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The apoplast and its significance for plant mineral nutrition

Author for correspondence:
Burkhard Sattelmacher
Tel: +49 431880 3189
Fax: +49 431880 1625
E-mail: bsattelmacher@plantnutrition.uni-kiel.de

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Burkhard Sattelmacher

Institute for Plant Nutrition and Soil Science, University Kiel, Oshausenstr. 40 D-24118 Kiel, Germany

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Summary

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It has only recently become apparent that the apoplast plays a major role in a diverse range of processes, including intercellular signalling, plant–microbe interactions and both water and nutrient transport. Broadly defined, the apoplast constitutes all compartments beyond the plasmalemma – the interfibrillar and intermicellar space of the cell walls, and the xylem, including its gas- and water-filled intercellular space – extending to the rhizoplane and cuticle of the outer plant surface. The physico-chemical properties of cell walls influence plant mineral nutrition, as nutrients do not simply pass through the apoplast to the plasmalemma but can also be adsorbed or fixed to cell-wall components. Here, current progress in understanding the significance of the apoplast in plant mineral nutrition is reviewed. The contribution of the root apoplast to short-distance transport and nutrient uptakes is examined particularly in relation to Na^+ toxicity and Al^{3+} tolerance. The review extends to long-distance transport and the role of the apoplast as a habitat for microorganisms. In the leaf, the apoplast might have benefits over the vacuole as a site for short-term nutrient storage and solute exchange with the atmosphere.

I. Introduction

When at the end of the 17th century Robert Hooke used a self-made microscope to study plant tissues, his observations led him to conclude that plants were made up of 'little boxes', or 'cells' as he called them. Since he conducted his initial work with dead plant material, such as cork, his cells consisted of cell walls only. It is interesting to recall that the plant compartment, which today is called the apoplast, has actually been known for a longer period of time than the symplast, and that it attracted the interest of biologists for many years before 'the dead excrusion product of the living protoplast' was forgotten, for almost three centuries. Cell walls were the subject of scientific interest mainly as a resource for industrial processing or in relation to animal or human health.

It was not before the mid 1800s that cell walls attracted the interest of a broader group of plant scientist (Schindler, 1993). It soon became evident that the term cell wall may be misleading since it is not appropriate to associate a highly complex matrix consisting of cellulose, hemicellulose, pectins and proteins (Sakurai & Nevins, 1993; Carpita *et al.*, 1996) that is highly flexible. By now we know that the chemical and physical properties of cell walls are not fixed but depend on a number of parameters including ontogeny (von Teichman & van Nyk, 1993; Cheng & Huber, 1997; Sakurai & Nevins, 1997; Steele *et al.*, 1997), environmental parameters such as temperature (Dawson *et al.*, 1995; Klein *et al.*, 1995; Siddiqui *et al.*, 1996), osmotic stress (Hirasawa *et al.*, 1997; Wakabayashi *et al.*, 1997), light (Parvez *et al.*, 1996; Cheng & Huber, 1997; Parvez *et al.*, 1997), heavy metal stress (Aidid & Okamoto, 1993; Degenhardt & Gimmler, 2000), and nutrient supply (Tan & Hogan, 1995; Findeklee *et al.*, 1997; Hay *et al.*, 1998). This is why it was suggested to replace the term 'cell wall' with the more precise term 'extracellular matrix' (Schindler, 1993). The more we learned about the extracellular matrix the more it became apparent that only few processes during growth and development of a plant do not involve cell walls (Sakurai, 1998).

It was the botanist Ernst Münch (Münch, 1930) who separated the plant into two principal compartments the 'dead' apoplast and the 'living' symplast. While Münch thought water and solute transport were the sole function of this new plant compartment, we know today that apoplastic functions are much more numerous. It has been suggested to consider 'the apoplast as the internal physiological environment of plant bodies' in which maintenance of homeostasis is essential (Sakurai, 1998). In this context it appears worth while to mention that in many cases environmental stimuli are not received directly by the cell but via changes within this internal environment (Hoson, 1998). As an example, which will not be considered any further, the response to phytohormones such as auxins (Tsurusaki *et al.*, 1997) or pathogen attack (Kiba *et al.*, 1997; Olivares *et al.*, 1997) may be taken.

From the viewpoint of plant mineral nutrition the apoplast appears to be of interest in many respects: nutrients do not simply pass through the apoplast on their way to the plasmalemma, but they may also be adsorbed or fixed to cell wall components which may be of significance for both nutrient acquisition (Thornton & Macklon, 1989; Ae & Otani, 1997) and tolerance against toxicity (Horst, 1995). Microorganisms colonize this compartment and may contribute directly to the nutrition of higher plants, for example by their ability to fix di-nitrogen (Kaile *et al.*, 1991).

These numerous functions require a broader definition than those given by Münch: according to present understanding all compartments beyond the plasmalemma constitute the apoplast (i.e. the interfibrillar and intermicellar space of the cell walls, the xylem as well as the gas and water filled intercellular space in its entirety). The border of the apoplast is formed by the outer surfaces of plants (i.e. the rhizoplane and the cuticle). Solutes or microorganisms adhering to these surfaces are not, however, apoplastic.

It is the objective of this article to review the processes and properties of the apoplast as far as they contribute to the mineral nutrition of plants. Examples will be taken from work being conducted in the scope of the priority research project of the German Research Association – 'The apoplast of higher plants: compartment for storage, transport and reactions' and especially from our own work.

II. The properties of the apoplast and its implication for solute movement

Although this is not a review on cell wall biochemistry it appears appropriate to consider briefly the physico-chemical properties of cell walls in order to consider implications for plant mineral nutrition (Brett & Waldron, 1996). Cell walls consist of a series of layers. The earliest layer is deposited at cell division and since the subsequent wall layers are laid down between the plasma lemma and the earliest layer, the oldest cell wall is found were the cell walls adjoins, the latest wall layer is found nearest to the plasma lemma. Three clear-cut layers differing in both chemical and physical properties can be distinguished: the middle lamella, the primary cell wall and the secondary cell wall.

1. The middle lamella

The middle lamella of dicot plants, and to a lesser degree of monocot, basically consist of pectins with different degree of methylation. Pectins are a very heterogeneous group, homogalacturonans and rhamnogalacturonans being just two prominent representatives.

2. The primary wall

The primary wall consists of a network of cellulose of a relatively low degree of polymerization, hemicellulose (xylans in monocot, xyloglucans in dicot) and glycoproteins. The

latter may represent between 5 and 10% of the cell wall dry weight (Cassab & Varner, 1988) demonstrating that cell walls are important sites for metabolism (VI The apoplast leaves).

3. The secondary cell wall

The secondary cell wall consists, to a higher degree than the primary wall, of cellulose of relatively high degree of polymerization, hemicellulose and protein content is considerably lower than is the case in the primary cell wall (Brett & Waldron, 1996). In both primary and secondary cell walls the cellulose/hemicellulose network consists of interfibrillar and intermicellar spaces which differ in size between 3.5 and 5 nm (Carpita *et al.*, 1979; Gogarten, 1988; Shepherd & Gootwin, 1989; Chesson *et al.*, 1997), and thus does not represent a major diffusion barrier even for larger molecules. However, due to friction and tortuosity, transport velocity may be hampered. For high molecular weight solutes such as fulvic acids, chelators or viruses, pore size prevents transport. Cell wall porosity may change with ontogeny (O'Driscoll *et al.*, 1993; Titel *et al.*, 1997) and cell differentiation (Lynch & Staehelin, 1995). The influence of environmental factors such as toxic metals remains an open question.

The hydraulic conductivity of cell walls is rather high and exceeds that of the plasmalemma by far. However, due to the larger cross-sectional surface of the symplastic pathway, the relative contribution of both components to water transport may be comparable; although in certain plant tissues the contribution of the apoplast appears to be rather low (Steudle & Frensch, 1996; Schulz *et al.*, 1997; Steudle & Peterson, 1998). The presence of aquaporins (Steudle, 1997) may increase the conductivity of the plasmalemma and thus the relative significance of the symplastic or trans root pathway. This has been convincingly demonstrated by Kaldenhoff *et al.* (1998). The authors decreased hydraulic conductivity of *Arabidopsis thaliana* roots by a factor of three by antisense expression of aquaporins. This was compensated for by the plant by increasing the size of the root system by a factor of five allowing the plant to cope with the reduced water permeability of the plasmalemma.

Since cell walls are normally found to have negative charges due to the predominance of free carboxyl groups of galacturonic acids of the pectins in the middle lamella and primary wall, movement of ions in cell walls is characterized by electrostatic interactions leading to an accumulation of cations in the apparent free space (AFS) in a nonmetabolic step (Marschner, 1995). (The term apparent free space has been chosen for the apoplastic space in order to stress the point that ion movement is not free but dependent on its interaction with the undiffusable anions of the cell wall.) The current view of ion movement in cell walls is highly influenced by the early work of Hope & Stevens (Hoson, 1987) as well as by that of Briggs & Robertson (1957). According to these authors the AFS is divided into the Donnan Free Space (DFS) and the Water Free Space (WFS). The Donnan Free Space is that part of the AFS where ion distribution is characterized by the presence of undiffusible anions, in the Water Free Space, however, ion movment is not restricted by electrical charges. The relative size of DFS : WFS is 20 : 80). Both cation exchange capacity (Demarty *et al.*, 1978; Bush & McColl, 1987) and electrical potential (Stout & Griffing, 1993) have been used to describe the physical properties of the DFS. However, we now know that rigid separation between the DFS and the WFS may be an oversimplification, because it is not possible to make any clear spatial differentiation between the two compartments (Platt-Aloia *et al.*, 1980; Starrach & Mayer, 1986) and the extent of the DFS is not fixed (Ritchie & Larkum, 1982). Nevertheless, the model has proved to be helpful especially in the understanding of uptake phenomena such as the apparent synergism between Ca^{2+} and $H_2PO_4^-$ (Franklin, 1969) or differences in the uptake of Zn^{2+} in ionic or chelated form (Marschner, 1995).

