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STOMATAL CONTROL ON PHOTOSYNTHESIS IN DROUGHT-TREATED SUBALPINE PINE SAPLINGS

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Summary: Stomata have a key role in the balance of gas exchange parameters. Stomatal closure limits transpiration under conditions of water deficit. It also suppresses photosynthesis due to limited CO_2-uptake. We examined the levels of stomatal control on the photosynthesis of *Pinus mugo* Turra, *Pinus peuce* Griseb. and *Pinus heldreichii* Christ. saplings during a two-year drought experiment. Plants were exposed to three different irrigation regimes (according to the annual course of precipitation): typical for the natural area of the species; for years with extremely dry periods during July as well as for years with drought during August. A species specific stomatal control on the rate of photosynthesis (An) under different irrigation regimes was observed. *P. mugo* saplings showed great resistance to drought, they kept similar An under the different irrigation schemes and increased intrinsic water-use efficiency (iWUE) under drought conditions. *P. peuce* saplings had reduced stomatal control on An, especially in the situation with late summer drought and unchanged iWUE. *P. heldreichii* saplings had very low An and stomatal conductance under all irrigation schemes accompanied by an increase in iWUE during early summer drought and a decrease in late summer drought. The relationships between photosynthesis and stomatal conductance under the three irrigation schemes were close to linear – photosynthesis did not reach asymptote in this species. These differences in the reaction of photosynthesis to stomatal conductance could be used as a good indicator for the adaptability of the studied species to summer drought.

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Keywords: Drought; intrinsic water-use efficiency; photosynthesis; pine saplings; stomatal control.

INTRODUCTION

In the Balkan Peninsula mountains, where the subalpine forests are often composed of specific mix of tree species (Panayotov et al., 2010), drought events are expected to increase in future (Stocker et al., 2013). This concerns the specific responses to drought of saplings from the most important or potentially vulnerable tree species for the Bulgarian subalpine zone. Among them the greatest concerns

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are focused on Mountain pine (*Pinus mugo* Turra), Bosnian pine (*Pinus heldreichii* Christ.) and Macedonian pine (*Pinus peuce* Grisb.) due to the endemic status and limited distribution of *P. heldreichii* and *P. peuce* and the importance of *P. mugo* for their regeneration. Furthermore, there is limited information for the regeneration of *P. peuce* and *P. heldreichii* and especially for the performance and survival of saplings of these two species in extreme climate conditions. To our knowledge, to date there are no specific studies on the physiological responses of these species in drought simulation experiments.

Photosynthesis is one of the most sensitive to drought physiological processes. The summer hot and dry period leads to soil and plant water deficit which results in stomatal closure to protect leaves from drying. Reduced rate of photosynthesis (An) can be caused by stomatal closure, and an altered pathway of the photosynthetic process (Assmann, 1988; Lawlor, 2002). Drought stress causes not only a substantial damage to photosynthetic pigments, but also leads to deterioration of thylakoid membranes (Huseynova et al., 2007; Yordanov et al., 2000). Photosynthetic capacity decreases quickly with leaf dehydration due to stomatal closure which leads to a shortage of CO_2 and lack of water, causing a decrease of sources for reducers (H^+) used in photosynthesis.

Stomatal conductance for CO_2 (g_{sc}) is a good indicator for determination of stomatal and nonstomatal limitations to photosynthesis (Xu and Zhou, 2008). Many studies based on greenhouse experiments revealed that changes in stomatal conductance were the main cause for decreased photosynthesis. Flexas and Medrano (2002) showed that An had a strong correlation with g_{sc} in both field-grown and potted grape wine plants. This strong relationship led to the assumption that the down-regulation of photosynthesis depends more on the availability of CO_2 in the chloroplast than on leaf water content or water potential (Flexas and Medrano, 2002; Galmes et al., 2011; Saibo et al., 2009). Yet Peri et al. (2002) showed that reduced photosynthesis caused by water-deficit conditions occurred at different leaf water levels in different species, even though at similar g_{sc}. Thus, it is likely that either water deficit has no effect on photosynthesis until a threshold is reached, beyond which it is impaired or a consistent suppression in metabolism is caused (Lawlor, 2002; Lawlor and Tezara, 2009). Moreover, some plants that survive in drought conditions often show high rate of photosynthesis, when water is not limiting (Flexas et al., 2014).

