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BRIEF COMMUNICATION

Changes in stomatal characteristics and photochemical efficiency during leaf development in six species of *Sorbus*

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Abstract

Measurements of *Sorbus* stomata size and density, maximal photochemical efficiency of photosystem II (F_v/F_m), variable-to-initial fluorescence ratio (F_v/F_0) and potential electron acceptor capacity ('area') were performed during leaf development in four parental diploid species, *S. aria*, *S. aucuparia*, *S. chamaemespilus*, *S. torminalis*, and two hybrid species, *S. hazslinszkyana* and *S. intermedia*. In fully expanded mature leaves, stomata lengths and densities were significantly larger in the shrub *S. chamaemespilus* than in the five tree species. The best performance of both the F_v/F_m and the F_v/F_0 ratio was recorded in *S. intermedia*, whereas *S. chamaemespilus* had the highest value of 'area'. From a physiological point of view, the results of this study showed that the photosystem II reaction centers remained intact functionally through all phenological stages of leaf expansion for all examined species of *Sorbus*.

Additional key words: phenological stages, photochemical efficiency, *Sorbus chamaemespilus*.

The genus *Sorbus* includes more than 250 species that are widespread mainly in temperate regions of the Northern Hemisphere (Phipps *et al*. 1990). Interest in this genus lies with both the vulnerability of the taxa *per se* and the evolutionary processes that have generated these taxa (Ennos *et al*. 2005). Flora Europaea lists 113 *Sorbus* taxa of various ranks in Europe. Among them, there are five diploid species (*S. aria*, *S. aucuparia*, *S. chamaemespilus*, *S. torminalis*, and *S. domestica*) which have been further classified into five different subgenera (Warburg and Kárpáti 1968). In addition, there are many local hybrids, morphologically intermediate between the subgenera. Generation of taxonomic novelty is believed to be driven primarily by a series of interspecific hybridizations and backcrosses among closely related taxa. The result is a series of new closely related taxa, microspecies and hybrids, that are reproductively isolated from each other, but which occasionally participate in further sexual hybridization events leading to a complex pattern of ongoing reticulate evolution of *Sorbus* (Robertson *et al*. 2010).

Stomata are structures in the epidermis of aerial organs that enable plants to adjust their gas exchange to suit the surrounding environmental conditions by modulating the aperture of a pore delimited by two guard cells. However, regulating the density and distribution of stomata in the epidermis is as important as pore opening and closure in providing optimal gas flow (von Groll *et al*. 2002). The positioning of stomata is highly variable between species but is regulated by a mechanism that maintains a minimum of one cell spacing pattern between stomata, which suggests positional signalling and cell-to-cell communications (Casson and Gray 2008, De Smet *et al*. 2009). In a number of species environmental factors such as light intensity, CO_2 concentration, water stress, and humidity have been shown to influence stomatal density.

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Abbreviations: Chl – chlorophyll; F_m – maximal fluorescence of dark-adapted state; F_v – variable fluorescence; F_0 – initial fluorescence of dark-adapted state; F_v/F_m – maximal photochemical efficiency of photosystem II; PSII – photosystem II; SEM – scanning electron microscopy.

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Chlorophyll (Chl) *a* fluorescence is an intriguing indicator that can assess photochemical efficiency and photoinhibition (Krause and Weis 1991). Furthermore, Chl fluorescence yields have been widely used in the monitoring of plant responses to environmental stress factors and climate change (Demmig-Adams and Adams 1992, Mohammed *et al*. 1995, Haisel *et al*. 2006). Also, it has been documented that both the stomatal and Chl fluorescence characteristics depend on the physiological age of the leaf (Šesták and Šiffel 1997, Nesterenko *et al*. 2006).

The main objective of this study was to characterize stomatal traits and photochemical efficiency during leaf development in six naturally occuring *Sorbus* species of central Europe.

