Leaf traits related to productivity in *Populus deltoides* during the post-flooding period.

MARÍA E. RODRÍGUEZ[1], FABIO G. ACHINELLI[12], VIRGINIA M. C. LUQUEZ[13]

1 - Instituto de Fisiología Vegetal (INFIVE), CCT CONICET La Plata – FCAyF UNLP, CC 327, 1900 La Plata, Argentina.

2 – CIC Buenos Aires, Argentina.

3: Author for correspondence. vluquez@agro.unlp.edu.ar. Tel: +54-221-423-6618.

FAX: +54-221-423-3698.

Abstract

Flooding stress induces changes in trees at plant and leaf level that can reduce growth and productivity. In this work, we explored changes in leaf traits related to productivity during the post-flooding period in three poplar clones with different degrees of flooding sensibility. Our hypothesis was that changes in leaf traits could lead to a higher photosynthetic activity in the post-flooding period to compensate for the reduction in carbon fixation under flooding.

Plants were grown in pots in a greenhouse. Flooding was induced by filling the pots with tap water up to 5 cm over the surface soil for 28 days. After this period, flooding ended and plant recovery was followed for 42 days.

Flooding caused changes at plant and leaf level, not only during flooding but also after the stress ended. During this post-flooding period, the formerly flooded plants of all clones produced leaves with increased area and thickness compared to the control plants, but the photosynthetic rate was not increased. The plants compensated for the reduced growth under flooding by substituting the leaf area loss instead of increasing the photosynthetic activity.

Key words: *Populus deltoides* – flooding – leaf traits – photosynthesis

Key Message:

After a flooding period, *Populus deltoides* plants compensate for the reduced growth under flooding by substituting the leaf area loss instead of increasing the leaf photosynthetic activity.

Introduction

The tolerance to flooding of woody plants varies according to species and genotypes, the age of the plant, the degree of covering by water, the flood duration and the conditions of the floodwater (Kozlowski 1997, Glenz et al. 2006). Among the most conspicuous responses to flooding, we can find growth reduction, development of hypertrophied lenticels, adventitious roots and aerenchyma formation; accelerated leaf senescence and abscission; changes in the absorption and availability of mineral nutrients; and several metabolic changes caused by hypoxic or anoxic conditions (Kozlowski 1997, Bailey-Serres and Voesenek 2008). During root hypoxia, photosynthetic activity can be reduced by stomatal closure in different poplar clones (Bejaoui et al. 2006, Gong et al. 2007, Guo et al. 2011).

In *Populus*, several morphological leaf traits are related to productivity: total leaf area (Rae et al. 2004, Monclus et al. 2005, Marron et al. 2005), number of leaves on the main stem (Rae et al. 2004), individual leaf area (Monclus et al. 2005, Marron et al. 2005), specific leaf area (Marron et al. 2005), and stomatal density (Al Afas et al. 2006). Some of these traits are affected by flooding: in *Populus trichocarpa* x *deltoides,* root hypoxia reduces leaf growth rate and final leaf size through the reduction of both cell size and cell number (Smit et al. 1989); in *Populus angustifolia*, flooding reduces leaf number and size (Rood et al. 2010); and in *Populus* plants with flooded roots, specific leaf weight increases (i.e., specific leaf area decreases, Liu and Dickman 1992).

These flood-induced leaf modifications will probably affect plant productivity. Under flooding, the combination of a reduced rate of leaf expansion and an acceleration of leaf senescence and abscission can reduce the photosynthetically active leaf area, thus decreasing plant growth (Luquez et al. 2012). This combined with a reduction in the photosynthesis rate due to stomatal closure results in a reduced availability of photosynthates for growth. In addition to that, there are changes in dry matter partitioning and a decrease in the root/shoot ratio (Kozlowski 1997).

In spite of the well-documented changes induced by flooding in leaf morphology and physiology, little is known about the effects of these modifications in the post-flooding period, although they are likely to affect growth recovery. These alterations cannot be neglected in a climate change scenario, where areas with extensive poplar plantations like the Lower Paraná River Delta will experience flooding events more frequently (Barros et al. 2006). Even when these flooding episodes do not cause plant death, they may alter plant and leaf traits, with potentially lasting effects on forest growth and productivity.

In a previous work, we identified three *Populus deltoides* clones planted in the Paraná Delta area with different degrees of growth reduction under flooding. The degree of growth reduction correlated with the overall reduction in total leaf area, individual leaf size and leaf expansion rate (Luquez et al. 2012). In the present work, we explored more extensively the changes experienced by these clones in the post-flooding period. We analyzed the changes induced by flooding in leaf traits that affect productivity by comparing three cohorts of leaves: the first cohort -L1- expanded before flooding induction, the second -L2- expanded during flooding, and the third -L3- expanded after the flooding episode. Our hypothesis was that changes in leaf architecture and biochemistry could lead to a higher net photosynthetic rate in the post-flooding period to compensate for the reduction in carbon fixation under flooding.

Material and Methods

Plant material, experimental design and stress treatment

The *Populus deltoides* W. Bartram ex Marshall clones used in this work were Alton, Stoneville 67 (ST67) and 149-82. These clones were selected because they showed different degrees of growth reduction under flooding in a previous experiment: Alton was tolerant, 149-82 was sensitive, and ST67 was sensitive but to a lesser degree than 149-82 (Luquez et al. 2012).

Two experiments were carried out. In the 2009 experiment, one-year-old cuttings of 60 cm long were planted in 7 L pots filled with clay loam soil on August 7, 2009. The pots were placed in a greenhouse in a completely randomized design, with 10 replicates for each clone and treatment. Irradiance inside the greenhouse on clear days reached a maximum value of 1282 μmoles m^{-2} s^{-1}. Bud flush occurred between August 20 and August 31, 2009. A slow-release commercial fertilizer (NPK 12:5:14 plus Mg, S, Ca, Zn, Fe, Mo and B) was added to the pots to ensure an adequate nutrient availability. The dose was 1 g of fertilizer per pot, and the fertilization treatment was repeated twice before the beginning of the flooding treatment. To avoid fungal diseases, the trees were treated once a week with two commercial fungicides (Benomyl 50% WP and Carbendazim 50% SC). Before the treatment, trees were pruned and only one shoot was kept, in order to minimize the variability induced by several shoots per tree. Flooding started when the shoots were 2 months old, and was induced by placing the potted trees inside a sealed 10 L pot filled with tap water up to approximately 5 cm above

soil level; water was added when necessary to keep this level. The control plants were watered regularly to field capacity. The flooding stress treatment started on October 28, 2009 and lasted for 35 days.