Independently of the cause for photosynthetic decrease, most plants tend to show an increase in water-use efficiency (WUE) when water deficit is mild (Anev et al., 2014). WUE depends on both stomatal conductance and the difference between water vapor pressure in leaf intercellular spaces and that in the air. As temperature affects the water vapor concentration in the leaf, temperature also has a pronounced effect on plant WUE. Therefore, iWUE defined as the ratio between An and stomatal conductance for water vapor (g_{sw}), is a better indicator for plant's physiological water use efficiency (Comstock and Ehleringer 1992). Higher iWUE may be caused by reduced g_{sw}, increased An or a combination of both (Xu et al., 2014).

iWUE increased in drought, primarily due to decreased stomatal conductance, and thus water loss declined more than carbon fixation (Edwards et al., 2012). This increase results in the non-linear relationship between An and g_{sc} (Gilbert et al., 2011), that is, water loss being restricted before, and more intensely, than photosynthesis. These findings are in confirmation with the hypothesis that plant evolution has brought about an optimization of carbon uptake versus water loss (Raven, 2002).

A linear relationship between stomatal conductance and photosynthetic rate was observed in experiments with varied light conditions (Wong et al., 1979), varied CO_2 ambient partial pressure (Wong et al., 1985a) and varied drought and photoinhibition (Wong et al., 1985b). However, paired measurements of daily maximal CO_2 assimilation rate and leaf conductance for some C3 species under long-term drought exhibited curvilinear relations (Hall and Schulze, 1980; Havranek and Benecke, 1978). Both sun and shade *Fagus sylvatica* leaves reached maximum photosynthetic rate at saturating stomatal conductance (Schulze and Hall, 1982). This response to drought found in C3 species probably had an adaptive significance since iWUE increased as the soil water supply decreased.

The aim of this study was to examine the level of stomatal control on the photosynthetic rate and iWUE of artificially irrigated individuals of *P. heldreichii*, *P. peuce* and *P. mugo* from subalpine locations in Bulgaria.

MATERIALS AND METHODS

Site description and plant material

The experiment was conducted in a rain shelter, provided with a blue plexiglass and located in the forest nursery of the University of Forestry in Yundola village, 1350 m a.s.l. (Fig. 1). 5–10 year-old saplings of three different species from genus *Pinus* were used in this experiment: *Pinus mugo* Turra, *Pinus*

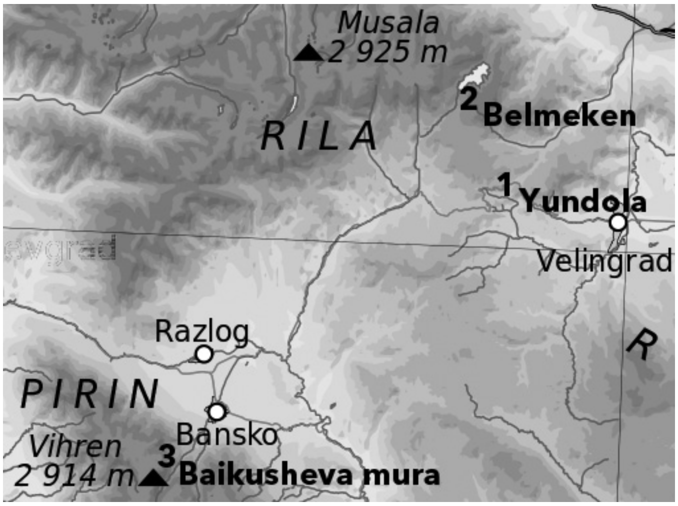

Figure 1. Location of the experimental plot Yundola (1) and the natural regions of the species used in the experiment: Belmeken for *Pinus mugo* (2) and Baikusheva mura for *Pinus peuce* and *Pinus heldreichii* (3).

heldreichii H. Christ., and *Pinus peuce* Grisb. The saplings with no disturbed soil substrata were moved from their natural environment (Pirin mountain, 1850 m a.s.l. for *P. heldreichii* and *P. peuce* and Rila mountain, 1900 m a.s.l. for *P. mugo*) to the forest nursery in the early spring of 2013. The saplings were grown during two years in 15-l containers with controlled irrigation.

The position of our experimental site was thus at a lower elevation and expectedly higher temperatures than the natural locations of the plants (Fig. 2), which coupled with decreased precipitation at the irrigation regimes (described below) imitated potential future warmer and drier summer conditions.

Drought simulation

The simulation of separate watering regimes was accomplished by an automatic system allowing water to trickle slowly (4.8 $l.h^{-1}$) to the roots of plants through a network of valves, pipes, tubing and emitters. We used as simulation targets usual precipitation amounts for mountain locations, drought in early summer and drought in late summer. Under the first water regime (C), the amount of water, supplied to the plants, was consistent with precipitation norms of the subalpine zone of the Bulgarian mountains Rila and Pirin, taken from Climatic book of Bulgaria (Kutchukova, 1983) (Table 1). The second water regime (J) included simulated rainfall amounts for 1993, characterized by drought in July, but abnormally high watering in September. The third watering regime (A) included simulated rainfall amounts for 2000, characterized by drought in the second half of the summer (August). In the period from October to May the saplings were moved out of the shelter, under natural canopy, where the rate of precipitation was uncontrolled.

