Ecophysiological adaptations of winter-hardened black spruce (*Picea mariana*) and tamarack (*Larix laricina*) seedlings to flooding.

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Abstract

Black spruce and tamarack are the predominant tree species in boreal peatlands. The effects of flooding on morphological and physiological responses were investigated in greenhouse grown (18 months old) black spruce (*Picea mariana*) and tamarack (*Larix laricina*) seedlings for 34 days. Flooding resulted in reduced root hydraulic conductance, net assimilation rate and stomatal conductance and needle electrolyte leakage in both species. Flooded tamarack seedlings maintained a higher net assimilation rate and stomatal conductance compared to flooded black spruce. Flooded tamarack seedlings were also able to maintain higher root hydraulic conductance compared to flooded black spruce seedlings at a comparable time period of flooding. Root respiration declined in both species under flooding. Sugar concentration in shoots increased while decreased in roots in both species under flooding. Needles of flooded black spruce appeared necrotic and electrolyte leakage increased over time with flooding and remained significantly higher than flooded tamarack seedlings. No visible damage symptoms were observed in flooded tamarack seedlings. Flooded tamarack seedlings developed adventitious roots beginning 14 days after the flooding treatment began. To investigate the possible physiological role of adventitious roots, their hydraulic conductivity was measured and compared with similarly sized flooded roots. Adventitious roots exhibited significantly higher root hydraulic conductivity. Flooded black spruce lacked any such morphological adaptation. These results suggest that tamarack seedlings were better able to adjust both morphologically and physiologically to a prolonged soil flooding than black spruce seedlings.

Key words: carbohydrate content, flooding, gas exchange, root hydraulic conductance, root respiration.

Introduction

Low oxygen concentration in the rooting medium due to waterlogging reduces root and shoot growth of trees by affecting many plant physiological processes, including net assimilation, transpiration, stomatal conductance (Anderson et al. 1984; Levan and Riha 1986; Pereira and Kozlowski 1977; Pezeshki and Chambers 1986), carbohydrate metabolism and respiratory activity (Pezeshki 1994).

Flooding triggers ethylene accumulation in plants (Tang and Kozlowski 1984a and b; Blake and Reid 1981). Evidence suggest that in some species morphological responses to flooding, such as hypertrophy of lenticels and formation of adventitious roots are associated with increased production of ethylene (Blake and Reid 1981). The close association of adventitious root formation and stomatal reopening in some species suggests that these aerenchymatous roots may play a key role in the recovery of gas exchange from flooding stress (Tang and Kozlowski 1984). In addition, flood-induced adventitious roots increase water absorption by roots (Jackson and Drew 1984; Tsukahara and Kozlowski 1985).

Root respiration has been shown to decline as a result of hypoxia in wheat (Huang and Johnson 1995), and aspen (Kamaluddin and Zwiazek 2002). Carbohydrate concentrations may remain the same or increase in roots and shoots under hypoxic conditions (Huang 1995). The rate and extent of increase in sugar content depends on the environmental conditions, species and even on genotypes within the same species (Setter et al. 1987). Soluble carbohydrates such as sucrose, glucose or fructose are of particular importance because of their direct relationship with respiration. Thus, carbohydrate allocation patterns appear to be critical for hypoxia tolerance (Huang and Johnson 1995).

Two conifers, black spruce (*Picea mariana* (Mill.) B. S. P.) and tamarack (*Larix laricina* (Du Roi) K. Koch), form open forest stands on peatlands in Alberta, Canada (Lieffers and Macdonald 1990). These organic sites have very low productivity and are characterized by high water table (Larsen 1982), poor soil aeration (Campbell 1980) and undergo significant variation in depth of water table during the growing season. Oxygen supply to tree roots decreases significantly when the water table rises close to the peat surface (Kozlowski 1984; Mannerkoski 1985).

Although black spruce and tamarack co-occur on moderately minerotropic peatlands, tamarack tends to dominate on wetter, minerotropic sites (Jeglum and He 1996; Tilton 1977; Kenkel 1987). Black spruce and tamarack possesses contrasting growth habits with the former one being evergreen with sclerophyllous leaves, while the later is deciduous with sclerophyllous leaves (Tyrell and Boerner 1987). Black spruce exhibits fixed growth i.e., it's growth is determined by the process of bud set in previous fall. Tamarack has determinate growth of short shoots but also possesses long shoots displaying indeterminate growth, thus allows a greater degreeof variation on annual growth in response to conditions during the growing season (Ref).

