DOI:10.1007/s10535-012-0121-3 BIOLOGIA PLANTARUM 56 (4): 749-756, 2012

Visualisation of xylem sap flow direction in isolated fine lateral roots and estimation of the xylem sap osmotic potential

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Abstract

Xylem sap outflow from fine lateral roots (FLRs) isolated from hydroponically grown young maize (*Zea mays* L.) plants was visualized by local brightening of test solutions contrasted with purified Indian ink particles. Flow into the vessels was indicated by the adsorption of Evans Blue in their walls. The fraction of the FLRs able to exude xylem sap in a mineral medium with 30 mM mannitol decreased with increasing incubation time. This change was strongly retarded, when the FLRs were incubated in a medium containing glucose instead of mannitol. There was a broad range of variation of the osmotic potential of the test solutions (Ψ_{so}), wherein the fraction of the FLRs showing an initially reversed flow of the xylem sap varied between zero and unity. A median (*M*) of the osmotic potential of the xylem sap in FLRs (Ψ_{sx}) was estimated. It represents the value of Ψ_{so} that was lower than Ψ_{sx} in half of the roots of a sample before their transfer to the test solutions (Ψ_{sxo}). *M* was dependent on the osmotic potential of the medium used for growth or pre-incubation of the FLRs. Its value was not dependent on the molecular size of the osmolytes used to adjust Ψ_{so}, including dextran 8, which is excluded from cell walls. In all of the studied plants, *M* was lower than the osmotic potential of the xylem sap collected from the root before isolation of the FLRs. To explain this finding it is assumed that FLRs with $\Psi_{sxo} > M$ had a higher hydraulic conductivity and a larger volume contributed to the exuded sap than those with $\Psi_{sx} < M$.

Additional key words: hydraulic conductivity, maize, radial water transport, root reflection coefficient, xylem loading, *Zea mays*.

Introduction

When isolated roots or root segments are incubated in a mineral medium the radial volume flux (J_v) is driven by a radial difference between the osmotic potential of the root medium (Ψ_{so}) and the osmotic potential of the xylem sap (Ψ_{sx}). This difference is sustained by a net solute flux (J_s) into the xylem vessels that depends on metabolism (Sabinin 1925, Arisz *et al*. 1951, Van Andel 1953, House and Findlay 1966a, Anderson *et al*. 1970). In the steady state the concentration of the solutes leaving the xylem vessels reflects the ratio between J_s and J_v. This ratio is not necessarily equal in all laterals and can vary along the axial path from the apical zone of a lateral root to the base of the main root. Hence, the osmotic potential of the xylem sap finally released from the root can differ from the local values in the zones of maximum water uptake (Anderson and Collins 1969, Arisz *et al*. 1951, Van Andel 1953, Kramer and Boyer 1995). Under certain conditions, the removal of ions from the xylem sap by their uptake into the basal or maternal root symplast can increase the osmotic potential of the finally exuded sap to a value even greater than Ψ_{so} (Eaton 1943, Klepper 1967).

In the branched root system of young maize plants the fine laterals are the major site of water uptake. Water uptake by the aerenchymatous main root axis (Michael *et al.* 1999, Lenochová *et al*. 2009) is negligible (Fritz *et al.* 2010). Although in an isolated FLR the local values of Ψ_{sx} may vary along its length, for each FLR there should be an isotonic medium with a defined value of Ψ_{so} that just compensates for the hydraulic effect of Ψ_{sx}. As the Ψ_{sx} of an individual FLR cannot yet be directly determined, we attempted to assess this value by determining a hydraulically balancing (isotonic) value of Ψ_{so}. After transferring a FLR into the isotonic medium, J_v will be zero for only a short time, whereupon Ψ_{sx}

Received 11 March 2011, *accepted* 28 July 2011.

Abbreviations: FLR - fine lateral root; J_s - solute flux [μmol root^{-1} s^{-1}]; J_v - volume flux [mm^3 root^{-1} s^{-1}]; L_p - hydraulic conductivity [mm^3 kPa^{-1} root^{-1} s^{-1}]; *M* - median of the osmotic potential in the xylem sap of the FLRs; Ψ_{so} - osmotic potential of the root medium [kPa]; Ψ_{sx} - osmotic potential of the xylem sap [kPa]; σ - radial reflection coefficient.

