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## Research paper

# Physiological and morphological responses to permanent and intermittent waterlogging in seedlings of four evergreen trees of temperate swamp forests

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Waterlogging decreases a plant's metabolism, stomatal conductance ( $g_s$ ) and photosynthetic rate ( $A$ ); however, some evergreen species show acclimation to waterlogging. By studying both the physiological and morphological responses to waterlogging, the objective of this study was to assess the acclimation capacity of four swamp forest species that reside in different microhabitats. We proposed that species (*Luma apiculata* [D.C.] Burret. and *Drimys winteri* J.R. et G. Forster.) abundant in seasonally and intermittently waterlogged areas (SIWA) would have a higher acclimation capacity than species abundant in the inner swamp (*Blepharocalyx cruckshankii* [H et A.] Mied. and *Myrceugenia exsucca* [D.C.] Berg.) where permanent waterlogging occurs (PWA); it was expected that the species from SIWA would maintain leaf expansion and gas exchange rates during intermittent waterlogging treatments. Conversely, we expected that PWA species would have higher constitutive waterlogging tolerance, and this would be reflected in the formation of lenticels and adventitious roots. Over the course of 2 months, we subjected seedlings to different waterlogging treatments: (i) permanent (sudden, SW), (ii) intermittent (gradual) or (iii) control (field capacity, C). Survival after waterlogging was high ( $\geq 80\%$ ) for all species and treatments, and only the growth rate of *D. winteri* subjected to SW was affected. *Drimys winteri* plants had low, but constant  $A$  and  $g$  during both waterlogging treatments. Conversely, *L. apiculata* had the highest  $A$  and  $g$  values, and  $g$  increased significantly during the first several days of waterlogging. In general, seedlings of all species subjected to waterlogging produced more adventitious roots and fully expanded leaves and had higher specific leaf area (SLA) and stomatal density (StD) than seedlings in the C treatment. From the results gathered here, we partially accept our hypothesis as all species showed high tolerance to waterlogging, maintained growth, and had increased  $A$  or  $g$  during different time points of waterlogging. Differences in leaf (SLA) and stomata functioning ( $g_s$ , StD) plasticity likely allows plants to maintain positive carbon gains when waterlogging occurs. The species-specific differences found here were not entirely related to microhabitat distribution.

**Keywords:** acclimation to waterlogging, carbon exchange, southern South America, stomatal density, transpiration.

## Introduction

Responding to and overcoming waterlogging is a challenge during all plant life stages, yet it is particularly difficult during germination and establishment. Plant tolerance to waterlogging influences tree species distributions (Pryor et al. 2006,

Piper et al. 2008) and abundance (Vreugdenhil et al. 2006). The knowledge of how seedlings respond to flooding is crucial to understanding the processes that determine plant community structure (Kozłowski 1997). Some waterlogging tolerant species from swamp forests of Central and North America are not

able to germinate, and their seedlings have low growth rates when exposed to waterlogging for days or weeks (Urquhart 2004). Waterlogging stress can be more damaging during the growth season of woody species than during the dormant period (Kozłowski and Pallardy 1997). During waterlogging, plants can be exposed to variable soil oxygen concentrations and, depending on the duration of water saturation, plants can experience hypoxia, anoxia and/or re-oxygenation (Blokhina et al. 2003).

Waterlogging decreases plant shoot metabolic rates, stomatal conductance, hydraulic conductance, transpiration, respiration and photosynthetic rates (Else et al. 1995, Visser et al. 2003). Reductions in photosynthesis are related to diffusional limitations due to stomatal closure and due to non-stomatal limitations (Pezeshki 2001). However, some species show tolerance (Peeters et al. 2002) or acclimation to waterlogging (Pryor et al. 2006, Piper et al. 2008). Tolerant woody species can maintain the water potential and photosynthetic activity of leaves after a period of acclimation (Sena Gomes and Kozłowski 1980, Li et al. 2004, Kogawara et al. 2006). Additionally, intermittent periods of waterlogging, unlike permanent waterlogging, could cause more stress due to the re-oxygenation of roots and subsequent production of reactive oxygen species in roots and shoots (Kalashnikov et al. 1994, Biemelt et al. 2000, Geigenberger 2003).

The gradual decrease of soil oxygen allows for the expression of particular plant morphological features related to the avoidance of or tolerance to hypoxia and anoxia. Examples of such features include the production of new adventitious roots that increase oxygen mobilization (Bloom et al. 1994) and the production of structural barriers to avoid oxygen loss at the root level (Voesenek et al. 2006). In addition, biochemical adjustments such as the activation of fermentation pathways (Ingamberdiev and Hill 2004) and/or de novo synthesis of some oxygen mobilizing enzymes (Hebelstrup et al. 2012) can occur during waterlogging exposure.

When flooding occurs gradually, or as a series of pulses increasing in intensity and/or duration, this allows for plant acclimation to the associated stress (Gibbs and Greenway 2003). In some herbaceous species such as maize, a minimum of 2 h of exposure to hypoxia leads to acclimation to waterlogging (Chang et al. 2000). Acclimation to waterlogging could imply internal signaling, such as the production or redistribution of hormones that increase tolerance to hypoxia and anoxia (Dat et al. 2004, Bailey-Serres and Voesenek 2008).

Waterlogging tolerance has been studied in tropical and subtropical forest species around the world (Fernández et al. 1999, Lopez and Kursar 1999, Fernández 2006, Kogawara et al. 2006, Mielke and Schaffer 2010). Studies related to forest management of seasonal flooding areas have revealed that some of the most tolerant species to waterlogging, *Alnus glutinosa*, *Salix alba* and *Taxodium distichum*, are found in the Northern Hemisphere (Anderson and Pezeshki 1999, Vreugdenhil et al. 2006). All of these species produce extensive adventitious

roots during waterlogging (Glenz et al. 2006) as also occurs in waterlogging tolerant species growing in Brazilian floodplains (Parolin 2009). Despite this, there is little information about the physiological mechanisms that confer tolerance to waterlogging in temperate forests of the southern hemisphere (Pryor et al. 2006), and there is even less information regarding evergreen swamp forest species from southern South America. From the one study that has been conducted, it is known that some species are capable of acclimating to waterlogging by not shedding their leaves when they are exposed to gradual waterlogging (GW) for periods of 2 weeks (Piper et al. 2008). However, there is no information about the key physiological and morphological adjustments that allow for this acclimation (such as: gas exchange maintenance and/or adventitious root formation). Likewise, it is unknown if other evergreen swamp forest species can acclimate to waterlogging.