The amount of nondiffusible cell wall anions are normally quantified by the cation exchange capacity (CEC) which is by far higher in dicot than in monocot species (Keller & Deuel, 1957). In most cases the CEC is determined with isolated cell wall material. In this context it appears noteworthy to state that due to spatial limitations only part of the exchange sites are accessible to cations leading to a much lower CEC *in vivo* (Marschner, 1995). The CEC of a plant tissue is not constant but is highly responsive to environmental factors. For example, salinity generally decrease the CEC (Bigot & Binet, 1986) which is regulated by enzymes such as pectin methylesterase (PME). This enzyme which demethylates pectins, generating pectic acid, and thus increasing the CEC may be affected by apoplastic polyamines (Charnay *et al.*, 1992; Berta *et al.*, 1997; Messiaen *et al.*, 1997) and thus by the N nutrition of the plants (Gerendás *et al.*, 1993). Manipulation of PME activity by means of molecular technology leads to changes in shoot growth rate as well as cation binding capacity (Pilling *et al.*, 2000). Relatively little is known on distribution and transport of PME in cell walls. However, the frequently observed accumulation of Ca^{2+} in the middle lamella of the junction zone (P. van Cutsem, pers. comm.) may be taken as an indication for a preferential transport of PME in this large intercellular spaces. Since the pH for H^+ of the cell wall is in the range of 4.3 (Baydoun & Brett, 1988) or lower (Richter & Dainty, 1989), a decreasing apoplastic pH may reduce the CEC (Allan & Jarrel, 1989). This is however, unlikely to occur under physiological conditions because apoplastic pH is highly regulated. Examples for this process in the leaf apoplast will be considered later (for the root apoplast see Felle, 1998).

The undiffusible anions have a strong influence on ion movement. For example, the existence of electrical bilayers may restrict movement of anions to the larger interfibrillar

spaces (Clarkson, 1991) while the velocity of cation movement (mainly Ca^{2+}) is reduced by interaction with the free carboxyl groups (Marschner, 1995).

III. The root apoplast – nutrient uptake and short-distance transport

Due to the negative charges in the root cell wall we observe an accumulation of cations and a repulsion of anions in the root apoplast (Clarkson, 1993). This is particularly clear for di- and polyvalent ions (Haynes, 1980). Although accumulation in the root apoplast is not an essential step in nutrient absorption, it does explain certain well-known phenomena such as differences in K : Ca ratio among plant species (Haynes, 1980). A very good example is also the preferential uptake of metals such as Zn and Cu in ionic over the chelated forms (Marschner, 1995). In the latter case however, one can not exclude that restriction of the relatively large chelate molecules by the cell wall pores is an important factor explaining the results.

A factor of little consideration is the property of water bound to gels such as pectins. Recent studies determining water relaxation in a model system however, suggest highly structured properties, quite different from those of free water (Esch *et al.*, 1999). Implications for the activity of enzymes and dyes frequently used in studies on ion relations in cell walls are far from being understood.

Binding of certain metal cations such as Cu (Thornton & Macklon, 1989), Mn (Bacic *et al.*, 1993), B (Matoh *et al.*, 1997), Zn (Zhang *et al.*, 1991b), or Fe (Zhang *et al.*, 1991a) to cell wall components may be quite specific. Cu, for example, may be bound in nonionic form to nitrogen containing groups of cell wall proteins (Harrison *et al.*, 1979; Van Cutsem & Gillet, 1982) while B is bound to diols and polyols, particularly *cis*-diols (Goldbach, 1997). In this context rhamnogalacturonan II is of special significance (O'Neill *et al.*, 1996; Kobayashi *et al.*, 1996). This binding leads to an accumulation of the relevant nutrient in the cell wall and it is tempting to speculate about the significance of this accumulation in the root apoplast for genotypic difference in mineral nutrient efficiency. A prominent example was given by Longnecker & Welch (1990) who argued that large amounts of apoplastic Fe in soybean roots contribute to Fe-efficiency. However, a critical evaluation showed that this Fe was basically adhering to the outer surface of the epidermis probably in particulate form (Fig. 1) and did not contribute significantly to the nutrition of the plant (Strasser *et al.*, 1999). This emphasizes the necessity to restrict the extension of the apoplast by the definition given in I Introduction.

An involvement of cell wall components of roots in the acquisition of sparingly soluble Fe phosphates in low fertility soils has recently been demonstrated for groundnut (Ae *et al.*, 1996). It has been suggested by the authors that this effect is due to a binding of Fe to root cell wall components and thus releasing phosphate. This view is supported by the fact that saturating the root before the test with Fe diminishes the phosphate mobilizing activity (PMA). This effect is reversible since removal of the Fe from the cell wall restores the PMA (Fig. 2). The precise mechanism is not yet understood (Ae & Otani, 1997). Nevertheless, this opens a new view on how plants may interact with the soil and influence nutrient availability within the vicinity of the apoplast.

Unstirred layers (USL) are defined as boundary layers of either liquids or gases in the vicinity of transport barriers. In these layers a complete mixing is not possible and, thus, concentration gradients are observed. USL are of principal importance for all transport processes across barriers such as the apoplast or the plasmalemma. As a consequence, it is not only the resistance of the barrier itself which determines transport rate, but also the diffusion across the USL (Zimmermann *et al.*, 1992). Transport resistance across USLs depends on the mobility of each solute as well as on the thickness of the USL. Since the thickness of USLs in the apoplast can be quite substantial (Thompson & Dietschy, 1980; Preston, 1982), it can be concluded that USLs are an important factor in transport processes in the apoplast.

As a consequence of the cell wall properties of roots, ionic relations in the vicinity of the plasmalemma can vary considerably from those in the rhizosphere (Franklin, 1969; Grignon & Sentenac, 1991). Such phenomena are of fundamental importance for the understanding of processes such as ionic antagonisms (Borst-Pauwels & Severens, 1984; Barts & Borst-Pauwels, 1985; Collier & O'Donnell, 1997) or apparent synergisms such as those between Ca^{2+} and $H_2PO_4^-$ (Franklin, 1969). However, ionic gradients can arise not only as a result of apoplastic properties or ion uptake (Kochian & Lucas, 1982; Henriksen *et al.*, 1990). Ionic fluxes into the apoplast may also be the result of efflux processes or of the activities of ionogenic pumps. For example, gradients may be formed in the vicinity of tissue with particularly high H^+ ATPase activity (Canny, 1993) or when Ca^{2+} is desorbed as a result of an increase in free H^+ concentration (Cleland *et al.*, 1990).

In the apoplast of roots the Casparian band represents the major diffusion barrier (Sanderson, 1983). Although it is generally considered to be completely impermeable to water and ions, recent results (Steudle *et al.*, 1993; Steudle, 1994) do suggest a certain degree of permeability. Depending on species and age the endodermis and exodermis contain cutin and suberin at different quantities (Schreiber, 1996; Zeier & Schreiber, 1998). Chemical composition of the Casperian band changes with ontogeny (Zeier & Schreiber, 1998) as well as with environment (Schreiber *et al.*, 1999). Adverse ionic relations, such as salt stress (Reinhardt & Rost, 1995), accelerate the formation of the endodermis which is understandable taking the significance of the Casparian band to prevent bypass flow into account. This significance is further emphasized by the observation that salt tolerant

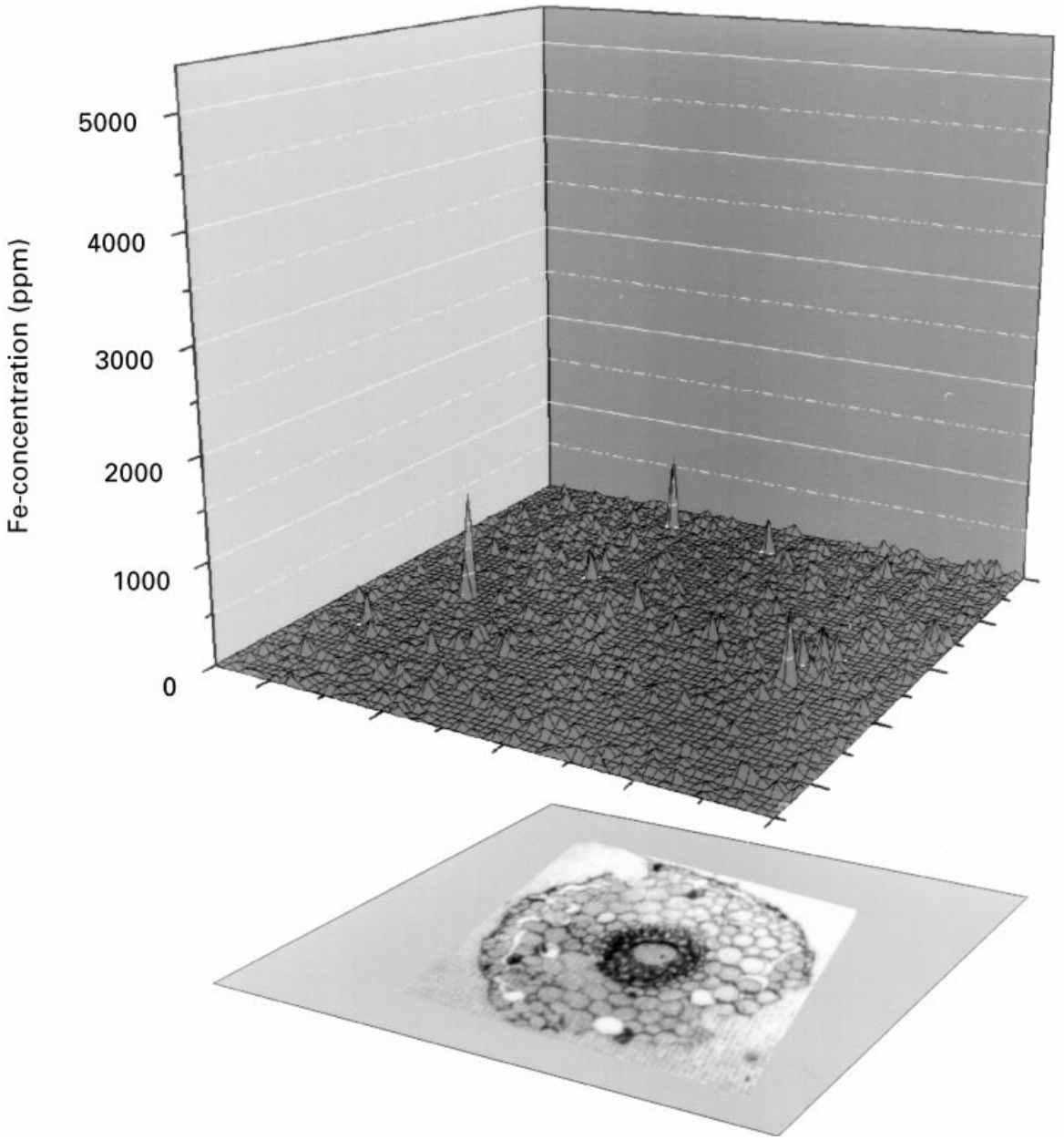

Fig. 1 Localization of Fe by Proton-Induced X-ray Emission in a cross section of root (7 μm thick) of a barley root grown in contact with soil. Soil was removed thoroughly by washing with water. Note the localization of Fe in the epidermis. *Courtesy of Strasser* et al., *1999*.

plant species in many cases reveal a thicker Casparian band than less tolerant ones (Plojakoff-Mayber, 1975). Schreiber *et al.* (1999) have described the chemical analysis of the cell walls of the endodermis.