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will decrease due to radial solute fluxes. Outflow will be restored rapidly even after transient flow reversal and reaches a new steady state at a decreased rate in a short time (Eaton 1943, Arisz *et al*. 1951, Van Andel 1953, House and Findlay 1966b). Therefore, Ψ_{sx} of individual FLRs cannot be determined by stepwise reducing Ψ_{so} until the flow of the xylem sap is zero. However, if the sap flow direction immediately after transfer of a FLR to a test solution with defined Ψ_{so} could be assessed, a statistical estimate of Ψ_{sx} might be obtained by studying the direction of the xylem sap flow in random samples of the FLRs at different Ψ_{so}.

We developed two methods to determine the xylem sap flow direction in FLRs prepared from branched roots of young maize plants. This enabled us to estimate a median value (*M*) for Ψ_{sx} in the FLRs and to compare the effects of osmolytes with different molecular size on the radial water transport.

Materials and methods

Three- to four-day-old maize (*Zea mays* L.) seedlings of the cultivar Badischer Landmais (*Treppens GmbH*, Berlin, Germany) with a seminal root length of 6 to 12 cm were cultivated for up to 8 d in polypropylene vessels containing *ca*. 40 cm^3 of the liquid medium. Diffusive aeration of the unstirred medium was achieved as described by Ehwald and Poers (2009) with modules of gas permeable polypropylene hollow fibres (*Accurel® 50/280*, Membrana AG, Wuppertal, Germany). Larger culture vessels (medium volume 500 cm^3), described by Fritz *et al*. (2010), were used when the plants were grown for a longer period (9 to 14 d). The oxygen concentration of the mineral medium as measured with a Clark-Electrode (*WTW*, Weilheim, Germany) remained at a high level (6 to 7 mg dm^{-3}) in both culture vessels. The plants were grown at 16-h photoperiod using *Philips SON-T AGRO 400* and *Philips LPI-T Plus* lamps with a photosynthetic photon flux density of 150 µmol m^{-2} s^{-1} (*Philips AG*, Brussels, Belgium). The plants were grown on two different media. Medium I was a solution prepared by dissolution of the macronutrients of the Knop solution in tap water. Its osmotic potential was *ca*. -70 kPa at the end of the culture period. When indicated, NaCl (25 mM) was added to medium I. Medium II was a complete Hoagland solution. Its osmotic potential was *ca*. -30 kPa at the time of harvest.

The following substances were used to adjust Ψ_{so}: Evans Blue, PEG 600 (*Sigma Aldrich*, Steinheim, Germany); Dextran 8, melibiose (*Serva Electrophoresis GmbH*, Heidelberg, Germany); glucose, mannitol, and NaCl (*Roth GmbH*, Karlsruhe, Germany). The Ψ_{so} was determined by means of a freezing point osmometer or, for solutions of dextran 8, with a vapour pressure osmometer (both *Dr. Knauer GmbH & Co. KG*, Berlin, Germany).

Indian ink method: In order to purify the carbon particles of commercially available Indian ink (*Artist discount GmbH*, Bad Segeberg, Germany) from soluble additives, one volume of the ink was mixed with 10 volumes of 96 % ethanol. The aggregated particles were centrifuged and the pellet re-suspended in a large volume of 40 % ethanol and washed twice in this manner. Subsequently the pellet was dispersed in a small volume of deionised water and boiled to remove ethanol. To prepare a stock solution, the concentration of the ink particles was adjusted to 100 g dm^{-3}. Aliquots of this solution were frozen at -20 °C. Before addition to the test solutions, the stock solution was sonified for 2 min with an *L-Converter BSP-6 of the Sonifier B12* (*Branson Sonic Power Company*, Danbury, USA). The final concentration of the ink particles in the test solutions was 1 g dm^{-3}. The osmotic potential of an ink particle suspension prepared with deionised water was above -0.1 kPa.

De-topped exuding root systems were transferred from the culture vessel to a large Petri dish filled with the culture medium, wherein the FLRs were detached with a razor blade. Thick branched laterals were excluded. Further selection was not carried out. The length of the studied laterals varied between 8 and 35 mm, and their diameter was between 150 and 350 µm. If not otherwise stated, the FLRs were kept 5 to 15 min in the root medium before their transfer to test solutions.

