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Research article

Transpiration, CO_2 assimilation, WUE, and stomatal aperture in leaves of *Viscum album* (L.): Effect of abscisic acid (ABA) in the xylem sap of its host (*Populus x euamericana*)

Peter Escher [a,*], Andreas D. Peuke [a], Peter Bannister [b], Siegfried Fink [c], Wolfram Hartung [d], Fan Jiang [e], Heinz Rennenberg [a]

[a] *Institute of Forest Botany and Tree Physiology, Chair of Tree Physiology, Albert-Ludwigs-University Freiburg, Georges-Köhler-Allee 053/054, D-79110 Freiburg i.B., Germany*
[b] *Department of Botany, University of Otago, P.O. Box 56, Dunedin, New Zealand*
[c] *Institute of Forest Botany and Tree Physiology, Chair of Forest Botany, Albert-Ludwigs-University Freiburg, Bertoldstrasse 17, 79085 Freiburg i.B., Germany*
[d] *Julius-von-Sachs-Institut für Biowissenschaften, Lehrstuhl für Botanik I, Julius-Maximilians Universität Würzburg, Julius-von-Sachs-Platz 2, D-97082 Würzburg, Germany*
[e] *Institute of Life Sciences, Beijing Normal University, Beijing, China*

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Abstract

Leaves of the mistletoe *Viscum album* (L.) show a high rate of transpiration, even when the host is under severe drought stress. The hypothesis that a strong control of ABA influx from the xylem sap of the host into the mistletoe prevents stomatal closure in mistletoe leaves was tested under the following conditions: sections of poplar twigs carrying a mistletoe were perfused with artificial xylem sap that contained different ABA concentrations and both transpiration and ABA levels were analysed in mistletoe leaves. Despite variation by a factor of 10^4, the ABA content of the host xylem did not affect ABA levels, leaf transpiration, CO_2 assimilation, WUE, or the degree of stomatal aperture in mistletoe leaves. These observations support the hypothesis of a strong control of ABA influx from the host of the xylem into the mistletoe, although degradation of ABA before it enters the mistletoe leaves cannot be excluded. This mechanism may ensure a water and nutritional status favourable for the mistletoe, even if the water status of the host is impaired.

Despite the lack of short-term sensitivity of ABA levels in mistletoe leaves to even strong changes of ABA levels in the xylem sap of the host, ABA levels in mistletoe leaves were relatively high compared to ABA levels in the leaves of several tree species including poplar. Since significant transpiration of the mistletoe leaves was observed despite high ABA levels, a diminished sensitivity of the stomata of mistletoe leaves to ABA has to be concluded. The stomatal density of adaxial *Viscum* leaves of 89 ± 23 stomata per mm is lower than those reported in a study performed at the end of the 19th century.

Keywords: ABA; Abscisic acid; Assimilation; Stomatal aperture; Transpiration; Xylem sap

1. Introduction

One of the most striking features of the mistletoe *Viscum album* (L.) is its high rate of transpiration, even when the host is under severe drought stress [23,54]. As a consequence, twigs of the host that are distal to the insertion of a mistletoe often dry out completely and die [53]. For several reasons, this observation is surprising. Excessive water loss of leaves under drought stress is usually prevented to some extent by regulating the degree of stomatal aperture [40,52] and abscisic acid (ABA) produced in the roots and allocated to the leaves with the transpiration stream is a central component of the

* Corresponding author. Tel.: +49 (0) 761 203 8300; fax: +49 (0) 761 203 8302.
E-mail address: peter.escher@ctp.uni-freiburg.de (P. Escher).

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signalling cascade that leads to stomatal closure [16,25]. *V. album* does not rely only on the water supplied by the xylem sap of its host, but also uses the xylem sap of its host as a source of carbon, nitrogen, and sulfur for growth and development [18,21,19,20]. Therefore, it would be expected that changes in the allocation of ABA in the xylem sap of the host would also regulate the stomatal aperture of mistletoe leaves. However this is obviously not the case and may be explained by (i) a very effective control of ABA influx from the host xylem into the mistletoe, or (ii) an ABA insensitivity of the stomata of mistletoe leaves.