Temperate swamp forests in Chile are patchy in distribution and are found from 35° to 45°S. The soils of these forests drain slowly (Schlatter et al. 2003) such that there is constant standing water 6–8 months of the year (Ramírez et al. 1996). In these areas, there is abundant winter rainfall (1500–2000 mm per year), and natural waterlogging is often an intermittent phenomenon associated with diurnal and/or seasonal variation in precipitation and evapotranspiration (Thiers et al. 2007). In Southern Chile, swamp forests are considered to be highly threatened and are highly vulnerable to the extreme decreases in precipitation and increases in temperature associated with climate change projected for this region (Montecinos and Aceituno 2003, Correa-Araneda et al. 2011). Increases in temperature could produce more damage during waterlogging due to lower oxygen solubility at lower temperatures (Jurczyk et al. 2016). Common evergreen tree species in swamp forests in Southern Chile include the Myrtaceae: *Myrceugenia exsucca*, *Luma apiculata*, *Blepharocalyx cruckshanksii* and the Winteraceae: *Drymis winteri*. The abundance of these species differs depending on the duration of waterlogging (Ramírez et al. 1996). *Myrceugenia exsucca* and *B. cruckshanksii* plants are abundant in areas where permanent waterlogging occurs. Contrastingly, *D. winteri* and *L. apiculata* grow along riverbanks that experience intermittent waterlogging (Ramírez et al. 1996, Donoso 2006). It is unknown how these species survive hypoxia or anoxia, whether or not they are capable of acclimating to GW and if physiological processes are maintained during waterlogging.

Our aim was to study waterlogging tolerance and acclimation in these four Chilean swamp tree species. To do so, we evaluated gas exchange parameters (photosynthesis, stomatal conductance) and some morphological responses (adventitious roots, lenticels, stomatal density) of individuals subjected to two controlled waterlogging treatments (sudden and gradual). We hypothesized that species more abundant in the forest border (*L. apiculata* and *D. winteri*) would have higher rates of survival and higher acclimation capacities compared with inner swamp

forest species (*B. cruckshanksii* and *M. exsucca*). We expected that the species from the forest border would maintain photosynthesis and transpiration during intermittent waterlogging. In contrast, we hypothesized that the species that are more abundant in the inner swamp forest would have a greater number of adventitious roots and lenticels in order to avoid low internal oxygen concentrations.

## Materials and methods

### Plant material and experimental design

Seeds were collected in 2010 from a swamp forest in San Martín, a private protected area belonging to Universidad Austral de Chile, 20 km north of Valdivia, Chile (39° 39' S, 72° 58' W). This site is located in the Valdivian rainforest ecoregion and shows a temperate rainy climate with Mediterranean influence, with a mean annual temperature of 12 °C and annual rain of 2300 mm<sup>3</sup>; these rains are produced mainly during May to September (Di Castri and Hajek 1976). Light intensity in the site where studied species live ranges from 0.5% to 10% of the open canopy, seedlings being mainly located in the range of 4–4.5% of the open canopy light intensity (J.P.Mora, personal communication). In this environment seeds were collected from 10 plants per species. Seedlings were generated in a common greenhouse. Pots containing organic soil were used to maintain 50 seedlings of each species for 1 year. The seedlings were kept at field capacity with initial fertilization (N, P and K). When the seedlings were 1 year old, three groups (12 seedlings per group) of each species were randomly selected for the application of the waterlogging treatments. Three treatments were used as follows: control (C) kept at field capacity, sudden waterlogging (SW) and GW. In order to study potential acclimation capacity, the entire root systems of the seedlings were submerged for 15 days for gradually and progressively longer periods of waterlogging following the method described by Piper et al. (2008). Briefly, we used short expositions to waterlogging (from 1 to 6 h) during the first 5 days of the gradual treatment, and then we increased the duration of this episodic waterlogging exposition to 12 h (next 6–10 days) or 24 h for each day (next 11–15 days), respectively. Then after 15 days, seedlings were kept permanently waterlogged for 45 days, after which the treatment was terminated. For the SW treatment, seedlings were waterlogged for 60 days straight. The water level at the base of the shoots was kept constant in both GW and SW; water was added when necessary to maintain the same shoot submersion level. Seedlings were maintained at greenhouse conditions, from 15 July 2011 to 15 September 2011; temperature and light were registered using a HOBO sensor (Pendant<sup>R</sup> temperature/Light Data Logger 64 k-(UA-002-64)), temperatures fluctuated between 8 and 25 °C, with a mean of 15 °C and the light intensity ranges from 60 to 350  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , with a mean of 130  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . After 2 months of treatment, the C, GW and SW seedlings were harvested and their dry weight was determined. Shoot/root ratio

was calculated for each species and treatment. In addition, survival rate at the end of the waterlogging and control treatments was recorded. At the beginning and the end of the each treatment, height was determined using 10 randomly selected seedlings of each species. Relative growth rate considering variation in biomass and height was determined. In order to determine dry weight, samples were dried at 60 °C on a stove (LSI-S MMM Medcenter, München, Germany) and then weighed using a Radwag AS 220/C/2 analytical balance (Radom, Poland).

### Leaf gas exchange determination

In order to determine daily variation in stomatal conductance, we used an ADC-LCA4 infrared gas analyzer (Analytical Development Co., Hoddesdon, UK). Three times a day, at: (i) 9:00–10:00 h, (ii) 12:00–13:00 h and (iii) 16:00–17:00 h, stomatal conductance (gs) was measured in ambient light, CO<sub>2</sub> and temperature in the greenhouse conditions. These determinations were performed on four clear days using five fully expanded leaves for each plant ( $n = 6$ ), before applying the waterlogging treatments. Light intensity ranged from 100 to 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  during these leaf gas exchange determinations. Before these determinations we built light responses curves to determine the saturating light intensity for each specie; this value was around 180–200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Considering the light response curves all photosynthetic determination were measured close to maximum photosynthetic response. During the waterlogging experiments described above (GW and SW), stomatal conductance was measured using the same method on Days 0, 6, 8, 21, 25, 29, 32, 40, 50 and 60. Using the same instrument and after 30 days of waterlogging treatment, photosynthetic rate (A), stomatal conductance (gs) and transpiration rate (E) were determined. Then water-use efficiency (WUE) was calculated using the A/E ratio. The correlation between A and gs for each species and for both the GW and SW treatments was also determined.

