

Physiological Responses of Four *Eucalyptus camaldulensis* Clones to Waterlogging in a Hydroponic System

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ABSTRACT

Waterlogging has a detrimental effect on environmental stress in *Eucalyptus camaldulensis* (Dehn.), as does calcareous soil, while together they act synergistically to induce stress in the plants. In the western region of Thailand, both conditions are deleterious to the eucalypt plantation industry. These stresses can reduce the biomass and yield of eucalypts at all stages of growth. To assist the eucalypt breeding program, powerful tools to screen a population are needed. Leaf gas exchange parameters can be used to study the photosynthetic capacity of plants during flooding, except in some flood-tolerant plants that have developed adaptive mechanisms to better maintain photosynthetic capacity. In this study, a physiological method was developed and applied to four *Eucalyptus camaldulensis* (Dehn.) clones to screen for the effects of waterlogging on net photosynthesis, maximum quantum yield, chlorophyll content and biomass. Four two-month-old eucalypt clones (C1 and C2 were calcicole clones and C3 and C4 were calcifuge clones) were grown in half-strength Hoagland's solution and subjected to waterlogging conditions for 16 days. Physiological parameters were monitored at days 0, 8 and 16 under a plant growth incubator. The net photosynthetic rate (A_{max}), stomatal conductance (g_s) and transpiration rate (E) rapidly reduced in C2 and C4 after 16 days of waterlogging. The maximum quantum yield (F_v/F_m), chlorophyll content and biomass of the four clones decreased in all cultivars under waterlogging at day 16. From measured physiological parameters, C2 and C4 suffered more severe stress under waterlogging than C1 and C3. Overall, a difference between the control and treated plants could be observed at 16 days after treatment. The physiological parameters of gas exchange and the F_v/F_m ratio were proven to be suitable indicators of waterlogging-tolerant traits.

Key words: maximum quantum yield, net photosynthetic rate, chlorophyll content, stomatal conductance, transpiration rate, cluster analysis

INTRODUCTION

Eucalyptus is a genus of native plants in Australia and includes many species of fast-growing economic trees. They have been introduced to many tropical and subtropical areas of the world. In Thailand, *Eucalyptus*

camaldulensis is planted as a part of the agro-industry to produce raw material for pulp production, furniture, poles and fuelwood. The export value to Thailand of publishing, packaging, pulp and paper material made from eucalypt was USD 1,027.69 million in 2005 (Department of Export Promotion, 2006). The plantation areas are

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mainly in the northeastern, eastern and western regions with some extension to the central region of Thailand. In the western region, approximately 70% of the plantation area is on soil defined as calcareous and located in the Kanchanaburi, Ratchaburi and Uthai Thani provinces. In general, calcareous soils occur in dry areas. However, in the west of Thailand, eucalypts have been planted in limestone-based valleys, which are rich in CaCO_3 as a parent material. Major problems usually occur during the rainy season when water runoff and erosion from the limestone mountains combines with waterlogging in the lowlands or valleys used for agriculture. It has been found that eucalypts can grow in calcareous soil under waterlogged conditions for a short time, but growth rates and biomass production are drastically decreased. For this study, the calcicole and calcifuge clones had been screened from various clones in the laboratory. These clones were selected as having both waterlogging and calcareous tolerance.

Waterlogging is a condition caused when air in the soil is replaced with water. Gas exchange between the soil and the air is decreased, because gas diffusion in water is 10,000 times less than in air (Marschner, 1995). When a region becomes inundated with water, most plants suffer from an oxygen deficiency under waterlogged conditions which can also reduce the capacity of the roots to take up and supply water and nutrients from the soil to the rest of the plant (De Simone *et al.*, 2002). Waterlogging has been shown to affect some major physiological functions. A variety of plants showed reduced stomatal conductance (Smith and Moss, 1998) in response to root-zone saturation and stomatal closure. This reduction was often accompanied by reduced CO_2 assimilation and water transpiration. Photosynthesis rapidly decreased, with a subsequent decrease in carbohydrate production and consequently the growth rate was reduced (Musgrave and Ding, 1998). In the long term, a reduction in

photosynthesis was partly traceable to changes in the carboxylation enzymes. Inhibited nutrient uptake led to a nutrient deficiency in the shoots that reduced the chlorophyll content of the leaves, causing early leaf senescence, abscission and chlorosis (Huang *et al.*, 1994) and reduced leaf area (Bradford, 1982). If the plants were grown under these conditions for a long period, the plants switched from an aerobic-respiration pathway to an anaerobic pathway (Kozłowski, 1984). The anaerobic respiration induced a fermentation pathway and ATP synthesis decreased. Under such conditions, plants reduced their photosynthesis and their growth rate (Bray *et al.*, 2000).