Early studies suggested that *V. album* lacked ABA [22,27] and was later disproved by Ihl et al. [29], who found low ABA levels in July and high levels in wintertime. Since low ABA levels in mistletoe leaves coincide with low rates of transpiration and low levels of ABA with high rates of transpiration [29], ABA insensitivity of mistletoe leaves does not seem to be responsible for the lack of stomatal closure under conditions of drought stress. The aim of the present experiments was to test the hypothesis that a strong control of ABA influx from the xylem sap of the host into the mistletoe prevents stomatal closure under drought stress in mistletoe leaves. Consequently, sections of poplar twigs bearing a mistletoe were perfused with artificial xylem sap that contained different ABA concentrations and both gas exchange and ABA levels were analysed in mistletoe leaves.

2. Methods

2.1. Plant material

The present experiments were performed with twig sections of *Populus x euamericana* carrying a mistletoe (*V. album* L.). Plant material was collected in the upper Rhine valley at a site near Rheinstetten (about 10 km south-west of Karlsruhe, Germany; co-ordinates 48°98'N, 08°33'E) in a 60–70 years old riparian lowland forest of *P. x euamericana* on the banks of a small water channel. Long-term annual air temperature in this region is 10.3 °C and annual precipitation is 770 mm [38]. On 22nd August 2005, a total of 20 twigs were collected with a lifting platform between 10 a.m. and 2 p.m. from three trees. The cut ends of the twigs were immediately transferred into a bucket filled with water to avoid embolism of the vessels.

2.2. Perfusion of poplar twigs

Poplar twigs carrying a mistletoe were perfused with artificial xylem sap [21]. For this purpose, twigs were cut off at both ends, resulting in a 10–15 cm section with a mistletoe attached. Approximately 2–3 cm bark was immediately peeled off at the proximal end and the wood was attached to the perfusion system as previously described [21]. Subsequently, the perfusion solution was pumped continuously through the twig for 20 h. In order to achieve close to natural osmotic conditions and a close to natural nutrient status, the perfusion solution contained 2 mM L-Gln, 2 mM K^+, 0.5 mM Mg^{2+}, 0.75 mM Ca^{2+}, 0.75 mM $H_2PO_4^-$, 0.5 mM SO_4^{2-}, 1.75 mM Cl^-. The pH was adjusted to 6.5 according to Refs. [7,8] to mimic the natural pH of the host xylem sap, to prevent dissociation of ABA and to keep ABA permeable across membranes. Solutions containing 0.01 μM (±)-*cis*/*trans*-abscisic acid (MoBi Tec, Göttingen, Germany) were applied for the initial perfusion for 3 h. Subsequently, the initial perfusion solution was replaced by solutions, which contained ABA at four different concentrations (0.01, 0.1, 1, 100 μM). Experiments were performed in a controlled climate chamber (Heräeus Vötsch HPS 1500, Hanau, Germany) at 25 °C, 50 ± 2% relative humidity, and ca. 430 $\mu E\, m^{-2}\, s^{-1}$ PPFD (provided by Philips TLD 58 W/550 and OSRAM L58 W/77 Fluora lamps).

2.3. Transpiration and CO_2 assimilation of mistletoe leaves

Transpiration and CO_2 assimilation of individual pairs of mistletoe leaves were measured at 3, 6, 13, and 20 h after beginning of the experiment with a GFS-3000 portable gas exchange fluorescence system connected to a 3010-S standard measuring head with a 3040-L LED light source (Heinz Walz GmbH, Effeltrich, Germany). In order to assess the specific transpiration (E) and CO_2 assimilation (A) of the mistletoe, the individual leaf areas were determined with an area meter (ΔT area meter, Delta-T Devices, Cambridge, UK). Water use efficiency (WUE) was calculated by the following equation:

$$\mathrm{WUE} = A\ \left[\mathrm{mmol\ CO_2\ m^{-2}\ s^{-1}}\right] / E\ \left[\mathrm{mmol\ H_2O\ m^{-2}\ s^{-1}}\right]$$