### Morphological measurements

After 2 months of treatment (GW, SW and C), the specific leaf area (SLA) was determined from harvested samples using the same leaves from which A was measured. Pictures of leaves were produced using the Imagem program and dry weight was determined as previously described. Specific leaf area was determined using freshly collected leaves (fully expanded leaves) produced before waterlogging (called old leaves) and leaves produced during the waterlogging treatments (new fully expanded leaves, called new leaves). Additionally, prints of fresh fully expanded new leaves were taken and observed under an Olympus CX 21 40 $\times$  optic microscope. Subsequently, the program Imagem was used to determine stomatal density (StD); values were expressed as per mm<sup>-2</sup> following methods described by Miller-Rushing et al. (2009).

The presence of adventitious roots and lenticels was evaluated in freshly harvested plants at the end of the waterlogging

treatments. Lenticel area from the base of the stem was measured and expressed as relative area considering the total height of the stem. The number of adventitious roots was recorded for each seedling for each waterlogging treatment. Also, the frequency of seedlings with adventitious roots and/or lenticels was measured.

### Statistical analysis

A one-way ANOVA followed by Tukey's a posteriori test were used to determine the effect of treatment on the seedlings of the four species studied. Pearson correlations were made for determining the relationship between A and gs measured at different times throughout both the GW and SW experiments. Significance was determined at  $P \leq 0.05$ .

Table 1. Survival rate (in brackets) and shoot/root ratio of *Drimys winteri* (D.w.), *Myrceugenia exsucca* (M.e.), *Blepharocalyx cruckshanksii* (B.c.) and *Luma apiculata* (L.a.) seedlings harvested after 2 months of being subjected to one of two waterlogging treatments (gradual or sudden) and control (field capacity). Each treatment (GW, SW and C) had 12 independent seedlings. There were no significant differences in survival rate among treatments. Chi-squared of survival:  $c^2_{\text{obs}} = 0.13$  and  $c^2_{\text{tab}} = 12.59$ . Lowercase letters denote significant differences in shoot/root ratio among treatments considering the same species.

	Control	Gradual	Sudden
D.w.	(100) $1.6 \pm 0.4$ a	(87) $1.3 \pm 0.1$ a	(93) $2.8 \pm 0.6$ a
M.e.	(100) $2.0 \pm 0.1$ a	(100) $1.8 \pm 0.2$	(100) $1.8 \pm 0.1$ a
B.c.	(100) $1.1 \pm 0.1$ b	(100) $2.3 \pm 0.5$	(100) $2.4 \pm 0.5$ a
L.a.	(100) $2.2 \pm 0.2$ a	(100) $1.7 \pm 0.1$ b	(100) $1.9 \pm 0.1$ ab

## Results

### Survival and growth during waterlogging

A large percentage, 78–100%, of all studied species survived 60 days of waterlogging. Furthermore, no significant differences in survival were found between treatments (Table 1). Specifically, 100% of all *D. winteri* and *M. exsucca* in all treatments survived, and seedling of all species grew under GW and SW. However, the relative height increment (RHI) of waterlogged (GW > SW) *D. winteri* was lower than that of *D. winteri* in the C treatment (Figure 1). Despite this, seedlings of *D. winteri* in the C and GW treatments had the greatest increment in height of all species and treatments; specifically the height of those in the C treatment increased by 110% and those in the GW treatment increased by 70% (Figure 1). In all treatments, seedlings of *B. cruckshanksii* had the lowest RHI (close to 20%), while *M. exsucca* and *L. apiculata* had intermediate RHI values (around 60%) (Figure 1).

All plants maintained their leaves during waterlogging, and shoot/root ratios ranged from 1.1 (*B. cruckshanksii*) to 2.8 (*D. winteri*) (Table 1). No species showed shoot/root ratio differences between GW and SW (Table 1). Both, *B. cruckshanksii* and *L. apiculata* showed significant differences on shoot/root ratios compared with the control. The shoot/root ratio in *B. cruckshanksii* seedlings had higher values during waterlogging (GW, SW) and lower values in *L. apiculata* seedlings under GW.

