Resilience of willows (*Salix* spp.) differs between families during and after flooding according to floodwater depth

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Abstract

Although the morphological and physiological responses of willows to flooding have already been characterized, less is known about their responses during the post-flooding period. After the end of the stress episode, plants may modify some leaf and plant traits to compensate for biomass loss. The aim of this work was to analyze the post-flooding responses of different willow genotypes under two different depths of floodwater. The hypothesis was that the growth recovery in the post-flooding period would be different according to the genotype and the floodwater depth. We analyzed three genotypes of five willow families (4 interspecific hybrids and one open-pollinated family). The treatments were: 1) Control: plants watered to field capacity; 2) T10: water covering 10 cm above soil level; 3) T65: water covering 65 cm above soil level. Both flooding treatments were followed by a period of recovery (without flooding). Growth was reduced by flooding in T65 but not in T10, while root-to-shoot ratio was reduced in both flooding treatments. The relative growth rate in height, leaf nitrogen concentration, stomatal conductance and electron transport rate changed in a different manner during the post-flooding period, depending on the treatment and genetic background. These results emphasize the need for evaluating a post - flooding recovery period for the breeding of willow genotypes destined for areas under risk of flooding. According to our results, *Salix matsudana* could be a source of flooding tolerance for willow breeding programs.

Key words: relative growth rate; root-to-shoot ratio; leaf nitrogen concentration

Key message: Willows differ in their post - flooding responses according to floodwater depth and genotype

Introduction

Willows (*Salix* spp.) naturally grow near riverbanks and floodplains, and they are considered as flood - tolerant forest trees (Karrenberg 2002). As a result, willow plantations can be developed in areas with high risk of flooding, either as a source of biomass, pulp and timber (Balatinecz et al. 2014), or with the purpose of restoring disturbed landscapes (Wang et al. 2017).

The morphological and physiological responses of willows to flooding have been studied extensively, and they vary according to the genotype, the length and frequency of the stress episodes, and the depth of the floodwater (Li et al. 2004, Markus - Michalczyk et al. 2016, Doffo et al. 2017, Rodríguez et al. 2018). Nevertheless, the responses of willows during the post - flooding period have received less attention (Jackson and Attwood 1996, Wang et al. 2017).

Global warming is expected to increase the occurrence of flooding episodes in several areas of the world (Kreuswieser and Rennenberg 2014, Garssen et al. 2015). In order to cope with the challenges imposed by this scenario, it will be necessary to develop new willow genotypes combining tolerance to flooding with improved growth and wood quality. To evaluate the tolerance of a species to flooding, it is necessary to analyze the responses not only during flooding, but also through the post - flooding recovery period (Striker 2012). For instance, submerged intolerant rice cultivars survive flooding, but suffer from water stress and desiccation upon de - submergence, leading to the death of the plants (Setter et al. 2010). The sudden exposure of previously submerged plants to air may be a stressful situation because of the abrupt raise in O_2 and irradiance, which cause an increase in Reactive Oxygen Species (ROS) or photoinhibition (Luo et al. 2009). Some willow species like *Salix variegata* develop an increased protection against the post - flooding oxidative damage under complete submergence (Lei et al. 2012).

Apart from the possible damage caused by post - anoxic injury, there are several traits related to productivity in willows that may be affected by flooding, like leaf area, specific leaf area and leaf nitrogen concentration (Robinson et al. 2004, Tharakan et al. 2005). In addition to that, flooding reduces the root - to - shoot ratio in willows (Markus - Michalczyk et al. 2016, Doffo et al. 2017). These morphological and physiological changes are likely to have an impact upon growth during the post - flooding period. Willows can be divided into two major ecological groups: riparian species adapted to periodically flooded environments, and wetland species that can grow in lowlands permanently covered with stagnant water (Dickmann and Kuzovkina 2014). In this work, we analyzed the progeny of five families, combining parents of *S. alba* (typically riparian), *S. nigra* (wetland species), *S. humboldtiana* (the only native willow species in South America, Dickman and Kuzokvina 2014), and *S. matsudana,* which is able to endure repeated periods of complete submergence (Wang et al. 2017). Since the parent's habitats experience a variety of flooding regimes, we expected to find different degrees of stress tolerance in the F1 progeny.