Fluorescence measurements can provide an indication of the photosynthetic performance as a means of measuring the stress tolerance of a plant. Under environmental stress, the plant disperses excess light in the form of heat and fluorescence. Fluorescence can represent the ability of plant to tolerate environmental stresses. In recent years, chlorophyll fluorescence has become a powerful tool in plant screening for environmental stress. Measuring chlorophyll fluorescence is a convenient, non-destructive and non-invasive method to monitor photosynthetic events at different functional levels (Smethurst and Shabala, 2003). Leaf-gas exchange parameters have been used to study the photosynthetic capacity of plants during flooding. Some flood-tolerant plants have developed adaptive mechanisms in order to maintain a better photosynthetic capacity (Liao and Lin, 2001). The most powerful tool to measure the photosynthetic activity was achieved by combining the fluorescence technique with other techniques, in particular, gas exchange measurements (Maxwell and Johnson, 2000).

Eucalypts are very sensitive to extreme waterlogging conditions, with reduced growth and development leading to poor biomass production. Identifying waterlogging-tolerant clones of eucalypts provides a challenge. Measurement of

the photosynthetic rate, transpiration rate, stomatal conductance, chlorophyll fluorescence and chlorophyll content can be used to develop a screening method for tolerance to waterlogging in the field and can provide three benefits: (1) it is a non-destructive method, (2) it saves time and (3) it has a low cost. The physiological response of eucalypts to waterlogging conditions studied in this research can be used to improve the tolerance-screening test for eucalypt clones and lead to efficiencies in the eucalypt breeding program.

MATERIALS AND METHODS

Plant materials and growth conditions

Plantlets from four clones of *Eucalyptus camaldulensis* (Dehn.) were selected for the experiment that was carried out in a greenhouse at the Tropical Vegetable Research Development Center (TVRDC) at Kasetsart University, in Nakhon Pathom, Thailand. C1 and C2 were classified as calcicole clones and C3 and C4 were identified as calcifuge clones by the Siam Forestry Company in the laboratory. Plantlets were propagated using a tissue culture technique. Two-month-old plantlets (20-25 cm in length) were planted in a hydroponic culture with half-strength Hoagland's solution with a pH of 5.75. The plantlets were acclimatized for two weeks before being subjected to a waterlogging treatment.

Experimental treatments

The plantlets were randomly allocated into two experimental groups; (1) control (C) and (2) waterlogged (W); The control plants were cultured with half-strength Hoagland's solution with a pH of 5.75 and supplied with air by a pump that kept the dissolved oxygen level in the range of 6.0-7.0 mgL⁻¹. The waterlogged treatment plants were cultured with half-strength Hoagland's solution with a pH of 5.75 and supplied with nitrogen bubbles at a flow rate of 5 Lmin⁻¹ that

kept the dissolved oxygen level in the range of 0.5-1.0 mgL⁻¹. The experiment was set up using a completely randomized design (CRD) with three replicates. Data on the net photosynthetic rate, stomatal conductance, transpiration rate, chlorophyll content and biomass were collected on days 0, 8 and 16.

Gas exchange parameters

Net photosynthetic rate (A_{max}), stomatal conductance (g_s) and transpiration rate (E) were measured on the youngest fully-expanded leaf in the fifth position from the tip with a LI-COR 6400 Portable Photosynthesis System (LI-COR Inc., Lincoln, NE, USA.) under a plant growth incubator with the following parameters: carbon dioxide concentration, 400 $\mu\text{mol mol}^{-1}$; photosynthetic photon flux density (PPF), 1,200 $\mu\text{mol m}^{-2}\text{s}^{-1}$; air flow rate, 400 $\mu\text{mol s}^{-1}$; relative humidity, in the range of 70-75% and temperature of the leaf chamber, around 30°C (Wadjinda, 2005).

Chlorophyll fluorescence

Chlorophyll fluorescence was measured using a PAM fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany). Chlorophyll fluorescence measurements were conducted under dark conditions during the period from 8 pm to 9 pm using a dark leaf clip (DLC-8) to protect the readings from the effects of temperature and PPF. The measurements were taken on days 1, 7 and 15 (Yingjajaval and Hiran-on, 2004). The F_0 value was measured under a low irradiance and the F_m value was measured under a high irradiance. The variable to maximum fluorescence ratio was then calculated as $F_v/F_m = (F_m - F_0)/F_m$.

Leaf chlorophyll content

From the beginning of the waterlogging treatment until just before the end of the experiment, sequential extractions of chlorophyll (Chl) *a* and *b* were made on days 0, 8 and 16. Leaf chlorophyll was extracted from two leaf discs (both

0.6 mm in diameter) from the youngest fully-expanded leaf using N, N-Dimethylformamide (DMF) 4 mL in the dark at 4°C for at least 48 h until the leaf disc color turned white. The absorbance was determined for each leaf sample using a spectrophotometer at wavelengths of 647 and 664 nm. Ratios of Chl *a* to *b* were calculated and compared with the stress treatment (Arnon, 1949).