In most plant species the hypodermis is converted into an exodermis – an outer Casparian band (Perumalla & Peterson, 1986; Peterson & Perumalla, 1990; Damus *et al.*, 1997) is formed which in many cases contain suberin deposition (Enstone & Peterson, 1997). The formation of the exodermis occurs later than that of the endodermis and depends largely on growing conditions (Barrowclough & Peterson, 1994) such as salinity stress (Reinhardt & Rost, 1995). The significance of the exodermis for water and ion uptake is discussed in the literature with some degree of controversy (Ferguson & Clarkson, 1976; Clarkson *et al.*, 1987; Peterson, 1988) and apparently depends largely on the ion under consideration (Enstone & Peterson, 1992). However, it was recently demonstrated that for nutrients such as K^+ it represents a

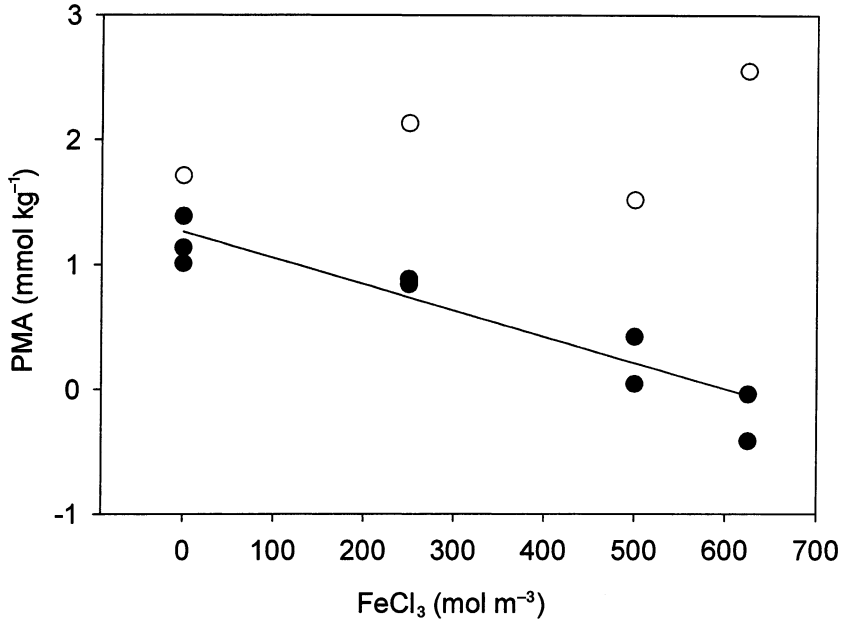

Fig. 2 Influence of $FeCl_3$ pre-treatment of groundnut cell wall on its phosphate mobilizing activity (PMA). Cell wall material, from which Fe^{3+} has been removed by washing with 0.5 M HCl, was incubated in a $FeCl_3$ solution for 30 min, carefully washed with deionized water and dried. Thereafter samples were divided: from one half Fe^{3+} was removed by washing with 0.5 M HCl before testing for PMA (open circles). The other half was used immediately for the PMA test (closed circles).

significant diffusion barrier (Gierth *et al.*, 1999) (Fig. 3). Prevention of dehydration in the case of more negative soil water potentials (Stasovsky & Peterson, 1991; Kamula *et al.*, 1994), control of solute exchange processes between symbiotic partners (Ashford *et al.*, 1989), as well as resistance against pathogen attack (Kamula *et al.*, 1995) are presently considered to be further functions. The exodermis does not form a continuous apoplastic barrier. Wounding, induced by lateral root development (Enstone & Peterson, 1997) together with the existence of passage cells (Peterson & Enstone, 1996), suggests that nutrients may diffuse into the AFS which occupies approx. 5% of the root volume (Shone & Flood, 1985) in spite of the existence of the exodermis. However, the significance of ions in the AFS for nutrient uptake must not be overestimated. The beneficial effect of an increase in absorbing surface area would be especially important for nutrients typically present in low concentration in the soil solution such as phosphorus or potassium. However, due to the uptake activity of epidermal cells including root hairs, concentration of the ions in the rhizosphere is often in the range of C_{min}, where influx is equal to efflux (Jungk *et al.*, 1982). Thus, the contribution of the AFS to nutrition of the plant with these nutrients is expected to be rather low.

The root apoplast is the plant compartment that first encounters adverse soil chemical conditions such as high Na^+ or high Al^{3+} concentrations. As shall be considered for Al^{3+} first, conditions in the root apoplast are determining for the response of the plant. As has been demonstrated for numerous plant species cessation of root growth is the first detectable symptom of for Al^{3+} toxicity (Horst & Goppel, 1986; Blamey *et al.*, 1993a). Pre-treatment of roots with silicon reduces the symptoms of aluminium toxicity (Corrales *et al.*, 1997). The interaction of Al^{3+} with cell wall components such as the pectin matrix (Clarkson, 1967; Blamey *et al.*, 1993b; Van *et al.*, 1994) could explain the phenomena of growth cessation. Pectins have great influence on cell wall properties such as hydraulic conductivity and, in connection with extensin, also on wall plasticity (Wilson & Fry, 1986). Recent findings demonstrating a correlation between pectin methylation and Al^{3+} tolerance support such a view (Schmohl & Horst, 1999). The immediate reduction of K^+ efflux (Horst *et al.*, 1992) as well as Ca^{2+} influx (Huang *et al.*, 1992; Rengel, 1992a,b) may be interpreted as being the result of an interaction of the trivalent cation with the plasmalemma (Horst, 1995; Kochian, 1995). There is evidence that Al^{3+} causes disturbance of cytoplasmic Ca^{2+} homeostasis, for example, in root hairs (Jones *et al.*, 1998). However, since physiological processes such as cytoplasmic streaming, which are extremely sensitive to any change in Ca^{2+} homeostasis (Plieth *et al.*, 1999), remain undisturbed by external Al^{3+} supply (Sattelmacher *et al.*, 1993), the suggested casuality (Rengel, 1992a,b; Lindberg & Strid, 1997) is not quite convincing (Kinraide *et al.*, 1994; Ryan *et al.*, 1994). Especially in the light of new findings suggesting that Al^{3+} rather prevents an increase of cytoplasmic Ca^{2+} brought about by high external H^+ (Plieth *et al.*, 1999).

The hypothesis that apoplastic processes are involved in Al^{3+} tolerance is emphasized by recent data suggesting that release of chelating substances such as organic acids into the apoplast is causally related with Al tolerance (Larsen *et al.*, 1998). This could also explain earlier findings demonstrating that form of N supply (NO_3^- vs NH_4^+) reveals a strong influence upon Al tolerance (Grauer & Horst, 1990). Recent

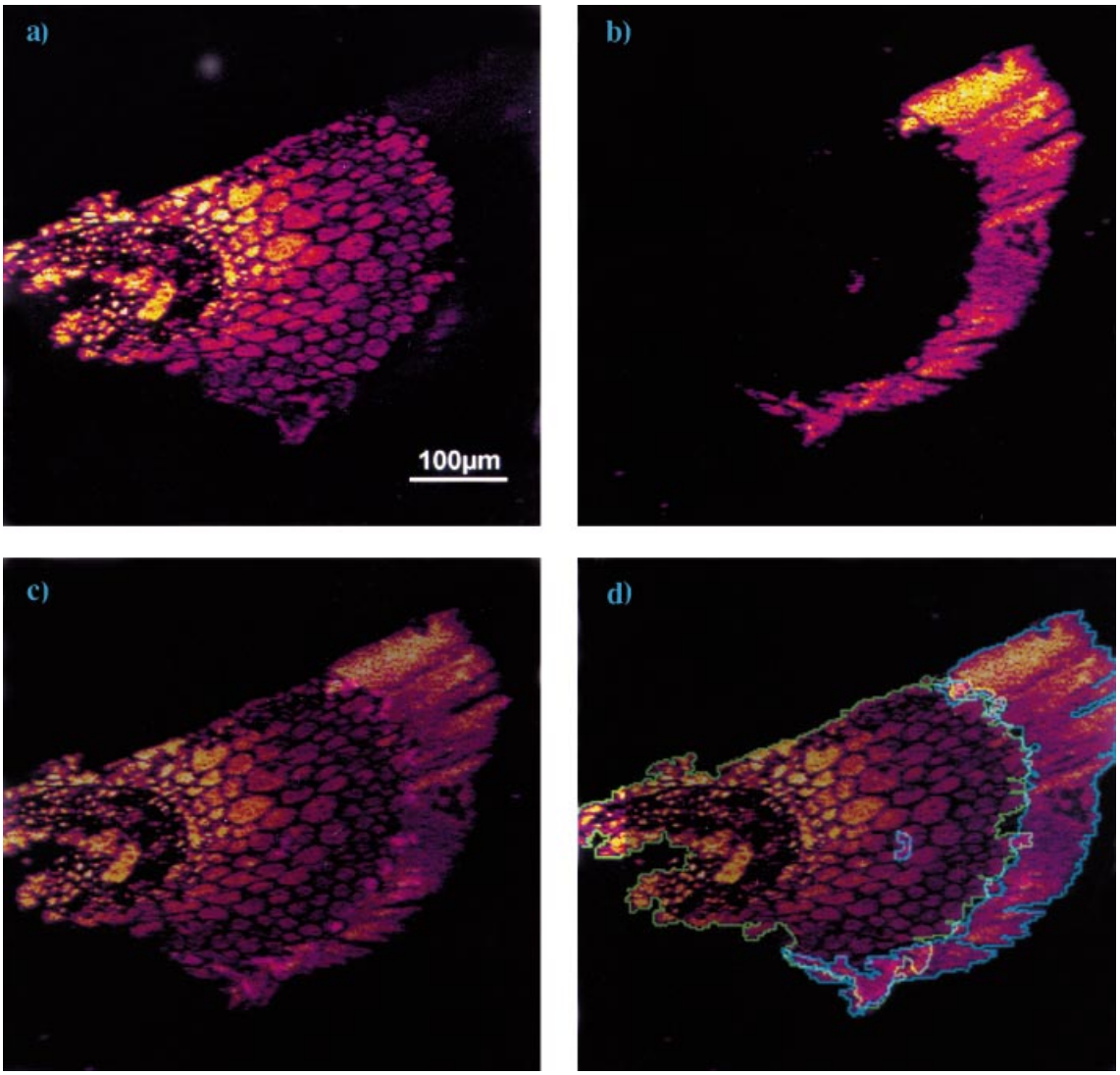

Fig. 3 Secondary ion mass spectroscopy (SIMS) images showing the distribution of $^{39}K^+$ and $^{85}Rb^+$ in a freeze-dried cryosection of a barley root. A droplet of a 60-mol m^{-3} RbCl solution was added to the basis of a nodal root of an intact transpiring plant 120 s prior to freezing the plant with liquid propane. (a) SIMS mapping of $^{39}K^+$ on a root cross section, imaging the cell contents of the cortex and the stele. Note that $^{39}K^+$ is absent from the surface adhering test solution, the cell walls and the xylem vessels in the stele. (b) The applied $^{85}Rb^+$ from the test solution exceptionally appears on the root surface but neither in the apoplast nor in the symlast of the root cortex and the stele, respectively. (c) Summarized SIMS images of both $^{39}K^+$ plus $^{85}Rb^+$ to show the total extent of the analysed cryosection. (d) Summarized SIMS images as in (c): The isotope distribution map of $^{39}K^+$ was framed by a green line and of $^{85}Rb^+$ by a light blue line. *Courtesy of R. Stelzer*.

findings demonstrate that Al^{3+} sensitivity is restricted to the distal part of the transition zone (Sivaguru & Horst, 1998; Mayandi *et al.*, 1999). Whether this is due to apoplastic pH gradients in the root tip region (Felle, 1998) or due to hampered IAA transport in cell walls (Kollmeier *et al.*, 1999) can presently not be judged on. The involvement of cell walls in the process of detoxification of Al species has been demonstrated for different plant species such as wheat (Maison & Bertsch, 1997).