In the 2011-2012 experiment, one-year-old cuttings of 20 cm long were planted in 4.5 L pots filled with a 1:1 soil-sand mix. The plants were treated as described above, except for fertilization. Pots were watered weekly with 50 ml of complete Hoagland solution (Legget and Frere 1971). Flooding was induced as described above, by placing the potted trees inside a sealed 6 L pot. The flooding stress treatment started on November 2, 2011 and lasted for 28 days. After that, the formerly flooded plants were removed from the sealed pots, water was allowed to drain, and the plants were measured for 44 days.

In the 2011-2012 experiment, three leaves were tagged in each plant, as described in Luquez et al. (2012): one leaf expanded before flooding (L1), one leaf expanded during the period of flooding (L2) and one leaf expanded after flooding ended (L3). Morphological, physiological and biochemical measurements were carried out on these leaves (see below). Unless otherwise stated, all data presented were measured in the 2011-2012 experiment.

Growth measurements and microscopic observations

Total shoot height was measured with a graduated stick. At the end of the experiment, all leaves were scanned and the total leaf area (TLA) was determined with the Image J software (http://rsbweb.nih.gov/ij/, Schneider et al. 2012). The individual leaf area (ILA) of leaves L1, L2 and L3 were determined in the same way. Dry mass was determined after drying leaves, shoots and roots at 65°C to constant weight. Specific leaf area (SLA, $cm^2\ g^{-1}$) was determined by taking a leaf disc of known area (2.27 cm^2) from each cohort and drying them to constant weight as described above. The Relative Growth Rate (RGR) for stem height growth was calculated according to Whitehead and Myerscough (1962).

Imprints were taken from the abaxial surface of leaves L1, L2 and L3 using clear lacquer and transparent tape. The imprints were fixed on glass slides, observed at 20x and photographed with a digital camera (Olympus Evolt E-330). Four pictures were taken for each imprint, each representing one observation field. The number of stomata per field (stomatal density) and the total number of epidermal cells per field (epidermal cell density) were counted using the Image J software

(http://rsbweb.nih.gov/ij/, Schneider et al. 2012), and the stomatal index (SI) was calculated according to Masle et al. (2005):

$$SI = (100 \times \text{stomatal density}) / (\text{stomatal density} + \text{epidermal cell density})$$

To determine leaf thickness, a piece of leaf around the main vein of leaves L1, L2 and L3 was fixed in FAA (formalin-alcohol-acetic acid). The leaves were cut by hand with a razor blade; seven cuttings were made of each sample. The cuttings were observed at 10x and photographed with a digital camera (Olympus Evolt E-330) and three measurements of thickness were performed on each side of the vein every 0.05 mm. Leaf thickness was calculated as an average of the six measurements made in all seven cuttings.

Gas exchange measurements

Photosynthetic activity (A), transpiration and stomatal conductance (gs) were measured with an IRGA CIRAS II, PP Systems in the experiment on the latest fully expanded leaf. Water Use Efficiency (WUE) was measured as the ratio between A and transpiration. The measurements were carried out between 10:00 am and 3:00 pm, under an irradiance of 1500 μmoles m^{-2} s^{-1}.

Chlorophyll and Rubisco content

One 5-mm-diameter leaf disc (chlorophyll) and two 10-mm-diameter leaf discs (Rubisco) were frozen in liquid nitrogen and stored at -80 °C until the determinations were carried out.

Chlorophyll content was determined using N,N-Dimethylformamide according to the method of Inskeep and Bloom (1985).

Rubisco content was determined by SDS-PAGE according to Laemmli (1970). Two 1-cm-diameter leaf discs were homogenized in 1X sample buffer (62.5mM Tris pH 6.8; 5% w/v SDS, 5% v/v glycerol, 5% v/v β-mercaptoethanol) and centrifuged at 10,000 rpm for 8 min at 4 ºC. For SDS-PAGE analysis, proteins in the supernatant were separated in 1.5 mm thick minigels with 12% of acrylamide concentration as in Laemmli (1970). A volume equivalent to 2.62 mm^2 of leaf area was loaded in each lane. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Gels were digitized and analyzed for background subtraction and banding density using the Image J software

(http://rsbweb.nih.gov/ij/). Three or four replicates per treatment were analyzed. The amount of Rubisco large Sub-unit (LSU) was calculated as a percentage of the initial content.

Statistical Analysis

The statistical analysis was carried out with R software version 2.8.1 (R Development Core Team, 2010). ANOVA and mean test were carried out using the **agricolae R** package.

Results

Dry matter partitioning was measured in the 2009 experiment (Fig. 1). Total dry weight was significantly reduced only in 149-82, but flooding altered dry matter partitioning in all clones. Root biomass was reduced in all clones and root/shoot ratio decreased in flooded plants compared to controls (Fig. 1, in italics). However, the loss of root biomass in Alton was lower than in the other clones. Its root biomass under flooding was reduced by 25% compared to control plants, while the reduction in the other clones was of 52% (ST67) and 66% (149-82). Consequently, the root/shoot ratio decreased by 35% in Alton flooded plants compared to controls, whereas it decreased by 50% in the other clones.

The periodical growth in height was similar in both experiments; therefore, only data from 2011 are presented. During the first two weeks of flooding, there were no differences in height between control and flooded plants, but marked differences began to appear among clones after three weeks (Fig. 2). Flooding did not reduce height in Alton, with no differences in RGR between both treatments (Fig.2, left hand side of the arrow). Flooded plants of 149-82 and ST67 (Fig. 2) reduced their height after the third week of flooding, but RGR was only significantly reduced in 149-82 (Fig.2, left hand side of the arrow). After four weeks, the flooding episode was ended and the plants were allowed to recover and measured for another 42 days. At the end of the recovery period, there were no differences in height between formerly flooded and non-flooded plants in Alton and ST67, while the levels of formerly flooded plants in 149-82 were still significantly lower than those of non-flooded plants. The RGR in the post-flooding period was significantly higher in formerly flooded plants of Alton and ST67, but not in 149-82 (Fig.4 right hand side).