The aims of this work were: 1) To analyze the morphological and physiological traits related to productivity in willows during the post - flooding period; and 2) To find out if these traits change differently according to the genotype and the depth of the floodwater. The hypothesis was that the growth recovery in the post - flooding period would be different according to the genotype and the depth of the floodwater.

Material and Methods

Plant material, growth conditions and stress treatment

Three genotypes of the F1 of each of five willow crosses were used in this work (15 genotypes in total); the parentage is detailed in Table 1. One family has a typically riparian mother (F9420), three families combine a riparian with a wetland species (F9408, F9802 and F13), and F9813 combines two wetland species. These individuals belong to the breeding program developed by the National Institute of Agricultural

Technology (INTA). The genotypes have already passed most selection steps of the breeding program, based on their growth, form and pest resistance.

One - year - old cuttings of 20 cm long were planted in 3.5 L pots, filled with a 1:1 mixture of soil and sand. Before planting, the cuttings were placed in water overnight, and treated with fungicides to avoid diseases. One cutting was planted per pot, and they were placed in a greenhouse with natural irradiance and under natural day length in La Plata (34° 59’ 09’’ S; 57° 59’ 42’’ W). The pots were watered daily, keeping the substrate at field capacity. Before the beginning of the treatments, plants were pruned leaving only one shoot per cutting, and fertilized twice with complete Hoagland solution (50 ml per pot, Leggett and Frere 1971).

Two flooding experiments were carried out: one with the water level at 10 cm above the soil surface (T10), and a deeper flooding treatment, with the water level at 65 cm above the soil surface (T65). In T10, only the root system was flooded, while in T65 most of the shoot was covered by water. The experiments were performed in different years (T10 during 2013 and T65 during 2014); each one had its own set of control plants (watered to field capacity) and differed in duration. Consequently, the statistical analysis was done separately for each of them. A scheme of each experiment is provided in Supplementary Fig.1. The variables measured, their abbreviations and units are detailed in Table 2.

For the T10 experiment, the cuttings were planted in pots on August 9, 2013. The treatments were: Control (watered to field capacity), and submerged in water 10 cm above soil surface (T10). Flooding started when the plants were 72 days old. The plants were flooded by placing them inside a bigger sealed pot, as previously described (Cerrillo et al. 2013). There were 6 replicates for each genotype and treatment, in a completely randomized layout (N=12 for each genotype, 36 for each family; 18 plants for control and 18 for T10 treatment). The flooding treatment started on October 21, 2013 and ended on December 20, 2013. After the end of flooding, a post - flooding recovery period of 30 days started, in which the pots were watered daily to field capacity. The final destructive measurements started on January 20, 2014, marking the end of the experiment.

In the T65 experiment, cuttings were planted on August 13, 2014. The control plants were watered daily to field capacity and the flooded plants were submerged to 65 cm above soil level (T65). The plants in the T65 treatment were placed in a pool filled with water; the water depth in the pool was checked every day and maintained at the same level by replacing the evaporated water when necessary. There were 6 replicates for each genotype and treatment, in a completely randomized layout (N=12 for each genotype, 36 for each family; 18 plants for control and 18 for T65 treatment).The flooding treatment started on October 16, 2014, when the plants were 62 days old, and lasted until November 19, 2014. After that date, it followed a post-flooding period until December 15, 2014, when the final destructive sampling started.

