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Title

Progressive wetting of initially hydrophobic plant surfaces by salts - a prerequisite for hydraulic activation of stomata?

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Introduction

The input of nutrients by aerosols is essential to forest ecosystems in the Amazon, Hawaii, and eastern North America, where particles reach by intercontinental aerosol transport (Newman 1995; Chadwick *et al.* 1999; Okin *et al.*, 2004). Phosphorus is especially important, but the input of other nutrients by particle deposition is also substantial and a wide range of ecosystems are affected (Grantz *et al.*, 2003). In spruce stands, up to 75 % of atmospheric nitrogen deposition did not reach the soil but was retained in the canopy, possibly accounting for a large part of the annual N requirement for growth (Schulze 2000, Tomaszewski *et al.* 2003, Gaige *et al.* 2007). Dry deposited particles usually accumulate on leaf surfaces with time, and residues from fog or dried intercepted rain droplets also contribute to the actual amount of particulate substances on leaf surfaces (Burkhardt, 2009). It is likely that the deposited amounts have globally increased by human influence, as nowadays the atmospheric aerosol composition contains 2.7 times more inorganic components of strongly increased hygroscopicity compared to pre-industrial times (Dentener *et al.* 2006; Tsigaridis *et al.* 2006).

The uptake of deposited nutrients may happen directly by the leaves. Differently from uptake via the soil, foliar uptake is a direct addition to plant metabolism and competes with no other process in supporting plant growth (Sparks 2009). The foliar uptake from the liquid phase is expected to be much more important than that from the gaseous phase (Harrison *et al.* 2000, Gaige *et al.* 2007, Sievering *et al.* 2007). Hygroscopic salts on the leaf surface will often be present in solution rather than in crystallised form, caused by leaf transpiration and the process of salt deliquescence, which for most salts happens considerably below 100% relative humidity (RH) (Pilinis *et al.* 1989; Burkhardt and Eiden 1994; Burkhardt *et al.* 1999). The ions in these concentrated solutions will be mobile and uptake/exchange with solutions inside the leaf is regulated by the respective concentration gradients and transport resistances.

There has been some debate in the past if uptake under natural conditions may solely happen across the cuticle or also via the stomata. The usually hydrophobic nature of pristine cuticles and stomatal geometry were assumed to completely prevent the intrusion of water droplets into the stomata (Schönherr and Bukovac 1972). Meanwhile, however, it has convincingly been shown that stomatal transport of liquid water, dissolved ions, and even dispersed nanoparticles can happen under natural conditions, i.e. without addition of surfactants (Eichert *et al.* 1998; Eichert and Burkhardt 2001; Burgess and Dawson, 2004; Zimmermann *et al.* 2007; Breshears *et al.* 2008; Eichert *et al.* 2008; Wilkinson and Davies 2008, 2009). It has also become clear that this process does not readily affect all stomata, but a certain threshold has to be overcome to 'activate' single stomata for liquid water transport (Burkhardt 2001). Once established, such a connection is likely to persist, enabling the transport of liquid water and dissolved or dispersed substances (Burkhardt and Eichert, 2001; Eichert *et al.*, 2008; Burkhardt 2009).

Due to its effect of establishing a liquid water layer, the process of establishment is called here 'hydraulic activation of stomata' (HAS). The exact mechanisms leading to HAS are still unclear. Several suggestions have been made, including fungal hyphae stretching into the stomata (Burgess and Dawson, 2004), mucilage growing in stomatal chambers (Zimmermann *et al.*, 2007; Westhoff *et al.*, 2009), and stomatal colonization by bacteria (Eichert *et al.*, 2008). In this paper we demonstrate the possibility of salts to spread on hydrophobic cuticles by repeated drying/wetting cycles as a model for the extension and establishment of salt bridges along stomatal walls. We also show the interaction of superficial salts with plant water relations, a possible consequence of HAS.

Methods

Bean (*Vicia faba*) plants were grown in hydroponic culture. Apple (*Malus domestica* Borkh. var. Golden Delicious) seedlings were grown in soil until about 12 leaf pairs were reached.