Observation chambers were prepared by sticking segments of the above mentioned gas permeable polypropylene hollow fibres onto a microscope slide in a parallel arrangement (Fig. 1*A*). The fibre segments (*Accurel® 50/280*, Membrana AG, Wuppertal, Germany) with an outer diameter of 380 µm supported the cover slide and supplied oxygen to the suspension. 400 mm^3 of a test solution with the ink particles was poured into the furrows between the fibres on the observation slide. The FLRs were shortly blotted on filter paper and distributed rapidly into the furrows between the fibres on the observation slide (up to 20 FLRs per slide). A large cover slide was applied and the FLRs were inspected at 50 times magnification under the microscope. The release of xylem sap by FLRs into the growth medium was directly observable by brightening at the cut ends, which had their origin in the stele (Fig. 1*B-D*). Axial inflow of the ink particles into the xylem vessels could be observed in real time and documented in cross sections. If not varied for the investigation of pre-incubation effects the period between dissection of the FLRs and their microscopic examination was held as short as possible (usually below 10 min). Counting of the FLRs showing outflow of xylem sap in random samples of 10 to 20 FLRs required about 3 min.

Evans-Blue method: Test solutions containing Evans Blue (5 or 10 g dm^{-3}) and the chosen osmolytes in

different concentrations were prepared in the growth medium and their osmotic potential was measured. The osmotic potential of an Evans Blue solution with a concentration of 5 g dm^{-3} in deionised water was 50 kPa. Random samples of FLRs prepared in the mineral medium as described above were shortly blotted on filter paper and transferred to small Petri dishes containing the test solutions. After an incubation period of 5 min the FLRs were washed briefly with tap water. When a series of parallel variants needed to be analysed, washed samples were kept in tap water before their transfer to the microscopic slide. The FLRs of a random sample (up to 30) were distributed by means of tweezers on a microscopic slide that was moistened with water. Subsequently, the FLRs were pressed between two microscopic slides using a wooden block to render the vessels visible (Fig. 2). An ocular micrometer was used to determine the length fraction of the FLR showing stained vessels. The osmotically driven inflow of the Evans Blue medium into the vessels was recorded, if the vessels were stained apart from the generally stained short region at the cut end (Fig. 2*A*).

To estimate Ψ_{sx}, random samples of the FLRs were incubated in three to four test solutions with different levels of Ψ_{so}, which were 50 to 150 kPa below the osmotic potential of the growth medium. The fraction of FLRs in these samples showing a radial water influx (ink method) or efflux (Evans Blue method) was plotted on Ψ_{so}. The value of Ψ_{so} corresponding to a fraction of 0.5 was assessed by interpolation. This value was regarded as median value for Ψ_{sx} (*M*).

Some FLRs that were still connected to the root system of a transpiring plant were decapitated directly behind the apex and a stock solution of ink particles (see above) was added to the mineral medium (dilution 1:250). After 2 h the FLRs were cut off, and the lateral vessels in hand slices at the root base were inspected under the microscope.

The xylem sap flow rate of the decapitated whole root systems in the mineral growth medium was recorded using the *SLG 1430-480* flow sensor (*Sensirion AG*, Staefa, Switzerland) as described in Fritz *et al*. (2010).

Arithmetic mean values are given with the confidence interval and/or the standard deviation. The significance level α was determined using the Student's *t*-test. Its value was given for differences between two mean values and for Pearson's correlation coefficient.