Biomass

Growth parameters for the plantlets were recorded at the beginning (day 0) and the end of the experiment (day 16). The plants were gently washed under running water and patted dry with

absorbent paper. Leaves, stems and roots were then separated and the fresh weights recorded. Plant material was dried at 65°C for 48 h to constant weight and then shoot and root dry weights were determined (Smethurst and Shabala, 2003).

RESULTS

Gas exchange

The net photosynthetic rate ($A_{\max}$), stomatal conductance (g_s) and transpiration rate (E) of the four clones are shown in Table 1. Parameters measured in the plant growth incubator were: carbon dioxide concentration, photosynthesis photon flux density (PPF), air flow rate, humidity

Table 1 Effect of waterlogging on net photosynthetic rate ($A_{\max}$), transpiration rate (E), stomatal conductance (g_s) and on F_o , F_m , F_v/F_m in four *Eucalyptus camaldulensis* clones after imposing waterlogging. ($A_{\max}$; $\mu\text{mol CO}_2\text{m}^{-2}\text{s}^{-1}$, E ; $\text{mmol H}_2\text{O m}^{-2}\text{s}^{-1}$, g_s ; $\text{mmol H}_2\text{O m}^{-2}\text{s}^{-1}$)

Day	Cultivar	Treatment	$A_{\max}$	E	g_s	F_v/F_m	F_o	F_m
0	C1	Control	24.9	4.98	589	0.838	548	3387
		Waterlogged	22.9	4.34	498	0.844	521	3347
	C2	Control	28.6	6.34	693	0.836	556	3382
		Waterlogged	23.4	4.52	548	0.840	522	3268
	C3	Control	30.1	6.97	793	0.831	589	3485
		Waterlogged	28.4	6.31	689	0.844	529	3401
	C4	Control	25.7	5.35	628	0.834	573	3450
		Waterlogged	28.1	6.45	681	0.837	567	3475
8	C1	Control	23.6	4.59	554	0.843	544	3475
		Waterlogged	20.2	3.78	395	0.838	539	3335
	C2	Control	24.2	4.87	568	0.835	527	3187
		Waterlogged	23.2	4.48	529	0.830	516	3041
	C3	Control	31.7	7.87	877	0.835	529	3203
		Waterlogged	30.9	7.52	826	0.830	533	3129
	C4	Control	31.2	7.68	866	0.836	544	3325
		Waterlogged	22.7	4.18	475	0.831	524	3100
16	C1	Control	27.6	5.57	654	0.835	557	3385
		Waterlogged	26.0	5.45	645	0.827	532	3068
	C2	Control	24.4	4.92	577	0.839	479	2983
		Waterlogged	13.3	1.33	119	0.823	523	2956
	C3	Control	33.5	7.95	883	0.831	533	3163
		Waterlogged	25.4	5.24	609	0.828	537	3123
	C4	Control	25.2	5.20	602	0.828	512	2982
		Waterlogged	8.2	0.77	64.5	0.820	510	2831

and temperature. In waterlogged plants, photosynthesis rates started to decline nine days after stress was imposed. C2 and C4 showed the most severe effect. The highest value of $A_{\max}$ in waterlogged plants was approximately $26.0 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ in C1 at the end of the experiment (Figure 1A). C4 recorded the lowest value ($8.2 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$) because the stomatal conductance of C1 was higher than that of C4 (Figure 1C). C2 and C4 had the lowest values. On day 16, the decline in $A_{\max}$ compared with the control was 45.5 and 67.5% for C2 and C4 respectively, with 24.2% for C3 and 5.8% for C1. The most severe effect over the whole 16 day period was noticed in C4, where the $A_{\max}$ of the stressed plants was only about 32.5% of the control (Figure 2).

Leaf transpiration (E) responded linearly with stomatal conductance (g_s) and also with net photosynthesis ($A_{\max}$) (Figure 1). It declined when stomatal conductance (g_s) was decreased. C1 and C3 had a higher level of leaf transpiration compared to C2 and C4 at day 16.

Chlorophyll fluorescence

The maximal photochemical efficiency or the maximum quantum yield (F_v/F_m) of the four clones was as high as 0.827 when averaged overall at the end of the experiment. With respect to the waterlogged plants, C1 and C3 had a higher level of F_v/F_m whereas C2 and C4 had lower values (Figure 3), but the values were not different among all cultivars. In the waterlogged treatment, all cultivars had a lower F_v/F_m than in the control plants at day 16. In this experiment, as the measurements were taken in the dark, there was no effect of temperature. F_0 increased whereas the maximum quantum yield (F_v/F_m) and F_m declined by day 16. In the waterlogged plants, F_0 was reduced in only C1. C2 and C4 had a higher F_0 than that of C1 and C3, when compared to the control plants. The F_m value demonstrated the maximum total fluorescence yield or the heat transfer from the leaf, which decreased

concomitantly with F_v/F_m in the waterlogging treatment at day 16 for all cultivars (Table 1).