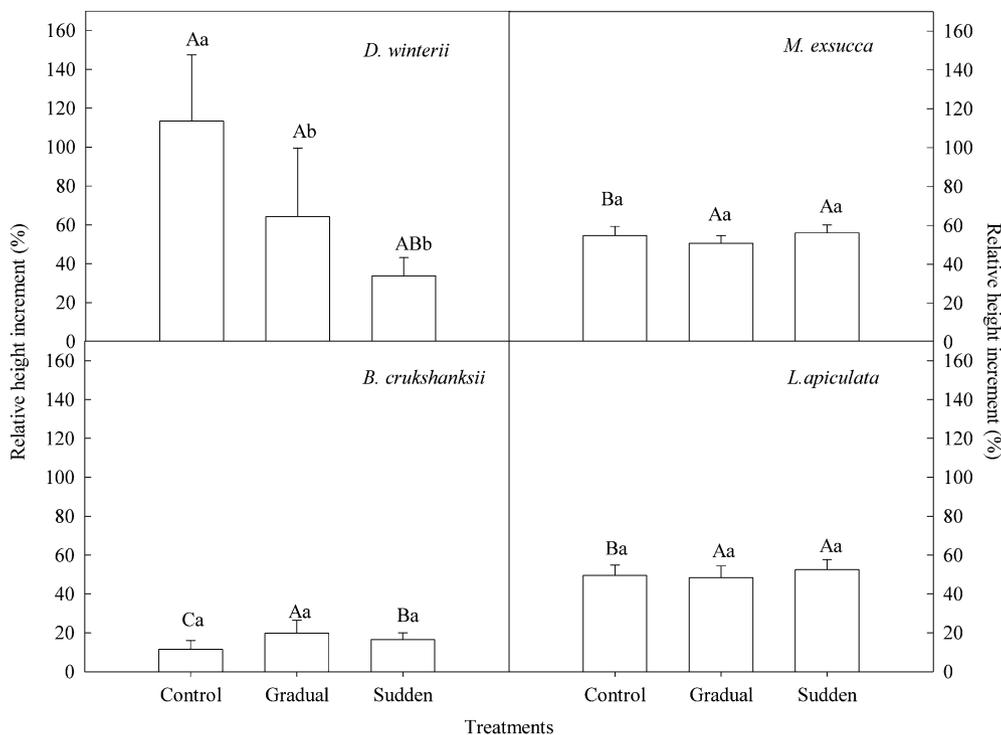


Figure 1. Relative height increment in *Myrceugenia exsucca*, *Blepharocalyx cruckshanksii*, *Luma apiculata* and *Drimys winterii* seedlings after waterlogging treatments (gradual, GW or sudden, SW) or control (C) conditions under field capacity. Capital letters show significant differences between species and lowercase letters shows significant differences between treatments ( $P < 0.05$ ).

### Morphological parameters during waterlogging

Seedlings of *L. apiculata* and *M. exsucca* did not produce lenticels under GW. However, the frequency of individuals of *B. cruckshanksii* and *D. winteri* with lenticels ranged from 67% to 100%, respectively. Seedlings of *D. winteri* in both the control and waterlogging treatments had lenticels (see Supplementary Data available at [Tree Physiology Online](#)). In the GW treatment, the area of the stem covered by lenticels was slightly higher for seedlings of *B. cruckshanksii* (14% of the total stem) than for seedlings of *D. winteri* (5% of the total stem) (see Supplementary Data available at [Tree Physiology Online](#)).

Adventitious roots were observed in all four waterlogged species, and the frequency of seedlings that had these structures ranged from 33% to 67%; seedlings of *B. cruckshanksii* subjected to GW and SW had the most adventitious roots with a mean of  $13 \pm 7$  of these structures per plant. Seedlings of the other species had approximately three adventitious roots per plant. Pictures of some of these structures are included in the Supplementary Data available at [Tree Physiology Online](#).

There were significant differences in SLA among the treatments; values ranged from  $170 \text{ cm}^2 \text{ g}^{-1}$  in *L. apiculata* (GW) to  $348 \text{ cm}^2 \text{ g}^{-1}$  in *B. cruckshanksii* (SW) (Table 2). The SLA of individuals in the control treatment (new leaf, Table 2) did not differ among species. However, the SLA of *B. cruckshanksii* from leaves produced under GW or SW was significantly higher than the SLA of similar leaves of the other studied species. Also, the SLA of new fully expanded leaves of *M. exsucca* and *B. cruckshanksii* was two to three times higher than the SLA of old leaves of these same species (Table 2).

Stomatal density differed significantly among species, ranging from 61 to  $537 \text{ mm}^{-1}$  (*D. winteri* in C and *L. apiculata* in SW, respectively) (Table 3). Fully expanded *D. winteri* leaves had the largest stomata, and *M. exsucca* had the smallest stomata (see Supplementary Data available at [Tree Physiology Online](#)). Compared with the other species, the StD of *L. apiculata* and *B. cruckshanksii* increased the most when subjected to GW and SW; the StD of leaves in the GW and SW treatments was two to three times higher than the StD of leaves in the control treatment (Table 3). In addition,  $g_s$  and StD were found to

be positively correlated ( $R = 0.7$ ;  $P \leq 0.005$ ) for all species and treatments.

### Transpiration and photosynthetic performance during waterlogging

In the control treatment, stomatal conductance differed significantly among species (Figure 2); *D. winteri* had the lowest  $g$  value ( $\sim 0.05 \text{ mol m}^{-2} \text{ s}^{-1}$ ) and *M. exsucca* had the highest ( $\sim 0.25 \text{ mol m}^{-2} \text{ s}^{-1}$ ). The stomatal conductance of all studied species was highest during the morning (9:00–10:00 h). In general, transpiration remained consistent throughout the day (Figure 2).

The A value of all species except *L. apiculata* was higher after 30 days of both waterlogging treatments compared with the control (measurements were made during the morning) (Figure 3). In general,  $g_s$  and E were similar among treatments and species. However, the  $g_s$  and E of *L. apiculata* subjected to the waterlogging treatments (SW) were significantly higher than that of the control (Figure 3). During the first week of both waterlogging treatments, A of *L. apiculata* and *M. exsucca* increased only under SW (Figure 4). However, *D. winteri* and *M. exsucca* maintained their A at the end of the SW treatment.

Additionally, we measured variation in stomatal conductance over the course of two months of the waterlogging experiment. Differences were found among species and treatments. During GW, the  $g_s$  of *D. winteri* and *B. cruckshanksii* was lower and

Table 3. Waterlogging effects on stomatal density of ( $n^\circ$ stomata/ $\text{mm}^2$ ) *Drimys winteri* (D.w.), *Myrceugenia exsucca* (M.e.), *Brepharocalyx cruckshanksii* (B.c.) and *Luma apiculata* (L.a.) seedlings after 2 month of the waterlogging treatments (gradual or sudden) and control (field capacity). Values represent the mean of six independent samples from fully expanded leaves during each treatment. Capital letters show significant differences between species and lowercase letters show significant differences between treatments ( $P < 0.001$ ).

Species	Control	Gradual	Sudden
<i>D.w</i>	$61.3 \pm 4.5$ Ca	$90.7 \pm 12.8$ Ca	$93.3 \pm 16.7$ Ca
<i>M.e</i>	$248 \pm 18.5$ Aa	$257.3 \pm 5.6$ Ba	$374.7 \pm 52.5$ Ba
<i>B.c</i>	$223.3 \pm 5.6$ ABc	$324.7 \pm 12.3$ ABb	$438 \pm 18.2$ ABa
<i>L.a</i>	$186.7 \pm 2.3$ Bb	$363.3 \pm 36.4$ Aab	$537.3 \pm 9.9$ Aa

Table 2. Effect of waterlogging on leaf morphology of *Drimys winteri* (D.w.), *Myrceugenia exsucca* (M.e.), *Brepharocalyx cruckshanksii* (B.c.) and *Luma apiculata* (L.a.) seedlings after 2 months of the waterlogging treatments (gradual or sudden) and control (field capacity). Specific leaf area (SLA = leaf area/leaf dry mass;  $\text{cm}^2 \text{ g}^{-1}$ ) of mature leaves expanded before the waterlogging treatment (old leaf) and leaves expanded during waterlogging (new leaf). Capital letters denote significant differences between species of the same leaf type, lowercase letters denote significant differences between treatments of the same leaf type, and asterisks show significant differences between leaf types of the same species ( $P < 0.05$ ).