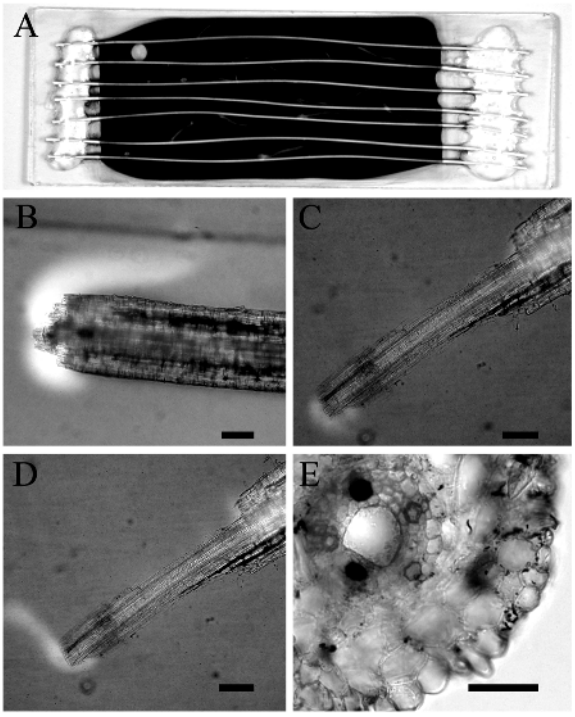

Fig. 1. Visualisation of xylem sap flow with the ink method. *A* - Observation chamber; the FLRs in the ink particle suspension under the cover slide were supplied with oxygen by polypropylene fibres. *B* - Brightening of the ink suspension by xylem sap efflux. *C* to *D* - Origin of the streaks at the stele. Photographs were taken at 10 s intervals. *E* - Accumulation of ink particles in the xylem vessels of decapitated FLRs by transpiration-induced axial flow *in situ*. Ink particle suspensions were made with the mineral medium I. *Bars*: 75 μm, (*B-D*) 50 μm (*E*).

Results

With the ink particle method the outflow of xylem sap into the test solution could be detected in real time (Fig. 1). The brightening of the ink suspension at the cut ends (Fig. 1*B*) could be destroyed by a slight movement of the cover slide. It recovered rapidly after its convective extinction (data not shown). When the tip was removed from the isolated FLRs, sap outflow occurred at both cut ends (data not shown). Removal of the cortical cells showed that the volume flow had its origin in the stele. In ink dispersions prepared with the root medium the brightening appeared and increased within seconds (Fig. 1*C*,*D*). When the osmotic potential of the test solutions (Ψ_{so}) was 50 to 150 kPa below that of the original medium, not all FLRs showed sap outflow. There was a significant decrease in the exuding fraction of FLRs during their incubation in an ink suspension with 30 mM mannitol. This change was smaller or absent at incubation in 30 mM glucose (Table 1).

Cross sections of the FLRs studied (Figs. 1*E*, 2*C*) generally showed a small diameter (*ca.* 10 μm) of the 5 to 10 peripheral xylem vessels. The larger central vessel was generally immature. Using root systems of plants in the four leaf stage (8 d of culture) volume fluxes through the small peripheral vessels were roughly estimated from the rate of xylem sap release by the whole root in the mineral solution used for hydroponics (15 to 30 × 10^{-3} mm^3 s^{-1}),

the number of the FLRs per root system (50 to 100), and the number of the vessels per FLR. According to this estimation volume fluxes through a single vessel were in the range between 15 to 60 × 10^{-6} mm^3 s^{-1} corresponding to axial flow velocities between 0.3 and 1.5 mm s^{-1}.

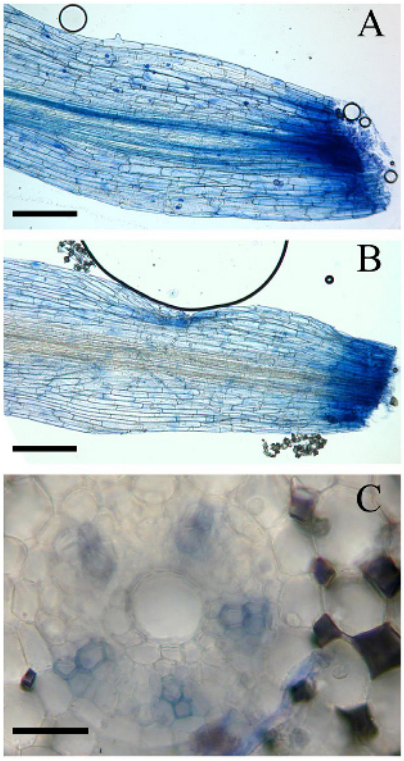

Fig. 2. Staining of xylem vessels after incubation of the FLRs in a hypertonic Evans Blue solution. *A* - Basal part showing non-selective staining of the cells close to the cut and staining of the vessels at a distance from the cut. The FLR was incubated in mineral medium I containing Evans Blue (5 g dm^{-3}) and mannitol (50 mM). *B* - Absence of staining at a distance from the cut after incubation in a hypotonic Evans Blue solution made up with mineral medium I. *C* - Free-hand section with stained vessels and gas-filled intercellular spaces. The detached FLR was incubated in a solution of Evans Blue (10 g dm^{-3}) and PEG 600 (Ψ_{so} -450 kPa) in tap water. *Bars*: 200 µm (*A,B*), 50 µm (*C*) .