In our previous study (Islam et al. 2002), we have shown that flooded tamarack seedlings were able to maintain higher root hydraulic conductance compared to flooded black spruce seedlings and had no visible flooding damage. However, the possible role of stored carbohydrate and root respiration on root hydraulic conductance was not investigated. Since the seedlings were only six-months old, the differences in response, which could occur from determinate, and indeterminate growth habit in these two species was absent. In this paper, we examined the effect of 34 days of soil flooding on gas exchange, root hydraulic conductance, membrane damage and electrolyte leakage and tried to explore the relationships between these parameters with carbohydrate metabolism and root respiratory activity in these two species at an age where determinate and indeterminate growth is in effect after winter hardening. We hypothesized that flood tolerance of tamarack is conferred by its greater morphological and physiological adaptation.

Materials and Methods:

Plant material and growing conditions:

Eighteen-month-old black spruce (*Picea mariana* (Mill.) B. S. P.) and tamarack (*Larix laricina* (Du Roi) K. Koch) seedlings were used in the flooding experiment. Seeds were obtained from the Alberta Tree Improvement and Seed Center (ATISC acc #4042). Seeds were stratified for three weeks at 4^0C and then germinated in Petri-dishes. One week after germination, seedlings were transplanted to Spencer-Lemaire (300ml) containers (Spencer-Lemaire Industries Ltd. Edmonton, AB. Canada) containing planting medium Pro-Mix BX (1:1:1; peat:moss:vermiculite; Premier Horticulture Inc., Riviere-du-Loup, Que. Canada) and placed in a growth chamber with $21^oC/18^oC$ day/night temperature, 65% relative humidity and 16-h photoperiod with photosynthetically active radiation (PAR) of 400 μmol m^{-2} s^{-1} provided by fluorescent lamps. Seedlings were watered to run off every other day and fertilized once a week with 30-10-10 N-P-K fertilizer (Plant-prod®- Plant Products Co. Ltd., ON, Canada).

After 24 weeks of growth we gradually reduced the growth chamber temperature and photoperiod in order to induce winter hardening. Plants were then placed in a cold room with 10^0C temperature and 10 h photoperiod for six weeks then in 4^0C and 8 h photoperiod for four months. After that seedlings were brought back to 15^0C temperature and 10 h photoperiod for two weeks. Thereafter seedlings were transplanted to 5” pots (with 1:1:1; peat:moss:vermiculite growing medium) and returned to the original growing conditions (21°C/18°C day/night temperature, 65% relative humidity and 16-h photoperiod) and were grown for 26 weeks.

Treatment application and experimental design:

Treatment application commenced when the seedlings flushed and were actively growing. Seedlings from both black spruce and tamarack were randomly placed in buckets where flooding was imposed by submerging the seedlings to root-collar level. Perforated buckets were used for control (non-flooded) seedlings to ensure adequate drainage. There were seven bucket for each treatment and twelve seedlings (six from each species) were randomly assigned in each bucket. Data were collected on day 2, 5, 8, 12, 16, 21, 27 and 34 after treatment imposition. On each measurement day, different physiological parameters were measured on five randomly selected seedlings from each species x treatment combination. A total of 160 seedlings (N) were used in this experiment. The experiment was a split-split-plot design (between subjects: flooding, within-subjects: species and time) and buckets were considered nested within treatments.

The statistical model is was following:

$$Y_{ijklm} = \mu + F_i + B_{j\,(i)} + S_k + FS_{ik} + B_{j(ik)} + T_l + TF_{il} + TS_{kl} + TFS_{ikl} + E_{ijklm}$$

Where, Y_{ijklm} = Physiological parameters (e.g., net assimilation, stomatal conductance, electrolyte leakage etc.), μ = overall mean, F = flooding treatment ($i = 1,2$), B = bucket within treatment ($j = 1,2,\ldots..14$); S = species ($k = 1,2$); T = time ($l = 1,2, \ldots 8$); E = error term ($m = 1,2,\ldots 160$).

Data were analyzed using analysis of variance (using SAS version 8.1; SAS Institute Inc., Cary, NC. 1996) to determine the main and interactive effects of flooding, time and species. Means were compared for significant differences at $P < 0.05$ using Tukey's test.

Measurements:

Gas exchange:

Net assimilation (NA), Stomatal conductance (g_s) and water use efficiency (WUE; net carbon assimilation rate divided by transpiration rate) of the flooded and non-flooded black spruce and tamarack seedlings were measured using an infrared gas analyzer (CIRAS 1: PP Systems, Haverhill, MA, USA) equipped with a automatic conifer cuvette (PLC(C), PP Systems Inc.). Leaf temperature was maintained at 20^0C and relative humidity at 40%. The flow rate was 250 ml min^{-1} and light level was maintained at 1050 µmol m^{-2} s^{-1}. Net assimilation and stomatal conductance rate were expressed on a leaf area basis. The uppermost shoots of a randomly selected seedling from each species and treatment combination were placed in the cuvette for gas exchange measurements. Gas exchange and all other physiological measurements (except root respiration and carbohydrate concentration) were taken on days 2, 5, 8, 12, 16, 21, 27, and 34 after flooding imposition. Needles were carefully detached from the stem and their surface areas were measured after computer scanning (Sigma Scan 3.0, Jandel Scientific, San Rafael, CA, USA).