All plants remained in the growth chamber (20°C, 50% RH at day; 18°C, 70% RH at night, photosynthetically active radiation (PAR) 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) where they were fully watered and nourished. Stomata-free cuticles from tomato fruits (*Solanum lycopersicum* L) were isolated and prepared using the procedure described elsewhere (Hunsche and Noga 2008). They were hydrophobic with mean contact angles of about 90°. For gas exchange measurements of bean, leaf pairs of an untreated leaf with the directly adjacent K_2HPO_4 treated leaf, respectively, were compared. Gas exchange was measured using a photosynthesis system (Li-6400, Lincoln, USA), with a CO_2 concentration of 400 $\mu\text{mol mol}^{-1}$, PAR of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$. A low humidity value of 25 % RH was set in order to stimulate transpiration.

Salts were applied to bean and apple leaf surfaces by solutions prepared from p.a. chemicals. K_2HPO_4 is slightly hygroscopic and has a deliquescence point (DRH) of 92%. DRH of NaCl and NaClO_3 are quite similar at about 75% (Pilinis *et al.* 1989). Bean leaves were treated applying droplets of K_2HPO_4 solution. 30 droplets of 6 μl with 10 mM solution were applied per leaf, usually every second day, with nine treatments altogether summing up to three weeks. Leaves were not visually damaged by the treatments. Gas exchange was measured before and after the last treatment with eight replicates, respectively. Treatments with NaClO_3 droplets (10 mM) on apple leaves happened only once. Droplets were applied either on the stomatous adaxial (upper) or on the abaxial (lower) side, respectively. 30 droplets of 6 μl with 10 mM solution were applied per leaf. Gas exchange was measured before treatment and on the second day after treatment with five replicates, respectively. At the time of the second measurement, some of the abaxially treated leaves already started to turn into brownish colour in the area where droplets had been applied. These changes became more pronounced after the measurements, leading to necrotic parts. Adaxially treated leaves were unaffected.

Single salts of NaCl and NaClO_3 were ground to heterogeneous particles of approximate diameters between 1 and 50 μm and were then dispersed on isolated tomato cuticles. Studies were conducted using an environmental scanning electron microscope (ESEM XL 30 FEI-Philips, Eindhoven, Netherlands) equipped with an EDX system (EDAX, Ametek GmbH, Meerbusch, Germany). The Genesis 4000 software was used to display and evaluate the collected EDX spectra. The samples were cooled in the ESEM to 7°C or 5°C, in order to allow relative humidity to increase to high values with small amounts of water vapour. The first wetting cycle usually started at 3 Torr (400 Pa), then humidity was increased by steps of 0.1 Torr (13 Pa). The next step, respectively, was triggered after any visual changes had ceased, which usually took between 30 s and 1 min. When most of the salt was deliquescent, but before the entire surface was covered by water, humidity was slowly decreased again. In this way, a complete wetting/drying cycle needed between 30 and 50 minutes.

Results

Apple leaves where NaClO_3 had been applied to the abaxial side showed a significant decrease of water use efficiency already two days after a single treatment, while stomatous adaxial leaves did not show any changes compared to the control, respectively. However, water use efficiency mainly decreased due to a decrease in photosynthesis and not due to an increase in transpiration (Fig. 1). After the measurements, most of the abaxially treated leaves developed necroses, while almost no damage was observed on adaxially treated leaves. Bean leaves showed significantly higher transpiration after repeated application of K_2HPO_4 droplets, compared to the control (Fig. 2). While photosynthesis remained unchanged, this led to a significantly lower water use efficiency of treated bean leaves.

K_2HPO_4 droplets on bean leaves left different marks. Most noticeable were the structures of crystals following the anticlinal walls between cells (Fig. 3a). Former droplets