Chlorophyll content

Chlorophyll content was strongly affected by waterlogging. In the waterlogged plants, the Chl *a* and *b* content had slightly decreased by day 8 and slightly increased by day 16 in C1 and C3. However, the Chl *a* and *b* content had slightly increased by day 8 and slightly decreased by day 16 in C2 and C4 (Figure. 4A, 4B). After 16 days of waterlogging all cultivars had a lower level of Chl *a*, *b* content and total chlorophyll compared with the control. At day 16, C1 and C3 showed a higher Chl *a*, *b* content and total chlorophyll than that of C2 and C4. The chlorophyll *a/b* ratio declined more drastically in the waterlogged plants than in the control (Figure 4D). Chlorosis symptoms were not observed in any of the cultivars. Only the chlorophyll content data for C1 and C4 is presented in Figure 4 as these clones showed the highest and lowest values of chlorophyll content and were more easily compared.

Biomass

The growth rate of the plants can be determined from the biomass values. As the biomass and plant size at the commencement of the experiment were not equal for all cultivars, in order to compare the plant growth rate, the biomass value was calculated using a normalized value based on the difference in biomass between day 16 and day 0. The normalized value showed the growth rate over the 16 days. Waterlogging caused a change in the plant growth characteristics. All cultivars showed no differential decrease in either root or shoot growth. In the waterlogged plants, the normalized value declined in all cultivars (Table 2). C1 and C3 had a higher value as a percentage of the control than either C2 or C4. No differences in growth responses among the cultivars were found after 16 days of waterlogging (Figure 5).