Species	Control		Gradual		Sudden	
	New leaf	Old leaf	New leaf	Old leaf	New leaf	Old leaf
<i>D.w.</i>	$220 \pm 32$ Aa	$172 \pm 58$ Aa	$177 \pm 32$ Ba	$156 \pm 32$ Aa	$223 \pm 31$ Ba	$162 \pm 39$ Aa
<i>M.e.</i>	$199 \pm 14$ Aa*	$81 \pm 3$ Ba*	$274 \pm 6$ ABa*	$102 \pm 12$ Ba*	$310 \pm 42$ ABa*	$98 \pm 7$ Ba*
<i>B.c.</i>	$232 \pm 19$ Ab*	$134 \pm 5$ ABa*	$292 \pm 31$ Aab*	$140 \pm 6$ ABa*	$348 \pm 17$ Aa*	$137 \pm 19$ ABa*
<i>L.a.</i>	$252 \pm 20$ Aa*	$126 \pm 12$ ABa*	$170 \pm 30$ Ba	$144 \pm 32$ ABa	$225 \pm 36$ Ba	$156 \pm 20$ ABa

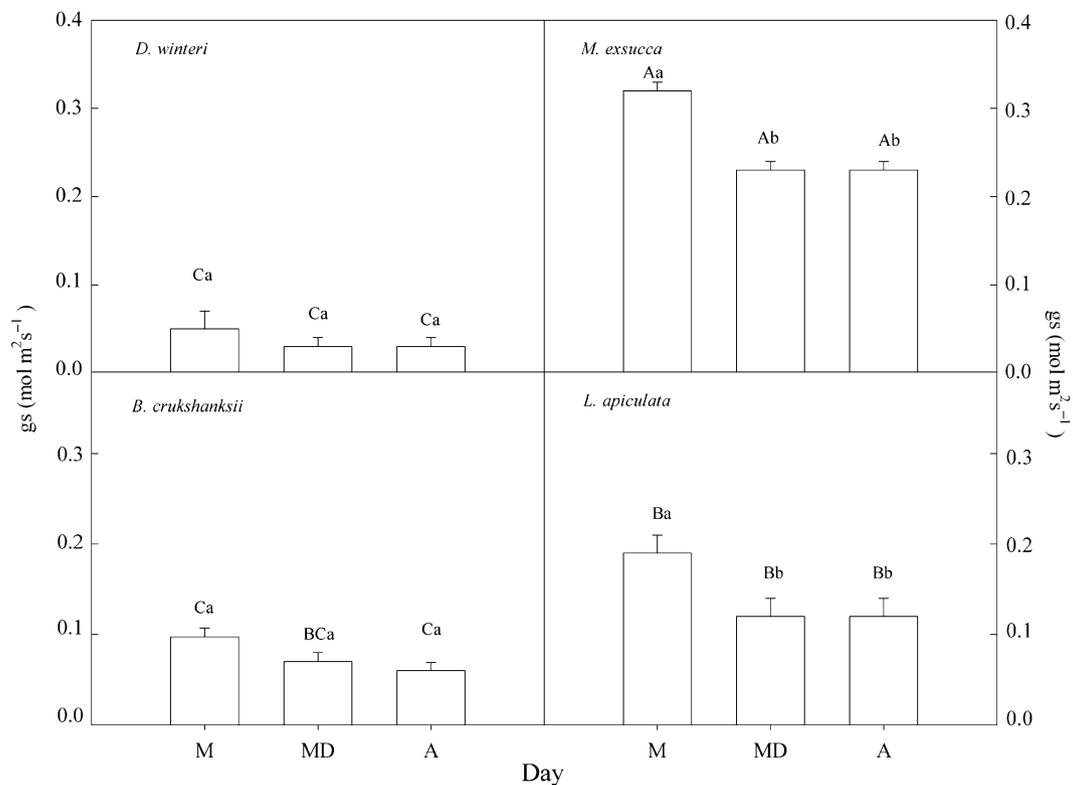


Figure 2. Daily stomatal conductance of four species *Myrceugenia exsucca*, *Blepharocalyx cruckshanksii*, *Luma apiculata* and *Drimys winterii*, taken at three time points: morning, 9:00–11:00 h (M), mid-day, 12:00–14:00 h (MD) and afternoon, 16:00–18:00 h (A). Each value is the mean of six independent determinations  $\pm$  standard error. Capital letters show significant differences between species at the same time of day, and lowercase letters show significant differences between time of day for each species ( $P < 0.001$ ).

more constant ( $\approx 0.2 \text{ mol m}^{-2} \text{ s}^{-1}$ ) than that of *M. exsucca* and *L. apiculata*. During both waterlogging treatments, the stomatal conductance of all species decreased, however this occurred at different time points (Figure 4). It should be noted that the stomatal conductance of *L. apiculata* increased during the first days of both GW and SW; the gs of this species was highest in the SW treatment ( $0.9 \text{ mol m}^{-2} \text{ s}^{-1}$ ), and this value was three times the initial value (before waterlogging). Additionally, a positive and significant correlation between photosynthetic rate (A) and stomatal conductance (gs) was only found for *L. apiculata* subjected to GW ( $R = 0.659$ ;  $P < 0.001$ ) (Figure 5).

We summarized the effect of both waterlogging treatments (GW, SW) on the four studied species, in survival, growth and both morphological and physiological parameters evaluated. In general, the effect of waterlogging was neutral or positive in all seedlings of different species, and depending of the duration to waterlogging exposition species showed different responses (Table 4).