Total leaf area (TLA) was measured discriminating the area developed in the post-flooding period from the area previously expanded (before/during flooding) (Fig. 3). After the 42-day-period of recovery, there were no significant differences in TLA between control and formerly flooded plants for any of the clones, but the relative number of leaves expanded before/during and after flooding was different (data not shown). There were no significant differences in leaf area expanded before/during flooding between control and flooded Alton, but it was significantly smaller in formerly flooded plants of 149-82 and ST67. The expanded area after the flooding period was significantly larger in formerly flooded plants than in the control treatment in all clones.

A and gs were measured throughout the flooding and the recovery periods (Fig. 4). Both variables were reduced by flooding in all clones, but the reduction was less marked in Alton. After the end of the stress, gs of formerly flooded plants recovered to similar values as control plants. There was a significant correlation between gs and A in control and flooded plants, but the relation was weaker in the post-flooding period, remaining significant only in ST67.

A, gs and WUE were measured in leaves L1, L2 and L3 when they reached their full expansion (Table 2); in the case of L2 and L3 it happened after the end of flooding. In the cohort expanded during flooding (L2), A did not differ between treatments. gs was significantly higher only in 149-82 flooded plants, while WUE decreased in all clones but only significantly in Alton. In the cohort expanded in the post-flooding period (L3), there were no differences in A, gs or WUE.

We determined ILA, SI, SLA and leaf thickness on the three cohorts, L1, L2 and L3 (Table 1). On leaf L2, ILA and SLA were not significantly affected by flooding in any of the clones. Flooding reduced SI in ST67 and increased leaf thickness in 149-82. In the cohort expanded during the post-flooding period (L3, Table 1), LAI increased in all clones, albeit not significantly in 149-82. There was no change in SLA, but leaf thickness increased significantly in all clones. SI decreased only in ST67.

We measured the chlorophyll and Rubisco content in all three cohorts of leaves (Table 3). We did not find significant differences between flooded and control plants in any of the clones.

Discussion

In *Populus* and other species, flooding causes the root system to die back, and the most tolerant genotypes develop new adventitious roots with aerenchyma (Kozlowski 1997, Cao and Conner 1999). Our results confirm this, since the genotype with more tolerance -i.e., less growth reduction under flooding- was Alton, which had a greater root biomass, newly developed roots with aerenchyma, and a root/shoot ratio less affected by flooding. The most sensitive clone, 149-82, developed neither hypertrophied lenticels nor adventitious roots (see additional figure 1). The variation in root biomass seems to be related to the growth recovery capability after flooding. The extensive root loss in 149-82 is the likely cause for the slow growth recovery in the post-flooding period. More roots imply a higher capability for water transport and nutrient absorption, allowing for the maintenance of a larger leaf area during flooding. In poplar, total leaf area often correlates with biomass accumulation (Rae et al. 2004, Monclus et al. 2005, Marron et al. 2005). In our experiment, 42 days after the end of the stress episode, TLA was not significantly different between control and formerly flooded plants. However, when discriminating between the areas developed before/during the flooding and post-flooding periods, a clear difference emerged. The formerly flooded plants developed a greater leaf area than the controls during the recovery period, thus compensating for the area loss under flooding due to an increased abscission. There was no difference in the number of leaves expanded after the end of the flooding stress period (data not shown); hence, the difference is due to the increase in the area of leaves expanded in the post-flooding period.

Growth rate depends ultimately on the carbon fixing capacity, and this can be reduced by flooding stress (Bejaoui et al. 2006, Gong et al. 2007, Guo et al. 2011). We found a significant correlation between gs and A during flooding, suggesting that the main cause for carbon fixation reduction is stomatal closure. But the correlation is weaker in the post-flooding period, suggesting that other factors could have an influence on A. Several leaf traits that correlate with biomass accumulation in poplar (Rae et al. 2004, Monclus et al. 2005, Marron et al. 2005) can be altered by different environmental factors and stresses, like root hypoxia (Smit et al. 1989) and increased CO_2 concentration (Ceulemans et al. 1995). There are also differences among genotypes, leaf side and leaf position in the canopy (Al Afas et al 2006, Dillen et al. 2008). It has been shown that a higher stomatal density can enhance photosynthetic capacity in *Arabidopsis* (Tanaka et al. 2013). These morphological and biochemical alterations of leaves could increase photosynthetic activity in the post-flooding period, thus compensating for the reduction of leaf carbon fixation under flooding due to leaf

249 area reduction and stomatal closure. To answer this question, we measured several leaf traits related
250 to productivity in cohorts of leaves expanded before, during and after the flooding period (L1, L2 and
251 L3, respectively), and measured gas exchange when these leaves reached their full expansion. The
252 gas exchange measurements in L2 and L3 were taken after the end of the flooding period, when gs
253 reached similar values as those of control plants. Consequently, any differences in photosynthetic
254 activity will be caused by alterations in the leaf architecture induced by flooding but not by a reduction
255 of gs.

256 The area of leaf L2 decreased but not to the same extent as in our previous work (Luquez et
257 al. 2012). The cause of this difference may lie on the length of the flooding period, which was shorter
258 than in the previous experiment. Regarding SLA, there were differences only at clonal level but not
259 between treatments. In those experiments with longer flooding periods, we found a reduction in SLA
260 on these same clones (data not shown), as reported by Liu and Dickman (1992) for hybrid poplar. As
261 for ILA, it is likely that the length of the flooding period influenced SLA, as it does to other plant
262 responses to this stress (Kozlowski 1997, Glenz et al. 2006). The lack of a clear trend of change in the
263 morphological data, mirrored what happened with gas exchange, Rubisco and chlorophyll data for L2,
264 i.e., it did not show any differences caused by flooding.

265 The leaf expanded in the post-flooding period (L3) showed clear trends regarding leaf area
266 and thickness, since both increased in the formerly flooded plants. SLA did not change, possibly
267 because both area and width increased at the same time. SLA modulates maximum photosynthetic
268 rate (A_{max}) and nitrogen use efficiency on leaves of an ample range of species: leaves with higher SLA
269 have a higher A_{max} per unit leaf N (Reich et al. 1998). Our results seems to fit in this broader pattern,
270 since the lack of change in SLA was accompanied with no change in the photosynthetic rate or the
271 fraction of leaf N involved directly in the photosynthesis, represented by Rubisco and chlorophyll
272 content. *P. deltoides plants growing* under different combinations of water and nitrogen availability,
273 shows moderate plasticity in leaf traits (Funk et al. 2007) and this seems to be the case in our results
274 as well. There were changes in leaf thickness and ILA, but most of the leaf traits did not change.