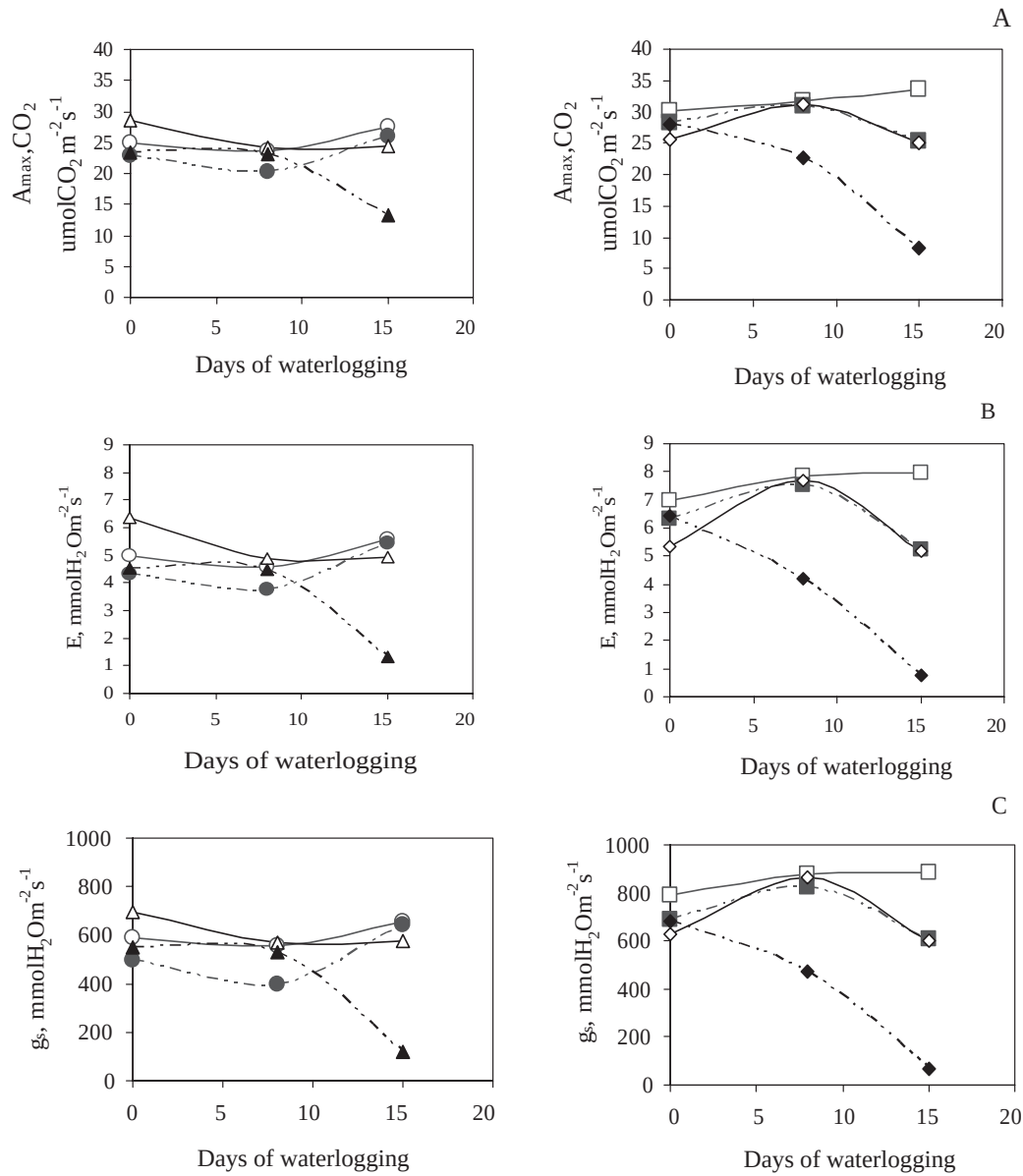


Figure 1 Effect of waterlogging on net photosynthetic rate (A); transpiration rate (B) and stomatal conductance (C) of four *Eucalyptus camaldulensis* clones over 16 days of waterlogging.

- C1 control
- -●- - C1 waterlogged
- △— C2 control
- -▲- - C2 waterlogged
- C3 control
- -■- - C3 waterlogged
- ◇— C4 control
- -◆- - C4 waterlogged

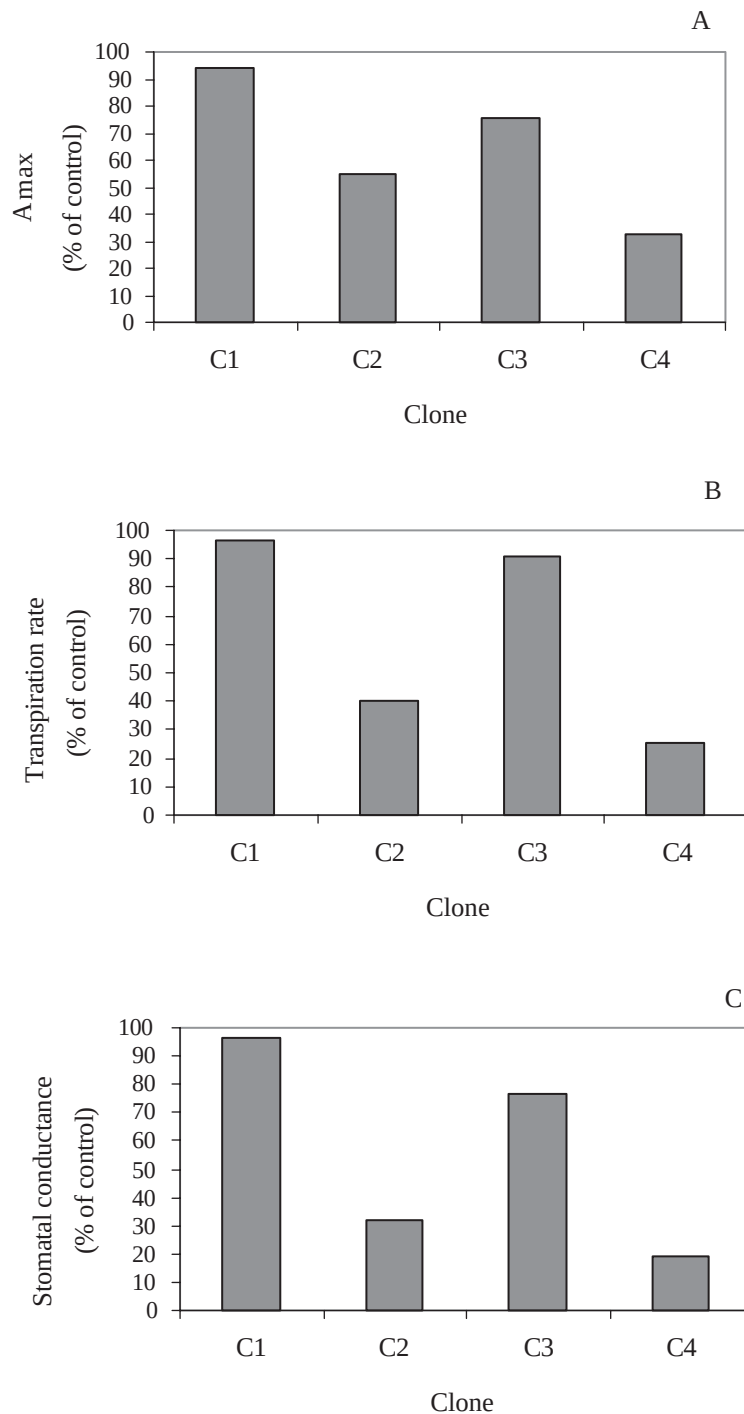


Figure 2 Effect of waterlogging on net photosynthetic rate (A); transpiration rate (B) and stomatal conductance (C) of four *Eucalyptus camaldulensis* clones (C1, C2, C3 and C4) over 16 days of waterlogging as a percentage of the control.

