Morphophysiological responses of *Alnus subcordata* (L.) seedlings to permanent flooding and partial submersion

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ABSTRACT

*Alnus subcordata* (L.), the Caucasian alder, is a pioneer riparian tree native in riparian zones and wetlands of the Middle East region of Asia. In the Hyrcanian forest of North Iran, one of the most valuable forest communities in this world region, the use of this species through afforestation practices is being considered to restore the forest cover in extensive wetland areas that have been recently altered by a significant increase in the duration of their waterlogged conditions. To assess restorers, the morphophysiological responses to flooding stress of *A. subcordata* were investigated during a 120-day *ex situ* experiment. Forty-eight one-year-old seedlings were subjected to three flooding treatments: 1) unflooded; 2) permanently flooded (i.e., 3 cm of flooding depth); and 3) partially submersed (i.e., 15 cm of flooding depth). Although survival of seedlings at the end of the experiment was almost complete, plants subjected to the two flooding stress treatments had a significantly lower growth. Other responses to flooding included the formation of hypertrophied lenticels and adventitious roots and a surprising higher allocation of biomass in roots. However, free proline concentration in leaves and roots was not affected by flooding. Overall, the results showed that *A. subcordata* is able to withstand long periods of waterlogged conditions by readjusting some morphological and physiological parameters.

Keywords: Adventitious roots, Caucasian alder, leaf area, proline, restoration, riparian tree.

1. Introduction

*Alnus subcordata* (L.), the Caucasian alder, is a pioneer, deciduous broadleaf and fast growing tree species widely distributed across the temperate areas of the Middle East region of Asia, including some regions of Iran and the Caucasus (Sabeti, 2006). Due to their ability to occupy riparian zones, the species of the genus *Alnus* (family *Betulaceae*) are known to develop important ecological functions in wetland ecosystems, such as soil stabilization and timber production (Stangl et al., 2009). In the Hyrcanian forest of northern Iran, which is one of the most ancient and unique forest communities in the world with 80 tree and 50 shrub species in less than two million hectares (Marvi-Mohajer, 2007), *A. subcordata* is frequently found as a native in wetlands, along streams and rivers and at the bottom of valleys and plains (Sabeti, 2006).

The extent of the Hyrcanian forest has strongly decreased during the last decades, mainly due to population growth and associated land reclamation for agriculture, road construction,
overgrazing and a higher frequency of fires. The loss of vegetation in the Hyrcanian forest has particularly affected the bottom of valleys and plains and the banks and floodplains of river courses where A. subcordata dominates, causing in these areas an increase in waterlogging and permanent flooding conditions for long and continuous periods of 5-6 months. Shortly, less forested uplands and more compact soils led to an increase in runoff, higher ground water levels and lack of adequate water drainage in the soil. Although constrained by the new environmental conditions, A. subcordata might be one of the few native tree species that can persist in the bottomlands of the Hyrcanian forest and outcompete the exotics Populus deltoides and Taxodium dischitum, the latter species especially well adapted to permanent flooded conditions (Day et al., 2006). Recently, restoration of the Hyrcanian forest bottomlands has become a major challenge for the forest management authorities. Active afforestation of A. subcordata is being considered as an alternative to restore the new waterlogged degraded areas in the forest, but science-based information on the responses of this species to flood stress is still missing.

Permanent flooding is a severe stress to trees that grow in wetland areas and affects normal plant functioning (Pezeshki, 2001; Jackson and Colmer, 2005). Plant responses to waterlogging are mediated by different morphological and physiological mechanisms (Pezeshki, 2001; Bailey-Serres and Voesenek, 2008; Parent et al., 2008). Flooding suppresses leaf formation and expansion of leaves, lead to premature leaf abscission, chlorosis and senescence, and reduces leaf size, area and number of leaves (Kozlowski, 1997). Waterlogging negatively affects shoot growth, produces death and decay of roots (Kozlowski, 2002), alters the proportions allocated to below- and above-ground biomass compartments (Gonzalez et al., 2010) and is often associated with massive decreases in overall plant biomass increment and productivity (Kozlowski, 1997, 2002), water and nutrient uptake and reduced metabolism (Dat et al., 2004). Other physiological adaptations include reduction in stomata conductance and photosynthesis as well as root hydraulic conductivity (Parent et al., 2008), and decrease in chlorophyll and protein levels (Kozlowski, 2002; Yordanova, 2007).