Table 1. The portions of FLRs showing xylem sap exudation after different periods of pre-incubation (PI) with 30 mM glucose or mannitol (Ψ_{so} = -115 kPa). For each experiment, the fine lateral roots dissected from at least five plants were pooled in the culture medium before random samples were incubated in glucose or mannitol media.

Exp.	PI	30 min	120 min	180 min	220 min
I	mannitol	0.67	0.44	0.12	-
	glucose	0.89	0.68	0.57	-
II	mannitol	0.71	0.70	0.50	0.34
	glucose	0.76	0.76	0.78	0.71

Table 2. Influence of the external osmotic potential Ψ_{so} [kPa] and the duration of incubations in Evans Blue (EB) and tap water (TW) [min] on the relative length of xylem staining in the FLRs. The seedlings were cultivated in medium I for 9 d (Exp. I) or 7 d (Exps. II and III). Means ± SE.

Exp.	Ψ_{so} of EB	Inc. EB	Inc. TW	Length
I (*n* = 9)	-197	10 - 15	10 - 15	0.33 ± 0.05
	-303	10 - 15	10 - 15	0.55 ± 0.04
	-383	10 - 15	10 - 15	0.66 ± 0.06
	-424	10 - 15	10 - 15	0.67 ± 0.07
II (*n* = 12)	-550	5	10	0.62 ± 0.06
	-550	10	10	0.58 ± 0.09
	-550	15	10	0.60 ± 0.07
	-550	20	10	0.64 ± 0.07
	-550	25	10	0.61 ± 0.08
III (*n* = 10)	-411	5 - 10	10	0.76 ± 0.05
	-411	5 - 10	20	0.75 ± 0.08
	-411	5 - 10	30	0.70 ± 0.07

Table. 3. Median value (*M*) of the osmotic potential in the fine lateral roots determined with different osmolytes. Seedlings were grown in medium 1 for 8 d. The FLRs were pre-incubated in mineral medium for 10 to 30 min with (Exp. I) or without (Exps. II and III) glucose (55 mM). Means ± SE, *n* = 5.

Exp.	Ψ_{so} [kPa]	Osmolytes	*M* [kPa]
I (ink)	-150	NaCl	-270 ± 20
	-150	mannitol	-280 ± 60
	-150	melibiose	-320 ± 60
II (EB)	-70	NaCl	-162
	-70	mannitol	-160
	-70	PEG 600	-167
	-70	dextran 8	-167
III (EB)	-70	NaCl	-153
	-70	PEG 600	-150
	-70	dextran 8	-147

When a transpiring plant with a decapitated FLRs was incubated in the ink particle suspension, the particles were sucked to the base of the FLR through the lateral xylem vessels (Fig. 1*E*). When detached FLRs were incubated in an ink suspension with an osmotic potential below -400 kPa, the ink particles moved from the cut end through a large part of the vessels, a process that could be observed in real time.

After incubation of the isolated FLRs in test solutions containing Evans Blue that were hypertonic to the xylem sap, vessels were selectively stained apart from the cut zone (Fig. 2*A,C*). Close to the cut end the dye accumulated in the dead cells and the cell walls bordering those intercellular spaces which had been infiltrated due to wounding. This non-selective staining also occurred in hypotonic dye solutions and was generally restricted to a short region. The FLRs were not uniform with respect to

their individual Ψ_{sx}. No selective staining of the vessels was found in Evans Blue media with Ψ_{so} that was equal or higher than that of the original medium. At decreasing Ψ_{so} a broad range in the variation of Ψ_{sx} was documented by a gradual increase in the fraction of FLRs with reversed flow direction, that occurred when Ψ_{so} of the test solutions was decreasing in the range from 50 to 200 kPa below Ψ_{so} of the original medium (Fig. 3).