Root hydraulic conductance:

Root hydraulic conductance (K_r) is defined as water flow rate (kg s $^{-1}$) per unit pressure drop (MPa) driving flow through the entire root system whereas root hydraulic conductivity (L_p) is obtained by dividing the K_r value by root volume and expressed in kg MPa $^{-1}$ s $^{-1}$ cm $^{-3}$ root volume. Root hydraulic conductance (K_r) was measured in intact roots using a High Pressure Flow Meter [HPFM] (Dynamax, Houston, TX, USA) as described by Tyree et al. (1995). The use of HPFM allows for measurements of intact roots since the water is applied under increasing pressure through an excised stem (around the root collar level) into the root system (Tyree et al. 1995). In this experiment, stems of seedlings were cut following gas exchange measurements two cm above the rot collar and flow rates were measured over a range of pressures from 0 to 2.75 MPa to obtain a linear pressure – flow relationship Tyree et al. (1995). Root hydraulic conductance of five root systems was measured from each species x treatment combination on each measurement day and was expressed in kg MPa $^{-1}$s^{-1}. Root hydraulic conductivity of adventitious roots and similarly sized flooded tamarack roots were also measured. Individual root volume was calculated by determining the radius and length of the roots.

Electrolyte leakage:

Electrolyte leakage, a measure of cell integrity and of cell membrane leakiness was measured using a conductivity meter (HI 8733 Hanna Instruments Inc., Woonsocket, RI, USA) as described by Zwiazek and Blake (1990) and Renault et al. (1998). Needles (approximately 0.5 g) were taken from five seedlings per species x treatment combination (after gas exchange and root hydraulic conductance measurements), washed with deionized water three times and placed in separate tubes, each containing 20-ml of deionized water. After 5 hours of incubation on an orbital shaker at 50 rpm, initial measurements were taken for electrical conductivity of the solution. Total electrolytes of the samples were obtained by autoclaving the samples at 121^0C for 15 minutes followed by freezing overnight at $–85^0$C. The samples were thawed by placing them in a water bath at room temperature for about 5 hours. Total electrolytes of the sample solution were then measured and electrolyte leakage was calculated as a percentage of the total electrolytes.

Root Respiration:

Root respiration of the whole root system was measured by recording oxygen uptake with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH). Root respiration was measured 2, 8, 16 and 27 days after flooding treatment was imposed. Root systems of the black spruce and tamarack seedlings (n = 5 per species x treatment combination) were placed in an airtight cylinder containing half-strength Hoagland's solution (Kamaluddin and Zwiazek 2002). Oxygen uptake rates were recorded every two minutes for 20 minutes. Respiration rate was expressed as average of oxygen uptake over time expressed in mmol L^{-1}.

Carbohydrate analysis:

Carbohydrates were extracted from shoots and roots three times with hot 85% ethanol at 95^0C. Carbohydrate concentrations were measured from both flooded and non-flooded black spruce and tamarack seedlings 2, 8,16 and 27 days after the treatment application. Soluble carbohydrate (sugar) concentrations were determined colorimetrically using phenolsulfuric acid as described by Smith et al. (1964). Starch was extracted from the same plant samples with NaOH and hydrolyzed by an enzyme mixture containing alpha-amylase (EC 3.2.1.1, ICN 190151, from *Bacillus licheniformis*) and amyloglucosidase (EC 3.2.1.3, Sigma A3514, from *Aspergillus niger*) and incubated for 41 h before colorimetric measurements with the glucoseoxidase/peroxidase-o-dianisidine reagent (Sigma Glucose Diagnostic Kit 510A) (Haisig and Dickson 1979). Absorbance readings were determined with an Ultrascope III spectrophotometer (Pharmacia LKB, UK) and sugar and starch concentration were calculated on a dry weight basis.

Results:

Morphological responses:

Visible damage of roots was observed in flooded seedlings of both species. Flooding reduced the root mass in both black spruce and tamarack seedlings compared to their non-flooded controls (not quantified, easily visible). Flooded tamarack seedlings developed adventitious roots beginning 16 days after the flooding treatment began. After this point in time, 86% of the flooded tamarack seedlings had adventitious roots (13 adventitious roots per seedling with an average length of 15 mm). Flooded black spruce lacked any such morphological adaptation. Needle chlorosis and necrotic needle tips were observed only in flooded black spruce seedlings.