Apoplastic processes are also involved in Na^+ toxicity (Wang *et al.*, 1997; Volkmar *et al.*, 1998). Although the mechanism is not fully understood an involvement of cell wall glycoproteins (Sun *et al.*, 1997) is presently debated. Additionally, in the case of Na^+ sensitive plants a considerable part of the Na^+ detected in the xylem has entered the stele apoplastically by the so-called bypass flow (active transport processes are not involved) (Steudle *et al.*, 1987; Yeo *et al.*, 1987; Frensch & Steudle, 1989). Interestingly, the activity of this process appears to be under genetic control (Garcia *et al.*, 1997). A displacement of Ca^{2+} from the boundary layer plasmalemma/apoplast is also involved in Na^+ toxicity (Lynch

& Läuchli, 1985; Rengel, 1992c; Yermiyahu *et al.*, 1997). This aspect will be further considered in the section entitled 'Ion relations in the leaf apoplast and sympterns of deficiency, and toxicity symptoms' (VI.5).

IV. The apoplast as a compartment for long-distance transport

Uptake from the soil solution into the root symplast and subsequent release into the xylem apoplast are two distinct processes (Pitman, 1972; Poirier *et al.*, 1991; Engels & Marschner, 1992). Restricted nutrient absorption by the root system may be due to either process (Engels & Marschner, 1992) – reduced activity of the uptake system into the symplast and reduced xylem loading. The precise implication for regulation of ion uptake rate is not yet fully understood but it is tempting to speculate that nutrient cycling (in this section) regulates ion uptake via the process of xylem loading (de Boer *et al.*, 1997; White, 1997) possibly by modulation of G-proteins (Wegner & de Boer, 1997b), while exogenous factors such as temperature or external ion concentration influence the influx into the root symplast.

Solute transport into the xylem of roots involves flux from the symplast into the apoplast (Läuchli, 1976). In earlier work it was thought that xylem loading was mediated by a passive leakage of solutes (Crafts & Broyer, 1938) but the involvement of metabolism in the process of xylem loading has later been demonstrated (DeBoer *et al.*, 1983). These physiological results were accompanied by cytological studies showing the existence of transfer cells in the paratracheal parenchyma (Kramer *et al.*, 1977). Contrary to findings suggesting an active transport mechanism for xylem loading (DeBoer *et al.*, 1983; Mizuno *et al.*, 1985) more recent data demonstrate a thermodynamic passive transport by ion channels (Wegner & Raschke, 1994). By now both inward (Wegner & de Boer, 1997a) and outward (Wegner *et al.*, 1997b) rectifying channels have been detected in this tissue contributing further to our understanding of xylem loading. The driving force is generated by H^+-ATPase which is expressed particularly in the paratracheal parenchyma cells (Jahn *et al.*, 1998).

Composition of the xylem sap is highly variable and depends on plant species, age (Prima & Botton, 1998), time of day (Schurr & Schulze, 1995; Urrestarazu *et al.*, 1995), location of sampling (Berger *et al.*, 1994), nutritional status, rooting medium (Förster & Jeschke, 1993), and last but not least on nutrient cycling within the plant (White, 1997). Mineral nutrient supply reveals a strong influence on xylem sap composition. As a rule, there exists a positive correlation between ion concentration in the external solution and in the xylem sap. Contrary to variation in rhizosphere pH the effect of nutrient uptake on xylem sap pH is not well studied and understood. While NH_4^+ nutrition always leads to an acidification of the rhizosphere due to a predominant uptake of cations (Marschner *et al.*, 1986) considerable discrepancies were found in the effect on xylem sap pH. In some studies an acidification (Allen & Raven, 1987) was observed, while others revealed no effect of N form at all (Zornoza & Carpena, 1992). These differences may be due to several factors including composition of amino acids, however further studies are required to elucidate this important aspect. In spite of the high puffering capacity of xylem vessel walls for H^+ (Mizuno & Katou, 1991), and the strong pH regulation which can be demonstrated impressively by perfusion experiments (Clarkson & Hanson, 1986), substantial variations in xylem sap pH has been observed (Urrestarazu *et al.*, 1995; Schurr & Schulze, 1996). These are due to changes in ion composition, and specifically selective ion transport into or out of the xylem. According to the strong ion difference (SID) concept which has recently been adapted to plants in general, and xylem sap in particular (Gerendás & Schurr, 1999) selective removal of K^+ decreases [SID] while selective removal of NO_3^- has the opposite effect. A decrease in [SID] leads to an increase in H^+ while an increase in [SID] decreases H^+ concentration. In this context it can be stated that in many cases pH in the xylem sap decreases in acropetal direction (Schill *et al.*, 1996). In general, an inverse relation between solute concentration and xylem flow rate is observed (Schurr & Schulze, 1995; Liang *et al.*, 1997). This is why data on the composition of xylem sap based on xylem pressure exudates has to be considered with some precautions.

Cation exchange capacity of xylem cell walls is rather high and has been estimated to be approx. 1000 mol m^{-3} for tomato (Wolterbeek, 1987). Interactions of cations with the nondiffusible anions lead to a separation of ion transport from water flow. The transport of cations may be compared with that in a cation exchange resin, while water is transported by mass flow (Wolterbeek, 1987; Marschner, 1995). The degree of retardation of ion translocation depends on the valence of the cation ($Ca^{2+} > K^+$), its own activity and surface charge, the activity of competing cations (Wolterbeek, 1987), the charge density of the nondiffusible anions, and the pH of the xylem sap (Wolterbeek, 1987). Consequently, the transport rate of di- or trivalent cations is enhanced significantly by complexation of the cation (Clark *et al.*, 1986). Cations may be complexed by organic acids (Senden *et al.*, 1994; Yang *et al.*, 1997), amino acids, or peptides (Mullins *et al.*, 1986; Senden *et al.*, 1994; Stephan *et al.*, 1996). It should be stressed that cation and anion transport are always linked to each other. Thus, if cation transport is enhanced by complexing molecules this also applies to anions.

It is often overseen that considerable amounts of organic compounds are transported in the xylem (Schneider *et al.*, 1994; Prima & Botton, 1998). Their significance is not, however, restricted to ion transport. High sugar concentrations in the winter (Schill *et al.*, 1996) or in the spring (Sauter, 1988; Ding & Xi, 1993) of perennial plant species or in maize

after silking (Canny & McCully, 1988; Engels *et al.*, 1994) may be taken as examples. It is by now well established that sugars in the xylem may contribute significantly to the osmotic pressure gradient (Pomper & Breen, 1995) and hence to long-distance transport. At least part of the organics have probably been passively leaked into the xylem. Numerous living late metaxylem vessels have been shown to exist even at a relatively large distance from the root tip (Wenzel *et al.*, 1989). At maturation the solute of the cytosol and the vacuole are released into the xylem. By this process up to 10% of shoot potassium demand may be released into the xylem by leakage (McCully *et al.*, 1987). The significance of this process for other nutrients such as Ca^{2+} is still obscure. But it should at least be mentioned that the mechanism by which high Ca^{2+} fluxes into the xylem at low cytoplasmic Ca^{2+} concentrations is still not understood.

Apoplastic phytohormones, mainly IAA, ABA and cytokinins are another important example for transport of organics in the xylem (Hartung *et al.*, 1992). It has been demonstrated that at least for IAA the apoplast is involved in synthesis (Tsurusaki *et al.*, 1997) and signal reception (Sakurai, 1998). While for ABA the significance of the apoplast is restricted to transport (Freundl *et al.*, 1998). There are several ways in which apoplastic phytohormones may affect ion absorption (Blatt & Thiel, 1993) and especially long distance transport in the xylem and, thus, the nutrition of plants: by mediating activity of ion channels (Bottger & Hilgendorf, 1988; Marten *et al.*, 1991; Blatt & Thiel, 1993; Wegner *et al.*, 1997a), by affecting CEC (Marschner & Ossenberg-Neuhaus, 1977), or by altering stomatal resistance (MacRobbie, 1995). Interestingly, the involvement of apoplastic anions such as malate or Cl^- (Hedrich & Marten, 1993) as well as cations, mainly Ca^{2+} (Atkinson *et al.*, 1990) in the regulation of ion channels in guard cells was demonstrated. Therefore, these ions may affect apoplastic transport processes in the xylem by regulating stomatal resistance.

Solutes transported in the xylem into the shoot do not necessarily reflect root uptake since a substantial part may have been redistributed via the phloem from the shoot to the root. This nutrient cycling is of particular importance for charge balance, especially in nitrate-fed plants (Gouia *et al.*, 1994; Marschner *et al.*, 1996, 1997), for compensation of short-term variations in root activity (Cooper & Clarkson, 1989) as well as for the osmotic potential required to maintain root pressure. Nutrient cycling is also of significance for regulation of nutrient uptake rate through the root system (Engels & Marschner, 1992; Herschbach & Rennenberg, 1994; Schneider *et al.*, 1994; Gebler *et al.*, 1999). Depending on the plant species and the nutritional situation, up to 60% of the nitrogen (Cooper & Clarkson, 1989), 30% of the sulphur (Larsson *et al.*, 1991), and 80% of the potassium (Jeschke & Pate, 1991b) found in the xylem may be allocated to the cycled fraction. Exogenous factors such as salt stress may reduce these figures significantly (Jeschke & Wolf, 1985; Jeschke *et al.*, 1992). There is good evidence that not only solutes but also the transport medium, water itself, may be cycled within the plant. Depending on the relative humidity of the ambient air up to 30% of the xylem water may have been re-translocated from the shoot to the root via the phloem (Tanner & Beevers, 1990).