In Evans Blue solutions with even lower Ψ_{so} vessels were stained along a considerable portion of the root length (Table 2). This portion increased with decreasing Ψ_{so} of the Evans Blue solution (Exp. I). It was independent of the incubation time (5 - 25 min) in the dye solution (Exp. II) and of the time of the final incubation in tap water (Exp. III). The product of the unstained length fraction of the vessels and Ψ_{so} was approximately constant, when Ψ_{so} was varied between -250 and -450 kPa (data not shown). In a hypertonic Evans Blue medium there was a rather high variability in the ratio between the length of vessel staining and the length of the FLRs. When this ratio was determined for 54 FLRs of different length (7 to 35 mm) after an incubation at Ψ_{so} of -550 kPa, its value was independent of the length (mean 0.61 ± 0.07).

When xylem sap was collected from an intact root and the median value of Ψ_{sx} in the FLRs taken from this root (*M*) was subsequently determined, *M* was significanly lower than the osmotic potential of the sap released by the whole root. In an experiment with 14 plants grown for 9 d in medium II this was found in all of the roots studied. The arithmetic mean of the osmotic potential of the exuded sap was -124 ± 11 kPa and that of the median values *M* of the FLRs amounted to -167 ± 14 kPa. In spite of the relatively narrow range of their variation, values for the exuded xylem sap showed a significant correlation with *M* (Pearson's correlation coefficient: 0.61). When plants were grown with 25 mM NaCl, *M* was significantly lower (-212 ± 15 kPa) than *M* of control plants (-156 ± 15 kPa).

Values of *M* obtained with the Evans Blue method and the ink particle method were not significantly different when the studied FLRs were taken from a common pool (not shown). After pre-incubation of the FLRs with additional glucose, *M* was decreased by a value close to the partial osmotic potential of the sugar in the medium (Table 3). *M* did not show a significant dependence on the molecular size of the osmolyte used to adjust Ψ_{so} in the test solutions. Values obtained with NaCl, mannitol and melibiose were not lower than those obtained with dextran 8, a polymer which cannot cross the endodermis (Table 3).

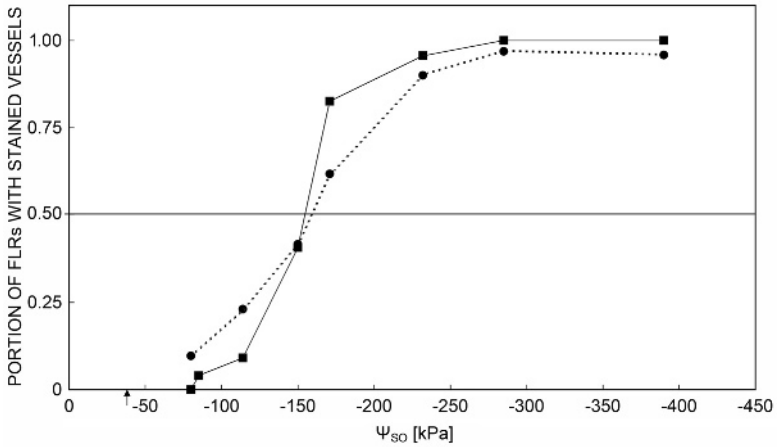

Fig. 3. Portion of the FLRs showing reversed flow (stained vessels) after their transfer from the culture medium (medium II) to Evans Blue solutions with various osmotic potentials. Different symbols designate two experiments carried out each with the FLRS from five plants that were pooled in the culture medium. The Ψ_{so} of the Evans Blue solution was adjusted with mannitol. Each point represents the fraction of at least 20 FLRs that showed selective staining of the vessels. *Arrow* - Ψ_{so} of the culture medium.