Physiological responses:

Flooding significantly reduced net assimilation, stomatal conductance, root hydraulic conductance and root respiration while increasing needle electrolyte leakage in both species. Flooding also increased sugar concentration in the shoot while reducing it in the roots in both species. A significant ($P < 0.0001$) reduction in net assimilation (NA) rate was observed in both black spruce and tamarack seedlings under flooding compared to their non-flooded controls (Figure 1a). There were no significant effects of buckets within treatments. Flooded tamarack seedlings maintained significantly ($P < 0.0001$) higher NA than flooded black seedlings on day 8, 12, 16, 27 and 34 during the experiment.

Similarly, a significant ($P < 0.0001$) reduction in stomatal conductance was observed in both species under flooded conditions compared to their non-flooded seedlings (Figure 1b). Stomatal conductance declined from day 2 in flooded black spruce and tamarack seedlings. However, the magnitude of reduction was higher in flooded black spruce seedlings. Flooded tamarack seedlings maintained significantly ($P < 0.0001$) higher stomatal conductance than flooded black seedlings throughout the experiment.

Flooding increased WUE in tamarack but decreased it in black spruce as compared to non-flooded seedlings (Figure 1c). Flooding significantly ($P < 0.0263$) reduced root hydraulic conductance in both black spruce and tamarack seedlings compared to their non-flooded controls (Figure 2a). Although there was significant species ($P < 0.0001$) and time ($P < 0.0209$) effect, the interaction effect of treatment*species*time was not significant. However, flooded tamarack had significantly ($P < 0.0080$) higher root hydraulic conductance than flooded black spruce seedlings on day 27 and 34. Mean root hydraulic conductivity of adventitious roots was significantly higher than the conductivity in similarly sized flooded tamarack roots (Figure 2b).

Electrolyte leakage increased significantly ($P < 0.0001$) in both flooded black spruce and tamarack seedlings (Figure 3a). There was no significant species effect. Flooded black spruce had significantly higher leakage than flooded tamarack on day 16 and 34.

Root respiration rate was significantly reduced ($P < 0.0001$) in both species under flooding (Figure 3b). However, flooded tamarack maintained higher root respiration than flooded black seedlings throughout the experiment.

Sugar content in shoots remained same until day 16, then increased significantly in both species compared to the non-flooded controls while the reverse was observed in roots (Figure 4a and b). Starch content in shoots of flooded black spruce remained the same until day 8 and then increased exponentially compared to their non-flooded controls while no such trend was observed in flooded tamarack seedlings (Figure 5a). However, root starch content declined in both species (Figure 5b).

Discussion:

The results of our study suggest that tamarack was relatively more tolerant to flooding than black spruce, in agreement with the results of our previous study (Islam et al. 2002). Net assimilation and stomatal conductance were reduced in flooded seedlings of both species. However, the magnitude of reduction was much less for both species than for six-month old seedlings subjected to flooding (Islam et al. 2002). Net assimilation in non-flooded (10.58-14.29 $\mu molm^{-2}s^{-1}$) and flooded tamarack (5.86-11.24 $\mu molm^{-2}s^{-1}$) was also higher compared to non-flooded and flooded black spruce seedlings in present study compared to those found when they were six-months old (Islam et al. 2002).

The early reduction in the rate of photosynthesis of flooded plants is correlated with stomatal closure, resulting in decreased CO_2 absorption by leaves (Pezeshki et al. 1996). Subsequently, photosynthetic processes may be adversely affected by the metabolic consequences of prolonged hypoxia (Bradford 1983a, 1983b). Flooding can also reduce net photosynthesis by reducing the amount or activity of carboxylation enzymes (Vu and Yelenosky 1992), by disruption of photosynthate transport, alteration in source-sink relationships, or reduced sink demand (Wample and Thornton 1984, Drew 1990). Stomatal conductance was also reduced in conjunction with reduced carbon assimilation in flooded highbush blueberry plants (Davies and Flore 1986), and in *Quercus falcata* and *Q. lyrata* under low soil redox conditions (Pezeshki et al. 1996). However, a reduction in root hydraulic conductivity coupled with reduced leaf water potential was the key factor for stomatal closure and slower leaf expansion in flooded *Ricinus communis* (Else et al. 2001).