Growth measurements and leaf traits

Height was measured with a ruler, and basal diameter with a digital caliper. The volume index was calculated as follows:

$$VI = [(\text{basal diameter})^2 \cdot \text{total height}]$$

The Flooding Tolerance Index (FTI, Fichot et al. 2009) was determined using the VI as follows:

$$FTI = (VI_{\text{stressed}} / VI_{\text{control}}) \times 100$$

The relative growth rates of the stems (RGR), either in height or basal diameter, were determined according to Whitehead and Myerscough (1962). The individual leaf area (ILA) and the specific leaf area (SLA) were determined on the latest expanded leaf at the end of the experiment. The leaf was scanned and the area determined with the software Image J (http://rsbweb.nih.gov/ij/, Schneider et al. 2012). At the end of the experiment, the total biomass for leaves, stem and roots was determined after drying the material at 65 °C to constant weight. Root - to - shoot ratio (RSR) was calculated with those data.

Leaf nitrogen concentration was determined on a pool of leaves, using the Kjeldahl method for total nitrogen (Brenmer 1996).

Stomatal conductance and ETR determinations

The stomatal conductance (gs) was determined with a Decagon SC1 porometer and the electron transport rate (ETR) with a modulated chlorophyll fluorescence meter (Hansatech FMSII, UK). The measurements were carried out between 10.30 and 13.30 h, on cloudless days, on the latest expanded leaf. The average irradiance during the measurements was 967 μmoles m^{-2} s^{-1}. Two measurements were carried out in the T10 treatment: one during late flooding (53 days after the start of flooding for gs, 54 days for ETR) and another during the post - flooding period (24 days after the end of the flooding treatment for ETR, 26 days for gs). For the T65 experiment, measurements were performed one day and 22 days after the end of flooding for ETR, and 9 days and 20 days after the end of flooding for gs.

Statistical Analysis

The statistical analysis was carried out with R 3.2.3 (R Development Core Team 2017), using the package agricolae. The aov function was used for the ANOVA, with clone and treatment as factors, and the post hoc analysis was carried out with the LSD test.

Results

The ANOVA results are depicted in Table 3, showing family, flooding and their interaction as factors. Since T10 and T65 were carried out in different years with their own control treatments, each experiment was analyzed separately.

After one week of flooding, all genotypes developed hypertrophied lenticels and adventitious roots in the submerged parts of the stem (Suppl. Fig.2).

Dry matter accumulation (TDW) and partitioning (RSR) were different in the T10 and T65 treatments (Fig. 1). In T10, TDW was not reduced by flooding, while in T65, it was significantly reduced in all families. The RSR was reduced by both flooding treatments, but the differences were not statistically significant in the T10 treatment for the F13 and F9420 families. In T10, there was a change in dry matter partitioning without total biomass reduction, while in T65 there was a reduction in total biomass plus a change in partitioning.

The relative growth rate in height during flooding (RGRh f, Fig. 2) was different in both treatments. In T10 there was no reduction, while in T65, RGRh f was significantly reduced in all families. In the post - flooding period, there were differences in the relative growth rate in height (RGRh pf) according to family and treatment (Fig. 2); F9408 increased RGRh pf in both T10 and T65, while F9802 did not. The other families showed different responses according to the treatment, increasing in some cases and without change in others, but there was no significant reduction in RGRh pf in any case.

The relative growth rate in basal diameter during flooding (RGRd f, Fig. 3) in T10 was similar or higher than in control plants, while in T65, it was similar or lower compared to the non - stressed treatment. In the post - flooding period, there were no significant differences in RGRd pf between control and flooded plants except for F9420 in T10.

In the leaves developed during the post - flooding period, there were no differences in SLA between control and flooded plants in neither T10 nor T65, but there were differences among families (Table 3). The size of the leaves developed during the post - flooding period (ILA) was affected by genotype and treatment (Table 3).

The electron transport rate (ETR, Fig. 4) did not change in T10, neither during flooding nor through the post - flooding period. For T65, no measurements were made during flooding because most leaves were covered by water. One day after the end of flooding, there was an increase in ETR that was statistically significant in three families. This increase did not last in the post - flooding period except for F9813.