The experiment was carried out on mature plants (at least 15-year-old ones) from four parental diploid species, *S. aria* (L.) Crantz, *S. aucuparia* L., *S. chamaemespilus* (L.) Crantz, *S. torminalis* (L.) Crantz, and two hybrid species, *S. hazslinszkyana* (Soó) Májovský and *S. intermedia* (Ehrh.) Pers., growing in the Arboretum Borová hora of the Technical University, Zvolen, Slovakia (48º35′N, 19º08′E, 322–345 m a.s.l.). The climate of the area is characterized by a mean annual temperature of 6.4ºC, minimum and maximum temperatures of –0.8 and 33.5ºC during the growing season, a mean annual precipitation rate of 532 mm, and a mean precipitation rate of 315 mm in the growing season. The main soil creative substrates are the slope loams of tufa materials with the admixture of loess loam. Measurements were taken from two representative plants per species when sun-exposed leaves of current-year stems were sampled from the outer crown (at 1–1.5 m height) at four different phenological stages of *Sorbus* leaf development as follows: 2nd stage – elongated bud, pigmented and unfolded at the apical end; 3rd stage – fully unfolded bud; 4th stage – growing young leaf; 5th stage – mature expanded leaf (Čaňová *et al*. 2008).

The leaves covered by trichomes were depilated. Stomatal characteristics (length, width, and density) were determined on 5–8 microrelief preparations sampled from 6 leaves per species at each phenological stage, using an *Olympus BH2* light microscope (*Olympus Europa*, Hamburg, Germany). Experiments were carried out in two replicates. For scanning electron microscopy (SEM) of stomata, fresh leaf samples were depilated, mounted on stubs, gold-coated in argon gas environment, and observed by low vacuum SEM using a *JEOL JSM-6390LV* instrument (*JEOL*, Tokyo, Japan) operating at 20 kV.

A portable fluorometer *Plant Efficiency Analyser* (*Hansatech Ltd*., Kings Lynn, UK) was used to determine Chl *a* fluorescence yields. Leaves were kept for 30 min under leaf clamps for dark adaptation. After the initial measurement of dark-adapted minimum fluorescence (F_0), leaves were exposed to a saturating irradiance of 2,100 μmol m^{-2} s^{-1} for 1 s to measure the maximal fluorescence of dark-adapted foliage (F_m). Maximal photochemical efficiency of PSII [$F_v/F_m = (F_m - F_0)/F_m$], variable-to-initial fluorescence ratio (F_v/F_0), and potential electron acceptor capacity ('area') were determined. Chl fluorescence measurements were made on both adaxial and abaxial surfaces of 20 leaves per species at each phenological stage. The measurements were accomplished within air temperature ranges of 9.5–12.3ºC at stage 2, 9.7–13.5ºC at stage 3, 10.6–17.1ºC at stage 4, and 18.8–21.3ºC at stage 5, respectively. Experiments were carried out in two replicates.

Data of stomatal traits were subjected to two-way analysis of variance (species and phenological stage were considered fixed effect-factors), whereas Chl fluorescence data were subjected to three-way analysis of variance (again, species, phenological stage and leaf-surface side were considered factors with fixed effects). *Duncan's* multiple range tests were used for pairwise comparisons of means.

At the beginning of leaf development (phenological stage 2), the highest stomata lengths and widths were found in *S. chamaemespilus* and then in *S. intermedia*. The lowest values were observed for the species *S. torminalis*, *S. hazslinszkyana* and *S. aucuparia* (Table 1). During leaf expansion (stages 3 and 4), different growth dynamics of stomata sizes, found predominantly in *S. aucuparia*, *S. chamaemespilus*, and *S. intermedia*, reflected a great variation of this trait behaviour in these species. More consistent results during stages 3 and 4 were determined in *S. aria* and *S. torminalis*. At the end of leaf expansion (stage 5), the largest stomata sizes were observed in *S. chamaemespilus* and then in *S. hazslinszkyana* (Table 1, Fig. 1*C*,*D*). The smallest stomata sizes were found in epidermises of *S. aria* (Table 1, Fig. 1*A*). The species *S. intermedia*, *S. aucuparia* and *S. torminalis* had intermediate phenotypes for this trait (Table 1, Fig. 1*B*,*E*,*F*). All six examined species of *Sorbus* had hypostomatous leaves with kidney-shaped guard cells and distinct perpendicular and parallel cuticular striations (Fig. 1*A*–*F*). The inner walls of guard cells were thicker than the outer walls (clearly seen in Fig. 1*F*). The abaxial epidermises were covered with large numbers of fibrous trichomes (predominantly in *S. aria*, Fig. 1*A*). However, the smoothest abaxial epidermal surface was displayed by *S. torminalis* (Fig. 1*F*).