Likewise in our previous study (Islam et al. 2002), we observed a gradual reduction in stomatal closure as well as root hydraulic conductance in both species presumably due to oxygen deficit on membranes in present study. Similar reductions in g_s and K_r were observed in flooded *Pyrus* species (Anderson et al. 1984) and *Vaccinium corymbosum* (Davies and Flore 1986). Wan et al (1999) have also shown strong positive correlation between root hydraulic conductance and stomatal conductance and transpiration rates. However, our results (with 18 month old seedlings) contrast with those of Reece and Riha (1991) in which root hydraulic conductance of tamarack and white spruce (2 year old) appeared to be unaffected after 10 days of flooding.

Although there was a gradual decline in stomatal conductance in flooded tamarack seedlings, there were apparent increases in root hydraulic conductance and WUE after 16 days in those seedlings whereas, both root hydraulic conductance and WUE were significantly lower in flooded black spruce seedlings. The increase of root hydraulic conductance in flooded tamarack seedlings coincided with the emergence of adventitious roots in those seedlings 16 days after flooding treatment began. Flooded black spruce seedlings did not have any adventitious roots. Morphological adaptations such as production of hypertrophied lenticels, aerenchyma tissue and adventitious roots have been attributed to the high nutrient-uptake efficiency of flood-tolerant species (Kozlowski 1997). It is reported that flood-induced adventitious roots increased water absorption by roots (Hook and Scholtens 1978, Jackson and Drew 1984, Tsukahara and

Kozlowski 1985). Our results suggest that adventitious roots increased total root hydraulic conductance as a result of their increased conductivity. Flooded tamarack seedlings were only able to develop hypertrophied lenticels but lacked the emergence of adventitious roots in six-month old seedlings (Islam et al. 2002).

Needle chlorosis and development of necrotic needle tips in flooded black spruce seedlings were possibly due to significantly higher membrane damage (i.e., increased needle electrolyte leakage). Needle chlorosis in black spruce might have been caused by a reduced supply of nutrients, which has also been observed in some flooded woody plants (Larson et al. 1992; Smith and Bourne 1989). Higher membrane damage could also explain the drastic reduction in NA in flooded black spruce seedlings. On the contrary, flooded tamarack seedlings showed less membrane damage and no visual symptoms of flooding damage.

Root respiration rates were reduced for both species under flooding, with a greater reduction for black spruce over the entire experiment. A reduction in root respiration was also observed in hypoxia sensitive *Triticum aesivum* cultivar ‘Coker 9835’ (Huang and Johnson 1995). Although it has been suggested that a low oxygen consumption rate can contribute to greater tolerance of hypoxia (Huang and Johnson 1995), our results contrast with that. Continued water uptake by roots is oxygen dependent because both the active ion uptake needed to maintain an osmotic gradient across the root (Rowe and Beardsell 1973) as well as membrane hydraulic conductivity (Glinka and Reinhold 1962) are known to require oxygen. Although flooded black spruce maintained low oxygen consumption in present study, it failed to maintain higher root hydraulic conductance, as did flooded tamarack.

Carbohydrates are the primary energy storage compounds in plants and their allocation pattern and translocation rate appear to be critical for hypoxia tolerance (Huang and Johnson

1995). Oxygen deficient conditions inhibit root elongation due to low carbohydrate reserves in the root tips (Webb and Armstrong 1983). The high accumulation of carbohydrates in shoots, compared to roots, in our study suggests that sufficient carbohydrates were available for metabolic activities but root zone hypoxia prevented their export to roots. Higher accumulation of carbohydrates was also observed in shoots of *Atriplex amnicola* (Galloway and Davidson 1993). A reduction in the translocation of assimilates in hypoxic plants (Schumacher et al.1985) leads to low carbohydrate availability in roots (Webb and Armstrong 1983). Barclay and Crawford (1983) have shown that tolerance of plants to flooding is dependent on the available carbohydrates reserves in the roots. In our study, although root sugars declined in both species, tamarack maintained higher sugar content throughout the experiment. Moreover, higher root respiration and root sugar content might have contributed to the higher root hydraulic conductivity in flooded tamarack seedlings. Root respiration has also been found to be dependent on stored carbon in soybean (Hansen et al. 1992).

In conclusion, our study demonstrated that tamarack exhibits greater flooding tolerance than black spruce seedlings. We suggest that membrane function and respiration dependent processes were responsible for the reduction in root hydraulic conductance in flooded plants. However, emergence of adventitious roots in flooded tamarack improved root water uptake. Morphological acclimations, maintaining high rates of root hydraulic conductance and respiration are likely the key processes that enable tamarack to withstand flooding effects better than black spruce.