Stomatal conductance (gs, Fig. 5) was not affected by the T10 treatment, except for family F9408, which experienced a reduction in the post-flooding period. T65 was measured only in the post - flooding period, and 9 days after the end of the stress episode, there was a significant increase in gs in the previously flooded plants in all families except for F13. This effect did not last long; 22 days after the end of flooding, gs was significantly higher only in F9813.

There were no differences in nitrogen concentration per unit leaf area in the T10 treatment compared to controls (N, Fig. 6), while in T65 it was only significantly increased in F13.

The flooding tolerance index for the volume index (FTI, Fig. 7) was determined at the end of the flooding treatment and again after the post-flooding recovery period. In the T10 treatment, flooded plants had a higher above - ground biomass than controls (FTI higher than 100), while in T65, growth was reduced by flooding (FTI lower than 100). For the T65 treatment, the family ranking was similar after flooding and during the post - flooding recovery period, while in T10 it was different.

Discussion

Effect of flooding depth on leaf traits related to productivity in willows.

In a previous work, we found that leaf nitrogen concentration increased in deeply flooded but not shallowly flooded plants (Rodriguez et al. 2018). These are interesting results, since leaf nitrogen concentration correlates with the photosynthetic rate (Reich et al., 1998) and this could enable a higher photosynthetic fixation rate in the post - flooding period. However, we did not find differences in nitrogen concentration between control and T65 plants after 26 days of recovery in four families (the exception being F13). The higher leaf nitrogen concentration did not last long after the end of the flooding episode. The increment occurred in deep flooded willows which experienced a reduction in growth, but not in shallow flooded willows that have a similar biomass as non - flooded plants (Rodriguez et al. 2018). It is possible to speculate that N uptake will continue in flooded willows, as it does in flooded *Populus tremula x P. alba* plants (Kreuzwieser et al. 2004). Thus, the increase in leaf nitrogen concentration is a consequence of the continuous uptake plus the transient reduction in growth, acting as a reserve that can be used for growth after the end of flooding (Warren et al. 2003).

In addition to leaf nitrogen concentration, individual leaf area (ILA) and specific leaf area (SLA) are traits that correlate with productivity in willows (Robinson et al. 2004, Tharakan et al. 2005). Both can be modified by flooding: SLA increases in leaves under submergence (Mommer and Visser 2005) and leaf size can be reduced by flooding (Cerrillo et al. 2013). In a previous work, we found that the deeper flooding treatment increased the SLA of leaves expanded during flooding (Rodriguez et al. 2018), but we did not find any effect of treatment in the SLA of leaves developed during the post - flooding period. On the other hand, leaf size had only a moderate correlation with dry mass accumulation in T65 ($r = 0.49$, $p \leq 0.001$, $n = 150$), and a low correlation in T10 ($r = 0.27$, $p \leq 0.01$, $n = 150$).

Floodwater depth and genotypes affect growth responses and dry matter partition in the post - flooding period.

The deeper flooding treatment (T65) was a more stressful situation for willows than shallow flooding (T10). Growth in height, and to a lesser extent in diameter, was reduced during flooding in T65, but not in T10. These results were similar to those reported for *Salix alba* and *S. viminalis* (Markus - Mychalzcyck et al. 2016) and *Alnus japonica* (Iwanaga and Yamamoto 2008), where growth was more reduced with an increase in the floodwater level. The restriction on gas exchange imposed by submergence caused a lower rate of carbon fixation that may explain the lower growth in the deep flooding treatment (Luo et al. 2009). However, the occurrence of non-stomatal limitations to photosynthesis could not be ruled out. It has been shown that both stomatal and non-stomatal limitations occur in flooded plants of sunflower (Guy and Wample 1984) and poplar (Bèjaoui et al. 2006).