275 Contrary to our hypothesis, there was no compensatory increase of the photosynthetic rate in
276 the post-flooding period. It seems that *Populus deltoides* plants increase their growth rate after
277 flooding by an increase in leaf area rather than by a higher photosynthetic capacity.
278

Conflicts of interest: the authors declare that they have no conflicts of interest.

Author contribution statement: MER carried out the most part of the experiments and the statistical analysis, FGA helped with the experimental part, VMCL did part of the statistical analysis and wrote the paper.

Acknowledgements:

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT PICT 487) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET PIP 269) to VMCL. Thanks to S. Martínez for helping with the microscopy work and to S. Cortizo for providing clone 149-82. VMCL is a researcher from CONICET. MER held fellowships from ANPCyT and CONICET.

References

Al Afas N, Marron N, Ceulemans R (2006) Clonal variation in stomatal characteristics related to biomass production of 12 poplar (*Populus*) clones in a short rotation coppice culture. Env Exp Bot 58: 279-286.

Bailey–Serres J, Voesenek LACJ (2008) Flooding Stress: Acclimations and Genetic diversity. Ann Rev Plant Biol 59: 313-339.

Barros V, Menéndez A, Natenzon C, Kokot R, Codignotto J, Re M, Bronstein P, Camilloni I, Ludueña S, González S, Ríos D (2006) Vulnerability to floods in the metropolitan area of Buenos Aires under future climate change. AIACC Working Papers No 26. http://www.aiaccproject.org/working_papers/working_papers.html. Accessed on April 3, 2014.

307 Bejaoui Z, Albouchi A, Abassi M, El Aouni MH (2006) Influence d'une hydromorphie modérée ou
308 severe sur la production de biomasse et les échanges gazeux de plants de peuplier euraméricain. Can
309 J For Res 36**:** 2654-2665.

310

311 Cao FL, Conner WH (1999) Selection of flood tolerant *Populus deltoides* clones for reforestation
312 projects in China. For Ecol Manag 117: 211-220.

313

314 Ceulemans R, Van Praet L, Jiang XN (1995) Effects of CO2 enrichment, leaf position and clone on
315 stomatal index and epidermal cell density in poplar (*Populus*). New Phytol 131: 99-107.

316

317 Dillen SY, Marron N, Koch B, Ceulemans R (2008) Genetic variation of stomatal traits and carbon
318 isotope discrimination in two hybrid poplar families (*Populus deltoides* S9-2 x *Populus nigra* Ghoy and
319 *P. deltoides* S9-2 x *P. trichocarpa* V24). Ann Bot 102: 399-407.

320

321 Funk JL, Jones CG, Lerdau MT (2007) Leaf and shoot level plasticity in response to different nutrient
322 and water availabilities. Tree Phys 27: 1731-1739.

323

324 Glenz C, Schlaepfer R, Iorgulescu I, Kienast F (2006) Flooding tolerance of Central European tree and
325 shrub species. For Ecol Manag 235: 1-13.

326

327 Gong JR, Zhang XS, Huang YM, Zhang CL (2007) The effects of flooding on several hybrid poplars
328 clones in Northern China. Agroforestry Syst 69: 77-88.

329

330 Guo XY, Huang Z, Xu A, Zhang X (2011) A comparison of physiological, morphological and growth
331 responses of 13 hybrid poplars clones under flooding. Forestry 84: 1-12.

332

333 Inskeep WP, Bloom PR (1985) Extinction coefficients of chlorophyll a and b in N,N-Dimethylformamide
334 and 80% acetone. Plant Physiol 77: 483-485.

335

336 Kozlowski TT (1997) Responses of woody plants to flooding and salinity. Tree Physiology Monograph
337 No 1. http://www.pucrs.br/fabio/fisiovegetal/Encharcamento.pdf. Accessed August 5, 2013.
338
339 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage
340 T4. Nature 227: 680-685.
341
342 Leggett JE, Frere MH (1971) Growth and nutrient uptake by soybean plants in nutrient solutions of
343 graded concentrations. Plant Physiol 41: 457-460.
344
345 Liu Z, Dickmann DI (1992) Responses of two hybrid *Populus* clones to flooding, drought and nitrogen
346 availability. I. Morphology and growth. Can J Bot 70: 2265 – 2270.
347
348 Luquez VMC, Achinelli F, Cortizo S (2012) Evaluation of flooding tolerance in cuttings of *Populus*
349 clones used for forestation at the Paraná River Delta, Argentina. Southern Forests 74: 61-70.
350
351 Marron N, Villar M, Dreyer E, Delay D, Boudouresque E, Petit JM, Delmotte FM, Guehl JM, Brignolas
352 F (2005) Diversity of leaf traits related to productivity in 31 *Populus deltoides* x *Populus nigra* clones.
353 Tree Physiol 25: 425-435.
354
355 Masle J, Gilmore SR, Farquhar GD (2005) The ERECTA gene regulates plant transpiration in
356 Arabidopsis. Science 436: 866 – 869.
357
358 Monclus R, Dreyer E, Delmotte FM, Villar M, Delay D, Boudouresque E, Petit J, Marron N, Bréchet C,
359 Brignolas F (2005) Productivity, leaf traits and carbon isotope discrimination in 29 *Populus deltoides* x
360 *Populus nigra* clones. New Phytol 167: 53 – 62.
361
362 R Development Core Team (2010) R: A language and environment for statistical computing. R
363 Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. http://www.R-project.org.
364 Accessed November 9 2010.

Rae AM, Robinson KM, Street N, Taylor G (2004) Morphological and physiological traits influencing biomass productivity in short-rotation coppice poplar. Can J For Res 34: 1488 – 1498.

Reich PB, Ellsworth DS, Walters MB (1998) Leaf structure (specific leaf area) modulates photosynthesis-nitrogen relations: evidences from within and across species and functional groups. Functional Ecol 12: 948-958.