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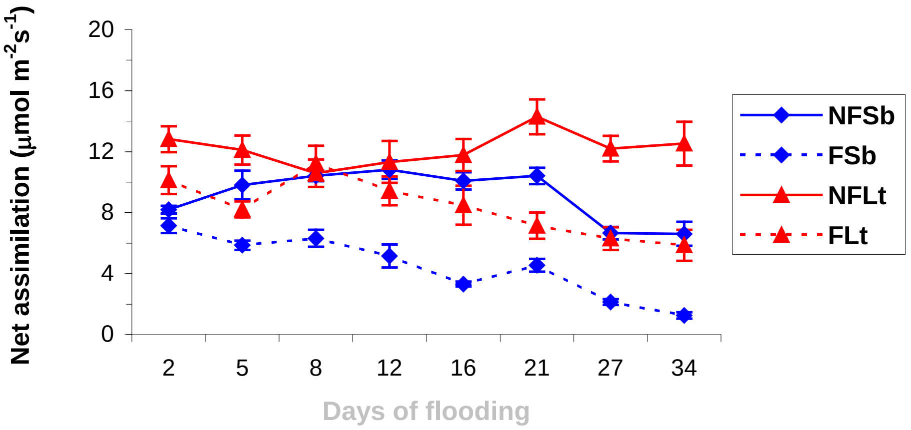
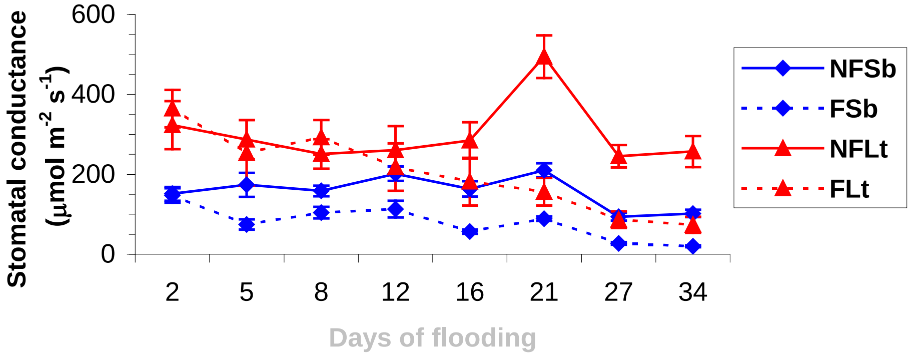
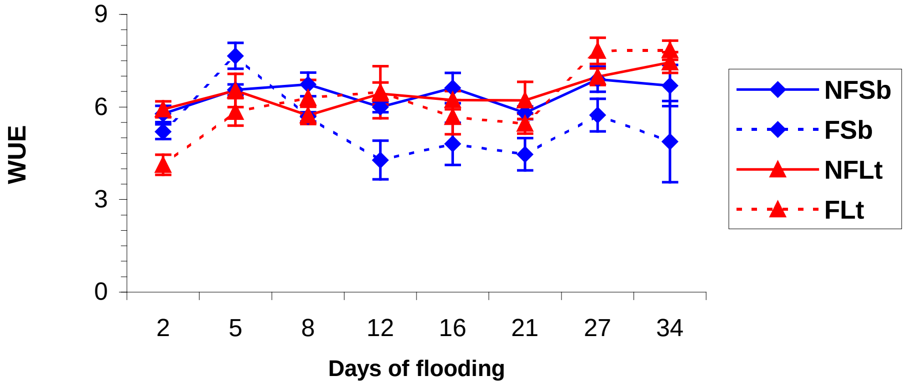
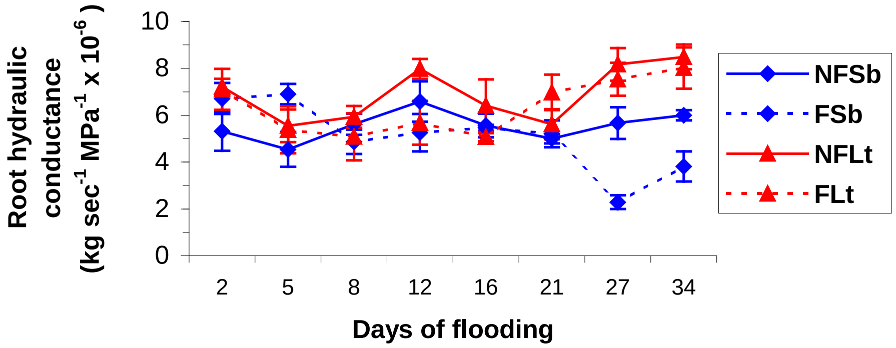
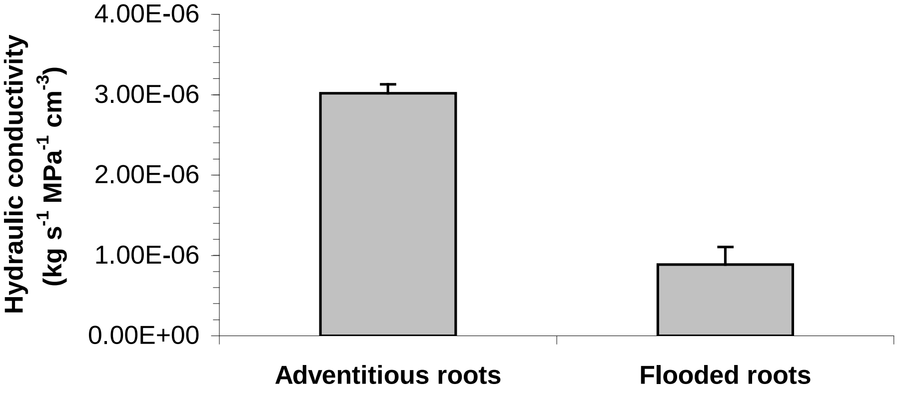
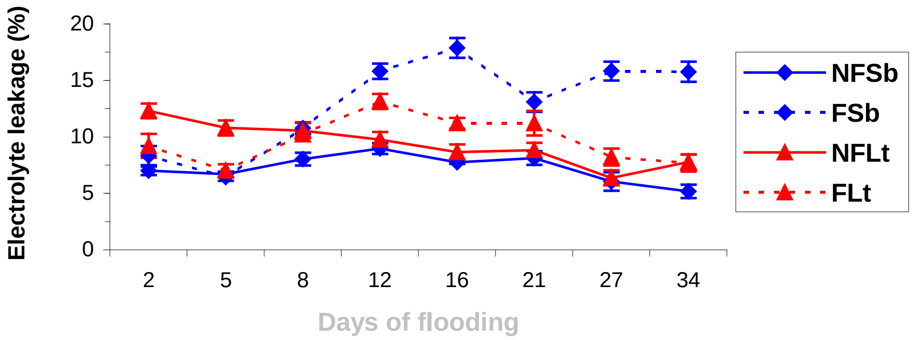