The relative growth rates in the post - flooding period were similar or higher in the previously stressed plants compared to the controls. This is probably related to the fact that stomatal conductance and photosynthetic activity (as ETR) during the post - flooding period were similar or higher in previously flooded plants compared to the control treatment. In flood - sensitive species, stomatal closure persist beyond the end of the hypoxia (Sojka 1992), but this is not the case for the *Salix* species analyzed in this work. It seems that willow leaves did not suffer an extensive damage during flooding, allowing for a fast recovery of gas exchange after the end of the stress episode. A similar behavior has been reported for other riparian species adapted to periodically flooded environments (Luo et al. 2009).

T10 and T65 both reduced the root - to - shoot ratio (RSR), because flooding arrest root growth (Jackson and Attwood 1996) and increases root mortality in willows (Markus - Michalczyk et al. 2016, Doffo et al. 2017). The difference between treatments is that T10 combines a reduced RSR with a similar dry matter accumulation as the control treatment, while in T65 there was a reduction in both RSR and total dry weight. In both flooding treatments, RSR still has not reached the same levels as the control plants after the recovery period.

In spite of the recovery of the relative growth rate in previously flooded plants of the T65 treatment, the biomass accumulation was still significantly lower compared to controls after 26 days of recovery, except for family F9813. It is possible that the other families need a longer period to recover to levels similar to those of the control treatment.

The responses of growth and leaf variables may be similar in both flooding treatments, but other responses differed among families. The tolerance index to flooding was calculated using volume index, because it showed a good correlation with total dry weight (r = 0.71 for T10 and r = 0.92 for T65). An interesting result is that the tolerance index rating for the families was different at the end of flooding and after the post - flooding period for T10, but it was similar for T65. This is not a major issue for the genotypes used here, since they are all tolerant to T10 conditions. But it is clear that tolerance differs among families for T65, and the genotypes that are more tolerant for T10 will not necessarily behave in the same way with a deeper floodwater level. This should be taken into account to recommend clones to be planted in flood - prone areas. On the other hand, the variation in response of the families analyzed show that it is possible to combine high growth with flooding tolerance in willows, and to select the best willow genotype according to the risk of flooding of the planting site.

Conclusions and perspectives

Our original hypothesis was accepted, since there were differences in the post - flooding responses according to the family and the depth of the floodwater. These results highlight the need to evaluate post - flooding responses, and not only the flooding period, in order to improve willow genotypes to be targeted to endure flooding conditions occurring in particular environments. For the deeper flooding conditions, the better performers were the families with *S. matsudana* as mother. These species could be a source of flooding resistance genes to improve willow genotypes destined to areas with risk of deep and prolonged flooding episodes.

Author Contribution Statement

GND, MER and FYO carried out the experimental work. TC selected the genotypes analyzed and contributed to the experimental design. VMCL designed the experiment, carried out the statistical analysis and wrote the paper.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1 - Plant material used in this work. *This clone is a spontaneous hybrid between a *Salix humboldtiana* mother and an unknown father.

Family	**Mother**	**Father**
F9408	*S. matsudana* NZ693	*S. alba* S7
F9813	*S. matsudana* NZ693	*S. nigra* C7-22
F9802	*S. matsudana* NZ692	*S. alba* SI58-004
F9420	*S. alba* SI64-004	Open - pollinated
F13	*S. matsudana*	S. x *argentinensis* cv "Galvete" * x *S. alba* "114-1"

Table 2 – List of variables measured in this work, with their abbreviations and units.

Variable name	***Abbreviations and Units***
Root to Shoot Ratio	RSR
Total Dry Weight	TDW (g)
Volume Index	VI (cm^3)
Relative Growth Rate for height	RGRh (cm day^{-1})
Relative Growth Rate for basal diameter	RGRd (mm day^{-1})
Leaf Nitrogen Concentration	N (μg cm^{-2})
Individual Leaf Area	ILA (cm^2)
Specific Leaf Area	SLA (cm^2)
Electron Transport Rate	ETR (μmol electrons m^{-2} s^{-1})
Stomatal Conductance	gs (mmol H_2O $m^{-2}s^{-1}$)

Table 3 – ANOVA table of the variables measured and estimated in this work. The values are those of P. The significant factors (P<0.05) are marked in bold.