Gas exchange measurements

The rate of photosynthesis (An, $\mu mol.m^{-2}.s^{-1}$), the stomatal conductance for CO_2 (g_{sc}, $molCO_2.m^{-2}.s^{-1}$) and for H_2O

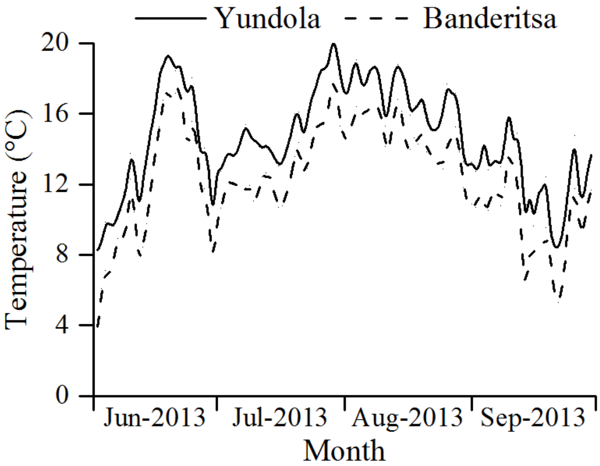

Figure 2. Daily average temperatures in the natural regions of the experimental saplings (Baikusheva mura) and in the shelter (Yundola) during the first year of the experiment.

Table 1. Monthly water amounts (L.m^{-2}) under three different irrigation regimes.

Regimes of irrigation	June	July	August	September
C	92.8	79.2	56.6	47.5
J	27.8	10.2	10.2	81.5
A	39.8	13.6	3.4	50.9

(g_{sw}, mol $H_2O.m^{-2}.s^{-1}$) were measured monthly within the two growth periods (May-October) on mature needles from the third of the top branch of 27 saplings, three per species at each irrigation regime. Measurements were done by a portable infrared gas analyzer LI-6400 (LI-COR Ltd., USA) with a conifer chamber (6400-05) at ambient conditions (Median ± median absolute deviation): rate of photosynthetically active radiation (204.0 ± 130.0 µmol.m^{-2}.s^{-1}), CO_2 concentration (383.5 ± 6.5 ppm), air temperature (21.5 ± 3.2°C) and relative humidity (51.5 ± 9.5 %). iWUE was calculated as the ratio of An to g_{sw}.

Data analysis

Because of random distribution of the data, the medians and quartiles of the studied physiological parameters were calculated for each case of measurements. A rectangular hyperbola (Eq.1) was used for non-linear regression between g_{sc} and An of saplings at the various regimes of irrigation:

$$A_n = \frac{\alpha . g_{sc}}{\beta + g_{sc}} \quad \text{(Eq. 1)}$$

The parameter α represents the maximum photosynthetic rate at maximum (saturating) stomatal conductance. The parameter β is the stomatal conductance at which An is half of α. Adjusted R^2 for regression and P-value were calculated. Tukey test was used for estimation of statistically significant differences between the regression coefficients and between iWUE for each species at the separate regimes of irrigation (Zar, 2010).

RESULTS

The saplings of *Pinus mugo* had the highest g_{sc} compared to the other species. Similar regressions between g_{sc} and A_n were observed in the different schemes of irrigation for this species. *P. heldreichii* saplings showed very low An and g_{sc} in most cases and these gas exchange parameters rapidly increased after watering. The relationships between the two parameters were close to linear and An did not reach asymptote (Fig. 3).

The saplings of *P. mugo* maintained similar stomatal control on An in the three irrigation schemes, independent of the water amounts (Table 2). Only in scheme J a slight reduction of the regression parameters was observed, which probably was due to greater variation in the gas exchange readings. In comparison with the well-watered saplings, the drought-treated *P. peuce* saplings decreased the stomatal control on An considerably in scheme J and drastically in scheme A. The levels of both An and g_{sc} in *P. heldreichii* saplings were extremely low. Moreover,