Each species also required a varying number of days after bud opening to reach maximum leaf development at stage 5. *S. aria* required 20 d, *S. torminalis* 24 d, *S. chamaemespilus* 28 d, *S. aucuparia* 29 d, *S. intermedia* 33 d, and for *S. hazslinszkyana* 34 d were required (Table 1).

With regard to stomatal densities at stage 2, *S. aucuparia* and *S. chamaemespilus* had the highest frequencies of stomata per mm^{-2} of leaf area, followed by *S. torminalis*, *S. hazslinszkyana* and then *S. aria*. The lowest frequency was found in *S. intermedia* (Table 1). Except of *S. chamaemespilus*, stomatal densities increased in the remaining five species of *Sorbus* during the leaf expansion period (stages 3 and 4). At the end of leaf

Table 1. Stomatal characteristics during leaf development in six examined species of *Sorbus*. Data represent means ± SE ($n = 60$ per species at each stage). Mean values followed by *the same letters*, a–f in the same column, are not significantly different at P=0.05. Numbers in parentheses indicate the number of days after bud opening within species and phenological stage.

Trait	Species	Stage 2	Stage 3	Stage 4	Stage 5
Stomata length [µm]	*S. aria*	22.46 ± 0.46^{c} (1)	22.25 ± 0.57^{c} (5)	23.51 ± 0.38^{c} (10)	26.33 ± 0.44^{e} (20)
	S. aucuparia	19.44 ± 0.83^{d} (1)	16.69 ± 0.47^{e} (4)	22.40 ± 0.37^{c} (15)	30.20 ± 0.54^{d} (29)
	S. chamaemespilus	35.42 ± 0.56^{a} (1)	31.83 ± 0.71^{a} (6)	31.06 ± 0.48^{a} (15)	37.38 ± 0.55^{a} (28)
	S. hazslinszkyana	19.75 ± 0.89^{d} (1)	27.24 ± 0.60^{b} (13)	30.33 ± 0.44^{a} (19)	35.13 ± 0.47^{b} (34)
	S. intermedia	29.23 ± 2.43^{b} (1)	26.05 ± 0.56^{b} (6)	26.73 ± 0.53^{b} (16)	32.83 ± 0.45^{c} (33)
	S. torminalis	20.50 ± 0.67^{cd} (1)	19.92 ± 0.63^{d} (7)	20.20 ± 0.37^{d} (12)	29.20 ± 0.50^{d} (24)
Stomata width [µm]	*S. aria*	20.25 ± 0.41^{c} (1)	19.31 ± 0.47^{d} (5)	18.43 ± 0.28^{c} (10)	18.23 ± 0.30^{c} (20)
	S. aucuparia	15.94 ± 0.56^{d} (1)	12.23 ± 0.25^{f} (4)	15.11 ± 0.25^{d} (15)	19.07 ± 0.37^{c} (29)
	S. chamaemespilus	28.22 ± 0.43^{a} (1)	24.77 ± 0.44^{a} (6)	19.94 ± 0.29^{b} (15)	23.54 ± 0.38^{b} (28)
	S. hazslinszkyana	15.91 ± 0.63^{d} (1)	23.18 ± 0.52^{b} (13)	21.97 ± 0.34^{a} (19)	24.57 ± 0.33^{a} (34)
	S. intermedia	24.92 ± 2.38^{b} (1)	20.85 ± 0.44^{c} (6)	19.32 ± 0.38^{b} (16)	22.60 ± 0.40^{b} (33)
	S. torminalis	16.75 ± 0.50^{d} (1)	16.37 ± 0.46^{e} (7)	15.54 ± 0.26^{d} (12)	19.13 ± 0.33^{c} (24)
Stomatal density [mm^{-2}]	*S. aria*	48.75 ± 3.59^{d} (1)	186.11 ± 8.14^{ab} (5)	215.77 ± 4.40^{a} (10)	177.08 ± 5.52^{b} (20)
	S. aucuparia	169.35 ± 11.86^{a} (1)	175.77 ± 5.40^{b} (4)	196.43 ± 3.88^{b} (15)	110.83 ± 2.99^{d} (29)
	S. chamaemespilus	151.50 ± 5.41^{a} (1)	202.08 ± 5.92^{a} (6)	169.85 ± 4.46^{d} (15)	226.15 ± 4.01^{a} (28)
	S. hazslinszkyana	73.84 ± 5.86^{c} (1)	142.03 ± 8.25^{c} (13)	172.39 ± 3.53^{cd} (19)	153.75 ± 3.75^{c} (34)
	S. intermedia	25.00 ± 0.00^{e} (1)	132.88 ± 5.73^{c} (6)	167.61 ± 3.45^{d} (16)	120.42 ± 2.82^{d} (33)
	S. torminalis	97.92 ± 6.07^{b} (1)	179.41 ± 6.93^{b} (7)	183.46 ± 5.48^{c} (12)	171.25 ± 6.05^{b} (24)