## Discussion

The results reveal that all of the species studied had high tolerance to waterlogging and high rates of survival; some species even

maintained growth after 2 months of waterlogging and experienced no loss of leaves. In this sense, our initial hypothesis is only partially accepted because no differences in survival were found among species. Only the Winteraceae, *D. winteri*, showed acclimation to waterlogging as we proposed for microsite swamp border areas. The capacity to acclimate to waterlogging has been detected in evergreen temperate forest species from southern South America such as *Nothofagus nitida*; this species sheds its leaves mainly in response to SW, though shedding does not occur during GW (Piper et al. 2008). Despite this being known for *N. nitida*, acclimation to waterlogging has not previously been detected in the Myrtaceae swamp forest species studied here. High tolerance to waterlogging has been observed in other Myrtaceae pioneers species such as *Malaleuca cajuputi* (Kogawara et al. 2006). These authors reported a high tolerance to hypoxia/anoxia; when seedlings were subject to hydroponic conditions provided by  $\text{N}_2$  as a source of air, this species maintained growth rate, photosynthesis, stomatal conductance and sugar translocation during short-term root anoxia (17 days), in opposition to *Eucalyptus camaldulensis*, a moderately flood-tolerant Myrtaceae species (Kogawara et al. 2006). It seems that that how fast waterlogging occurred led species to show different acclimation capacities. As an example, the maintenance of leaf expansion and growth have also been reported for several tropical South

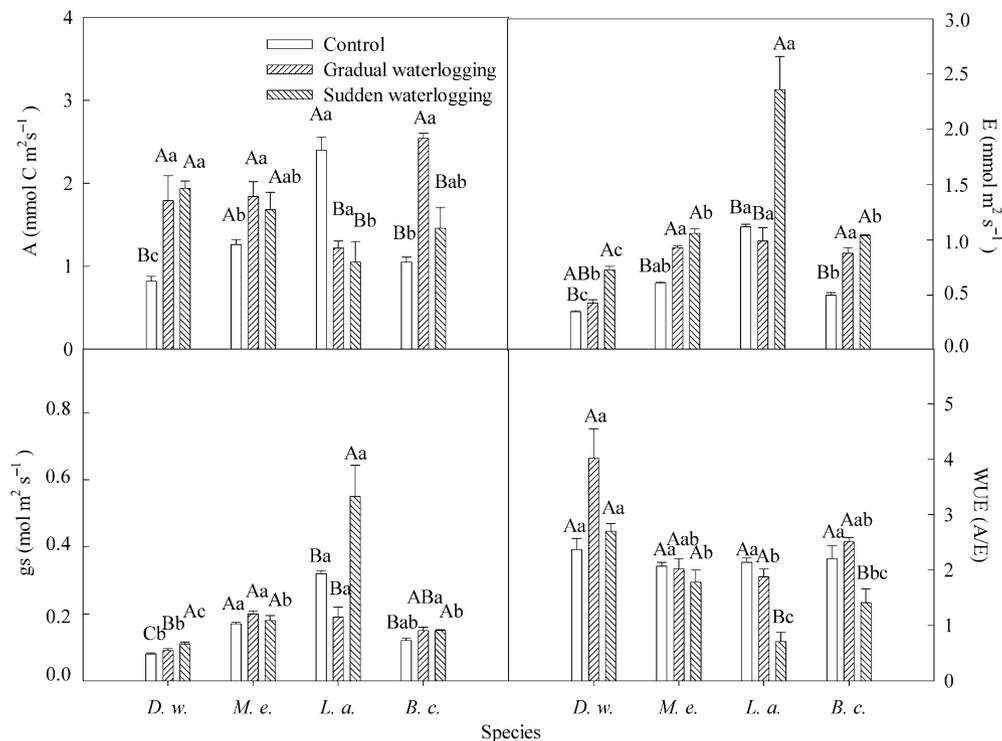


Figure 3. Effect of waterlogging on photosynthetic parameters of *Myrceugenia exsucca* (*M.e.*), *Brepharocalyx cruckshanksii* (*B.c.*), *Luma apiculata* (*L.a.*) and *Drimys winteri* (*D.w.*) seedlings after 1 month of the waterlogging treatments (gradual, GW or sudden, SW) and field capacity control (C). Shown are photosynthetic rate (A;  $\mu\text{mol of C m}^{-2} \text{s}^{-1}$ ), stomatal conductance (gs;  $\text{mol m}^{-2} \text{s}^{-1}$ ), transpiration rate (E;  $\text{mmol m}^{-2} \text{s}^{-1}$ ) and water-use efficiency (WUE; A/E). Capital letters show significant differences between treatments for the same species and lowercase letters show significant differences between species ( $P < 0.05$ ).

American waterlogging tolerant species that experience seasonal flooding with different depth water, without negative effect on growth (Parolin 2000, Herrera et al. 2008).

### Physiological responses to waterlogging

Considering the photosynthetic response of seedlings exposed to waterlogging for 1 month, acclimation was also detected in *D. winteri*, *M. exsucca* and *B. cruckshanksii*. This result is only partially in agreement with our predictions; we expected that border species (*L. apiculata* and *D. winteri*) would better maintain photosynthesis and would more readily acclimate to waterlogging than would inner swamp forest species (*B. cruckshanksii* and *M. exsucca*). Additionally, the photosynthetic rate of *B. cruckshanksii* was two times higher after 1 month of GW. This unexpected high value could represent photosynthetic acclimation to waterlogging such as that observed for the fully expanded leaves of other waterlogging tolerant species including *Campsiandra laurifolia*, *Symmeria paniculata* and *Eschweilera tenuifolia* from tropical South America. For these species, higher photosynthetic rates have been detected when river water rises 5 m compared with periods of initial rising water (Herrera et al. 2008). In this case, these increases in photosynthetic rate during extreme waterlogging are correlated with increased concentrations of soluble leaf protein and Rubisco carboxylation efficiency measured from leaves emerging above the water. It is likely that in our

experiment the seedlings had different acclimation capacities, and these were revealed at different time points during the waterlogging treatment. Unfortunately, we did not measure leaf protein concentrations, so we are unable to comment on the mechanisms involved in the maintenance or increase of carbon exchange. In general, in our experiment all of these species maintained net carbon assimilation (A) after 1 month of both waterlogging treatments, indicated varying degrees of photosynthetic and stomatal conductance plasticity. During the initial days of GW, the photosynthetic rate of *L. apiculata* and *M. exsucca* increased, though it later decreased. Conversely and even at the end of GW, the photosynthetic rate of *D. winteri* was low but positive. Similarly, the photosynthetic capacity of some *Lolium perenne* genotypes is upregulated during low-temperature waterlogging (Jurczyk et al. 2015); however, prolonged flooding decreases carbon exchange due non-stomatal limitations.

