Vitis 35 (2), 65–68 (1996)

Stomatal patchiness of grapevine leaves. I. Estimation of non-uniform stomatal apertures by a new infiltration technique

by

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S u m m a r y : Non-uniform stomatal behaviour of vine leaves is associated with a heterobaric leaf structure. A microscopical analysis of cross sections of glasshouse- and *in vitro*-grown Silvaner leaves indicates single airspaces which are pneumatically isolated by vessels, bundle sheath extensions and the abaxial and adaxial epidermes. A pressure-regulated infiltration technique is presented by which the infiltration process and the infiltration capacity (percentage of the surface area of infiltrated airspaces) can be estimated and photographed using a light microscope. The average surface area of airspaces ranged from 0.10 mm^2 (Regent) to 0.14 mm^2 (Silvaner), the number of stomata per airspace from 35 (Regent) to 42 (Silvaner). The infiltration capacity of turgid leaves is shown to be negatively correlated with the surface tension of the infiltrated liquid and positively with stomatal conductance and with infiltration pressure, except for very low stomatal conductances (e.g. 12 mmol H_2O m^{-2} s^{-1}). The latter relationship follows a saturation curve confirming heterogenous stomatal aperture over the leaf blade. The distribution of stomatal apertures does not appear to be bimodal but to follow a bell-shaped curve. There is some evidence for the stomata of an airspace to behave heterogenously as well.

K e y w o r d s : leaf anatomy, stomatal patchiness, stomatal conductance, infiltration.

Introduction

In the past gas exchange was generally believed to be uniform over the entire leaf surface bearing stomata, e.g. this assumption was made to estimate partial pressure of CO_2 in intercellular spaces (VON CAEMMERER and FARQUHAR 1981). There is much evidence now that the gas exchange of leaves of a number of species is non-uniform, i.e. some stomata of the leaf blade are fully open while others are in part or fully closed. As a consequence alterations of stomatal conductance as derived from gas exchange measurements can be due to changes of the ratio of open *vs.* closed stomata.

Meanwhile non-uniform stomatal behaviour („patchiness") has also been demonstrated to occur in leaves of grapevines which were subjected to water stress, low air humidity or after application of abscisic acid (DOWNTON *et al.* 1988 a, b; DÜRING 1992). Diurnal changes of patchiness under field conditions led to the assumption that non-uniform stomatal behaviour may be an ubiquitous phenomenon preferably of leaves belonging to the heterobaric leaf type (DÜRING and LOVEYS 1996). According to MOLISCH (1912) and NEGER (1918) „heterobarische" (heterobaric) leaves are characterised by air-tight compartments which can be visualised by infiltration of liquids into the intercellular spaces of leaves *via* open stomata.

In this paper we have investigated the anatomy of heterobaric grape leaves. A technique is presented by which the infiltration of liquids as a function of pressure can be visualised microscopically. A forthcoming paper deals with effects of ambient factors and spatial and dynamic aspects of heterogenous stomatal behaviour.

Material and methods

P l a n t m a t e r i a l : Three-year-old, ungrafted *Vitis vinifera* (Silvaner) or the interspecific variety Regent ((Silvaner x Müller-Thurgau) x Chambourcin) were cultivated as potted plants under glasshouse conditions. They were regularly supplied with water and mineral nutrients and protected against fungus diseases by sulphur application. *In vitro* plants were cultivated at 25 °C. Light (40–50 µmol quanta m^{-2} s^{-1}) was provided by Fluora-lamps (Osram, Germany) for 16 h per day.

M i c r o s c o p y o f c r o s s s e c t i o n s : Leaf sections of fully expanded but not senescent leaves of potted and *in vitro*-grown grapevines (variety: Silvaner) were placed in ethanol at -20 °C. The chlorophyll-containing ethanol was replaced weekly until the leaf sections had lost almost all chlorophyll. Within the following 4 d the samples were transferred stepwise into an imbedding solution (Historesin, Jung, Heidelberg, Germany). Finally a „hardener" (Jung, Heidelberg, Germany) was added to induce polymerisation. To optimize this process under low oxygen conditions the samples were transferred into an desiccator with an elevated CO_2 concentration. 14 d after the onset of polymerisation the samples were cut in slices (1.5–2.5µm) by microtome (Microm, Heidelberg, Germany) and stained by toluidine blue (1 %, Sigma, Deisenhofen, Germany) to visualize the lipophile parts of the sample under a light microscope (C. Zeiss, Germany).