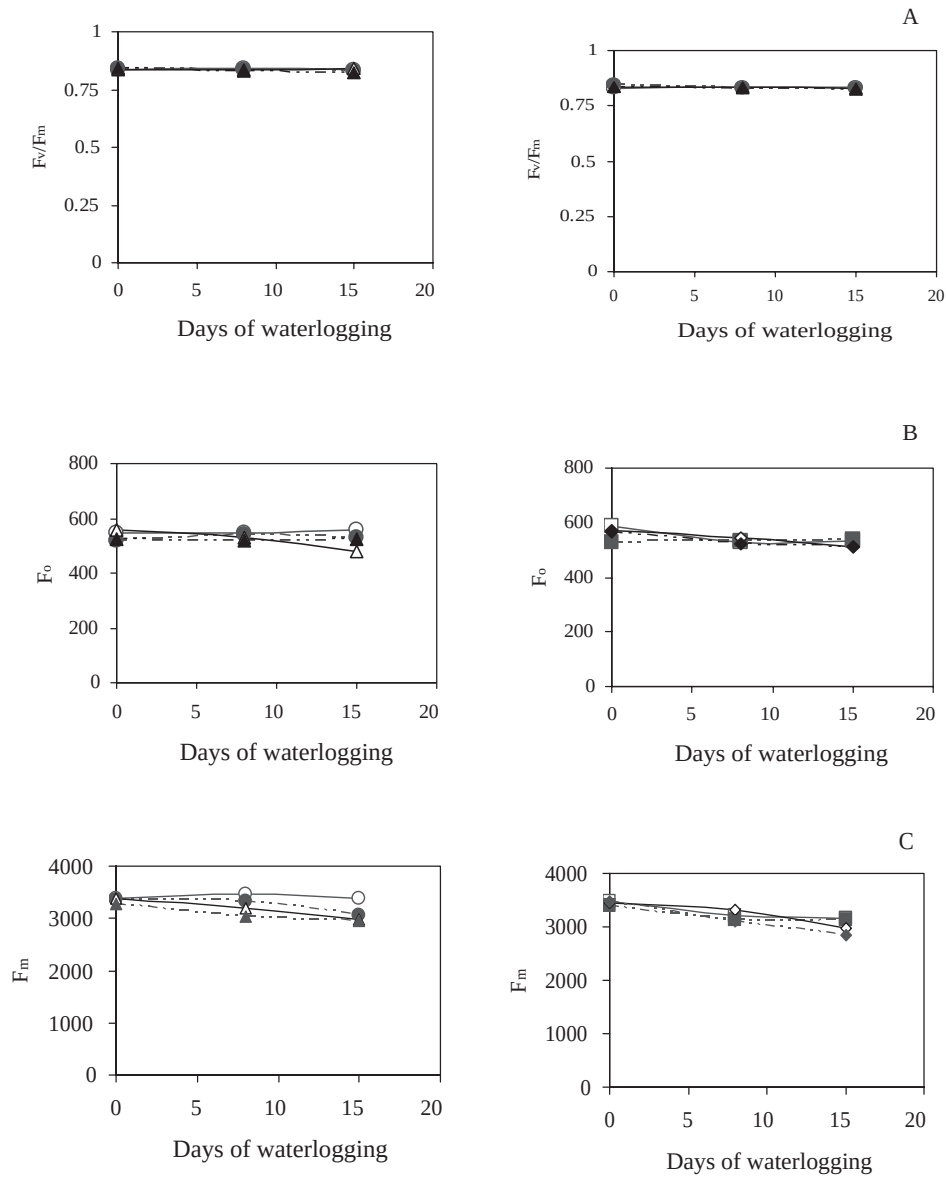


Figure 3 Effect of waterlogging on Fv/Fm(A), Fo (B) and Fm (C) over 16 days of waterlogging in four *Eucalyptus camaldulensis* clones.