Parenchyma cells, the so-called paratracheal parenchyma, surround the xylem elements. Due to absorption and/or release of solutes from or into the xylem, composition of the xylem sap may vary with increasing distance of transport (Sauter & van Cleve, 1992; Berger *et al.*, 1994). The absorption may be transient or permanent. While the former represents a storage process the latter is considered as detoxification (Marschner, 1995). Absorption and release may occur simultaneously. This is why, with certain plant species other than legumes, a decrease in NO_3-N and an increase in amino acid concentration in the xylem sap may be observed with increasing transport distance. This is due to the absorption of NO_3-N and a release of reduced nitrogen compounds (Pate *et al.*, 1990). In this context it should not be forgotten that transfer cells present in the paratracheal parenchyma may also mediate the exchange of solutes between xylem and phloem (Jeschke & Pate, 1991a). The significance of this process especially for nitrogenous compounds is often underestimated (van Bel, 1990). The combination of these processes – absorption from the xylem, release into the xylem and transfer into the phloem – may lead to strong concentration gradients in the xylem sap (higher in the base and lower in the apical region (Berger *et al.*, 1994)). This is often correlated with changes of the xylem sap pH (Schill *et al.*, 1996).

V. The apoplast – habitat for microorganisms

Although the presence of microorganisms inside healthy plant tissue has been known of since the beginning of this century, at least (Perotti, 1926), and despite numerous reports on indigenous endophytic bacteria in various plant tissues including tubers, shoots (Fig. 4), roots and fruits (for review see Hallmann *et al.*, 1997), it was mainly considered as the result of insufficient surface sterilization. It is only recently that it has been demonstrated that nonpathogenic bacterial endophytes may stimulate plant growth by increasing resistance to abiotic (Hallmann *et al.*, 1997) and biotic stress (Pleban *et al.*, 1995), as well as by contributing to the nutrition of its host (Hecht-Buchholz, 1998). With the availability of molecular methods to detect endophytes in plant sap (Reinhold-Hurek *et al.*, 1998) or visualize them even on a tissue basis (Katupitiya *et al.*, 1995) new powerful tools became available in endophyte research.

Endophytes may enter the plant via natural openings like stomates or lenticels (Hallmann *et al.*, 1997) or by wounds induced by natural processes such as dying of epidermal cells, lateral root formation (Shishido *et al.*, 1995) or root growth

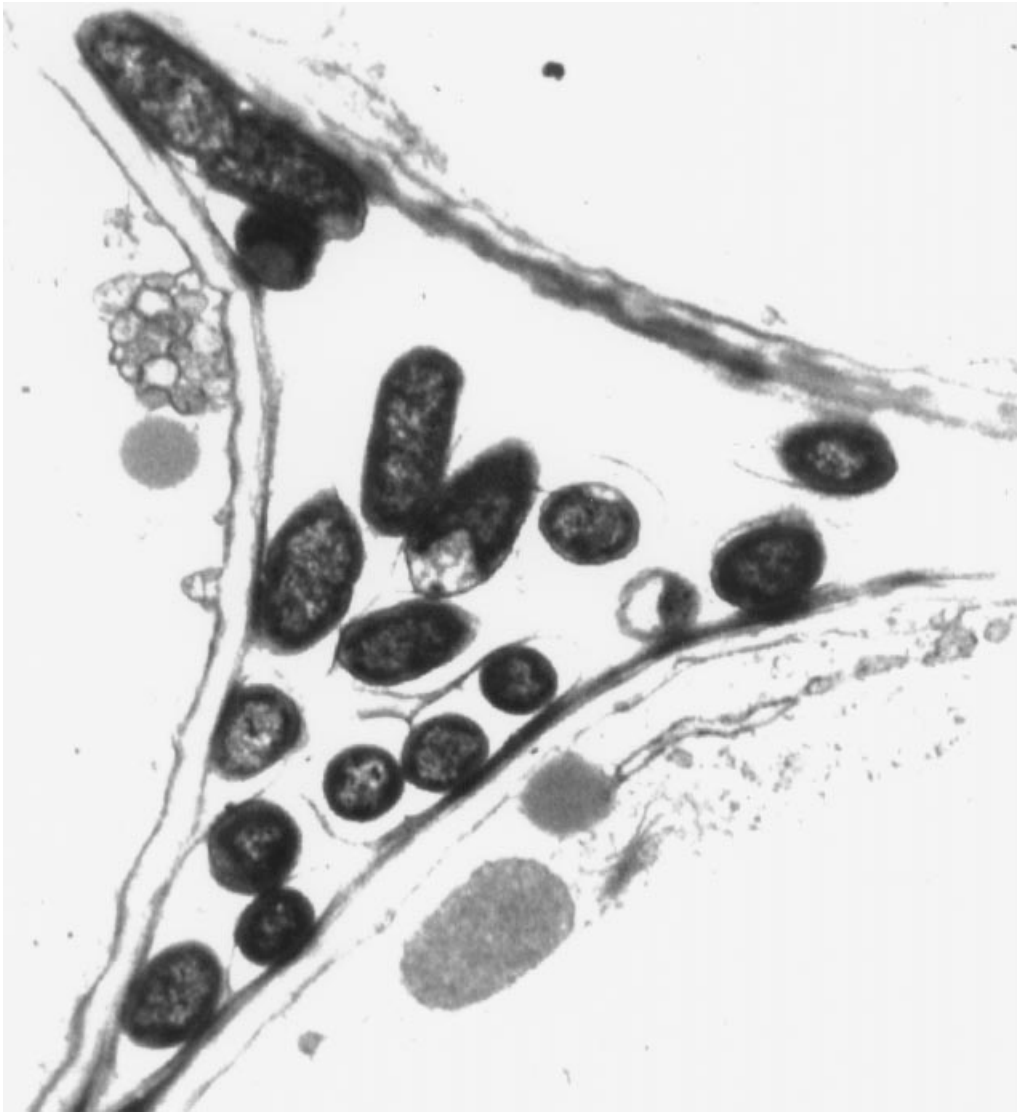

Fig. 4 Endophytic bacteria colonizing the intercellular space of a maize stem. *Courtesy of C. Hecht-Bucholz*.

through the soil. Lesions induced by pathogenic microorganisms or nematodes (Hallmann *et al.*, 1998) may ease endophytic colonization. However this process does not depend on natural or artificial wounds. Even if grown in a water culture, colonization before the formation of laterals have been reported (Quadt *et al.*, 1997) indicating an active penetration (James & Olivares, 1998). The assumption of such a mechanism is further supported by the presence of cellulytic and pectinolytic enzymes produced by numerous endophytes (Reinhold *et al.*, 1993). However the significance of this mechanism for field colonization is controversial (Hallmann *et al.*, 1997). The colonization of the stele, and especially the xylem vessels, is difficult to understand without the assumption of active penetration, if one does not assume that colonization does occur via the root apex where the vascular tissue is insufficiently differentiated (Hurek *et al.*, 1987). Once inside the plant the endophyte may be transported via the xylem (James & Olivares, 1998) or inside the intercellular space (Hurek *et al.*, 1994). Although transport velocity is much higher in the xylem enabling a systemic colonization of the plant, the presence of bacteria in xylem vessels, as impressingly demonstrated by James *et al.* (1994), is somewhat surprising because one would expect that they would cause xylem vessel cavitation.

Endophytes may support plant growth in many instances – as already mentioned by increasing resistance to biotic or abiotic stress factors – the biotic factors being by far better documented (Hallmann *et al.*, 1997), by changing root anatomy (Malinowski *et al.*, 1999) as well as by contributing directly to plant mineral nutrition. It is only this latter

aspect which shall be considered in the scope of this review. Diazotropic endophytes have been reported for numerous plant species (Hecht-Buchholz, 1998), sugar cane and rice (Boddey *et al.*, 1995) probably being the most prominent ones. While their presence is not in question, their ecological significance is under debate. For sugar cane a fixation rate of up to 150 kg N ha^{-1} yr^{-1} has been reported (Hecht-Buchholz, 1998). Figures for other crops are much lower but still significant (Boddey *et al.*, 1995; Hecht-Buchholz, 1998). The principal question to be answered is whether or not these data obtained under more or less artificial conditions are of relevance for agriculture. In order to contribute to this question the following considerations raised by Dong *et al.* (1994) for sugar cane may be helpful: assuming that the apoplastic fluid in sugar cane occupies 3% of the stems we would end up with 3 t ha^{-1} of apoplastic fluid from a harvested crop of 100 t ha^{-1} (Dong *et al.*, 1994). Since conditions for bacterial activity, such as pH (approx. 5.5), sugar (approx. 10%), mineral content, as well as temperature, are near the optimum, 3000 l of broth should be sufficient to explain a biological di-nitrogen fixation in the range given in this section. However, there far no experimental evidence for biological nitrogen fixation in a relevant amount by non-legumes. Published data for sugar cane reveal a conflicting picture: data obtained with the ^{15}N dilution technique lead to significantly lower figures than those obtained with the N-balance method (Urquiaga *et al.*, 1999). Unfortunately there is no convincing study applying the ^{15}N natural abundance method which should allow a reliable estimate. In any debate on the significance of diazotrophic endophytes it should, however, not be overlooked that biological di-nitrogen fixation is, carbon wise, an expensive approach. If we assume a carbon consumption of 10 g C per 1 g fixed N (Kappen *et al.*, 1998) we would require 1.5 T Carbon or approx. 3 T of sugars or approx. 10% of the sugar yield for the fixation of 150 kg N ha^{-1} yr^{-1}.

VI. The apoplast of leaves – a compartment of storage and of reactions

1. Transport routes in the leaf apoplast

Through the petioles, the xylem stream enters into the leaf where it is predominantly transported in the veins to sites of rapid evaporation, such as leaf margins or leaf teeth. If the veins are mechanically ruptured, as may occur under natural conditions (e.g. through insect attack) the ruptured site is rapidly bypassed possibly by an increased rate of transport in minor veins (W. Merbach pers. comm.).

The fate of the xylem sap in the leaf apoplast was subject of a debate between supporters of apoplastic and of symplastic routes for water transport (Canny, 1990c). According to current knowledge the predominant route depends mainly on the driving force: hydrostatic pressure gradients support transport through the apoplast, whereas osmotic gradients mainly favour symplastic routes (Westgate & Steudle, 1985). According to the Hagen-Poisseuille law the volume flow is affected by the tube diameter to the fourth power. One would, thus, expect transport to be restricted to the major veins. However, contrary to the number of vessels per vein, diameter of xylem vessels is rather independent of vein size (Canny, 1990a). This is not true for the smallest veins where large vessels are absent and accumulation of solutes was observed accordingly (Canny, 1990a). Since mass flow is difficult to imagine outside the xylem vessels, and flux by diffusion is just effective over very short distances (Canny, 1990b), intercostal fields are rather small and in most cases do not exceed seven cell layers. For the particular cell wall zones in which diffusion takes place, Canny has suggested the term 'nanopaths' (Canny, 1988).