2.4. Stomatal density and degree of stomatal opening

The upper adaxial surface of one leaf of each mistletoe was coated with UHU Hart (UHU GmbH & Co. KG, Bühl, Germany) immediately after the experiment. The cured glue conserved imprints of the leaf surfaces, enabled the analysis of the degree of stomatal opening at the end of experiment, and restricted possible shrinking effects [28]. Subsequently, hardened glue was observed with a microscope using the differential interference contrast techniques (DIC) (Zeiss Axiophot, equipped with a CCD-camera Axiocam MRc 5). After focusing, five pictures of each leaf were taken at 20 times magnification with 1292 × 968 pixel and saved as 8 bit greyscale tif files (Fig. 2). Subsequent image analyses were performed with ImageJ [47].

For the determination of stomatal density, one picture of each leaf was used. Stomata were identified and marked manually to obtain the number of stomata per frame and the spatial distribution (2D) data. To normalise the results and to make data comparable, euclidian distances were calculated between stomata to access mean euclidian distance as a leaf parameter. To describe stomatal distribution statistically [24], the degree to which the observed pattern departs from a random pattern [R] was calculated according to Ref. [13] with the limits $R = 0$, i.e. aggregated pattern individuals are

at same locus, distance to the nearest neighbour is 0; $R = 2.1491$, i.e. perfectly uniform pattern with maximal hexagonal spacing; and $R = 1$, i.e. pattern with random distribution.

For determination of stomatal aperture, the perimeters of plainly visible stomata were marked manually in five pictures per leaf. Subsequently, area, bounding rectangle perimeter, and minor and major axis of a fitting ellipse of the stomata were measured simultaneously with ImageJ [47]. Due to the large variation of the lengths of stomata, the area of aperture is not directly comparable. To avoid these difficulties, "relative stomatal aperture (RSA)" according to Ref. [32] as a size independent variable was calculated. RSA [%] is specified as $(400 \times A)/(\Pi \times L^2)$, where A is the aperture area and L is the length of pore ≈ major axis of a fitting ellipse.

2.5. Determination of ABA concentrations in leaves

Three leaves were cut from each mistletoe before and directly after the experiment, immediately frozen with liquid nitrogen, and stored at −80° until further analysis. For determination of ABA content, leaves were lyophilised, ground with mortar and pestle, and aliquots of the three leaves of each mistletoe were cumulated. Freeze-dried tissue samples were homogenised and extracted in 80% aqueous methanol. Extracts were passed through a Sep Pak C_{18}-cartridge. Methanol was removed under reduced pressure and the aqueous residue was partitioned three times against ethyl acetate at pH 3.0. The ethyl acetate of the combined organic fractions was removed under reduced pressure. The newly obtained residue was taken up in TBS-buffer (tris buffered saline; 150 mmol/L NaCl, 1 mmol/L $MgCl_2$ and 50 mmol/L tris at pH 7.8) and subjected to an immunological ABA assay (ELISA) as described earlier [37,43]. The accuracy of the ELISA has been verified in earlier investigations [43]. Recoveries of ABA during the purification procedures were checked routinely using radioactive ABA and found to be higher than 95%. The immunochemicals were generously supplied by Prof. Weiler, Ruhr Universität Bochum (Germany).

2.6. Data analysis

Transpiration rates of one selected pair of leaves of each of the 20 mistletoes were measured repeatedly at 3, 6, 13, 20 h. One mistletoe, which did not show any significant transpiration during the experiment, was discarded from further data analysis as we did also for severe (negative) outliers in assimilation and WUE. Analysis of variance was done by GLM-ANOVA with Tukey–Kramer multiple-comparison post hoc test. All statistical analyses were carried out with NCSS 2004 [26]. In boxplots, the top and the bottom of the box represent the 25th and 75th percentile. The length of the box is equal to the interquartile range (*IQR*). Lines drawn in the box represent the median. T-shaped lines are the adjacent values ≤75% (≥25%) percentile + 1.5 *IQR* (−1.5 *IQR*).