Variable		***T10***			***T65***	
	Family	**Flooding**	**Interaction**	**Family**	**Flooding**	**Interaction**
RSR	0.422	**0.0001**	0.126	**0.0001**	**0.0001**	0.855
TDW	**0.0001**	0.153	0.871	**0.0007**	**0.0001**	0.1444
VI	**0.0192**	**0.0001**	0.8106	**0.0001**	**0.0001**	0.159
RGRh f	0.0784	**0.0169**	0.6736	0.1289	**0.0001**	**0.0253**
RGRh pf	0.0532	**0.0076**	0.2906	**0.0139**	**0.0001**	0.1450
RGRd f	**0.0196**	**0.0035**	0.4969	0.4481	**0.0012**	0.0549
RGRd pf	0.501	0.490	0.221	**0.0477**	0.1006	0.9509
N	0.771	0.255	0.650	**0.0307**	0.2683	0.4131
ILA	**0.0001**	**0.0004**	0.0838	**0.0001**	**0.0001**	**0.0355**
SLA	**0.0001**	0.103	0.857	**0.0095**	0.0867	**0.0156**
ETR [1]	0.3776	0.6830	0.0818	0.396	**0.0001**	0.724
ETR [2]	0.113	0.678	0.321	0.358	0.231	0.336
gs [1]	**0.0139**	0.6414	0.5176	**0.0123**	**0.0001**	0.4427
gs [2]	0.0657	0.2035	0.1893	**0.0093**	0.9096	**0.0209**

[1]: late flooding for T10 and early post-flooding for T65.

[2]: post - flooding for T10 and late post-flooding for T65.

LEGENDS TO THE FIGURES

Fig.1 Total Dry Weight (TDW) and Root - to - Shoot Ratio (RSR) in the T10 and T65 treatments in five willow families. Means followed by the same letter do not differ according to LSD test ($p < 0.05$). N=15 for each family and treatment

Fig.2 Relative Growth Rate for height (RGRh) for treatments T10 and T65 during flooding (f) and in the post - flooding period (pf) in five willow families. Means followed by the same letter do not differ according to LSD test ($p < 0.05$). N=18 for each family and treatment

Fig.3 Relative Growth Rate for diameter (RGRd) during flooding (f) and post - flooding (pf) in treatments T10 and T65 in five willow families. Means followed by the same letter do not differ according to LSD test ($p < 0.05$). N=18 for each family and treatment