Net assimilation (μmol m^{-2}s^{-1})
20
16
12
8
4
0
2
5
8
12
16
21
27
34
Days of flooding
NFSb
FSb
NFLt
FLt

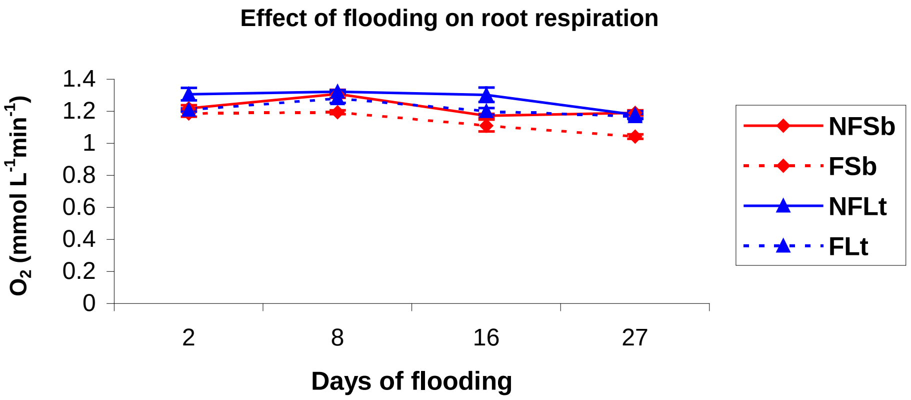

Stomatal conductance (μmol m^{-2} s^{-1})
600
400
200
0
2
5
8
12
16
21
27
34
Days of flooding
NFSb
FSb
NFLt
FLt

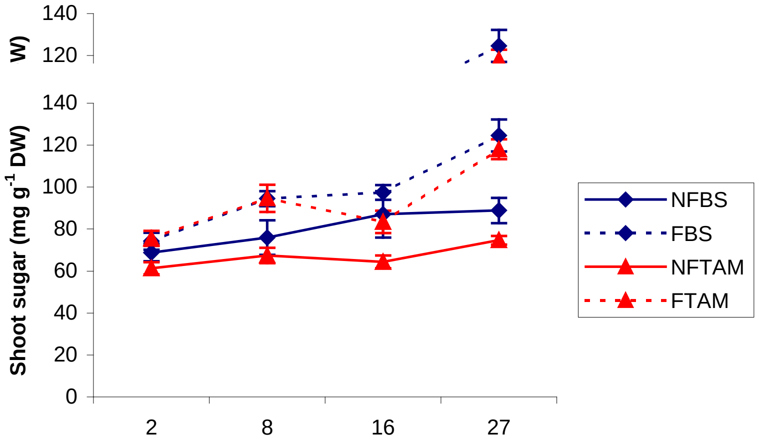

WUE
9
6
3
0
2
5
8
12
16
21
27
34
Days of flooding
NFSb
FSb
NFLt
FLt


Root hydraulic conductance (kg sec^{-1} MPa^{-1} x 10^{-6})
10
8
6
4
2
0
2
5
8
12
16
21
27
34
Days of flooding
NFSb
FSb
NFLt
FLt