Partial and total submersion, that are extreme cases of flooding, may also induce in flood-tolerant species the formation of adventitious roots, hypertrophied lenticels and aerenchyma development (Kozlowski, 1997, 2002; Dat et al., 2004; Glenz et al., 2006). In fact, the height of flood waters during plant submersion is an important issue for plant development. Flooding depths may affect the expression of the stress adaptability by means of change in growth, morphology and physiological processes in plants, as noted by Iwanaga and Yamamoto (2008) for A. japonica.

The variety of plant strategies to cope with waterlogging conditions, however, may vary enormously among closely related species (Kozlowski, 1997; Sakio, 2005; Voesenek et al., 2006). For example, Populus nigra a riparian tree that dominates the forest of many river courses in Southwestern Europe, is generally considered as a high flood-tolerant species, while one of its natural competitors, P. alba, might be better classified as an intermediate flood-tolerant (Glenz et al., 2006). In the case of the genus Alnus, previous studies have examined the tolerance to permanent flooding of A. glutinosa (Siebel and Blom, 1998), A. incana (Hughes et al., 1997; Kaelke and Dawson, 2003; Francis et al., 2006), A. japonica (Iwanaga and Yamamoto, 2008), A. maritima (Schrader et al., 2005), A. rubra Bong and A. viridis Sinuate (Batzli, 1997). The overall result of those studies is that Alnus spp. are high tolerant to permanent flooding but some species such as A. glutinosa may be relatively more than others in the genus. However, to our knowledge, there is neither study concerning the
flood-tolerance of *A. subcordata* nor any information about the effects of flooding depth on growth, morphology and physiology of this species in the existing literature.

The purpose of the present study was to investigate the effects of permanent flooding and partial submersion on *A. subcordata* using some morphological and physiological parameters that may be involved in the plant strategy to cope with waterlogging conditions and partial submersion. The results of this work could be useful to assess the species’ adaptation to the new environmental conditions in the Hyrcanian forest of Iran and thus helping guiding future afforestation and restoration projects.

### 2. Materials and Method

#### 2.1 Plant material and experimental design

In March 2009, seeds of *A. subcordata* were collected from natural populations in the Hyrcanian forest, 25 km south from the city of Amol (36°34'N, 52°17'E, 76 m a.s.l.), located in the Mazandaran province (N Iran). The seeds were immediately brought to the commercial nursery of Koloudeh, situated approximately 12 km from the city of Amol, to grow 15-months old seedlings in a nursery open field.

In May 2010, plants were transplanted into 23 cm side cubic pots (one plant per pot), previously filled with a homogeneous mixture soil (sand: clay-loam = 1:5 by volume, pH = 8.4) taken from a typical *A. subcordata* recruitment site in the Hyrcanian forest and without any added fertilizer. Twenty days after the transplantation, 48 of the pots containing surviving and healthy plants of similar size were transported to the Natural Resources campus located in the Tarbiat Modares University in the city of Noor (36°35'N; 52°02'E, -25 m a.s.l.), Mazandaran province to be used in an experiment carried out outdoors at an open site.

The 48 plants were randomly divided into three groups of 16 plants that were placed in three concrete pools (3.5 m length x 2.5 m wide x 0.5 m deep). Then, on 13 June 2010, each group was subjected to the following three flood treatments: 1) control (C): soil water content at field capacity, 2) permanent flooding with no submersion (F), with the water level 3 cm above the soil surface and 3) permanent flooding with partial submersion (S), with the water level 15 cm above the soil surface. The flood treatments were maintained with no variation during 120 days until the end of the experiment on 15 October 2010. The flood treatments were based on a range of flood depths observed in the recruitment sites for *A. subcordata* of the Hyrcanian forest. Water was periodically added whenever the water level in the pools decreased due to evapotranspiration. Mean daily minimum and maximum air temperatures and cumulative precipitation registered during the 120 days of experiment were 19°C, 40°C and 71 mm, respectively (Mazandaran Meteorological Office).