I m p r e s s i o n s o f e p i d e r m i s : Impressions or replicas of the leaf surface were obtained by slightly pressing „Dentalpaste" (Bayer, Leverkusen, Germany) to the abaxial epidermes of Silvaner and Regent leaves. As a

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secondary replica colourless nail polish was used which, after hardening, was examined under a light microscope to estimate stomatal frequency.

Infiltration under the microscope: The precision of the infiltration technique described earlier (DÜRING and LOVEYS 1996) was further developed by using a metal pressure chamber closed by two safety glasses on the upper and lower side (Fig. 1). The pressure chamber was connected to an inverse microscope (IM 25; C. Zeiss, Germany) with a camera. After inserting the leaf segment into the water-filled pressure chamber the intercellular airspaces were evacuated by pulling outward the syringe piston (-60 kPa). A micrometer screw connected to a glass syringe (30 ml) was used to raise or lower water pressure in the system; stepless pressure changes were recorded by two digital pressure meters, one for positive (0 to 300 kPa), and one for pressure below ambient (0 to -100 kPa) (Wallace and Tiernan, Günzburg, Germany). All experiments were carried out at room temperature (24 ± 1 °C). To test the effect of various surface tensions (st) on infiltration bidistilled water (st = 0.07275 N m^{-1}), 25 % methanol (st = 0.04638 N m^{-1}) and 96 % ethanol (st = 0.02304 N m^{-1}) were used (WEAST 1987, in: BEY-SCHLAG *et al.* 1992).

Determination of gas exchange: Stomatal conductance (g_{H_2O}) was estimated using a "Miniküvettensystem" (Walz, Effeltrich, Germany) at constant leaf temperature (25 °C), dew point (13 °C), light intensity (900 µmol quanta m^{-2} s^{-1}) and ambient CO_2 partial pressure (350 µbar) (for details: DÜRING 1991).

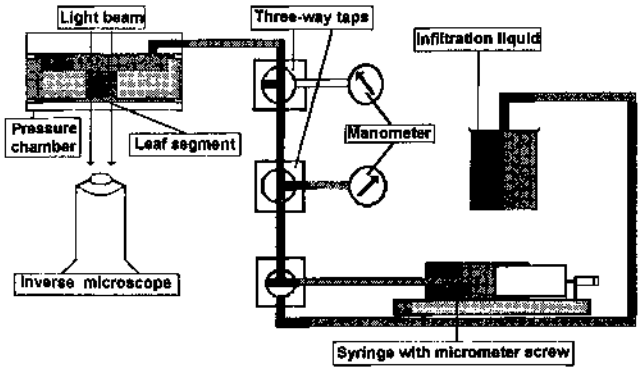

Fig. 1: Experimental arrangement for the infiltration of liquids into leaf segments by stepless variation of pressure (-70 kPa to 300 kPa). The process of infiltration can be observed microscopically and photographed.

Results

Microscopic cross sections: Like many other dicotyledons grapevines have leaves which are characterised by a netted or reticulate venation with bundle sheath extensions separating the mesophyll into small airspaces („Luftkammern" according to NEGER 1918). As is shown in Fig. 2 A the airspaces of glasshouse-grown grapevine leaves (variety: Silvaner) are pneumatically isolated by cell layers such as vessels, bundle sheath extensions and adaxial and abaxial epidermes. Thus, in contrast to homobaric leaves there is no lateral diffusion of gases in the mesophyll. The gas exchange between these airspaces and the atmosphere is predominantly limited to the stomatal pores of each airspace. Besides glasshouse-grown leaves we also studied the anatomy of *in vitro*-grown leaves. Cross sections indicate that *in vitro*-grown Silvaner leaves are characterized by a smaller leaf thickness, a lower number of mesophyll cells and shorter and thicker palisade cells compared to glasshouse-grown leaves. Here again we found internal airspaces which were isolated from each other by bundle sheath cells and their extensions (Fig. 2 B).