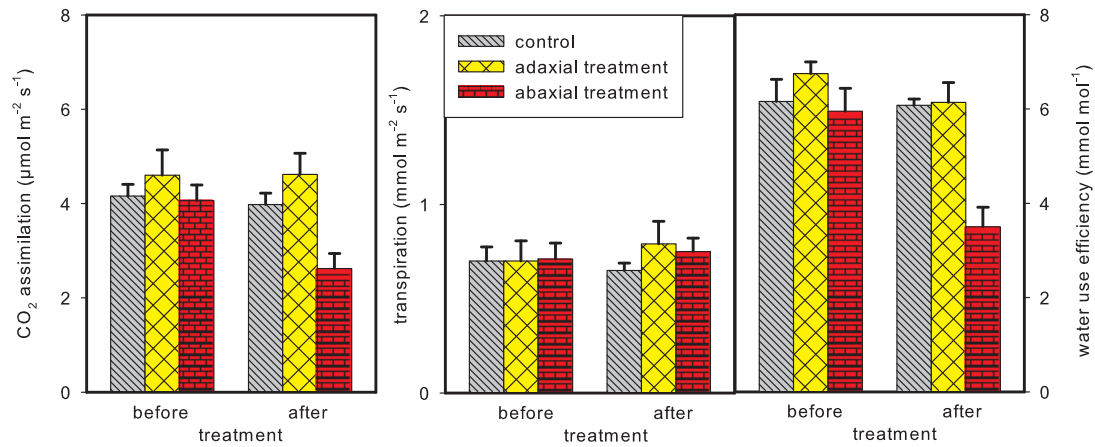


Fig. 1: CO₂-assimilation, transpiration, and water use efficiency of apple leaves treated with NaClO₃ droplets, measured the day before and two days after droplet application.

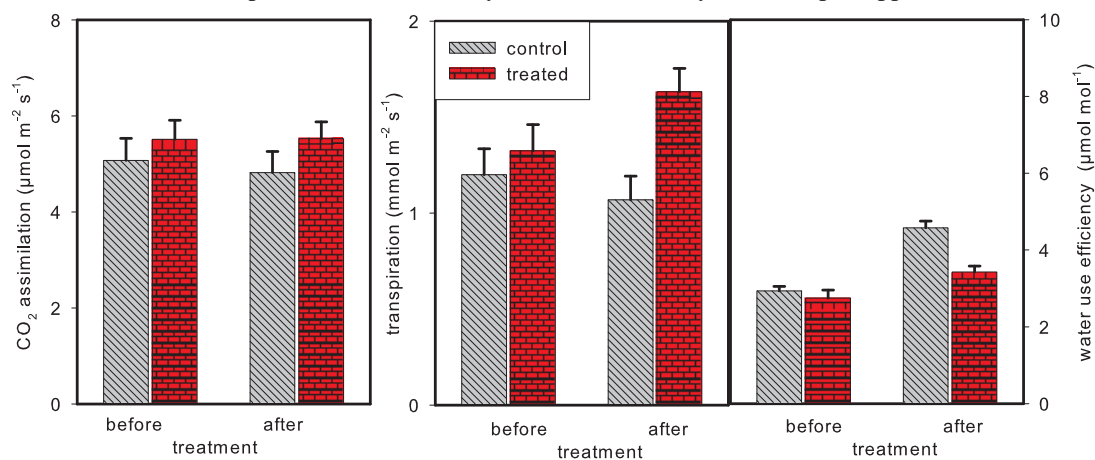


Fig. 2: CO₂-assimilation, transpiration, and water use efficiency of bean leaves treated with K₂HPO₄ droplets, measured the day before and the day after last droplet application.