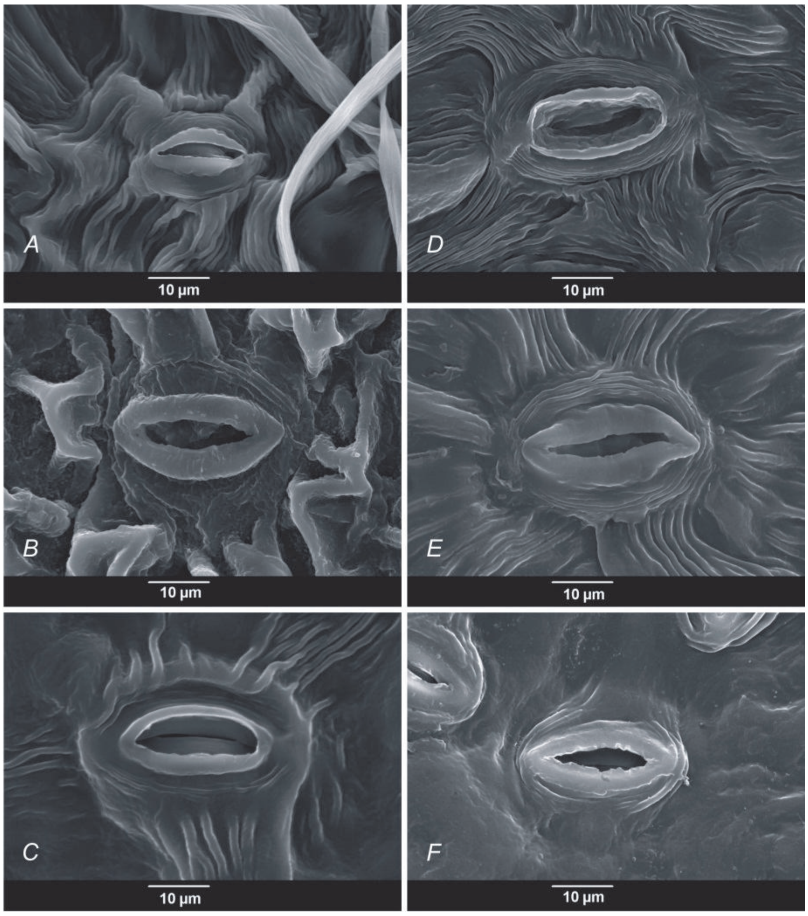

Fig. 1. SEM images of stomata taken from fully expanded hypostomatous leaves of six species of *Sorbus* (samplings made at phenological stage 5). *A* – stoma of *S. aria*. *B* – stoma of *S. aucuparia*. *C* – stoma of *S. chamaemespilus*. *D* – stoma of *S. hazslinszkyana*. *E* – stoma of *S. intermedia*. *F* – stoma of *S. torminalis*. Scale bars = 10 µm.