Rood SB, Nielsen JL, Shenton L, Gill KM, Letts MG (2010) Effects of flooding on leaf development, transpiration and photosynthesis in narrowleaf cottonwood, a willow-like poplar. Photosynthesis Res 104: 31-39.

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH image to Image J: 25 years of image analysis. Nature Methods 9 (7) 671-675.

Smit B, Stachowiak M, Van Volkenburgh E (1989) Cellular process limiting leaf growth in plants under hypoxic root stress. J Exp Bot 40**:** 89-94.

Tanaka Y, Sugano S, Shimada T, Hara–Nishimura I (2013) Enhancement of leaf photosynthetic capacity through increased stomatal density in Arabidopsis. New Phytol 198: 757-764.

Whitehead FH, Myerscough PJ (1962) Growth Analysis of plants. New Phytol 61: 314-321.

388 Table 1 - Individual Leaf Area (ILA, cm^2), Stomatal Index (SI), Specific Leaf Area (SLA, $cm^2\ g^{-1}$) and
389 Leaf Thickness (μm) in three cohorts of poplar leaves. The first cohort (L1) completed its expansion
390 before flooding induction, the second cohort (L2) expanded during the period of flooding, and the third
391 cohort (L3) expanded after the end of the stress treatment. Means followed by the same letter do not
392 differ significantly ($p<0.05$ LSD). C: control, F: flooded.

393

394

Treatment	Cohort	ILA	SI	SLA	Thickness
Alton C	L1	66.3 a	8.2 a	175 b	265 a
149-82 C	L1	89.3 b	9.0 b	181 b	221 b
ST67 C	L1	68.2 a	8.8 ab	222 a	221 b
Alton C	L2	102.3 a	8.8 a	95 b	309 b
Alton F	L2	98.1 a	9.0 a	94 b	315 b
149-82 C	L2	106.8 a	8.5 a	112 a	278 a
149-82 F	L2	98.5 a	8.9 a	106 a	295 c
ST67 C	L2	95.1 a	10.1 b	114 a	272 a
ST67 F	L2	103.1 a	8.8 a	113 a	278 a
Alton C	L3	78.9 c	7.7 a	99 b	310 d
Alton F	L3	112.5 ab	8.3 ab	107 ab	323 b
149-82 C	L3	102.8 ab	8.1 ab	110 ab	297 a
149-82 F	L3	116.2 a	7.7 a	114 a	326 b
ST67 C	L3	87.9 c	10.1 c	117 a	277 a
ST67 F	L3	127.6 b	8.9 b	118 a	289 c

395
396
397
398

399

Table 2 – Net Photosynthesis (A, μmoles CO_2 m^{-2} s^{-1}), Stomatal Conductance (gs, mmoles H_2O m^{-2} s^{-1}) and instantaneous Water Use Efficiency (WUE) in three cohorts of poplar leaves. The first cohort (L1) completed its expansion before flooding induction, the second cohort (L2) expanded during the period of flooding, and the third cohort (L3) expanded after the end of the stress treatment. Means followed by the same letter do not differ significantly ($p<0.05$ LSD). C: control, F: flooded.

Clone / Treatment	*Leaf Cohort*	*A*	*gs*	*WUE*
Alton C	L1	17.3 a	322 a	3.78 a
149-82 C	L1	14.2 a	311 a	2.93 b
ST67 C	L1	16.4 a	262 a	3.93 ab
Alton C	L2	12.6 ab	144 ab	3.12 ab
Alton F	L2	14.2 b	196 b	2.65 bc
149-82 C	L2	11.2 a	123 a	2.74 ab
149-82 F	L2	10.7 a	191 b	2.16 c
ST67 C	L2	12.3 ab	116 a	3.20 a
ST67 F	L2	12.2 ab	157 ab	2.96 ab
Alton C	L3	15.0 a	98 b	6.85 a
Alton F	L3	13.7 a	89 ab	5.07 a
149-82 C	L3	16.3 a	94 ab	6.52 a
149-82 F	L3	13.1 a	77 ab	5.27 a
ST67 C	L3	14.4 a	71 a	6.15 a
ST67 F	L3	13.6 a	81 ab	5.43 a

412 Table 3 - Chlorophyll (Chl, μg cm^{-2}) and Rubisco content (as percentage of the initial content) in three
413 cohorts of poplar leaves. The first cohort (L1) completed its expansion before flooding induction, the
414 second cohort (L2) expanded during the period of flooding, and the third cohort (L3) expanded after
415 the end of the stress treatment. Means followed by the same letter do not differ significantly ($p<0.05$
416 LSD). C: control, F: flooded.

417

418

Clone / Treatment	*Leaf Cohort*	*Chl a*	*Chl b*	*Total Chl*	*Rubisco LSU*
Alton C	L1	31.6 a	11.6 a	44.5 a	100
149-82 C	L1	29.7 a	11.6 a	41.3 a	100
ST67 C	L1	32.5 a	33.4 a	44.5 a	100
Alton C	L2	25.2 ab	10.1 ab	35.2 ab	73 a
Alton F	L2	26.5 a	10.3 a	36.8 a	82 a
149-82 C	L2	24.8 ab	9.9 ab	34.8 ab	88 a
149-82 F	L2	26.6 a	10.4 a	37.0 a	91 a
ST67 C	L2	23.5 bc	9.9 ab	33.3 bc	72 a
ST67 F	L2	21.4 c	9.4 b	30.8 c	66 a
Alton C	L3	27.1 ab	10.8 ab	37.9 ab	82 a
Alton F	L3	27.1 ab	10.9 ab	38.0 ab	76 a
149-82 C	L3	28.7 bc	11.2 bc	39.4 bc	100 a
149-82 F	L3	30.2 c	11.8 c	42.0 c	108 a
ST67 C	L3	25.0 a	10.3 a	35.3 a	85 a
ST67 F	L3	27.4 ab	11.3 bc	38.7 abc	87 a

419
420
421

422

Legends to the figures

Fig. 1 – Dry matter partitioning between roots, stem and leaves in three *Populus deltoides* clones - Alton, 149-82 and ST67-, in the 2009 experiment. The root system of the plants was flooded (F) for 35 days, while the control plants (C) were maintained under well-drained conditions. Means with the same letter do not differ significantly ($p<0.05$ LSD) for total dry matter. In italics: root/shoot ratio for each treatment and clone (shoot = stem + leaves).