Because, *L. apiculata* is abundant along riverbanks (Ramírez et al. 1996), it is possible that this species is able to maintain positive carbon exchange during intermittent waterlogging. Additionally, *L. apiculata* has been shown to increase its photosynthetic rate during the next 15 days after waterlogging during a recovery period (Zúñiga-Feest et al. 2011); however, this species is not able to maintain photosynthesis after 2 months of waterlogging. It has been suggested that the point at which waterlogging down-regulates photosynthetic and stomatal

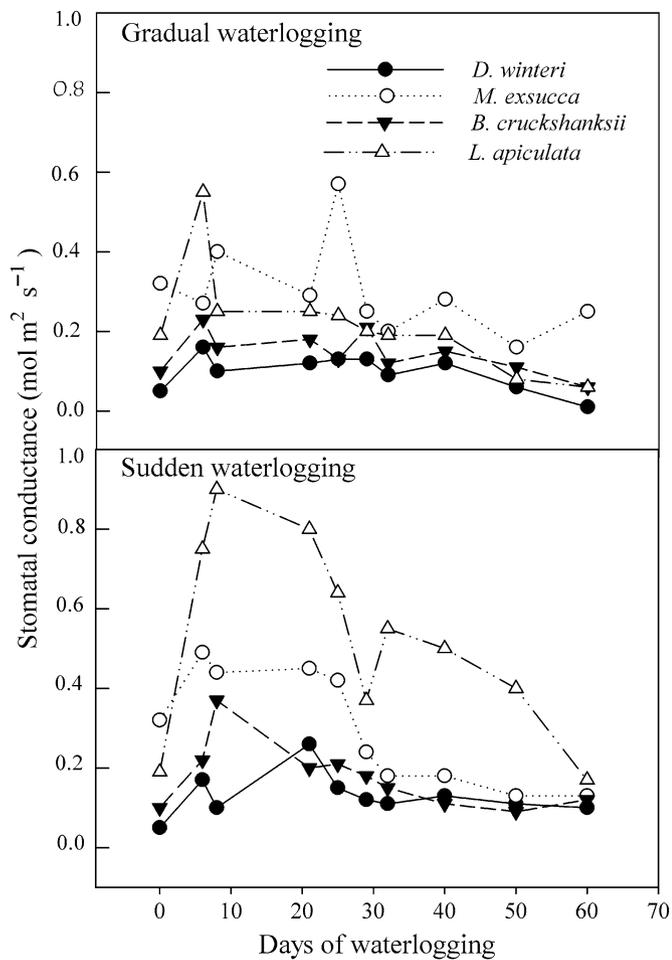


Figure 4. Kinetic leaf conductance (g) of four swamp forest species: *Myrceugenia exsucca*, *Blepharocalyx cruckshanksii*, *Luma apiculata* and *Drimys winterii*. Each value is the mean  $\pm$  standard error of three independent determinations from fully expanded leaves at day light intensity. See details in Materials and methods. Lowercase letters show significant differences between different days of the experiment ( $P \leq 0.05$ ).

conductance depends on the intrinsic anatomical differences, leaf age and rubisco content of waterlogging tolerant species (Herrera et al. 2008). Photosynthetic rate and stomatal conductance were positively correlated only in *L. apiculata* subject to GW ( $R = 0.659$ ;  $P < 0.001$ ). Additionally, this species had the highest internal carbon leaf concentration ( $C_i$ ) and the lowest rate of  $C_i$  use, compared with the other studied species when was measured on a light response curve (A. Bustos-Salazar, personal communication). These results suggest that this species is capable of fine control of its mesophyll conductance and/or rubisco carboxylation efficiency. However, further research is needed to explain the observed differences in photosynthetic performance of leaves of different swamp forest species.

The maintenance of a positive carbon balance has been indicated as a key mechanism that explains the differential survival of two riparian tree species subjected to waterlogging (Li et al. 2015). Flood-tolerant species such as *T. distichum* and *Fraxinus pennsylvanica* sustain photosynthesis even when in conditions hypoxic to roots (Kogawara et al. 2006). During waterlogging, photosynthesis is generally reduced by stomatal closure triggered by root hypoxia (Mielke et al. 2003, Islam and Mac Donald 2004); however, resistant species might maintain stomatal functioning during exposure to waterlogging, as generally occurs in the temperate swamp forest species studied here. *Ulmus laevis* (resistant to waterlogging) exhibits pulses of increments in photosynthesis and stomatal conductance during the first 20 days of exposure to a waterlogging treatment. However after 60 days of exposure, photosynthesis and stomatal conductance decrease (Li et al. 2015). Under similar experimental conditions the photosynthetic rate and stomatal conductance of *Ulmus minor*, a species sensitive to waterlogging, progressively decrease over the course of 30 days, however  $C_i$  is maintained constant. These reductions of photosynthetic rate during long

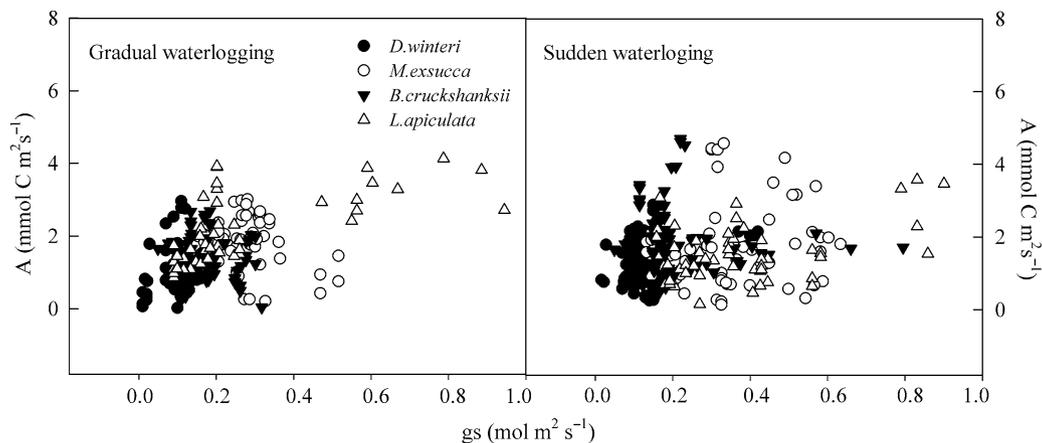


Figure 5. Pearson's correlation of photosynthetic rate (A;  $\mu\text{mol C m}^{-2} \text{s}^{-1}$ ) and stomatal conductance (gs;  $\text{mol m}^{-2} \text{s}^{-1}$ ) of *Drimys winterii* (filled circles), *Myrceugenia exsucca* (open circles), *Blepharocalyx cruckshanksii* (filled triangles) and *Luma apiculata* (open triangles); species were subjected to gradual and sudden waterlogging treatments for 2 months, as described in Materials and methods.