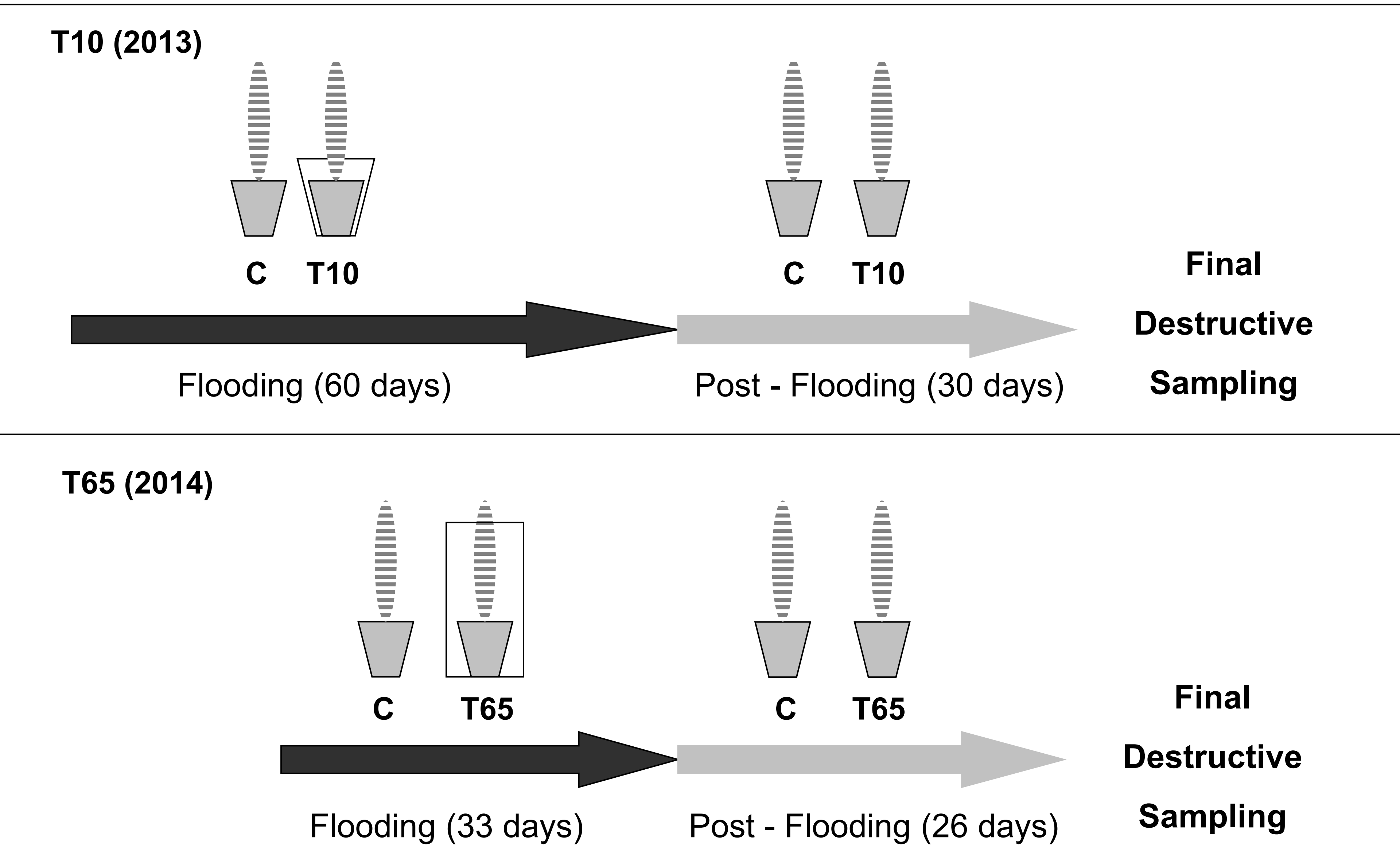
Fig.4 Electron Transport Rate (ETR) during flooding and post - flooding for treatment T10 and early and late post - flooding for treatment T65, in five willow families. Means followed by the same letter do not differ according to LSD test ($p < 0.05$). N=15 for each family and treatment