Surprisingly little information is available on this path of apoplastic transport in the leaf tissue. Since a study by Strugger (1939), most authors assume there is transport in the intercellular spaces and/or in the water films covering the outer surfaces of cell walls. This concept may not, however, be realistic since the wettability of cell walls, at least in the leaf apoplast, is thought to be low (Ursprung, 1925; Ray *et al.*, 1958; Schönherr & Bukovac, 1972). The formation of such an 'inner cuticle' depends on both plant species and environmental conditions. If this holds true apoplastic transport in the leaves would be restricted to the 'cell wall apoplast' (Canny, 1995), the interfibrillar and intermicellar space. Since the water content of cell walls is rather high (Hardegree, 1989), being lower in older than in younger plant tissue (Goldberg *et al.*, 1989), this is not difficult to imagine, and it has indeed been demonstrated for roots (Bayliss *et al.*, 1996). We do not yet have any good information about whether such 'internal cuticle' covers the entire internal leaf surface or only certain areas, such as the substomatal cavity (Schönherr & Bukovac, 1972; Edington & Peterson, 1977). Its existence however, would explain how in the process of guttation, xylem sap can be excreted from the leaf by root pressure without flooding the entire leaf apoplast. Experiments with stable isotopes demonstrating equilibrium within rather short time periods suggests the 'internal cuticle' is not a major diffusion barrier. Its nature, if existing, is not understood, but is presumably the result of methylation rather than of cutin incrustation (Sitte, 1991).

2. Methods of studying apoplastic solutes

One of the major problems in any approach to study apoplastic ion relations is the method by which apoplastic solution is obtained. For leaves several indirect methods have been suggested, including elution procedures (Long & Widders, 1990), the vacuum perfusion of leaf discs (Bernstein, 1971), a pressure technique (Jachetta *et al.*, 1986), and different centrifugation techniques (Dannel *et al.*, 1995; Mühling

Table 1 Estimates of ($[Ca^{2+}]_{apo}$) obtained with different methods

$[Ca^{2+}]_{apo}$	Method	Reference
300–800 μM	Infiltration / centrifugation	Mühling & Sattelmacher (1995)
170 μM	Ca^{2+}-selective microelectrodes null point method	Cleland *et al.* (1990)
100 μM	(Stomata-aperture)	De Silva *et al*. (1998)
10–30 μM	Fluorochrome BTC	Mühling *et al.* (1997)
< 10 μM	Aequorin luminescence	C. Plieth & B. Sattelmacher (unpublished)

& Sattelmacher, 1997). All these methods have special advantages of their own. For example the infiltration centrifugation technique allows the use of solutions differing in exchange strength (Mühling & Sattelmacher, 1995) and thus to differentiate between free and adsorbed cations, contributing significantly to current knowledge on ionic relations in this plant compartment. However, in spite of the fact that ionic conditions in the apoplast are dynamically regulated, ionic conditions in the leaf apoplast are highly variable – both temporal and spatial concentration gradients exist. This is why conventional methods leading to an average concentration, which does not exist in most locations, are inadequate to describe a complex situation.

Due to the fact that inexpensive equipment is required, the infiltration / centrifugation methods are probably those most widely used in apoplast research. While concentrations of ions such as K^+ or Mg^{2+} and in most cases determined correctly there is evidence that those of Ca^{2+} may be overestimated (Table 1). Cytoplasmic contamination has been frequently considered as one factor affecting the apoplastic washing fluids. However, if the experiments are restricted to healthy non-stressed plants cytoplasmic contamination is unlikely to occur. Even at high centrifugation forces composition is relatively little affected (Lohaus *et al.*, 2000). One problem in any application of the infiltration / centrifugation technique is the precise determination of the air- and water-filled apoplastic spaces (Leidreiter *et al.*, 1995) in order to convert concentration in the washing fluid correctly into concentration in the apoplastic fluid (Lohaus *et al.*, 2000). It should not be overseen that with this technique and in most cases ion- or element concentration, and not ion activity, is determined. It is thought that this may be one factor explaining differences especially in Ca^{2+} obtained with different methods (Table 1).

A more direct approach to study ionic relations *in situ* is achieved by X-ray microanalysis (Pihakaski-Maunsbach & Harvey, 1992) or the application of ion-selective microelectrodes (Blatt, 1985). However, X-ray microanalysis requires complex preparation of the specimen, which is likely to disturb ionic relations, especially if mobile ions such as K^+ or H^+ are being considered. Recent progress in the preparation technique (Gierth *et al.*, 1998) however, leads to promising results. Ion-selective microelectrodes (Felle, 1993) give access only to the apoplast in the immediate vicinity of injured cells or in the substomatal cavity. The use of ion selective dyes offers the possibility of determining ionic activity at a high temporal and spatial resolution with a minimum of invasive disturbance (Bright *et al.*, 1989). Its application has significantly contributed to our knowledge on apoplastic processes – the high temporal and spatial variability of ion relations in this plant compartment became apparent (Hoffmann *et al.*, 1992; Hoffmann & Kosegarten, 1995; Mühling & Sattelmacher, 1995; Mühling & Sattelmacher, 1997; Mühling *et al.*, 1997).

3. Solute relations in the leaf apoplast

Our knowledge of solute concentration in the leave apoplast is rather restricted. Such knowledge is important for, amongst other things, the understanding of transport processes such as phloem loading, enzymatic reactions as well as cell expansion (Grignon & Sentenac, 1991; Dietz, 1997).

As first suggested by Cram (1999), plants may employ the apoplast to adjust cell turgor. This may be of special significance in the process of adaptation to salt stress (Clipson *et al.*, 1985) or increasing cell osmotic pressure. As an example, of the latter sugar beet roots may be taken which maintain turgor over the vegetation period in spite of a large increase in cell osmotic pressure (Tomos *et al.*, 1992). The precise mechanism remains uncertain but it has been suggested that K^+ may be involved in this process (Tomos & Leigh, 1999).

With the exception of the impressive data of Nielsen & Schjoerring (1998), demonstrating that NH_4^+ in the leaf apoplast is highly regulated (Kronzucker *et al.*, 1998) and that of Mimura *et al.* (1992) suggesting a similar mechanism for P_i, no evidence for an ion homeostasis in the apoplast exists although it has been suggested several times (Dietz, 1997). In the following paragraph available indications for such a mechanism will be considered. Apoplastic Ca^{2+} ($[Ca^{2+}]_{apo}$) has been chosen as an example. Any debate on homeostasis of $[Ca^{2+}]_{apo}$ requires precise information on the concentration range normally encountered in this plant compartment. Such information however, is lacking. Data taken from the literature vary from 1000 μM to 10 μM (Table 1). The factors responsible for this discrepancy are numerous including extraction and determination methods (Lohaus *et al.*, 2000). It is suggested that the noninvasive aequorin method gives the most reliable estimates. This

would indicate that $[Ca^{2+}]_{apo}$ may be much lower than commonly anticipated.

There are indications that similar to $[Ca^{2+}]_{cyt}$, $[Ca^{2+}]_{apo}$ is involved in the regulation and differentiation of plant growth and development and thus tightly regulated. For example, in leaves $[Ca^{2+}]_{apo} > 500\ \mu M$ induce stomatal closure (De Silva *et al.*, 1985, 1998). An involvement of apoplastic Ca^{2+} in controlling cell expansion (Cleland *et al.*, 1990; Arif & Newman, 1993) and regulation of gravitropic roots curvature (Bjorkman & Cleland, 1991; Cleland *et al.*, 1990; Suzuki *et al.*, 1994) has been described. Experiments of Roberts & Haigler (1990) suggest an involvement of apoplastic Ca^{2+} in cell differentiation such as tracheary-element development and there may be little doubt of the involvement of $[Ca^{2+}]_{apo}$ in fruit ripening (Burns & Pressey, 1987; Almeida & Huber, 1999) and pollen tube growth (Fan *et al.*, 1997; Ma & Sun, 1997).

The assumption of $[Ca^{2+}]_{apo}$ homeostasis is supported by the fact that under certain circumstances variation of $[Ca^{2+}]_{apo}$ results into a change of $[Ca^{2+}]_{cyt}$ (Felle, 1991; Gilroy *et al.*, 1991). This is understandable in the light of the existence of several Ca^{2+} conducting cation channels (Smolders *et al.*, 1997; Geitmann & Cresti, 1998; Li *et al.*, 1998). The existence of apoplastic calmodulin is difficult to explain without the assumption of a homeostasis of $[Ca^{2+}]_{apo}$ if one does not assume passive processes as the responsible factor. This however, appears unreasonable because calmodulin-specific binding proteins have been detected in the cell wall (Song *et al.*, 1997; Sun *et al.*, 1998; Ma *et al.*, 1999) and exogenous application of calmodulin induces such diverse effects as stimulation of cell division (Sun *et al.*, 1994), increase of both cell wall regeneration (Sun *et al.*, 1995) and pollen tube growth (Ma & Sun, 1997). The latter system appears to be specially suited to study the significance of extracellular calmodulin. Recent data suggest the involvement of G proteins in the transduction of the calmodulin signal (Ma *et al.*, 1999).

The observation that, especially in calcicole plants, $[Ca^{2+}]_{apo}$ in the leaf may differ quite drastically from those in the xylem (De Silva *et al.*, 1996; De Silva & Mansfield, 1999) as well as the fact that $[Ca^{2+}]_{apo}$ does respond to environmental stimuli such as temperature and mechanical stimulation (C. Plieth & B. Sattelmacher, unpublished) may be taken as strong indications for a homeostasis of $[Ca^{2+}]_{apo}$. Possible ways for its regulation will be considered below.

4. Concentration gradients in the leaf apoplast

As mentioned in VI 3. Solute relations in the apoplast, ion relations in leaf apoplast are highly variable. Spatial gradients may be the result of several factors among others differences in rate of uptake, delivery by mass flow or efflux from the symplast, and have been considered in greater details by Canny (1990b). For the former, an example is provided by the H^+ concentration gradients in the vicinity of leaf teeth (Canny, 1987; Wilson *et al.*, 1991), and for the latter the accumulation of K^+ in the vicinity of stomata may be taken (Grignon & Sentenac, 1991; Mühling & Sattelmacher, 1997).

Emphasis should be placed on the fact that at least for C_4 plant species one common apoplast does not exist in leaves (Keunecke & Hansen, 1999). Bundle sheath cells are connected to mesophyll cells via numerous plasmodesmata (Evert *et al.*, 1977; Botha, 1992), but their apoplastic compartments are separated by a suberin lamellae (Evert *et al.*, 1977; Hattersley & Browing, 1981; Botha *et al.*, 1982; Evert *et al.*, 1985; Canny, 1995). Thus, ionic conditions in the two apoplastic compartments may differ significantly. Although up to now no direct evidence exists this may be concluded from the great difference in pH optima of K^+ channels in the two compartments (Keunecke & Hansen, 1999). Since a similar situation has been described for wheat (Dietz, 1997) it may be anticipated that a separation of the apoplast of leaves into smaller compartments by diffusion barriers is a more common phenomena in the plant kingdom.