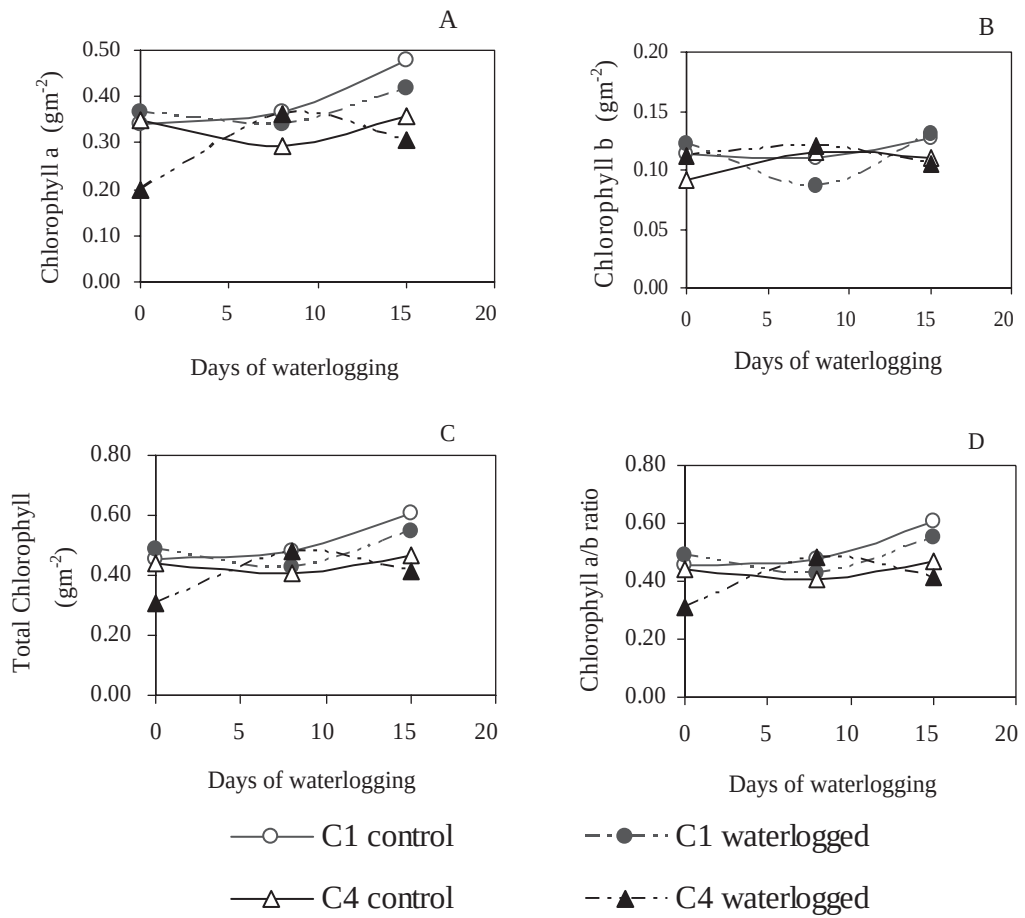


Figure 4 Effect of waterlogging on leaf pigment composition in two *Eucalyptus camaldulensis* clones, C1 and C4 over 16 days of waterlogging.

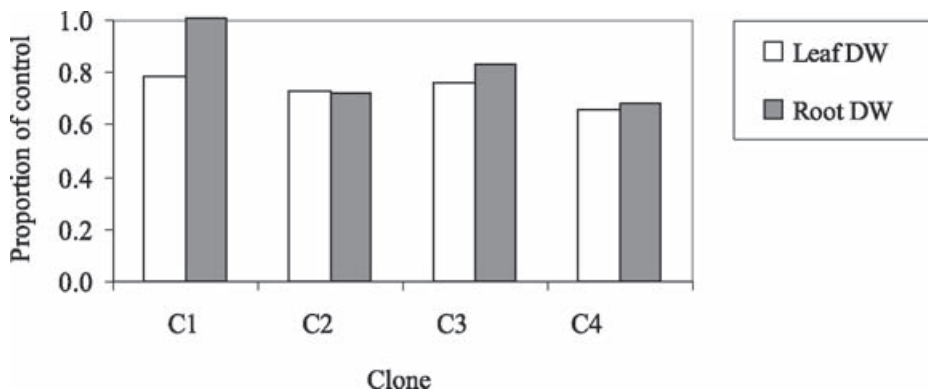


Figure 5 Effect of waterlogging on leaf and root dry weights on four *Eucalyptus camaldulensis* clones as a percentage of the control after 16 days of waterlogging.

Table 2 Effect of waterlogging on biomass characteristics of four *Eucalyptus camaldulensis* clones. Root and shoot fresh and dry weights are shown as normalized values after waterlogging was imposed.

Clone	Treatment	Shoots		Roots	
		Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
C1	Control	10.78	3.31	5.59	0.52
	Waterlogged	6.25	2.11	2.67	0.48
C2	Control	14.52	4.18	5.37	0.61
	Waterlogged	8.34	2.65	4.78	0.32
C3	Control	12.71	3.91	4.63	0.59
	Waterlogged	8.49	2.24	4.13	0.28
C4	Control	22.97	6.79	9.57	0.88
	Waterlogged	12.48	4.47	7.08	0.54

DISCUSSION

Four clones of *Eucalyptus camaldulensis* were grown in half-strength Hoagland solution (control) and subjected to waterlogging conditions for 16 days. The effect of waterlogging on net photosynthetic rate ($A_{\max}$), stomatal conductance (g_s), transpiration rate (E), chlorophyll fluorescence, chlorophyll content and biomass in calcicole and calcifuge clones were studied. In all clones, $A_{\max}$, g_s and E were lowered following exposure to waterlogging, especially in the C2 and C4 clones.