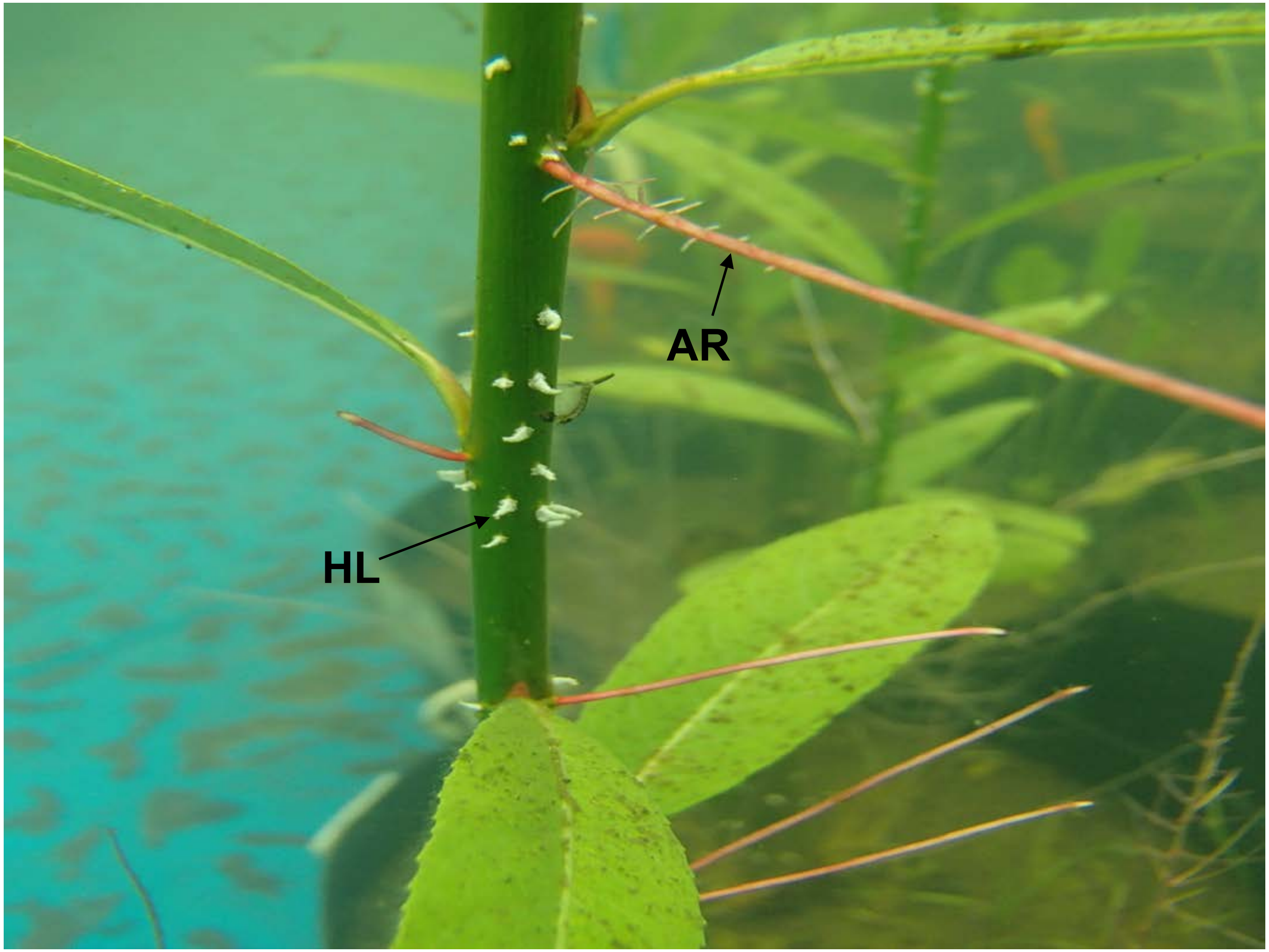
Fig.5 Stomatal conductance (gs) during flooding and post - flooding for treatment T10 and early and late post - flooding for treatment T65, in five willow families. Means followed by the same letter do not differ according to LSD test ($p < 0.05$). N=15 for each family and treatment

Fig.6 Leaf Nitrogen concentration per unit leaf area (N) at the end of the T10 and T65 experiments, for five willow families. Means followed by the same letter did not differ according to LSD test ($p < 0.05$). N=12 for each family and treatment

Fig.7 Flooding Tolerance Index (FTI) of the five families, calculated with the Volume Index for both experiments (T10 and T65) at the end of flooding (flooding) and at the end of the post-flooding recovery period (post - flooding). The value was calculated with the average Volume Index for each treatment and family

Suppl. FIG.1. An outline of the experiments carried out in this work. T10: Plants submerged 10 cm above soil level. T65: Plants submerged 65 cm above soil level.





Suppl.FIG.2. Hypertrophied Lenticels (HL) and Adventitious Roots (AR) developed in the submerged parts of the stems of willow plants.