expansion (stage 5), the highest number of stomata per unit leaf area was observed in *S. chamaemespilus*, followed by *S. aria*, *S. torminalis* and *S. hazslinszkyana*. The species *S. intermedia* and *S. aucuparia* had the lowest stomatal densities in fully expanded mature leaves (Table 1).

Distinct stomatal characteristics found in fully expanded mature leaves of *S. chamaemespilus* (the largest stomata lengths and the highest stomatal density) might be related to the growth form. This species is a small shrub, whereas the remaining five species are medium-size trees. The values of stomatal characteristics in the tree species were found to be significantly lower than those of *S. chamaemespilus*. Variations in stomatal density caused by species intrinsic characteristics in relation to their life/growth forms were reported previously by Tay and Furukawa (2008). These authors observed that different life/growth forms of Japanese vine species differed significantly in stomatal density, distribution, stomatal index and epidermal cell density under the developmental constraint of leaf surface. In addition, stomatal density was found to be greater in shrubs than in trees based on the reanalyses of Salisbury's data (1927) for stomatal number, which were accomplished by Kelly and Beerling (1995). These authors concluded that interspecific differences in stomatal density were not associated with the suggested differences in plant 'exposure' that encompasses the effects of humidity surrounding the leaf, wind speed, and irradiance on stomatal density.

During leaf development, there was a clear trend toward an increase in the F_v/F_m ratio from stage 2 to stage 5. At stage 2, *S. hazslinszkyana*, *S. intermedia* and *S. aria* had the highest values of F_v/F_m, whereas the poorest performance was recorded in *S. torminalis*. For the expanded leaves coming from stage 5, the best performance in F_v/F_m was found in *S. intermedia*. The species *S. torminalis* and *S. chamaemespilus* had the lowest values of the ratio, not reaching the value 0.80 (Table 2). The influence of the leaf-surface side on the ratio was not significant (*F*-test = 1.37, df = 1, P=0.2428).

With regard to the F_v/F_0 ratio at stage 2, the highest values were found in *S. hazslinszkyana*, *S. intermedia* and *S. aria*, whereas the lowest value was recorded in *S. torminalis*. During stages 3 and 4, different fluorescence dynamics of F_v/F_0 ratio were found predominantly in *S. aucuparia*, *S. hazslinszkyana* and *S. chamaemespilus*. At the end of leaf development, the highest yield of F_v/F_0 was observed in *S. intermedia*. Alternatively, the species *S. chamaemespilus*, *S. hazslinszkyana* and *S. torminalis* had the lowest values of the ratio (Table 2). The influence of the leaf-surface side on the ratio was not significant (*F*-test = 2.09, df = 1, P=0.1483).

In the case of 'area' at stage 2, the performances of *S. aria* and *S. chamaemespilus* dominated among the six examined species. The lowest values of 'area' were recorded in *S. aucuparia*, *S. hazslinszkyana* and *S. torminalis*. At stage 5, the best performance was found in *S. chamaemespilus*, whereas *S. aucuparia* exhibited the poorest performance (Table 2). Significantly higher values of 'area' were recorded on the adaxial leaf surface (*F*-test = 900.06, df = 1, P=0.0001).