#### 2.2 Non-destructive morphological measurements

Height, diameter and number of leaves of all plants were recorded at the beginning and at the end of the experiment. Height was measured on the main stem, from the base to the apex and to the nearest mm. Diameter was measured using a digital caliper at the base of the main stem and also to the nearest mm. The differences in height, diameter and number of leaves between Day 1 and Day 120 were used to calculate the height, diameter and leaf number increments as indicators of plant growth. Only living leaves were counted, but they included senescent and damaged leaves. Throughout the experiment, any visible effects of flooding
stress on root, stem, and leaf morphology (i.e., adventitious roots, hypertrophied lenticels and leaf yellowing and shedding) also were recorded.

### 2.3 Destructive morphological measurements

At the end of the experiment, four randomly selected plants of each of the three treatments were carefully removed from the pots and separated into root, stem and leaf components. Then, five fully developed leaves were collected from each plant, scanned and analyzed with an UTHSCSA Image Tool analysis system (University of Texas Health Science Center, San Antonio, Texas, USA) to estimate leaf area. Roots were washed to remove the soil and divided into original and adventitious roots. Root nodules containing nitrogen-fixing bacteria were visually assessed during harvest. All the material was dried in the oven at 70 °C during 48 h and weighted to calculate root, stem and leaves biomass. From these measurements, root : shoot biomass ratio and specific leaf area (leaf area divided by leaf biomass, cm$^2$ g$^{-1}$), were subsequently calculated.

### 2.4 Physiological measurements

Leaf samples of the four selected plants per treatment were also used to determine chlorophyll content (mg g$^{-1}$ of fresh weight, FW) following Arnon (1949) procedure. Also at the end of the experiment on Day 120, proline was extracted with sulfosalicylic acid (3%) from fresh leaf and root tissues of the 12 plants that had been selected for the destructive morphological assessments and was quantified according to the protocol described by Bates et al. (1973). Proline is known as the most important osmolyte accumulated under environmental stress in plants (Roger, 2003).

### 2.5 Data analysis

All the measured variables were subjected to a one-way ANOVA with flooding treatment as the independent factor (3 levels: control, flooding and submersion). Differences between means were analyzed by the post hoc LSD multiple range test. Assumptions of normality and equality of variances were checked for all variables using Shapiro-Wilk’s and Levene’s test respectively. In all cases a level of significance of $P < 0.05$ was used to reject the null hypothesis. All statistical analyses were conducted using the statistical software package SPSS 17 for Windows.

### 3. Results

All the plants survived the 120 days of permanent flooding and partial submersion treatments, while only one of the 16 control plants died at the end of the experiment. However, substantial differences in most of the morphological and physiological parameters were found between the three treatments.

#### 3.1 Non-destructive morphological measurements

Both height and diameter growth were much reduced under flooded conditions compared to control treatment (Figure 1). Flooding also influenced enormously the number of leaves (Figure 1). An absolute loss of more than one half of the leaves under the flooded conditions contrasted significantly to the great increase in leaf number experienced by the unstressed plants. Moreover, the leaves of flooded plants were in a worse health state (i.e., yellowing...
and shedding were very common among the surviving leaves) than control plants. Hypertrophied lenticels started to appear on submerged stems after 15 days of flooding.

**Figure 1:** Non-destructive morphological measurements on *Alnus subcordata* 15-month old seedlings after 120 days of three different flooding treatments. Each bar represents the mean of 16 seedlings subjected to control (soil water content at field capacity), permanent flooding (water level at 3 cm above the soil surface) and partial submersion (water level 15 cm above the soil surface) conditions and are denoted in black, white and gray colors, respectively. C – Control, F – Flooding, S – Submersion. The error bars represent ±1 standard error of the mean. Lowercase letters indicate differences between flooding treatments (post hoc LSD multiple range test $P < 0.05$). The F statistics resulting from one-way ANOVA and their P values are also shown for each variable.