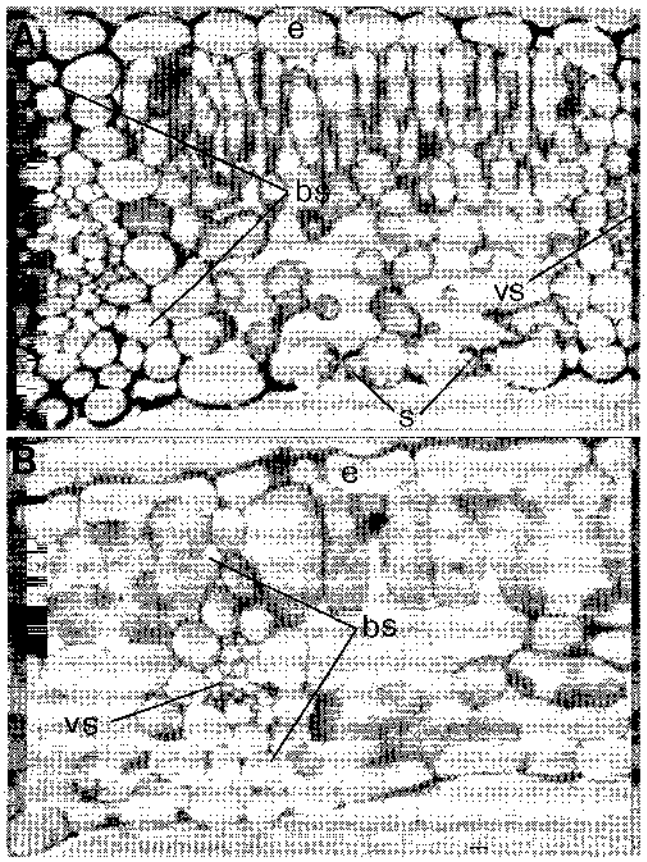

Fig. 2: Cross-sections of Silvaner leaves stained with toluidine blue; 440x. e: epidermis, s: stomata, bs: bundle sheath extension, vs: vascular system. **A**: glasshouse-grown, **B**: *in vitro*-grown leaf.

Infiltration capacity: It has been shown earlier that airspaces with open stomata can be distinguished from those with closed stomata by infiltration of water or other liquids (MOLISCH 1912, NEGER 1918, FRÖSCHEL 1951, BEYSCHLAG and PFANZ 1990; grapevines: DÜRING 1992, DÜRING and LOVEYS 1996).

In order to study the infiltration of liquids into airspaces in detail a microscopical, pressure-regulated infiltration technique was developed. This technique enabled us to estimate the average surface area of airspaces which was 0.14 mm^2 (± 0.01 c. l., p ≥ 5 %) for Silvaner and 0.10 mm^2 (± 0.01 c. l., p ≥ 5 %) for the variety Regent. The average number of stomata per airspace was 42 (Silvaner) and 35 (Regent).

Immediately after estimating stomatal conductance of a leaf section by gas analysis the infiltration capacity of this section, i.e. the percentage of the surface area of infiltrated airspaces, was determined.