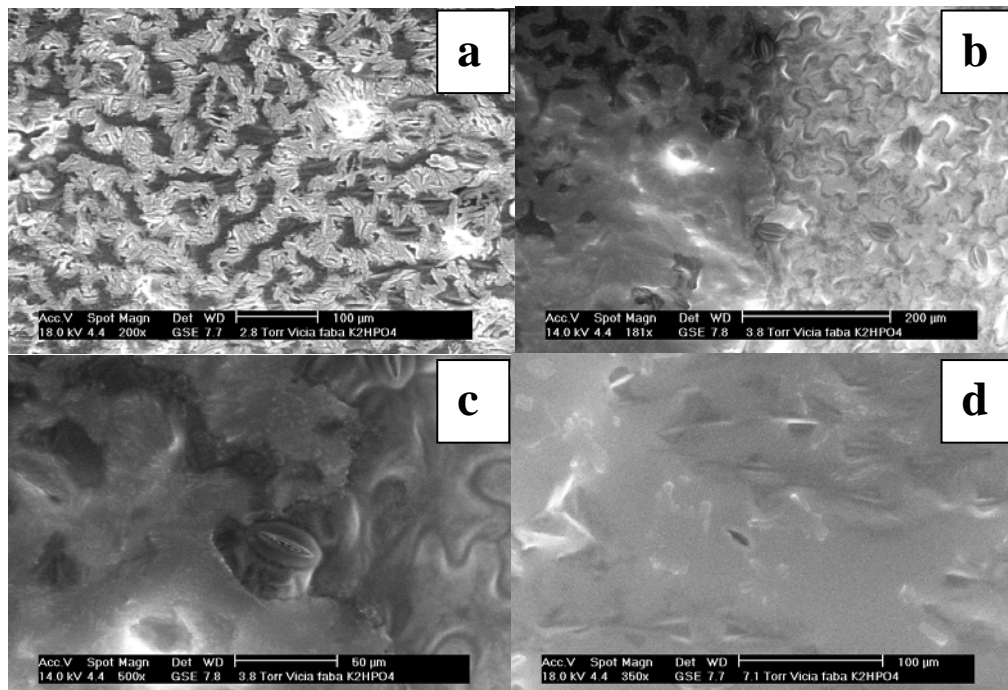


Fig. 3: K₂HPO₄ salt on the surface of *Vicia faba* leaves. a)-c) show dry salt at 37% RH (a) and 52% RH (b,c), respectively. d) Deliquescent salt, 95% RH.