Table 4. Summarized effects of waterlogging treatments (GW, SW) on different parameters in seedlings of four swamp forest species, as follows: *Drimys winteri*, *Myrceugenia exsucca*, *Brepharocalyx cruckshanksii* and *Luma apiculata*. Negative effect indicated as (-), neutral effect (O) or positive effect (+), comparing with the C. The intensity of each effect was denoted by a higher number of (++) or (+++).

	<i>D. winteri</i>	<i>M. exsucca</i>	<i>B. cruckshanksii</i>	<i>L. apiculata</i>
Survival	O	O	O	O
Growth (height)	- <sup>1</sup>	O	O	O
A (1 month)	+	+	+ <sup>2</sup>	-
gs (1° 15 days)	O	++	+	+++
gs (1 month)	O	O	O	+
SLA	O	+	++	O
Stomatal density	O	O	++	+ <sup>1</sup>
Shoot/root	O	O	+	O <sup>2</sup>
Adventitious roots	+	+	++	+
Lenticels	+	O	+	O

<sup>1</sup>Effect only observed in SW treatment.

<sup>2</sup>Effect only observed in GW.

periods of waterlogging could be explained by the reduction of CO<sub>2</sub> carboxylation by rubisco. In addition, this mechanism has been proposed for genotypes of *Citrus* that are moderately tolerant to waterlogging (Hossain et al. 2009). Reduction of mesophyll conductance has been proposed as a phenomenon that limits photosynthesis rate when stomatal closure has not yet occurred (Galmés et al. 2007) and could occur during water limitation (drought) or water excess (waterlogging).

Like other mechanisms to alleviate waterlogging stress, biochemical adjustments could also occur in herbaceous species, where activation of fermentation pathways has been identified during waterlogging (Bailey-Serres et al. 2012). In swamp forest species tolerant to waterlogging, the production of photoassimilates likely maintains the transport of sugars from the mature leaves (source) to the roots and new leaves (sink), and this likely continues even during waterlogging in opposition to sensitive to waterlogging species, which close their stomata, thereby decreasing carbon gains and halting the transport of sugar to sink organs (Kogawara et al. 2006). It is probable that in our experiment the seedlings maintain carbon gains and sugar transport during waterlogging, because it is in agreement with the lack of reduction in biomass growth rates observed here and additionally, with the increase in the shoot/root ratios of *D. winteri* and *B. cruckshanksii* during SW. Also, the notable production of new leaves by *M. exsucca* indicates that growth is not limited during waterlogging (see Supplementary Data available at [Tree Physiology Online](#)).

### Morphological responses to waterlogging

We initially hypothesized that inner swamp species would have more adventitious roots and lenticels than border swamp species. However, all studied species had adventitious roots, and only *B. cruckshanksii* and *D. winteri* had lenticels. The relative area of the shoot with lenticels and the mean number of adventitious

roots were highest in *D. winteri* and *B. cruckshanksii*. In addition, *D. winteri* seedlings had the highest frequency of lenticels, both in the control and when subjected to SW. This suggests that these structures could be a constitutive response of this species in wet habitats. Additionally, leaves expanded during waterlogging (new leaves) had high SLA and high StD; probably this anatomical change on the leaves lead an increase on it water transport compared to old leaves (produced before waterlogging).

The seedlings of species abundant in the inner swamp forest (*B. cruckshanksii* and *M. exsucca*) had the greatest increments in SLA during SW. This is contrary to what is found for Cloud Mountain trees, which have reduced leaf area during waterlogging due to hypoxia, however the leaves of these trees do maintain stable transpiration (Santiago et al. 2000). We also observed significant differences among the leaves of the studied species; all Myrtaceae seedlings had smaller stomata than did the Winteraceae. Through fine stomatal conductance regulation, small sized stomata have been related to fast and efficient responses to environmental changes (Franks et al. 2009). When grown in different environments, Myrtaceae species are known to have high plasticity in terms of StD (Cao 2000, Gamage et al. 2003). Meanwhile, large stomata have been reported in species with stomata that have a weak capacity to respond to changes in water availability, as probably occurs in *D. winteri*.

The four swamp tree species studied had high tolerance to waterlogging because they not shed their leaves and do not decrease their foliar expansion. In contrast, it has been widely reported that waterlogging-sensitive species shed their leaves and stop leaf expansion (Else et al. 1995, Lambers et al. 2008). Additionally, no variation in leaf water potential has been detected in the four studied species in similar experiments carried out under similar greenhouse conditions (F.Alves and V. Martínez, personal communication). In contrast, waterlogging has been found to significantly decrease the leaf water potential of sensitive species of Chilean temperate rainforests such as *Embothrium coccineum* and *Nothofagus dombeyi* (A.Z.-F., personal observation).

The original hypothesis made here is only partially accepted because all species survived and grew in both waterlogging treatments (GW and SW), and stomatal conductance and photosynthesis were maintained during short exposure to waterlogging (0–20 days). Also, all species studied here had adventitious roots and adjusted leaf structures, leading to increased water transport during waterlogging (higher SLA, StD). Furthermore, *L. apiculata* was highly plastic in terms of its stomatal functioning during GW; this is likely related to its distribution in swamp forest environments. The knowledge of the physiological mechanisms employed by these swamp forest species to tolerate and/or acclimate to waterlogging would help us to better understand swamp forest regeneration dynamics. This, in turn, would provide us with relevant information to decide what species would be most appropriate for restoration and forest recovery programs.

## Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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## Conflict of interest

None declared.

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