3. Results

3.1. Transpiration, assimilation, and WUE of mistletoes at increasing ABA concentrations in the host xylem

Specific transpiration of mistletoe leaves (E) was highest in controls at 3 h exposure (2.5 ± 1.2 mmol $H_2O\ m^{-2}\ h^{-1}$) (Fig. 1) and subsequently declined significantly to 0.8 ± 0.4 mmol $H_2O\ m^{-2}\ h^{-1}$ at 13 h exposure. But there was no significant effect of the ABA concentration in the xylem sap of the host ranging from 0.01 to 100 μM, on transpiration of the mistletoe leaves during the entire experiment (Fig. 1). Assimilation rates (A) of the mistletoe leaves ranged from 2.3 to 10.2 mmol $CO_2\ m^{-2}\ s^{-1}$ with an overall mean of 4.5 ± 3.5 mmol $CO_2\ m^{-2}\ s^{-1}$ (Fig. 2). There were no significant differences due to the time of exposure or the different ABA concentrations in the xylem sap of the host. The WUE of C assimilation was calculated from the individual ratio between A and E for each mistletoe; it increased in controls from 3 to 6 h of exposure, then remained constant, and was not affected by the ABA concentration in host xylem sap (Fig. 3).

3.2. ABA concentrations in leaf material

ABA concentrations in mistletoe leaves ranged between 6.9 and 31.8 μmol g dw^{-1} (Fig. 6). Despite the wide range of ABA concentrations applied, the ABA concentration in the mistletoe leaves was not significantly affected by the ABA concentration in the xylem sap of the host.

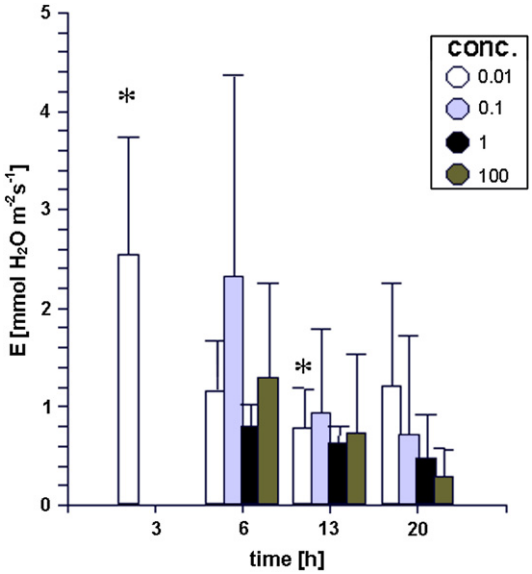

Fig. 1. Transpiration rate E (mmol $m^{-2}\ s^{-1}$) of the leaves of mistletoes exposed to different ABA levels via the xylem sap of the host. E was measured at 3, 6, 13 and 20 h after starting the perfusion experiment at 25 °C and 50% relative humidity. In controls, 0.01 μM ABA was fed into host xylem during the entire experiment. In the other series, the perfusion solution containing 0.01 μM ABA was replaced after 3 h by solutions containing 0.1, 1 or 100 μM ABA. Significant differences in E between treatments (ABA levels) were not observed at any individual time. * Indicates significant differences in controls after different times of perfusion.