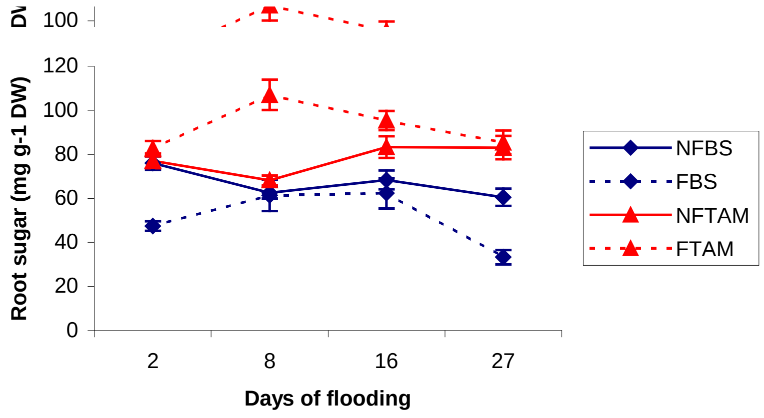

Hydraulic conductivity (kg s^{-1} MPa^{-1} cm^{-3})
4.00E-06
3.00E-06
2.00E-06
1.00E-06
0.00E+00
Adventitious roots
Flooded roots


Electrolyte leakage (%)
20
15
10
5
0
2
5
8
12
16
21
27
34
Days of flooding
NFSb
FSb
NFLt
FLt

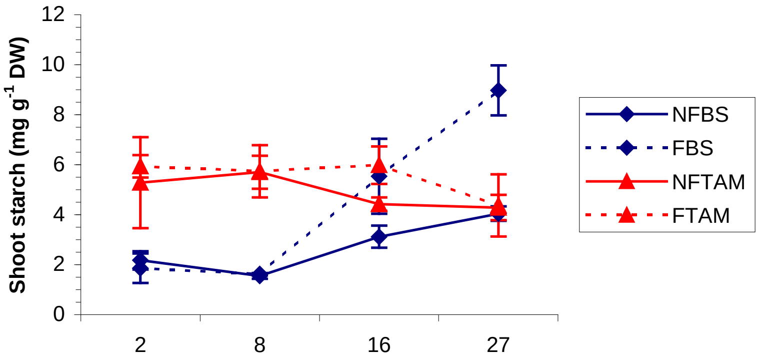

Effect of flooding on root respiration
O_2 (mmol L^{-1}min^{-1})
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
8
16
27
Days of flooding
NFSb
FSb
NFLt
FLt



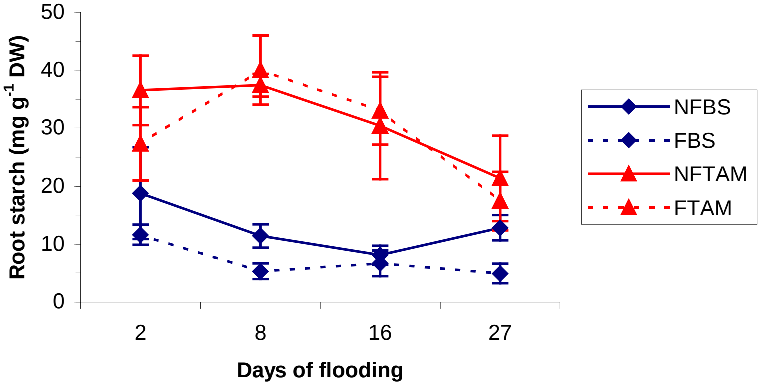
W)
140
120
Shoot sugar (mg g^{-1} DW)
140
120
100
80
60
40
20
0
2
8
16
27
NFBS
FBS
NFTAM
FTAM



DW
100
Root sugar (mg g-1 DW)
120
100
80
60
40
20
0
2
8
16
27
Days of flooding
NFBS
FBS
NFTAM
FTAM



Shoot starch (mg g^{-1} DW)
12
10
8
6
4
2
0
2
8
16
27
NFBS
FBS
NFTAM
FTAM



Root starch (mg g^{-1} DW)
50
40
30
20
10
0
2
8
16
27
Days of flooding
NFBS
FBS
NFTAM
FTAM