F_v/F_m is one of the most important traits used to assess the photosynthetic status of plants in ecophysiological research. The F_v/F_0 ratio estimates the efficiency of the water-splitting complex on the donor side of PSII. The variable 'area', *i.e.* area above the induction curve between F_0 and F_m, determines the potential capacity for electron transport during the primary processes of photosynthesis. The F_v/F_m ratio, typically ranging between 0.75–0.85, is directly proportional to the effectiveness of

Table 2. Chlorophyll *a* fluorescence yields during leaf development in six examined species of *Sorbus*. Data represent means ± SE (*n* = 40 per species at each stage). Mean values followed by *the same letters*, a–d in the same column, are not significantly different at P=0.05. Numbers in parentheses indicate the number of days after bud opening within species and phenological stage.

Trait	Species	Stage 2	Stage 3	Stage 4	Stage 5
F_v/F_m	*S. aria*	0.770 ± 0.002^{a} (1)	0.756 ± 0.002^{c} (5)	0.777 ± 0.004^{c} (10)	0.811 ± 0.002^{b} (20)
	S. aucuparia	0.753 ± 0.003^{b} (1)	0.766 ± 0.002^{b} (4)	0.785 ± 0.003^{ab} (15)	0.803 ± 0.004^{bc} (29)
	S. chamaemespilus	0.758 ± 0.005^{b} (1)	0.752 ± 0.005^{cd} (6)	0.780 ± 0.006^{bc} (15)	0.792 ± 0.006^{d} (28)
	S. hazslinszkyana	0.774 ± 0.005^{a} (1)	0.775 ± 0.004^{a} (13)	0.792 ± 0.005^{a} (19)	0.804 ± 0.004^{bc} (34)
	S. intermedia	0.771 ± 0.005^{a} (1)	0.773 ± 0.004^{a} (6)	0.792 ± 0.006^{a} (16)	0.820 ± 0.005^{a} (33)
	S. torminalis	0.726 ± 0.006^{c} (1)	0.749 ± 0.005^{d} (7)	0.775 ± 0.006^{c} (12)	0.797 ± 0.007^{cd} (24)
F_v/F_0	*S. aria*	3.389 ± 0.041^{a} (1)	3.070 ± 0.056^{c} (5)	3.525 ± 0.072^{b} (10)	4.293 ± 0.042^{b} (20)
	S. aucuparia	3.064 ± 0.044^{b} (1)	3.288 ± 0.036^{ab} (4)	3.646 ± 0.074^{b} (15)	4.127 ± 0.102^{bc} (29)
	S. chamaemespilus	3.050 ± 0.069^{b} (1)	3.009 ± 0.050^{c} (6)	3.542 ± 0.059^{b} (15)	4.078 ± 0.060^{c} (28)
	S. hazslinszkyana	3.443 ± 0.059^{a} (1)	3.249 ± 0.073^{b} (13)	3.849 ± 0.062^{a} (19)	4.052 ± 0.030^{c} (34)
	S. intermedia	3.379 ± 0.035^{a} (1)	3.415 ± 0.037^{a} (6)	3.840 ± 0.053^{a} (16)	4.558 ± 0.045^{a} (33)
	S. torminalis	2.681 ± 0.050^{c} (1)	3.008 ± 0.040^{c} (7)	3.462 ± 0.039^{b} (12)	3.968 ± 0.080^{c} (24)
'Area' [bit 10^8 s^{-1}]	*S. aria*	0.188 ± 0.012^{a} (1)	0.175 ± 0.009^{c} (5)	0.223 ± 0.011^{c} (10)	0.317 ± 0.015^{bc} (20)
	S. aucuparia	0.134 ± 0.017^{c} (1)	0.187 ± 0.017^{bc} (4)	0.207 ± 0.200^{cd} (15)	0.289 ± 0.023^{c} (29)
	S. chamaemespilus	0.171 ± 0.018^{ab} (1)	0.204 ± 0.019^{ab} (6)	0.263 ± 0.021^{b} (15)	0.360 ± 0.024^{a} (28)
	S. hazslinszkyana	0.134 ± 0.015^{c} (1)	0.190 ± 0.022^{bc} (13)	0.313 ± 0.019^{a} (19)	0.335 ± 0.028^{ab} (34)
	S. intermedia	0.168 ± 0.017^{b} (1)	0.223 ± 0.026^{a} (6)	0.310 ± 0.021^{a} (16)	0.327 ± 0.025^{ab} (33)
	S. torminalis	0.116 ± 0.016^{c} (1)	0.140 ± 0.017^{d} (7)	0.187 ± 0.018^{d} (12)	0.311 ± 0.019^{bc} (24)