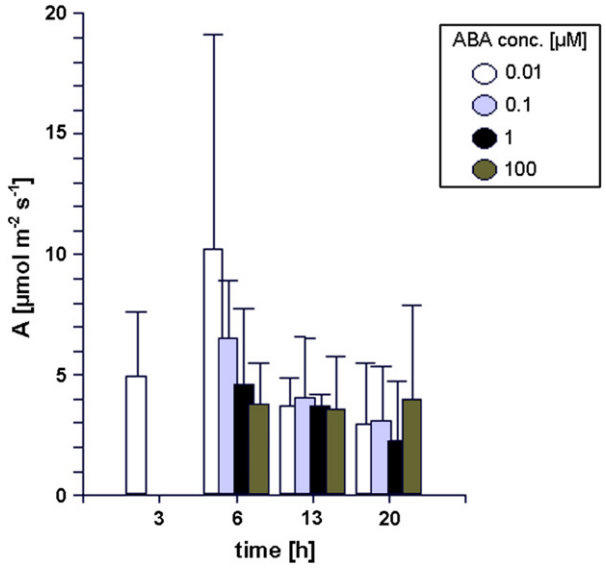

Fig. 2. Assimilation rate A (µmol m^{-2} s^{-1}) of the leaves of mistletoes exposed to different ABA levels via the xylem sap of the host. A was measured at 3, 6, 13 and 20 h after starting the perfusion experiment at 25 °C and 50% relative humidity. In controls, 0.01 µM ABA was fed into host xylem during the entire experiment. In the other series, the perfusion solution containing 0.01 µM ABA was replaced after 3 h by solutions containing 0.1, 1 or 100 µM ABA. Significant differences in A between treatments (ABA levels) at any individual time or at the same treatment after different times of perfusion were not observed.

3.3. Stomatal density and stomatal aperture

Mean stomatal density of *Viscum* leaves (Fig. 4) was 89 ± 23 stomata per mm^{-2} with a range between 36 and 121 stomata

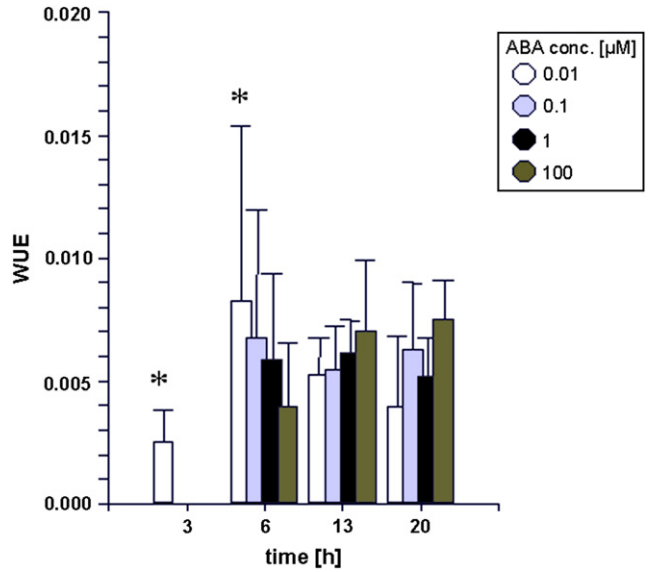

Fig. 3. Water use efficiency (WUE) of the leaves of mistletoes exposed to different ABA levels via the xylem sap of the host. WUE was calculated at 3, 6, 13 and 20 h after starting the perfusion experiment at 25 °C and 50% relative humidity. In controls, 0.01 µM ABA was fed into host xylem during the entire experiment. In the other series, the perfusion solution containing 0.01 µM ABA was replaced after 3 h by solutions containing 0.1, 1 or 100 µM ABA. Significant differences in WUE between treatments (ABA levels) were not observed at any individual time. * Indicates significant differences in controls after different times of perfusion.

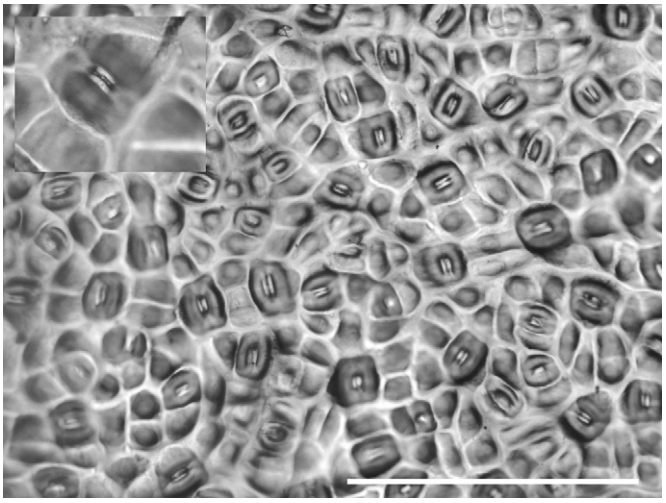

Fig. 4. Gray-scale image of stomata on the leaf surface of *Viscum album*. The images were obtained by light microscopy of glue imprints. The white bar represents a distance of 400 µm. For measuring stomatal aperture, pictures with a 20-fold higher magnification to this picture were used (for example see the not scaled inset in the left up corner).