When the environmental conditions under which the study was carried out were kept constant, the stomatal conductance (g_s) controlled leaf transpiration (E) and $A_{\max}$ by regulating the influx of carbon dioxide into the leaf. A higher g_s value led to CO_2 diffusion into the leaf at a higher rate, resulting in a higher photosynthetic rate. At this time transpiration of the water from the leaf to the air occurred. Both $A_{\max}$ and E declined when the stomata closed and $A_{\max}$ decreased in the waterlogged plants. C1 and C3 had higher levels of $A_{\max}$ while C2 and C4 had lower values, because the stomatal conductance of C1 and C3 was higher than that of C2 and C4.

Leaf transpiration (E) responded linearly

with stomatal conductance (g_s) as did net photosynthetic rate ($A_{\max}$), showing a reduction when g_s was decreased. Under waterlogging conditions, the plants could not take up water and nutrients from the soil, so they alleviated the induced stress by closing their stomata to protect against a loss of water. C1 and C3 had a higher level of leaf transpiration compared to C2 and C4 in the waterlogged treatment at day 16. This result was in agreement with Ahmed *et al.* (2002), who reported that photosynthesis, stomatal conductance and transpiration rate were reduced in mungbeans under waterlogging.

It seemed that the reduction in photosynthesis by waterlogging might have been associated with stomatal closure. On the other hand, the chlorophyll fluorescence measurement showed a decrease in F_v/F_m during the same period, indicating that the early photosynthesis reduction was caused by the damage of the photosystem II (PSII) (Ahmed *et al.*, 2002). Measurement of the chlorophyll fluorescence of the leaf in the dark can be used to determine the maximum photosynthetic efficiency or dark-adapted quantum yield, because all the reaction centers of PSII are in an active state. The data showed that the leaf could use the absorbed light for the photochemical process. In optimum

conditions, the dark-adapted quantum yield should be around 0.83 (Maxwell and Johnson, 2000). Previous studies used a sustained decrease in the dark-adapted F_v/F_m and an increase in F_o to indicate the occurrence of photoinhibitory damage in response to high temperature and water stress (Epron *et al.*, 1992). This study showed that the dark-adapted quantum yield and F_m had been reduced whereas F_o had been increased after imposing waterlogging. This was consistent with results reported for wheat (Webb and Fletcher, 1996), mungbeans (Ahmed *et al.*, 2002) and lucerne (Smethurst and Shabala, 2003), that showed the maximal quantum efficiency was reduced after imposing waterlogging. In this experiment, the maximum quantum yield (F_v/F_m) was measured at night and so there was no effect due to temperature or PPF, but only due to the stress.

Chlorophyll content is one parameter that can be used to determine the effect of waterlogging in the plant. Some reports concluded that waterlogging can cause a decrease in the chlorophyll content, with *Brassica rapa* showing more than a 50% reduction in total chlorophyll after seven days of waterlogging (Daugherty and Musgrave, 1994). Huang *et al.*, (1997) also demonstrated that waterlogging reduced the chlorophyll content of wheat by 20% in waterlogging-tolerant wheat and by 40% in waterlogging-sensitive wheat. In the current study, in the waterlogged plants, all cultivars except for C4, showed an increase in the Chl *a*, *b* and total chlorophyll after 16 days of waterlogging when compared with the control. These results did not agree with Smethurst and Shabala (2003), who observed that Chl *a*, *b* and total chlorophyll had declined in all their lucerne cultivars by day 15 of waterlogging. A reduction in photosynthesis affected the accumulation of biomass in the plant. Smethurst and Shabala (2003) compared four lucerne cultivars differing in their tolerance to waterlogging and found that the four cultivars

showed no differences with regard to their biomass reduction. In this study, the normalized value declined in the waterlogged plants of all cultivars. However, no differences in growth responses among the four eucalypt cultivars were found after 16 days of waterlogging. This result is consistent with work carried out in wheat (Huang *et al.* 1997) and lucerne (Smethurst and Shabala, 2003).

CONCLUSION

In all cultivars studied, waterlogging caused changes in leaf gas exchange, net photosynthesis, chlorophyll fluorescence and biomass. Chlorophyll fluorescence did not differ between any cultivars. However chlorophyll fluorescence is a non-destructive method for measuring the effect of stress. The most powerful applications of fluorescence do not use this technique alone, but combine it with gas exchange measurements. Using this approach, the four eucalypt clones could be categorized into four groups: (1) C1 is classified as a calcicole and waterlogging-tolerant clone, (2) C2 is classified as a calcicole and waterlogging-susceptible clone, (3) C3 is classified as a calcifuge and waterlogging-tolerant clone and (4) C4 is classified as a calcifuge and waterlogging-susceptible clone.

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