### 3.2 Destructive morphological measurements

Most of the destructive morphological variables: leaf area, specific leaf area, leaf biomass, stem biomass, root (original and total) biomass and total biomass were significantly lower in the plants subjected to flood stress than in the control plants (Figure 2). Only adventitious roots (first appearing on Day 20) and root : shoot biomass ratios were greater in the flooding and submersion plants compared to the control plants. These latter two variables also differed between the permanent flooding and the partial submersion treatments. In particular, submerged plants had greater adventitious roots and higher root : shoot ratios than plants.
permanently flooded. No nodules were observed in the permanent flooding and partial submersion treatments, contrasting to their frequent presence in the roots of control plants.

Figure 2: Destructive morphological measurements on *Alnus subcordata* 15-month old seedlings after 120 days of three different flooding treatments. Each bar represents the mean of 4 seedlings (16 seedlings for leaf area) subjected to control (soil water content at field capacity), permanent flooding (water level at 3 cm above the soil surface) and partial submersion (water level 15 cm above the soil surface) conditions and are denoted in black, white and gray colors, respectively. C – Control, F – Flooding, S – Submersion. The error bars represent ±1 standard error of the mean. Lowercase letters indicate differences between flooding treatments (post hoc LSD multiple range test $P < 0.05$). The F statistics resulting from one-way ANOVA and their P values are also shown for each variable.

3.3 Physiological measurements

Chlorophyll content of *A. subcordata* seedlings decreased significantly with increasing flooding depth (Figure 3). However, no significant differences in proline concentration were found between the three treatments, either in leaf or in root tissues (Figure 3).
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Figure 3: Physiological measurements on *Alnus subcordata* 15-month old seedlings after 120 days of three different flooding treatments. Each bar represents the mean of 4 seedlings subjected to control (soil water content at field capacity), permanent flooding (water level at 3 cm above the soil surface) and partial submersion (water level 15 cm above the soil surface) conditions and are denoted in black, white and gray colors, respectively. C – Control, F – Flooding, S – Submersion. The error bars represent ±1 standard error of the mean. Lowercase letters indicate differences between flooding treatments (post hoc LSD multiple range test P < 0.05). The F statistics resulting from one-way ANOVA and their P values are also shown for each variable.

4. Discussion

Continuous flooding for 120 days induced several responses in *A. subcordata* plants that were similar to those reported in the literature for other wetland plants, including the reduction in growth and vitality and the formation of hypertrophied lenticels and adventitious roots (Kozlowski, 1997; Pezeshki, 2001; Kozlowski and Pallardy, 2002; Dat et al., 2004; Parent et al., 2008). However, the extreme flood stresses imposed here did not considerably affect the survival of seedlings. This surprising result was consistent with the findings of Schrader et al. (2005), who reported that total flooding of *A. maritima* did not reduce its survival rates. Therefore, it seems that morphological and physiological adaptations to flood stress in *A. subcordata* could well increase the likelihood of survival. Our results also showed that flooding greatly decreased the height growth, diameter growth, number of leaves and their area in *A. subcordata* seedlings. In fact, decreases in plant growth, leaf area and number of leaves are common responses to flooding reported for other species of *Alnus* spp. (Hughes et al., 1997; Schrader et al., 2005; Francis et al., 2005). These responses to flood stress occur
due to a variety of factors including anaerobic respiration of the root and the disrupted translocation of root metabolites (Kozlowski, 1997; Pezeshki, 2001).

Changes in biomass allocation between organs are another strategy to cope with environmental stress in wetland plants (Yin et al., 2009). Flood-tolerant species have various ways to survive and recover from flooding impacts, ranging from low rates of biomass loss and low recovery to relatively high rates of biomass loss and quick recovery (Visser et al., 2000; Van Eck et al., 2004). In this experiment, flooding conditions reduced biomass in all plant compartments, suggesting that *A. subcordata* would fit better in the latter group of species, although an eventual recovery after flood stress remains untested. Reduction in *A. subcordata* biomass as a consequence of flooding was largely the result of decreased shoot growth, lost of leaves, root decay and inhibition of root initiation and growth.