per mm^{-2} (Table 1). Mean euclidian distance (ED) varied between 352 and 446 µm. R calculated with minimum ED and the number of stomata per unit area showed a mean value of 1.53 ± 0.13, indicating that stomatal pattern is about half between random distribution and perfectly uniform hexagonal spacing. Despite the broad range in the observed leaf parameters, outliers were not found (Mahalanobis distance) and the leaf material of the examined mistletoe specimen was relatively homogenous. Relative stomatal aperture (RSA) ranged between 12.4% and 14.2% and was not significantly affected by the concentration of ABA supplied in the xylem of the host (Fig. 5).

4. Discussion

The present perfusion experiments show that differences in the ABA content of the host xylem do not cause short-term effects in mistletoe leaves on ABA levels, leaf transpiration, CO_2 assimilation, WUE, or the degree of stomatal aperture. For two reasons this result is surprising. On one hand, the ABA concentrations were chosen to simulate water stress [12] and should induce a fast [11,35] stomatal response in plant leaves [3,7]. On the other hand, the ABA concentrations in the xylem sap of the host were varied 10^4-fold and were several orders of magnitude higher than ABA concentrations reported for the xylem sap (0.2–2.2 µM) of *Populus* species

Table 1
Parameters of the spatial distribution of stomata on the adaxial side of mistletoe leaves (median euclidian distance, ED; mean euclidian distance; minimum euclidian distance; maximum euclidian distance; stomatal density, n (mm^{-2}); and R)

Median ED (µm)	Mean ED (µm)	Min. ED (µm)	Max. ED (µm)	n (mm^{-2})	R
411 ± 19	424 ± 19	42 ± 16	996 ± 59	89 ± 23	1.53 ± 0.12

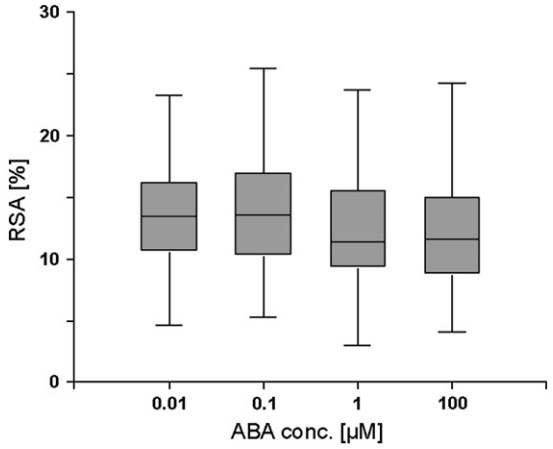

Fig. 5. "Relative stomatal aperture (RSA)" [%] of mistletoe leaves after 20 h perfusion of the host xylem with different ABA concentrations. Significant differences in RSA between treatments (ABA levels) were not observed.

[11,10]. Apparently, short-term fluctuations in ABA production by the host roots, its allocation to and action in the host leaves, and drought stress, do not affect transpiration of the mistletoe leaves. Hence, the severity of drought stress for the host is greatly increased by the presence of mistletoes. It may therefore be concluded that the hypothesis tested in the current experiments is valid, i.e. that the flux of ABA from the host xylem into the mistletoe is strongly controlled by the mistletoe and ensures a favourable water and nutritional status for the mistletoe, even if the water status of the host is impaired. However, the present experiments cannot exclude the possibility that this mechanism of control is located at the level of ABA breakdown [30] before it enters the mistletoe leaves, rather than at the level of ABA influx at the haustorium. However such a breakdown in the apoplastic space seems to be unlikely.