Fig. 2 – Growth in height of three *Populus deltoides* clones: Alton, 149-82 and ST67. The treatments were control (well-drained, black circles) and flooded (white circles). The arrows indicate the end of the flooding treatment. The asterisks indicate statistically significant differences between control and flooded plants of the same clone. Relative Growth Rate (RGR) values are multiplied by 10^3. c: control, f: flooding; pf: plants previously flooded.

Fig.3 - Total leaf area and area expanded after the end of flooding of three *Populus deltoides* clones: Alton, 149-82 and ST67. The treatments were control (C) and flooded (F). In the 2011 experiment and after 28 days of flooding, the plants were allowed to drain and their recovery was followed for 42 days. Means with the same letter do not differ significantly ($p<0.05$ LSD). Vertical bars: standard error of the mean.

Fig. 4 - Net Photosynthesis (A, μmoles CO_2 m^{-2} s^{-1}) and Stomatal Conductance (gs, mmoles H_2O m^{-2} s^{-1}) of three *Populus deltoides* clones: Alton, 149-82 and ST67. The treatments were control (well-drained, black circles), flooded (white circles) and plants flooded after the end of the stress treatment (grey circles). r: Pearson correlation coefficient. The asterisk indicates statistically significant differences ($p<0.05$).

FIG.1

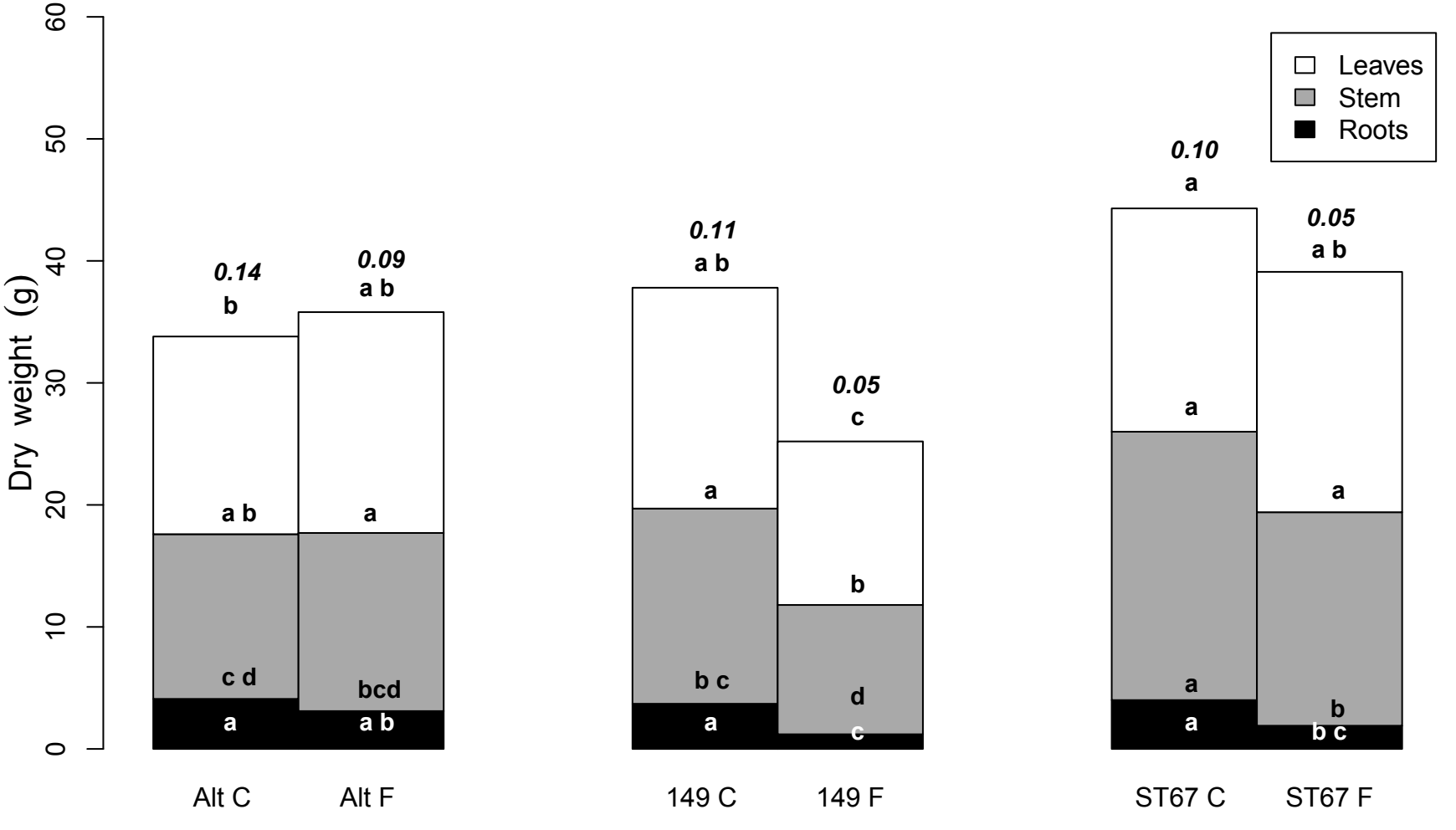

Dry weight (g)
0
10
20
30
40
50
60
Leaves
Stem
Roots
0.14
b
a b
c d
a
0.09
a b
a
bcd
a b
0.11
a b
a
b c
a
0.05
c
b
d
c
0.10
a
a
a
a
0.05
a b
a
b
b c
Alt C
Alt F
149 C
149 F
ST67 C
ST67 F