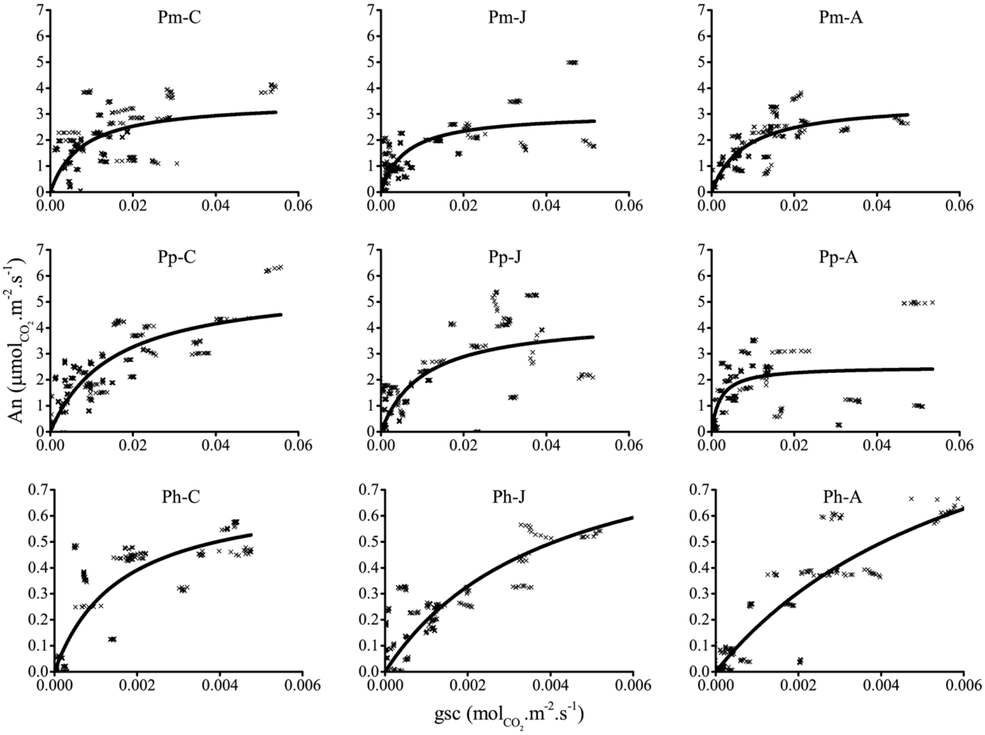

Figure 3. Photosynthetic rate (An) in relation to stomatal conductance (g_{sc}) in *Pinus mugo* (Pm), *Pinus peuce* (Pp) and *Pinus heldreichii* (Ph) saplings under well-watered (C), July-drought (J) and August-drought (A) scheme of irrigation.

Table 2. Regression analysis of stomatal control on photosynthesis in *Pinus mugo* (Pm), *Pinus peuce* (Pp) and *Pinus heldreichii* (Ph) sapling under well-watered (C), July-drought (J) and August-drought (A) scheme of irrigation. The regression parameters are shown with their standard errors. For each species the same symbols indicate that the regression coefficients under the three different irrigation schemes are not significantly different. AdjR^2 shows the adjusted coefficients of determination of the regressions and the asterix symbols indicate the level of significance: *** - *p*-value < 0.001.

Variants of experiment	α ± SE	β ± SE (x 10^{-3})	Adj. R^2
Pm-C	3.50 ± 0.21^a	7.59 ± 1.20^a	0.51***
Pm-J	3.04 ± 0.15^a	5.94 ± 0.78^a	0.36***
Pm-A	3.48 ± 0.16^a	8.07 ± 0.89^a	0.65***
Pp-C	5.72 ± 0.31^a	15.08 ± 1.70^a	0.57***
Pp-J	4.37 ± 0.30^b	10.28 ± 1.92^b	0.52***
Pp-A	2.51 ± 0.12^c	2.11 ± 0.42^c	0.38***
Ph-C	0.70 ± 0.04^a	1.59 ± 0.24^a	0.73***
Ph-J	0.99 ± 0.09^b	4.02 ± 0.60^b	0.71***
Ph-A	1.33 ± 0.16^b	6.71 ± 1.26^c	0.85***