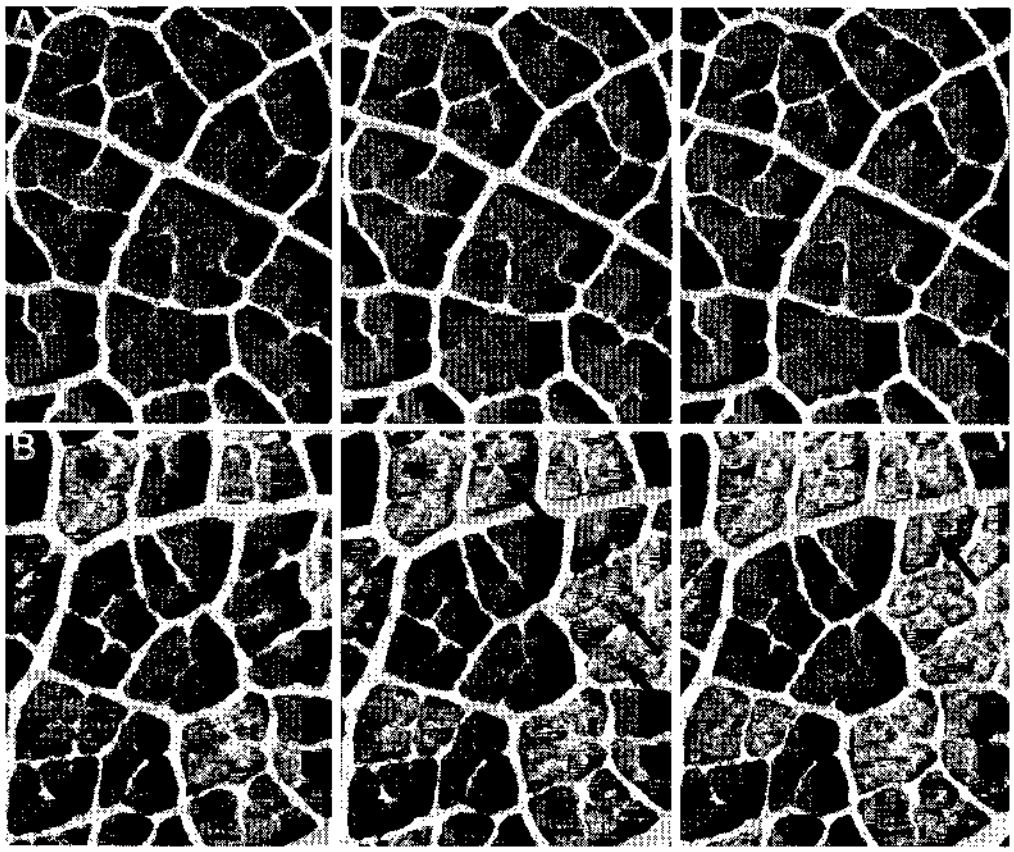

Fig. 3: Infiltration of distilled water at increasing pressure (left to right: 0, 100, 300 kPa) into intercellular spaces of Silvaner leaves at two stomatal conductances. **A**: 12 mmol H_2O m^{-2} s^{-1}; **B**: 160 mmol H_2O m^{-2} s^{-1}, arrows indicate infiltration of additional airspaces. 35x.

As is shown in Fig. 3 at very low stomatal conductance (12 mmol H_2O m^{-2} s^{-1}, induced by high ambient CO_2 partial pressure) and at high leaf water potential (-0.05 MPa) a continuous increase of pressure from 0 to 300 kPa does not cause any infiltration, while at higher stomatal conductances an increase of pressure leads to an increasing number of infiltrated airspaces. At very high stomatal conductance (305 mmol H_2O m^{-2} s^{-1}) only a small pressure (50 kPa) is necessary to infiltrate 84 % of the airspaces. Except for very low stomatal conductances the infiltration *vs.* pressure curves are not linear but follow „saturation curves" indicating a heterogenous pattern of stomatal apertures (Fig. 4).

At increasing pressure infiltration of water into single airspaces of leaves of *in vitro*-grown plants confirms the results we had obtained from cross sections: airspaces are

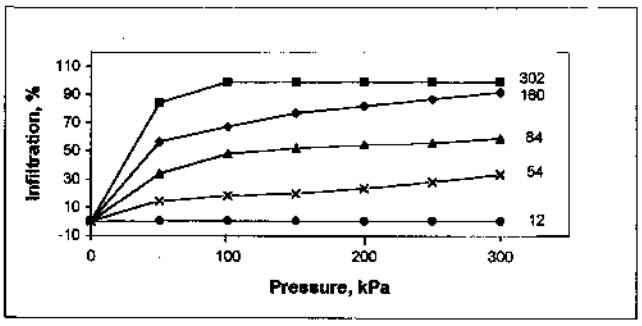

Fig. 4: Infiltration of distilled water as a function of infiltration pressure at various stomatal conductances (12, 54, 84, 160, 302 mmol H_2O m^{-2} s^{-1}). Variety Silvaner.