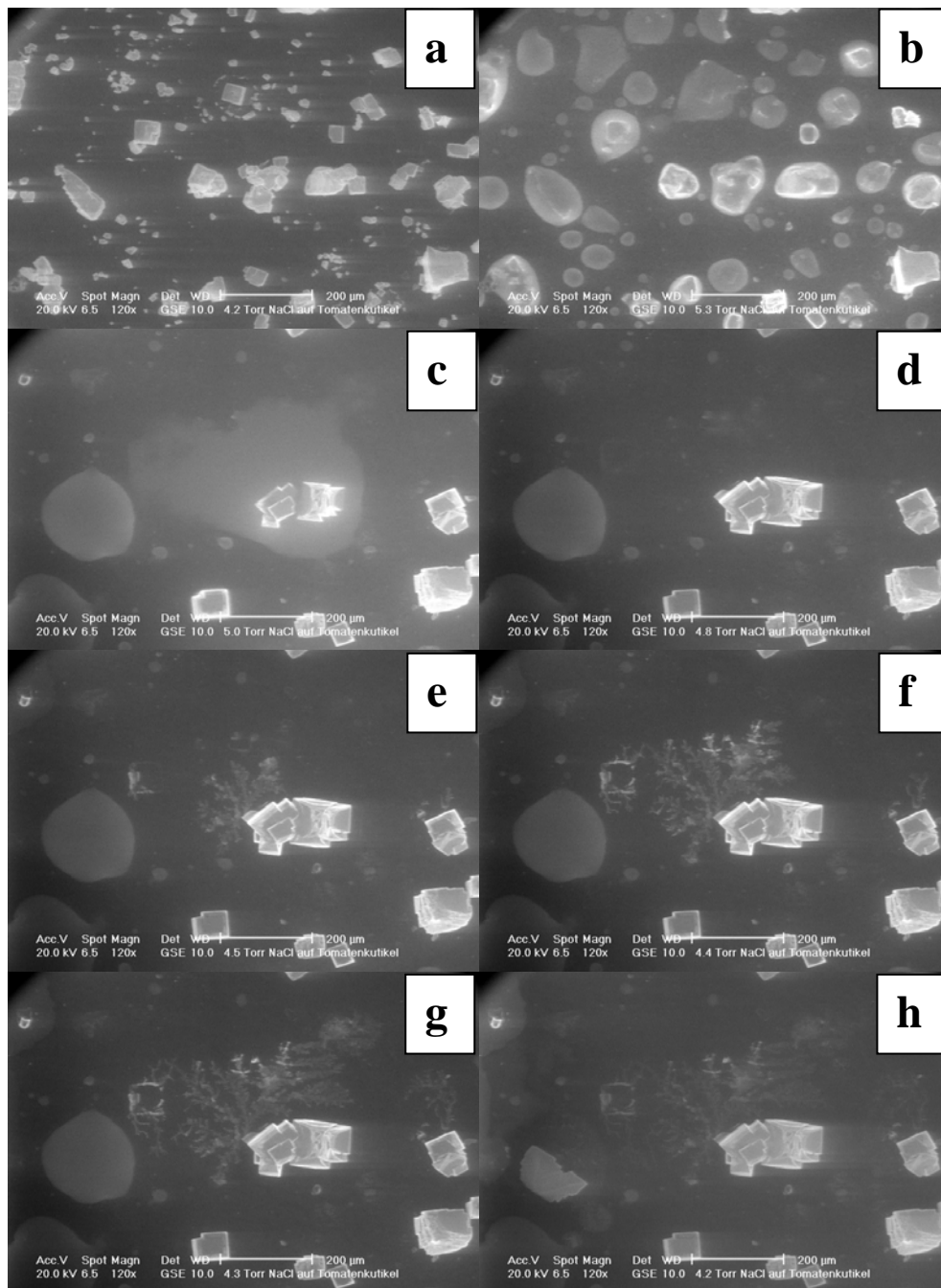


Fig. 4: NaCl salt on isolated tomato cuticles. a) initial state of ground dry particles, 65% RH. b) first wetting, 82% RH. c)-h) fifth drying, after appr. 4 hours, detailing at 77%, 73%, 67%, 66%, and 65% RH, respectively.

left thicker crusts, in some places also covering stomata. Some areas without recognizable crystals but of darker appearance compared to droplet free regions could be noted (Fig. 3b,c). When humidity exceeded the deliquescence point of the salt, droplets dissolved and covered larger areas (Fig. 3d).

NaCl crystals on tomato cuticles became deliquescent when RH increased above the deliquescence point (Figs. 4a,b). After completion of each of the first four wetting/drying cycles not much changes could be observed compared to the initial situation, although some formation of larger crystals was observed. During the drying phase of the fifth cycle, salt started to form dendritic patterns, originating from the base of larger crystals (Fig. 4c-h). This