Root growth is typically reduced to a higher level than stem growth, resulting in lower root:shoot ratio in flooded than in unflooded seedlings (Kozlowski, 1997). However, we unexpectedly observed that this ratio increased with greater flooding depths. According to Polomski and Kuhn (1998) observations in *Salix, Populus* and *Alnus* spp, the dieback of the original root system due to flooding may favor the development of younger roots, in turn attenuating the decrease in root biomass and ultimately leading to higher root: shoot ratios. Similar processes might have occurred in our experiment. However, the abundant production of adventitious roots under the two flooding treatments could also contribute to those unexpectedly high root:shoot ratios found here. In fact, if adventitious roots had not been used for root: shoot ratio calculations, only the ratio in the submersion treatment would have been significantly higher than the control (0.85 vs. 0.58, respectively). The production of adventitious roots either on the original root system or on the submerged portions of stems in flooded seedlings has been described as an adaptive mechanism against flooding (Kozlowski and Pallardy, 2002; Glenz et al., 2006). Examples of *Alnus* species that produce adventitious roots in response to flooding include *Alnus glutinosa, A. rubra* and *A. japonica* (Kozlowski, 1997; Glenz et al., 2006; Kozlowski and Pallardy, 2002; Iwanaga and Yamamoto, 2008). This morphological adaptation to flood stress and others also observed in our experiment, such as the formation of hypertrophied lenticels, increase the uptake of $O_2$ by aerial tissues and promote its transport into the root system (Glens et al., 2006).

*Alnus* spp. are known to have a symbiotic relationship with arbuscular mycorrhizal fungi, which allows individuals to acquire and utilize nitrogen and phosphorus more effectively (Monzon and Azcon, 2001). They also produce root nodules containing the nitrogen-fixing actinomycete *Frankia* spp. However, flooding stress may cause the death of these valuable bacteria (Kaellkel and Dawson, 2003). Thus, while root nodules containing nitrogen-fixing were frequently observed in the control, we did not observe any nodules on the roots of flooded seedlings.

As expected, chlorophyll decreased in leaf tissues as a result of flooded conditions (Kozlowski, 2002; Yordanova, 2007). The reduction in chlorophyll content due to flooding may be due to either slow synthesis or fast breakdown of chlorophyll pigments (Ashraf, 2003). However, we did not observe any significant changes in free proline levels after applying the flooding stress. This observation contrasts to the findings of Garcia-Sanchez (2007), who reported non-significant effects of flooding on proline levels in leaves but an increase in roots in two species of *Citrus* (*C. sinensis* and *C. resnhi*).
Finally, it should be pointed out that the lack of substantial differences between our permanent flooding and partial submersion treatments may be due to the fact that once the soil is flooded; the depth has little significance until a substantial part of the plant foliage is covered. Our water level of 15 cm (below the limit of the lower foliage of seedlings) was probably not enough to observe more differences with the 3 cm treatment, besides a higher adventitious root biomass and associated root : shoot ratios, and a lower chlorophyll content.

5. Conclusion

Restoration of degraded riparian and bottomland forests usually involve the planting of native pioneer tree species to create a more effective riparian and floodplains buffer (Harper et al., 1999; Webb and Erskine, 2003). However, afforestation practices very often fail due to a lack of science-based knowledge about the growth, morphological and physiological adaptations of the target species to the local hydrological regime. Ideally, species planted in restoration schemes must be able to tolerate a wide range of hydrological conditions and to grow rapidly, so as to consolidate the substrate and provide a habitat for later successional desired species (Francis et al., 2005). According to the findings of this experiment, A. subcordata is a flood-tolerant species that has the capacity of withstanding long periods of waterlogged conditions by reducing plant growth and readjusting some morphological and physiological parameters. Therefore, this species is a priori suitable to be included in afforestation plans in the bottomlands and riparian zones of the Hyrcanian forest and other wetland environments with similar hydrological constraints. However, this conclusion must be taken with caution as the reduction in growth and biomass rates were substantial and the recovery of plants after the cessation of the stress was not tested in this experiment. Pilot planting tests in the field are strongly recommended to complement the results of this study as an assessment for the effectiveness of future restoration projects based on afforestation practices.

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6. References


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