FIG.2

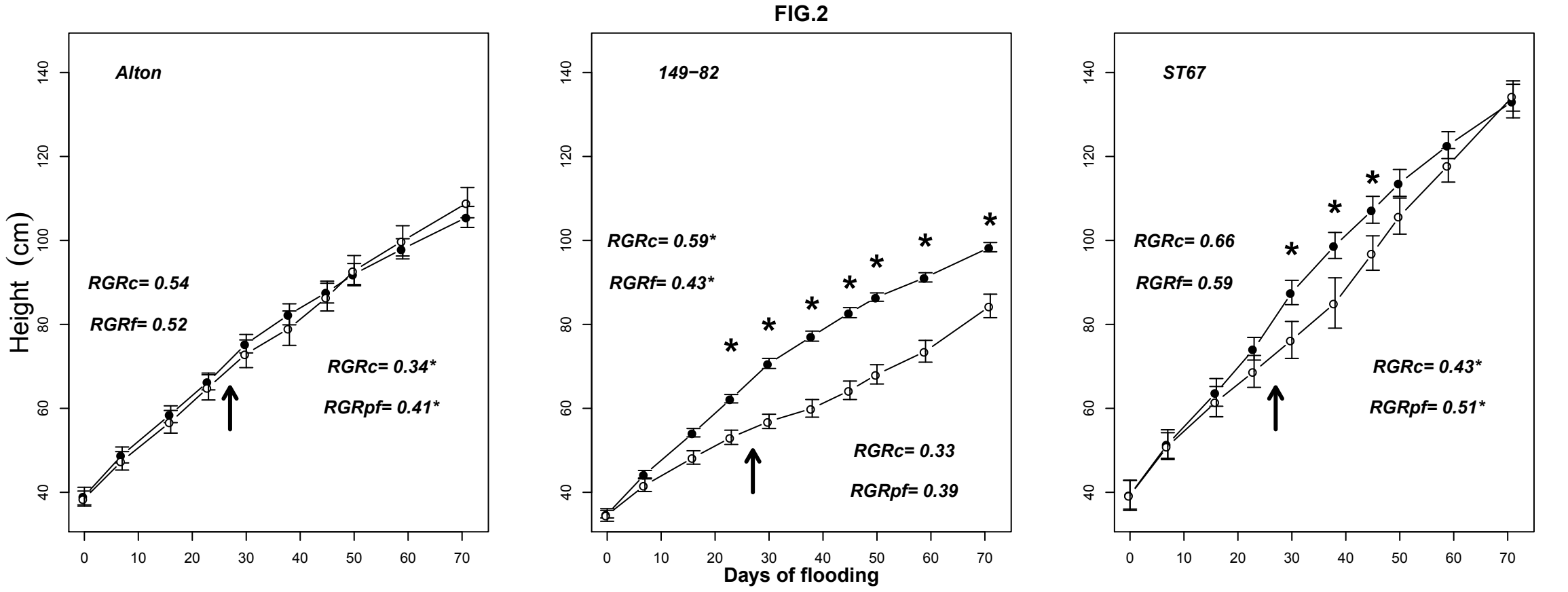

Alton
RGRc= 0.54
RGRf= 0.52
RGRc= 0.34*
RGRpf= 0.41*
149−82
RGRc= 0.59*
RGRf= 0.43*
RGRc= 0.33
RGRpf= 0.39
ST67
RGRc= 0.66
RGRf= 0.59
RGRc= 0.43*
RGRpf= 0.51*
Height (cm)
Days of flooding

