively high

levels of antioxidant activity (Fig. 6) protect the leaf from the harmful effects of oxidative stress.

The fast growth and rapid accumulation of biomass make *C. citriodora* a highly effective wood crop species in areas of high water availability. However, *C. citriodora* is relatively intolerant of drought stress, leading to impaired photosynthesis and the frequent loss of both seedlings and young trees (Fig. 1) (Agaba *et al.*, 2010; Brilli *et al.*, 2013). *Z. nummularia* possesses physiological (Figs. 1 and 6) and morphological adaptations (Clifford *et al.*, 1998; Arndt *et al.*, 2001; Haworth and McElwain, 2008) to the negative effects of drought stress. The apparent capacity of *Z. nummularia* leaves to fully exploit favourable growth conditions, combined with the ability to tolerate periods of water deficit, are highly desirable in a biomass crop species grown in drought prone rain-fed marginal lands. Nonetheless, further work is required to fully assess the relative impact of drought on rates of biomass accumulation in *Z. nummularia* (eg. Monclus *et al.*, 2006), as this is the critical criterion in any successful hard or fuel-wood crop. Nonetheless, the results of this study suggest that the ability to tolerate drought through effective stomatal control, maintenance of  $G_s$  at low levels of soil water availability and enhanced protective mechanisms against oxidative stress in *Z. nummularia* may permit its potential exploitation as a highly effective wood plantation species in rain-fed marginal lands in drought prone areas.

In conclusion, this study has shown a clear distinction between the respective anisohydric and isohydric drought responses of *C. citriodora* and *Z. nummularia*. *Z. nummularia* reduced  $G_s$  at higher FTSW levels than *C. citriodora*, but was able to maintain higher rates of  $G_s$  at lower levels of soil water availability. Foliage of *Z. nummularia* exhibited less compositional change in terms of the ratio of carbon isotopes and nitrogen concentration in response to water deficit than *C. citriodora*. Leaves of *Z. nummularia* also possessed higher levels of the antioxidant POX than *C. citriodora*, possibly as a pre-emptive protective mechanism against the harmful effects of the frequent drought events that occur in the native habitat of *Z. nummularia*. This apparent ability to sustain  $G_s$  during episodes of reduced water availability, combined with elevated antioxidant mechanisms that confer tolerance to the harmful physiological effects of drought, may permit the use of *Z. nummularia* as an effective hard and fuel-wood plantation species in drought prone areas. While *C. citriodora* displays rapid growth, ideal for use as a wood crop species in areas where drought events are relatively infrequent, the results of this and other studies suggest that it is relatively intolerant of water-deficit conditions, exhibiting reduced gas-exchange and growth. The observations of previous studies may suggest that the attributes required for high rates of photosynthesis and accumulation of biomass under optimal growth conditions may be mutually exclusive to the attributes that confer drought tolerance. However, the high rates of  $G_s$  and foliar nitrogen concentration would

suggest that *Z. nummularia* is capable of sustaining high rates of leaf-level photosynthesis. This adaptation may permit *Z. nummularia* to fully exploit brief intervals where conditions are favourable to growth.

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