light utilization under standard conditions of CO_2 fixation and to the quantum yield of photochemical processes (Demmig and Björkman 1987). A decline in F_v/F_m is a sensitive and early indicator of change in both photosynthesis and in the physiological status of the plant in general, resulting from various environmental stresses. During leaf development, the values of F_v/F_m in the six species of *Sorbus* were far higher than the threshold value of 0.725 that indicates the onset of reversible changes in reaction centers of PSII (Bolhàr-Nordenkampf and Götzl 1992). At each phenological stage, there was no sign of significant decrease in F_v/F_m for any examined species. This result suggests that the PSII reaction centers remained intact functionally through all phenological stages of leaf expansion. Similar results were also reported for different leaf developmental stages in *Trifolium repens* (Yoo *et al.* 2003) and green-leaved *Fagus sylvatica* cultivars (Čaňová *et al.* 2008).

Photosynthesis is also one of the most temperature-sensitive processes in plants. The temperature response of the CO_2 assimilation rate is described by a parabolic curve with an optimum temperature, and thus CO_2 assimilation is inhibited at both low and high temperatures (Yamori *et al.* 2005, Mathur *et al.* 2011). Within the photosynthetic apparatus, PSII is the most thermolabile component of the electron transport chain. Among partial reactions of PSII, the oxygen-evolving complex is particularly heat sensitive (Georgieva *et al.* 2000). Low temperature often causes over-reduction of photosynthetic electron transport carriers and subsequent photoinhibition (Huner *et al.* 1998, Yamori *et al.* 2011). In this study, the relationships between air temperature and Chl *a* fluorescence yields at different phenological stages were significant ($y = 0.719 + 0.004\ x$, $P<0.001$, $R^2 = 0.56$ for F_v/F_m; $y = 2.193 + 0.098\ x$, $P<0.001$, $R^2 = 0.62$ for F_v/F_0; $y = 0.022 + 0.015\ x$, $P<0.001$, $R^2 = 0.64$ for ‘area’). Lower values of Chl fluorescence yields were recorded at colder temperatures confirming the results of previous studies (Larcher 2000, Ogaya *et al.* 2011). Rising temperatures resulted in increased values of Chl fluorescence variables. However, no photoinhibition was observed, and the physiological range of temperature was maintained through all phenological stages.

Chl fluorescence has also been used in the discrimination of various taxonomic groups of plants, including marine algae (Drinovec *et al.* 2011), filmy ferns (Parra *et al.* 2009), gymnosperms (Major *et al.* 2007, Offord 2011) and angiosperms (Cavender-Bares *et al.* 2005, Calatayud *et al.* 2007). In the case of *Sorbus*, Ďurkovič *et al.* (unpublished results) examined Chl *a* fluorescence yields for *S. aria*, *S. aucuparia* and *S. chamaemespilus* directly in the subalpine natural habitat. The shrub *S. chamaemespilus* had a significantly lower F_v/F_m ratio than *S. aria* and *S. aucuparia*, which is in agreement with the result presented here for the artifically planted *Sorbus* plants. Percival and Sheriffs (2002) also recorded high values of F_v/F_m for nonstressed leaves of *S. aucuparia* (0.811) as well as for *S. aria* (0.821) (Percival 2005).

Taken together, from a physiological point of view, the results of this study showed that the PSII reaction centers remained intact functionally through all phenological stages of leaf expansion in all examined species of *Sorbus*. There was no sign of significant decrease in F_v/F_m for any species.

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