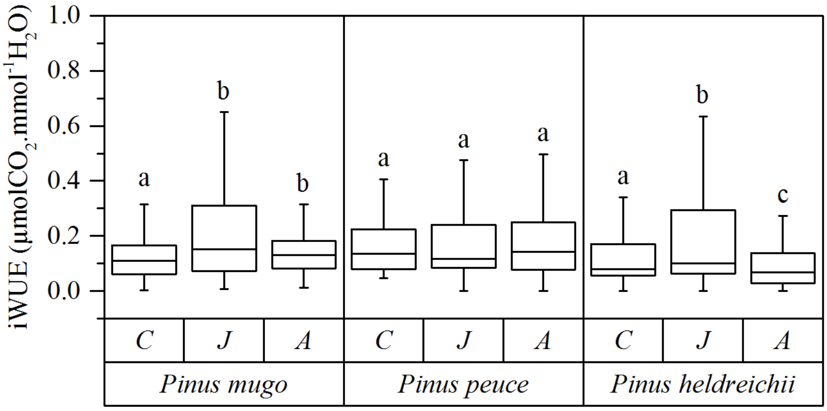

Figure 4. Intrinsic water use efficiency (iWUE, µmol CO_2.mmol^{-1} H_2O) of the experimental saplings under well-watered (C), July-drought (J) and August-drought (A) scheme of irrigation. The medians and quartiles for the experimental period are shown. The same letters indicate the absence of statistically significant differences ($p < 0.05$, Tuckey test) between iWUE of the saplings from each species under the three different irrigation schemes.

unlike *P. peuce*, drought-treated saplings increased these regression parameters.

P. mugo responded to both early and late summer drought with an increase of iWUE. This increment was better expressed in respect to scheme J when compared to scheme A. *P. peuce* did not show sensitivity to the irrigation scheme with respect to iWUE. The saplings of *P. heldreichii* reduced the water loss in scheme J resulting in increased iWUE, but during late summer drought reduced iWUE even below the level in scheme C (Fig. 4).

DISCUSSION

Pinus mugo, *Pinus peuce* and *Pinus heldreichii* have a great importance for the subalpine forests of the Balkan Peninsula. In the subalpine area tree species are exposed to several stress factors such as low temperature, summer and/or winter drought and potentially high level of ozone pollution (Tzvetkova et al., 2011). The studied tree species showed different relations between rate of photosynthesis and stomatal conductance. *P. mugo* saplings showed a flexible response to drought, evident by the absence of significant differences in stomatal control on An among schemes C, J and A. Probably this is a sign for drought resistance within the experimental reduction of water amounts. This assumption is consistent with the significant increase of iWUE in particular in scheme J, which can be explained as an ability of this species to a rapid reduction in water loss during summer drought. As a typical high-altitude species that grows naturally in the subalpine regions of Central and Southeast Europe, *P. mugo* is a very flexible species that is considered to be a pioneer colonizing areas that are unfavorable for other tree species. Aučina et al. (2011) showed that *P. mugo*, moved into quite distinct habitats, was able to adapt a suite of ectomycorrhizal symbionts that sufficiently supported growth and development of this species and allowed natural seedling regeneration. Furthermore, *P. mugo* is resistant to cavitation which helps plants during the winter period when soil is frozen and they are exposed to severe dehydration (Mayr et al., 2006). The saplings of *P. peuce* showed a weak stomatal control on An, especially in the situation with late summer drought. The absence of significant changes in iWUE among the schemes of irrigation may be associated with the delayed response of *P. peuce* to the shortage of soil moisture. Using dendrochronological methods Panayotov et al. (2010) have found high sensitivity of *P. peuce* to temperature regimes in August. Drought stress in this species probably accumulates and during a long dry period permanently decreases stomatal control on photosynthesis. The close to linear relationships between stomatal conductance and photosynthesis in *P. heldreichii* under the drought schemes are an indicator for enhanced stomatal control on photosynthesis. The very low levels of An and g_{sc} could explain the previously established slow growth rate in this species (Panayotov and Yurukov, 2007). The increase in iWUE during early summer drought indicates that *P. heldreichii* relies on the limited water loss even in the period of intensive growth. However, the reduction in iWUE in scheme A probably is due to severe water stress. The continuous increase in iWUE could not prevent the decrease in tree growth due to the negative effects of some factors, such as climate change (Xu et al., 2014) or anthropogenic environmental pollution (Ignatova et al., 2012; Damyanova et al., 2014). The strategy for water save to protect from drying is uneconomic in the longer term and is part of the survival strategies of only a few high drought-tolerant species (Schulze et al., 2005).

In conclusion, the small differences in stomatal control on An in *P. mugo* saplings among the schemes of irrigation could be interpreted as an evidence for wide ecological flexibility to soil moisture, which was supported by the increment in iWUE. The decrease of An in *P. peuce* during the drought could be considered as a marker for high sensitivity to changes in soil moisture; moreover, iWUE remained unchanged in drought conditions. The variable response of *P. heldreichii* saplings to soil moisture in respect to iWUE may be a reflection of the lower increment of An and the more rapidly enhanced g_{sw}. In general, among the three examined sub-alpine species *P. mugo* and partially *P. heldreichii* demonstrated higher physiological potential for adaptation.

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