FIG.3

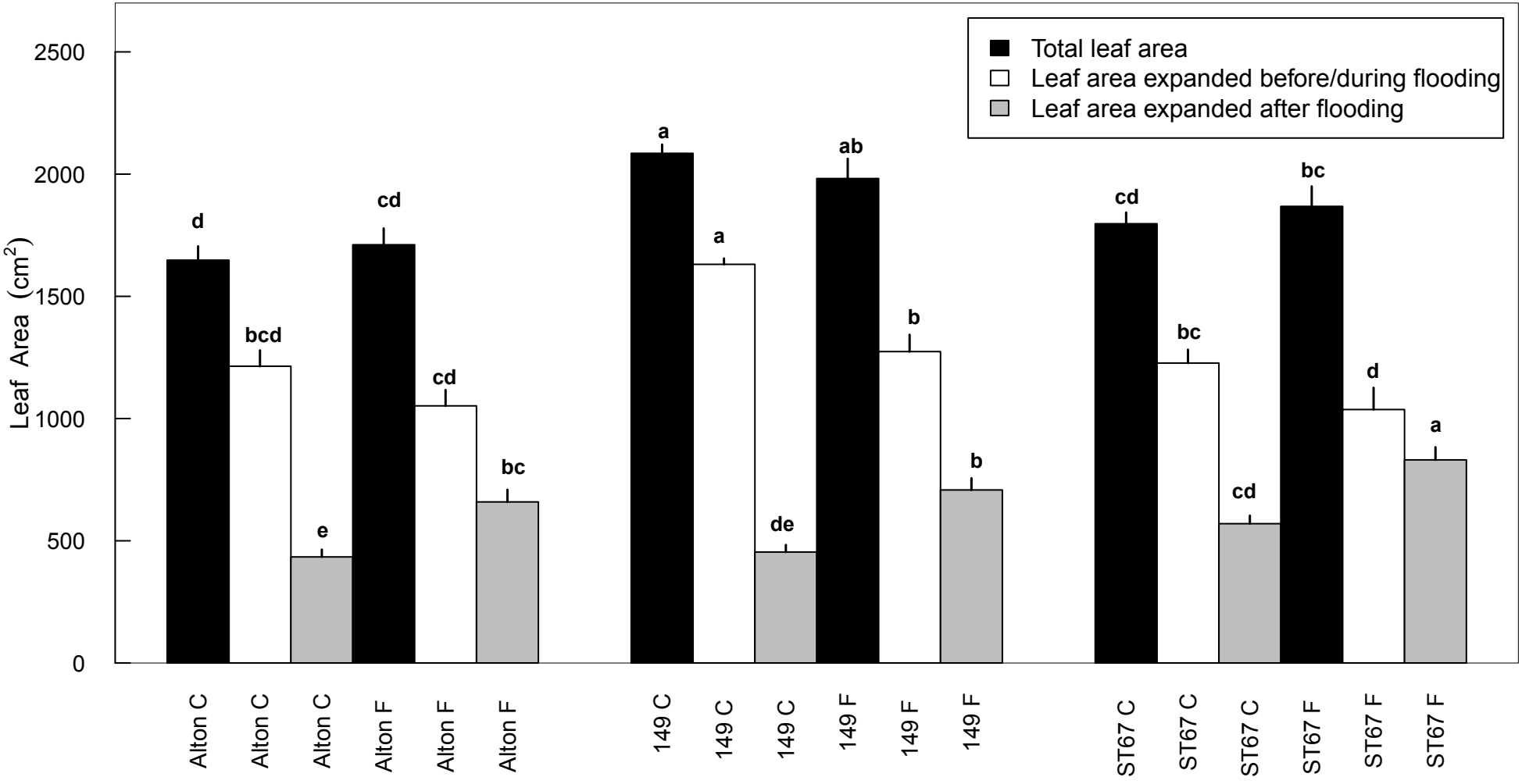
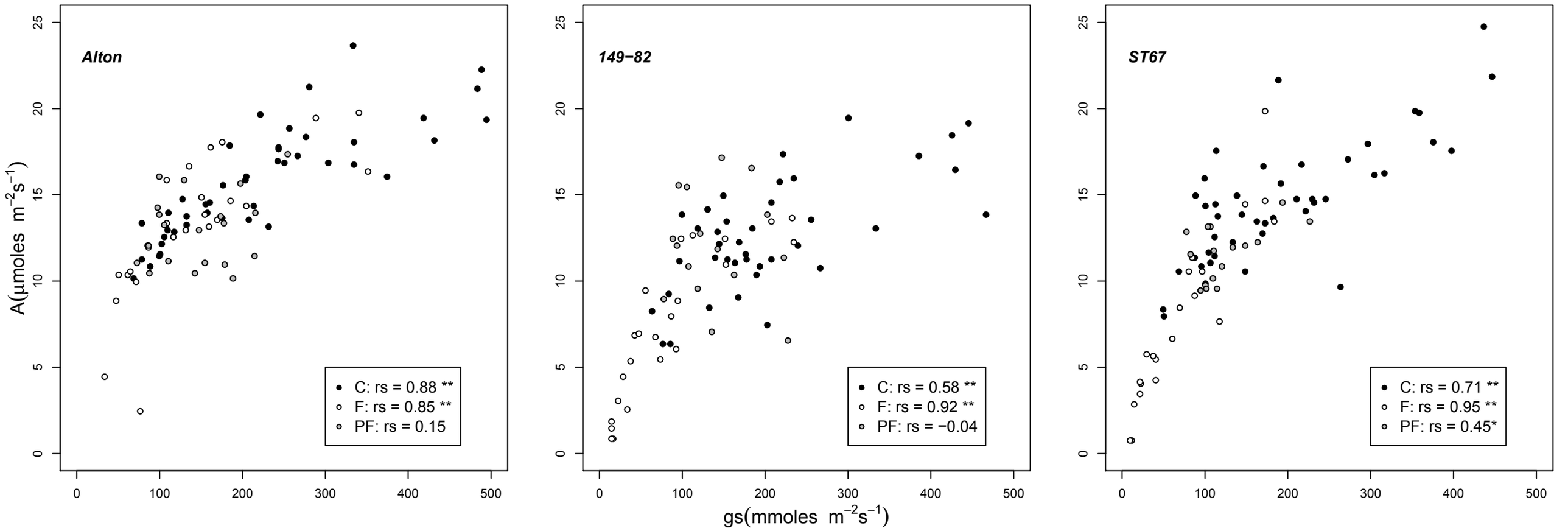
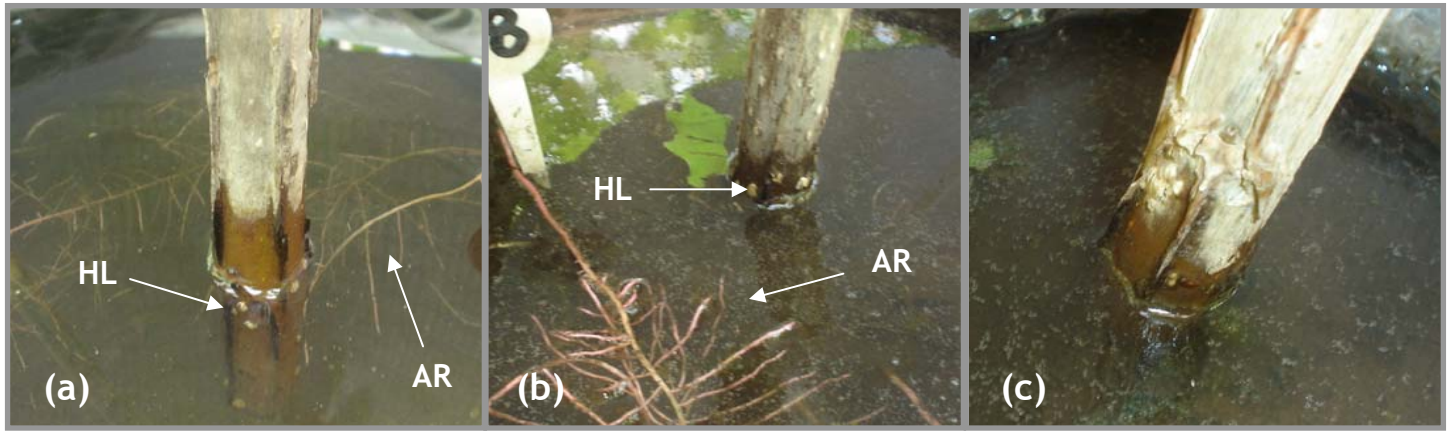

Total leaf area
Leaf area expanded before/during flooding
Leaf area expanded after flooding
Leaf Area (cm2)
2500
2000
1500
1000
500
0
d
bcd
e
cd
cd
bc
a
a
de
ab
b
b
cd
bc
cd
bc
d
a
Alton C
Alton C
Alton C
Alton F
Alton F
Alton F
149 C
149 C
149 C
149 F
149 F
149 F
ST67 C
ST67 C
ST67 C
ST67 F
ST67 F
ST67 F

FIG.4


Alton
C: rs = 0.88 **
F: rs = 0.85 **
PF: rs = 0.15
149−82
C: rs = 0.58 **
F: rs = 0.92 **
PF: rs = −0.04
ST67
C: rs = 0.71 **
F: rs = 0.95 **
PF: rs = 0.45*
A(μmoles m−2s−1)
gs(mmoles m−2s−1)
0
5
10
15
20
25
0
100
200
300
400
500

Supplementary Figure 1

A - Cuttings of the clones used, showing that Alton (a) and ST67 (b) developed hyperthrophied lenticels (HL) and adventitious roots (AL), while 149-82 (c) did not.



B - The adventitious roots had aerenchyma (marked with an arrow) that developed only in flooded plants. Lenght of the bar: 100 μm. C: Control. F: Flooded.