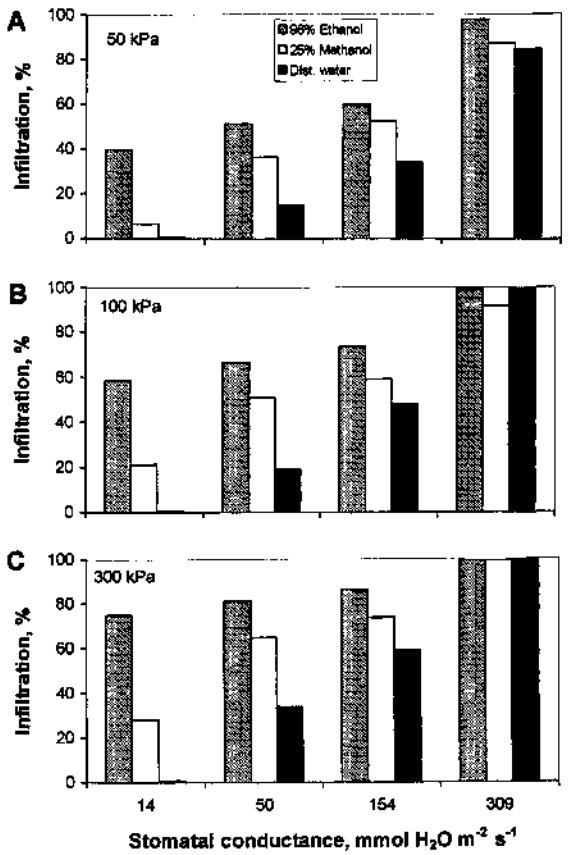

Fig. 5: Infiltration capacity (percentage of the surface area of infiltrated airspaces) as a function of infiltration pressure, stomatal conductance and surface tension of the infiltration liquid. Variety: Silvaner.

pneumatically isolated and the stomatal apertures are heterogenous over the leaf blade.

It is interesting to note that the infiltration capacity obviously depends on the leaf water status as well. At lower leaf water potentials, e.g. -1.1 MPa, the infiltration capacity increased at pressures >100 kPa although stomatal conductance was very low. Thus, the determination of heterogenous stomatal behaviour by infiltration appears to be restricted to fully turgid leaves.

In a series of experiments the effect of surface tension of liquids was investigated. Fig. 5 demonstrates the infiltration capacity increasing with pressure, stomatal conductance and decreasing with increasing surface tension of the infiltrated liquid. During the experiments we tried to answer the question if the stomatal apertures of a single airspace are homogenous or not. Observations of a single airspace during infiltration showed that infiltration did not occur in all parts of the airspace at the same time. Instead, at increasing pressure infiltration started in a certain part of the airspace and from there the liquid spread out into other parts of the airspace. The liquid is supposed to have entered the airspace via the most widely opened stomata, thus we can assume heterogeneity of stomatal apertures of an airspace.

Discussion

The results presented in this paper clearly demonstrate the anatomical basis for heterogenous gas exchange of grapevine leaves. Nevertheless, from a series of publications it can be derived that non-uniform photosynthesis does occur in homobaric leaves as well (Review: TERASHIMA *et al.* 1988). However, bundle sheath extensions obviously enhance non-uniform photosynthesis in heterobaric leaves (TERASHIMA 1992).

Cross sections of glasshouse-grown and *in vitro*-grown grapevine leaves indicated distinct differences with respect to leaf anatomy. This can be attributed to the extremely different cultural conditions, namely with regard to light intensity and air humidity. The observation that leaves of *in vitro* vines had different stomatal apertures at the abaxial part of their leaf blade was somewhat surprising as stomatal aperture of *in vitro*-plants in general is believed to be not changeable. E.g., *in vitro* apple plants were unable to close their stomata under conditions which normally induce stomatal closure (BRAINERD and FUCHIGAMI 1982). However, meanwhile there is evidence from gas exchange measurements for stomata of *in vitro*-grown grapevines to respond to changes of ambient conditions confirming the results obtained by infiltration (DÜRING, in prep.).

Our results indicate that the infiltration capacity is affected - besides other factors - by the leaf water potential. It can be assumed that a decline of the leaf water potential which is associated with a decline of the epidermal turgor is transmitted to the subsidary cells of the stomata. E.g., we confirmed results of RASCHKE (1970) that a reduction in water supply to leaves causes the subsidiary cells surrounding the stomata to collapse within 60–90 s (DÜRING 1993). As a consequence stomatal resistance to increasing infiltration pressure will be limited.

Infiltration of water into leaf blades implies that, due to its specific surface tension „a certain minimum (threshold) aperture of a stoma is necessary to allow water flow through the pores at a given infiltration pressure" (BEYSCHLAG and PFANZ 1990). Therefore this method does not provide information on the stomatal aperture outside that relative to the threshold unless either liquids of various surface tension or various infiltration pressures are applied (MOLISCH 1912; FRY and WALKER 1967). Our experiments with liquids differing in surface tension and those with increasing infiltration pressure do not indicate a linear relationship between infiltration pressure and infiltration capacity. Thus it is concluded that the distribution of stomatal apertures is not bimodal but follows a bell-shaped curve. Work is in hand to demonstrate that single airspaces behave autonomously, possibly adapting rapidly to alterations of endogenous and/or ambient conditions.

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Received March 20, 1996