Temporal variations in apoplastic ion relations are the results of changes of metabolic activity, caused, for example, by processes involved in day/night transition. A dark/light transition leads to a bi-phasic apoplastic pH response (Mühling & Sattelmacher, 1995): alkalization observed immediately after onset of the light treatment is considered to be a reflection of the onset of photosynthetic electron transport leading to an alkalization of the stroma which is compensated for by H^+ uptake from the cytosol, and the apoplast, respectively. It can be suggested that this is the first indication of the involvement of physical pH state in pH maintenance in leave tissue and demonstrate the significance of the leaf apoplast as a transient ion reservoir. Temporal variation in apoplastic ion relations may also be the result of changing environmental conditions. Exposure of leaves to NH_3 (Hanstein & Felle, 1999; Hanstein *et al.*, 1999) or to stress may be taken as examples (Behl & Hartung, 1986; Daeter & Hartung, 1995).

5. Ion relations in the leaf apoplast and symptoms of deficiency and toxicity

Ionic conditions in the leaf apoplast are of significance for the occurrence of deficiency as well as for the toxicity symptoms. Following are just a few examples for each situation. (1) Fe-deficiency: it has been reported that under certain conditions, leaves revealing Fe deficiency symptoms may have higher total Fe contents than control leaves (Mengel *et al.*, 1984). This has been interpreted as the result of Fe immobilization in the leaf apoplast (Mengel & Geurtzen, 1988) due to high apoplastic pH (Hodson & Sangster, 1988; Smolders *et al.*, 1997; Kosegarten *et al.*, 1999). In spite of the fact that, so far, evidence for high apoplastic pH in relevant degree are missing (Mühling & Sattelmacher, 1995), a point has been made that Fe content in leaves should only be compared if leaf size is comparable (Hanstein *et al.*, 1999), which may

not have been the case in the above mentioned study. This point is of significance because at heavy Fe deficiency leaf expansion may be hampered which leads to a concentration effect. However, independent data demonstrate that cell wall Fe may not be completely remobilized (Zhang *et al.*, 1996) thus limiting Fe use efficiency (i.e. dry matter production per mol Fe acquired). (2) Ca-deficiency: in most cases Ca deficiency can not be related to leaf elemental content (Murtadha *et al.*, 1988). In many cases tissues revealing Ca-deficiency symptoms had higher Ca contents than control tissue (Foroughi & Kloke, 1974; Steenkamp *et al.*, 1983). Higher Ca influx into the necrotic tissue as well as concentration effects due to losses of dry matter have been discussed as possible explanations (Wissemeier, 1996). The significance of apoplastic Ca^{2+} for the characterization of the nutritional status of plant tissue could be first demonstrated by Behling *et al.* (Wissemeier & Horst, 1991). Wissemeyer (pers. comm.) could demonstrate that Ca^{2+} activity but not content in the leaf apoplast of potato correlated with Ca^{2+} supply as well as with genotypic differences in Ca efficiency. Different contents in chelating substances such as organic acids in the apoplastic fluid have been discussed as being responsible for the difference between content and activity. (3) Mn-toxicity: large differences in respect to critical Mn content between and within plant species have been reported (Wissemeier & Horst, 1991) which cannot be explained by differences in exclusion ability but rather by differences in tissue tolerance (Horst, 1983). Mn-tolerant genotypes may reveal higher Mn contents without any toxicity symptoms than Mn-sensitive ones (Burke *et al.*, 1990). It could be demonstrated that Si plays a key role in the process of Mn tissue tolerance (Jucker *et al.*, 1999). This is apparently correlated with apoplastic processes – application of Si reduces the amount of free Mn^{2+} in the apoplastic fluid (Maier, 1997). But according to this author the key component for Mn tolerance is apoplastic organic acids which reduce Mn^{2+} activity. (4) Salt toxicity: it was first suggested by Oertli (1968) that accumulation of salt in the leave apoplast may be one factor for the syndrome of salt toxicity. So far only a few studies have dealt with the relation between leaf apoplastic ion concentrations and salt tolerance suggesting an inverse relationship between the two factors (Speer & Kaiser, 1991) and thus supporting the so called 'Oertli hypothesis' (Kinraide, 1999). However, it has been suggested that the increase of apoplastic solute content may be due to damage of membrane integrity rather that a primary response to salinity (Niu *et al.*, 1995). Recent results suggest that not only the osmotic relationhsip but also tolerance of apoplastic enzymes are of significance (Thiyagarajah *et al.*, 1996). As considered in VI 9. Apoplastic ion balance in greater detail, ion accumulation in the leaf apoplast does occur only if xylem import exceeds phloem export. The significance of phloem export in the process of salt tolerance has, thus, been recently stressed (Lohaus *et al.*, 2001).

6. Ion relations in the leaf apoplast – influence of nutrient supply

The impact of nutrient supply to the rooting medium on the ionic relations in the leaf apoplast depends strongly on the nutrient under consideration. While apoplastic K^+ concentration is a reflection of K^+ supply (Mühling & Sattelmacher, 1997), apoplastic Ca^{2+} remains relatively stable. The rapid decrease in apoplastic K^+ that occurs well in advance of a decrease in total tissue K^+ demonstrates the sensitivity of this parameter. Since K^+ is the most abundant cation by far (Mühling & Sattelmacher, 1995) it may be anticipated that a decrease in K^+ has far reaching consequences for the composition of the apoplastic solution. The influence of the form of N supply (NO_3^- vs NH_4^+) on apoplastic pH in leaves has been debated controversially. It has been argued that NO_3^- nutrition leads to an alkalization while NH_4^+ induces an acidification (Hoffmann *et al.*, 1992; Mengel *et al.*, 1994). Our own work suggest that NO_3^+ nutrition may lead to an alkalization depending on NO_3^+ concentration in the xylem sap, which is dependent, among other factors, on the NO_3^+ concentration in the nutrient solution and the NO_3^+ reductase activity in the root. However, NH_4^+ nutrition should normally reveal little influence on apoplastic pH in leaves, since at least at low N concentration in the rooting medium, NH_4^+ concentration in the leaf apoplast is unaffected by the form in which N is supplied. This has been questioned by Finnemann & Schjoerring (1999) who reports relatively high NH_4^+ concentrations in the xylem sap and in the leaf apoplast, the latter is probably due to photorespiration and is highly regulated (Kronzucker *et al.*, 1998; Nielsen & Schjoerring, 1998). While the form of N supply to the rooting medium has relatively little effect on apoplastic pH, this is not true for foliar application of NH_4^+ which leads to an immediate acidification (Peuke *et al.*, 1998), while fumigation with NH_3 decreases H^+ activity (Hanstein & Felle, 1999; Hanstein *et al.*, 1999).

7. The leaf apoplast – compartment for transient ion storage

The function of the leaf apoplast as a reservoir for ions such as K^+ has been demonstrated in the vicinity of guard cells or motor cells (Bowling, 1987; Freudling *et al.*, 1988). The apoplast has several advantages over the vacuole in respect to storage of cations and also anions (Grignon & Sentenac, 1991; Mühling & Sattelmacher, 1995). Therefore, a more general role of the apoplast as a short-term reservoir for ions may be anticipated. The advantages are mainly the high CEC of the cell walls (VI 3. Secondary ion balance) and the ease with which ions can be taken up from the apoplast. As has been discussed in greater detail by Grignon & Sentenac (1991) ions are taken up easily, because the nondiffusible anions can be neutralized by H^+. An H^+/cation exchange thus leads to a reduction of the negative charge and increases the

electrochemical gradient. Because H^+ is osmotically inactive, the osmotic gradient increases simultaneously. Due to the very small size of the leaf apoplast even small amounts of ions can cause significant changes in osmotic potential (Blatt, 1985). In this context it is tempting to speculate that the ionic conditions in the leaf apoplast and the above mentioned mechanisms may be one reason for the differences between plant and animal cells in respect to the ion species pumped for the generation of the electrochemical gradient: while animal cells mainly pump Na^+, plant cells utilize H^+.

8. Ion fluxes between apoplast and symplast

Little information is available on nutrient fluxes from and into the apoplast of leaves. This is partly due to the fact that basic information such as ionic concentrations in this plant compartment cannot be readily obtained from the literature. As already considered for Ca^{2+} (Table 1) available information on the concentration of other ions such as K^+ varies widely – from 50 μM (Blatt, 1985) to 100 mM (Teng & Widders, 1988; Long & Widders, 1990). It has been suggested that next to circadian variations (Teng & Widders, 1988; Mühling & Sattelmacher, 1995), and partially questionable methodological approaches, concentration gradients within the leaf apoplast (Canny, 1990b; Wilson *et al.*, 1991) are responsible for this strong variation.

So far, ion channels and co-transporters have been identified. The former mainly in guard cells (K^+ and Cl^- channels) showing that large fluxes are induced in response to a closing or opening signal (Schroeder *et al.*, 1984; Hosoi *et al.*, 1988; MacRobbie, 1988; Hedrich & Dietrich, 1996; Roelfsema & Prins, 1997; Felle *et al.*, 2000). Apoplastic Ca^{2+} is thought to play a key role in this process (Schulz & Hedrich, 1995). The latter in mesophyll cells for peptides (Jamai *et al.*, 1994), and anions such as P_i (Mimura *et al.*, 1992).

Regulation of stomatal conductance is a good example of the significance of the apoplast as a compartment of signal transduction. Since guard cells and neighbouring cells are not connected symplasmatically, all solutes involved are transported in the apoplast. Apoplastic signalling is a fascinating subject which is unfortunately beyond the limits of this review (Carpita *et al.*, 1996; Dietz, 1997).

As discussed in VI 1. Transport routes in the leaf apoplast, it has been suggested that ions are absorbed into the symplast mainly in the vicinity of the minor veins. In this context, ion conductance in the paratracheal parenchyma is of special interest. Information available demonstrates the presence of ion channels for the xylem contact cells (Keunecke *et al.*, 1997). At present, two classes of channels, differing in pH optima, have been identified (Keunecke & Hansen, 1999). It is suggested that they are separated by a cutin lamella. While acidification stimulates K^+ conductance in the bundle sheath a decrease is found in the mesophyll cells (Keunecke & Hansen, 1999). The characteristics of these channels and the influence of ionic concentration show that they are in the range of the uptake system II that is characterized by high uptake rate, but low selectivity. These characteristics are advantagous for the conditions in the leaf apoplast: (1) ions in this plant compartment have, in most cases, been absorbed selectively from the rooting medium and translocated into the xylem and have thus passed at least two membranes; (2) taking the dimension of the leaf apoplast into account, selectivity of ion uptake from the leaf apoplast would lead to a rapid accumulation of those ions acquired with less preference; (3) a high uptake rate is required to prevent their accumulation if the supply of ions into the leaf apoplast by the transpiration stream is high.