Despite the lack of short-term sensitivity of ABA levels in mistletoe leaves to even strong changes of ABA levels in the xylem sap of the host, ABA levels in mistletoe leaves were relatively high (Fig. 6) compared to ABA levels in the leaves of several tree species, including poplar [1,11,14,40]. This was also observed for the hemiparasite *Rhinanthus* in relation to its host *Hordeum vulgare* [30,31]. Significant transpiration of the mistletoe leaves was maintained (Fig. 1) indicating a diminished sensitivity of the stomata of mistletoe leaves to ABA. Such a diminished sensitivity may have been caused by external pH values unfavourable for ABA action on stomatal aperture [46,45], and/or low calcium and high potassium concentrations, previously reported for mistletoes in general and *Viscum* in particular [6,17,22,33,34,39,44]. High calcium levels play an important role in the signalling cascade [2,25,49] leading to stomatal closure and, thus, for ABA sensitivity of this process [25,48]; high potassium levels may counteract the response on stomatal aperture mediated by calcium [15,48]. Thus, in addition to an effective control of short-term changes in ABA levels, relative ABA insensitivity seems to mediate high transpiration by mistletoe leaves that is largely independent of environmental changes.

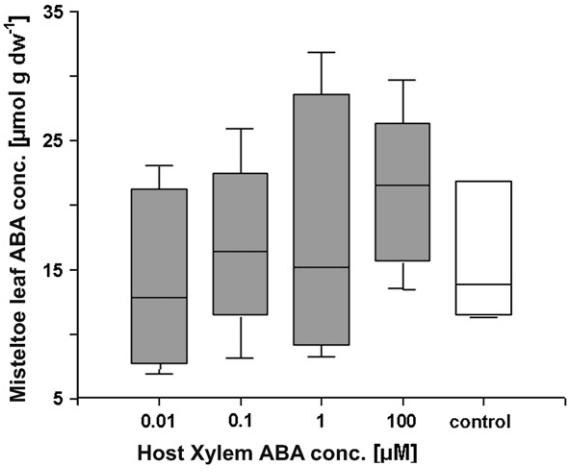

Fig. 6. ABA concentrations in leaves (µmol g dw^{-1}) of mistletoe leaves after 20 h perfusion of the host xylem with different ABA concentrations. Significant differences in RSA between treatments (ABA levels) were not observed.

The adaxial stomatal density of *Viscum* leaves of 89 ± 23 stomata per mm determined in the present study is lower than those reported in a study performed at the end of the 19th century (100 per mm^2 adaxial and abaxial) [36]. One possible explanation is that stomatal densities of leaves of *V. album* also decrease with CO_2 enrichment in the atmosphere as previously reported for other plants [8,55] in order to reduce their expense of transpirational water loss [42,55]. For xylem-tapping mistletoes, for which it is vital to have a more negative water potential than hosts, the reduction of the stomatal density seems to be absurd. But it could make sense if (i) a transpirational gradient is established mainly by the insensitivity of mistletoe stomata at higher VPD [17], or if (ii) temperate mistletoe establishes the difference in water potential mainly osmotically [5,51] or if (iii), due to the increased WUE the host is less water stressed [5] and the relative water potential gradient to the host could be maintained with lower stomatal densities and transpiration rates [9]. Investigations of historical mistletoe material would provide additional evidence on stomatal densities.

We did not analyse stomatal densities of the host leaves. But since previously for *P. x euamericana*, a stomatal density of 170–180 stomata per mm has been reported [50] and other *Populus* species exhibited stomatal densities from 170 to 288 stomata per mm [41], it is likely that the stomatal density of the *V. album* leaves in our study is also considerably lower than those of its host. This would be in agreement with the findings on a range of New Zealand mistletoes, which have in general lower stomatal densities in leaves than its hosts [4]. Further studies have to elucidate whether the mistletoe/host quotients of the stomatal densities in general and particularly for *Viscum* are related to their heterotrophic carbon gain and the quotients mistletoe/host of the photosynthetic capacity.

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