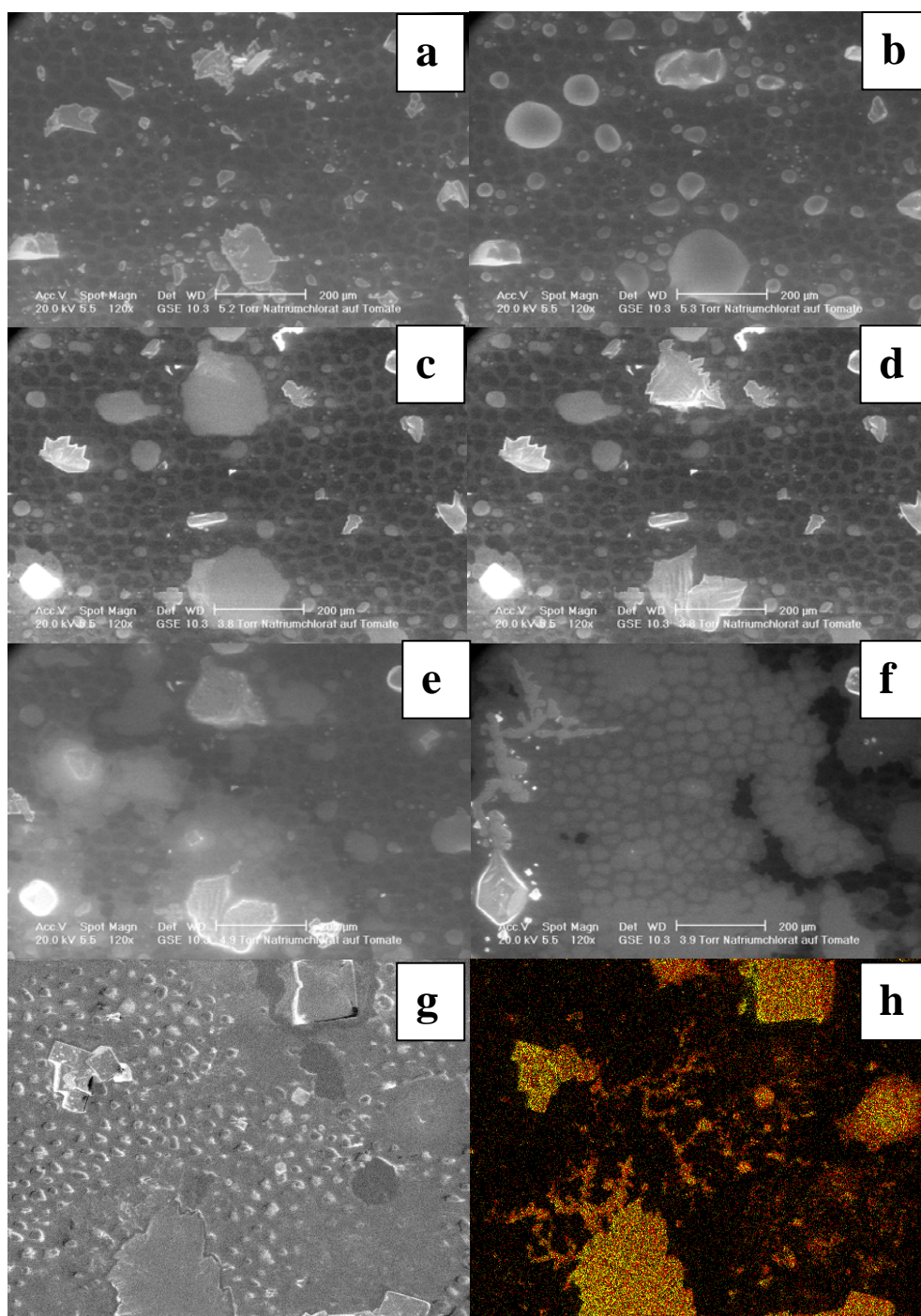


Fig. 5: a)-f) ESEM pictures of NaClO_3 salt on isolated tomato cuticles. a) initial state of ground dry particles, 80% RH. b) first wetting, 82% RH. c)-d) first drying, 58% RH. e) second wetting, 75% RH. f) second drying, 60% RH, about 70 minutes after starting first cycle. g) low vacuum SEM picture of NaClO_3 on tomato cuticle after two wetting/drying cycles. h) Same site, overlay of characteristic X-ray emission of Na and Cl.

was about four hours after initialisation of the measurements. NaClO_3 crystals on tomato cuticles became for the first time deliquescent above 80% RH (Fig. 5a,b). They showed a marked hysteresis with efflorescence starting at around 60% RH (Fig. 5 c,d). The second wetting started earlier, at 75% RH, giving already the impression of liquid layers covering large areas in regions far from the crystals (Fig. 5e). During the next drying cycle, dendritic

growth of crystals into almost all regions of the cuticular surface could be observed (Fig. 5f). Formation of dendritic crystals starting from large crystals was also visible from EDX images of Na and Cl (Figs. 5g,h). In this case, the formation of dendritic crystals covering large areas already started about 70 minutes after initialisation.

Discussion

NaClO₃ caused a significant decrease of photosynthesis two days after a single treatment, but only if the droplets were applied to the stomatous side of the apple leaves. This indicates an involvement of stomata, although a different type of cuticular properties on upper and lower sides of the leaf can not be ruled out. K₂HPO₄ did not damage the leaf even after multiple application of droplets. However, the accumulation was strong enough to increase transpiration. Increased transpiration by application of some salts and inert particles to leaves has early been reported (Duggar and Cooley 1914; Wagner 1939; Beasley 1942; Gmur *et al.* 1983; Burkhardt *et al.* 2001). An involvement of stomata has been assumed (Beasley 1942), e.g. by a sort of ‘wick’ formation (Burkhardt *et al.* 2001; Burkhardt, 2009). Thus, both results point to an involvement of stomatal transport, with likely different processes of stomatal activation. While for K₂HPO₄ possibly the high accumulation was the most important factor, it showed relatively compact crystallisation on bean leaves in generally well defined areas (Fig. 3). The behaviour of NaClO₃ on cuticles was different as it rapidly spread over large areas. This happened already during the second wetting/drying cycle, much faster than for NaCl under the same conditions. A special crystallisation mechanism of NaClO₃ has been described earlier (Sears 1957), and the well known herbicidal activity of NaClO₃ may be a result of this behaviour.

We have shown here for the first time a general way for the hydrophilization of pristine hydrophobic leaves. This is likely to be a model for processes happening during HAS by drying/wetting cycles of salts. This process together with the mentioned biotic processes may therefore serve as a ‘door opener’ for the efficient transport of nutrients, water, and signals/information along a transstomatal hydraulic connection.

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