9. Apoplastic ion balance

If ion transport into the leaf apoplast exceeds uptake into the symplast, any ions that cannot be retranslocated via the phloem may accumulate in the leaf apoplast (Flowers & Yeo, 1986; Flowers *et al.*, 1991; Speer & Kaiser, 1991). Attempts to calculate ion balances of the leaf apoplast at different nutritional situations for *Ricinus communis* (Komor *et al.*, 1989; Schobert & Komor, 1992; Zhong *et al.*, 1998) as well as for *Zea mays* (Lohaus *et al.*, 2000) stress the significant role of the phloem export. An accumulation of ions in the leaf apoplast has been reported for salt stress (Flowers & Yeo, 1986; Flowers *et al.*, 1991) as well as for Mn toxicity (Wissemeier & Horst, 1990).

The following mechanisms may contribute to the avoidance of toxic ion concentration in the leaf apoplast under such conditions, the relative significance varying with plant species as well as with the solute under consideration: removal from the equilibrium by precipitation as calcium oxalate either in the apoplast (Fink, 1992) or in the vacuoles of idioblasts (Ruiz & Mansfield, 1994); guttation (Zornoza & Carpena, 1992); leaching from the leaf apoplast (Arens, 1934); incorporation into the epidermis (Sangster & Hodson, 1986) and the trichomes (De Silva *et al.*, 1996; Zhao *et al.*, 2000); vs abscission of the entire leaf. While the relevance of most of these parameters for stress avoidance is well documented, the role of leaching from the leaf for apoplastic solute balance is still debatable (Pennewiss *et al.*, 1997). Leaching from the leaf apoplast has attracted interest mainly in relation to forest decline (Mengel *et al.*, 1987; Pfirrmann *et al.*, 1990; Turner & Tingey, 1990) or nutrient cycling in nutrient-limited ecosystems (Tukey *et al.*, 1964, 1988). However, in older literature the so-called 'kutikuläre Exkretion' (Arens, 1934) was considered to be an important mechanism for avoiding high salt concentrations in the leaf. The influence of misting in stimulating growth as observed by Pennewiss *et al.* (1997) is in general agreement with more recent findings with *Picea* (Leisen *et al.*, 1990). Experiments with maize revealed a beneficial effect of leaching only at rather high concentrations of NaCl (Pennewiss *et al.*, 1997). In our own experiments on the effects of misting on growth, large differences

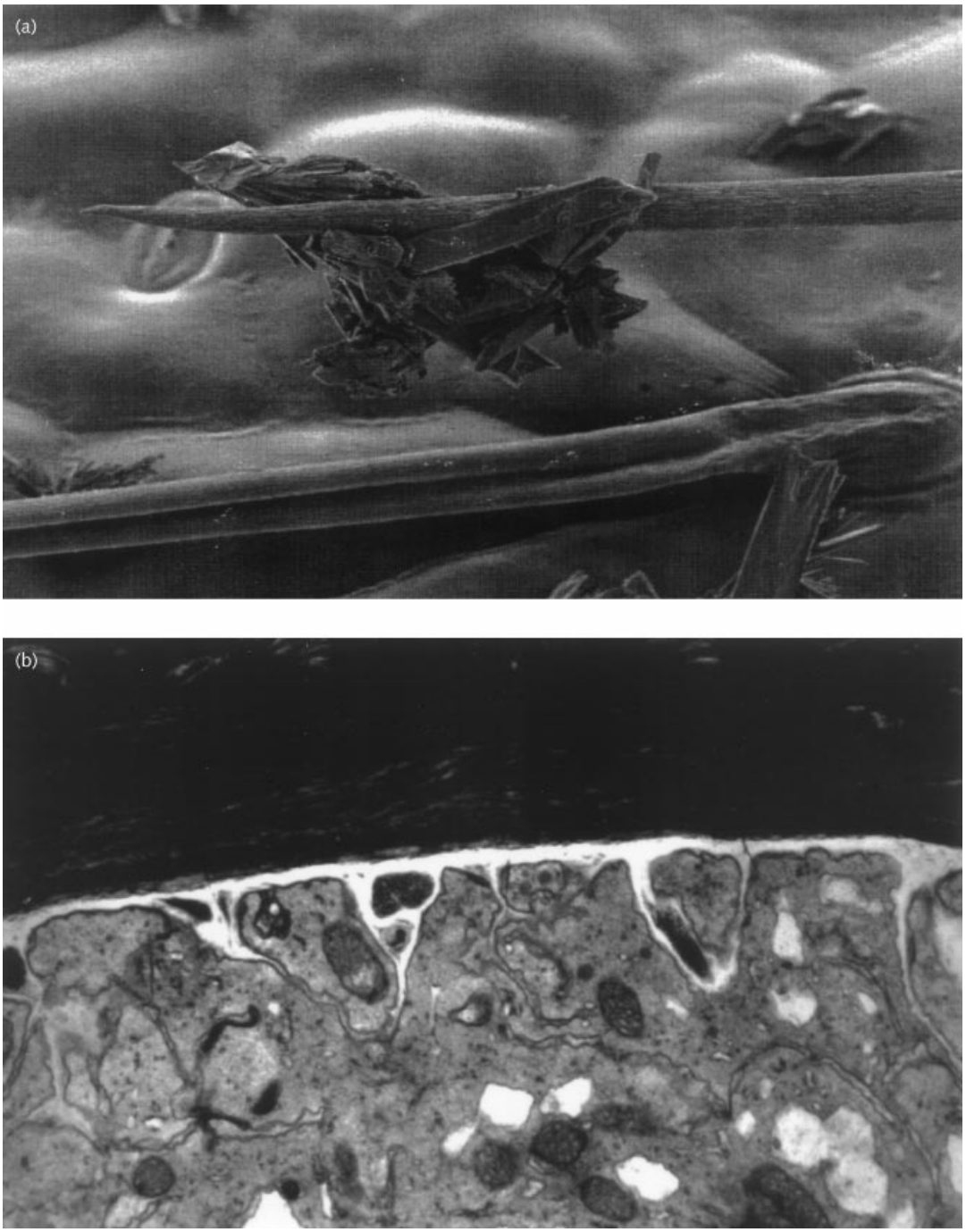

Fig. 5 Calcium crystals adhering to trichomes of *Lupinus luteus* after cultivation in a nutrient solution containing 15 mM Ca^{2+} (a) and transfer-cell-like structures of the base cell of the trichomes shown above (b). *Courtesy of W. H. Schröder (a) and E. Landsberg (b).*

between plant species were observed: while *Lupinus luteus* responded with a dramatic increase in growth, in plants such as *Gossypium hirsutum* growth was impeded (Pennewiss *et al.*, 1997). Although the leaf water status was improved by misting this cannot explain the observed growth phenomena (Stirzaker *et al.*, 1997). From the data obtained it is not possible to explain the precise path by which ions are leached from the leaf apoplast, but one may speculate that stomates or hydathodes and trichomes are significant. Calcium-containing crystals adhering to the trichomes of *L. luteus* at high Ca^{2+} concentration in the nutrient solution (Sattelmacher & Mühling, 1997) and the discovery of transfer cell-like structures in the base cell of *L. luteus* trichomes may be taken as support for such an assumption (Fig. 5).

10. Leaf apoplast – interaction with the atmosphere

The leaf apoplast connects the plant with the atmosphere. Together with the atmospheric conditions the properties of the cuticle, stomates, and conditions in the leaf apoplast, determine the exchange processes in either direction. Even though it is not of direct significance in this context, it may be of general interest that ozone is detoxified in the leaf apoplast by cell wall-bound peroxidases (Langebartels *et al.*, 1991; Luwe *et al.*, 1993). The interaction of atmospheric pollutants with the leaf apoplast are considered here only in relation to the mineral nutrition (for N and S).

As demonstrated for NH_3, plants may represent both a sink and a source for ammonia (Husted & Schjoerring, 1996). Exchange properties depend largely on physiological conditions in the leaf apoplast (Mattsson *et al.*, 1998). In this context the pH of the apoplastic solution is of special interest (Husted & Schjoerring, 1995). Nitrogen nutrition apparently has less influence than stomatal conductance (Husted & Schjoerring, 1996) and the NH_3 concentration in the atmosphere (Hanstein *et al.*, 1999). It is not surprising that NH_3 volatilization may be especially high at plant maturity (Husted & Schjoerring, 1996).

The pollutant N_2O originating from anthropogenic sources may be absorbed in relatively large amounts through stomates (Wellburn, 1990). After detoxification, in which ascorbate may be involved (Ramge *et al.*, 1993), it can be used as an N source, and beneficial effects on plant growth have been reported (Hufton *et al.*, 1996). The significance of endophytic chemolithoautotrophic nitrite oxidizers for the N_2O emission of plants has recently been suggested for *Picea* in a natural ecosystem (H. Papen, pers. comm.). This result is interesting since data on plants as N_2O sources is rather obscure.

SO_2 is oxidized autocatalytically or by peroxidation into SO_4^{2+} in this plant compartment (Pfanz *et al.*, 1992) and consequently is absorbed in this form by the cells (Polle *et al.*, 1994).

VII. Conclusions

Apoplastic properties greatly influence all aspects of plant mineral nutrition: chemical composition of root cell walls may be involved in both efficient use of nutrients, such as Zn or Cu, and tolerance against toxic ions, such as Na or Al. Ion, relations in the apoplast may differ quite significantly from those in the rhizosphere, which may explain uptake phenomena such as apparent synergisms. Although an exodermis is formed in most plant species, this does not apparently represent an absolute diffusion barrier for nutrients. The significance of the apparent free space for nutrient absorption however, is questionable.

The chemical composition of the xylem is highly variable and the mechanisms involved in ion exchange require further elucidation. Due to the high cation exchange capacity of the cell walls polyvalent cations are mostly transported in chelated form. Organics in the xylem sap may contribute significantly to the osmotic potential in some plant species. Beacause of cycling of both nutrients and water within the plant, the composition of the xylem sap does not necessarily represent root activity.

The apoplast of all plant parts may be colonized by endophytes that might contribute to the mineral nutrition, as demonstrated for diazotrophic organisms.

Ion relations in the leaf apoplast are very variable; however, they may be regulated, as discussed in greater detail for Ca^{2+}. Nutrient supply to the rooting media influences composition of the apoplastic fluid. The leaf apoplast may reveal certain benefits over the vacuole as a site for short-term nutrient storage, in addition to its importance as a site for solute exchange with the atmosphere.

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