Discussion

Both presented methods were found to be useful in the study of the dynamics of xylem sap flow in the narrow xylem vessels of fine lateral roots. With the ink particle method the direction of radial and axial volume could be repeatedly assessed in real time with the same FLRs. This was an advantage when studying the ability of the FLRs to exude xylem sap at a reduced Ψ_{so}. The decrease in the fraction of exuding FLRs that took place during prolonged pre-incubation in the mannitol containing medium (Table 1) was most likely due to sugar starvation, since this was absent or much weaker in the glucose medium. There is no significant metabolism of mannitol in maize (Fritz and Ehwald 2011), whereas glucose is a good substrate for respiration, growth, and maintenance of the sucrose pool in maize roots (Göring and Gerlach 1966). Stabilisation of the exuding fraction

in the glucose medium confirmed previous findings of the strong dependence of the radial ion transport activity on sucrose provision from the shoot *via* the phloem (Bowling *et al.* 1985). The observed instability of Ψ_{sx} in the isolated FLRs must be considered for the determination of its median value (*M*). In order to obtain a value that approaches the situation *in situ*, it is recommended to keep the freshly isolated FLRs for not more than 15 min in the mineral growth medium before transfer to the test solutions as described in Materials and methods.

The axial flow of the ink particles (diameter *ca*. 0.5 - 1 μm) through the vessels of the FLRs (Fig. 1*E*) proved the absence of cell wall (pith) barriers on the axial flow path. Hence, in spite of their small diameter (*ca.* 10 μm) the lateral xylem vessels in which the flow through the FLRs took place were not tracheids.

Selective staining of the vessel walls by Evans Blue is probably due to the slow diffusion of the large tetravalent anion (Stokes' diameter 2.2 nm) along the cell walls between the living xylem parenchyma cells surrounding the vessels. Staining of the denatured protoplasts and walls close to the cut showed that the cell walls were not completely impermeable to Evans Blue and that all cell walls could adsorb the dye (Fig. 2). The independence of the stained length fraction of the vessels on the staining time (Table 2) proved that the convective flow of the medium into the vessels ceased within 5 min and that dye adsorption to the vessel walls was a rapid process. During the flow of the dye solution into the xylem vessels, solute excretion to the vessels and the entrance of the hypertonic solution decreased Ψ_{sx} until it became almost isotonic to the test solution. Subsequently, Ψ_{sx} was decreased further due to the solute flux to the vessels and this enabled the restoration of axial outflow (Eaton 1943, Van Andel 1953, Arisz *et al.*1951, House and Findlay 1966b). Since the stain was not rapidly washed from the vessels (Table 2) one can assume that outflow of the Evans Blue solution did not affect staining. Hence, with this method it can be determined whether or not the xylem sap of individual FLRs is initially hypotonic or hypertonic to a given test solution.

In a hypertonic Evans Blue solution a certain ratio between the stained length and the whole length of the vessels (r) should represent the transient isotonic phase with the maximal depth of medium entrance, if mixing of the stained medium in the vessels with the original xylem sap could be neglected. This ratio should decrease with the initial value of Ψ_{sx} and it should increase with the value of Ψ_{so} according to formula: $r = 1 - \Psi_{sx}/\Psi_{so}$.

The length ratio between the stained part of the peripheral vessels and the root length showed the expected increase with decreasing Ψ_{so} (Table 2). The variance of Ψ_{sx} is expressed by the variance of this ratio. Since the length ratio did not show a significant dependence on root length, it was justified to use FLRs of different length for the estimation of the median value of Ψ_{sx} as described in Materials and methods.

The observation of sap efflux with the ink method did not unambiguously indicate the initial flow direction. In a small fraction of the samples, a slight brightening of the ink particle suspension 5 min after the transfer of the FLRs to the test solution did not exclude initial sap influx, as the phenomenon could be due to restoration of outflow. Considering the broad range of variation of Ψ_{sx} discussed above this uncertainty could not have a strong influence on the estimated median value (*M*). However, as the Evans Blue method allowed for the almost simultaneous assessment of the initial flow direction in a large number of random samples, this method is preferable for the determination of *M*.

As expected, *M* reflected the salt concentration of the growth medium. The transition from one steady state of sap outflow to another one induced by a moderate decrease of Ψ_{so} required less than 4 min in the young root systems of maize (Fritz *et al*. 2010) or *Luffa cylindrica* (Zhu *et al*. 1986). This can explain the strong reduction of *M* by pre-incubation at reduced Ψ_{so} (Table 3, Exp. I).

Klepper (1967) found that the osmotic potential of xylem sap exuded from primary maize roots grown on mineral media with different concentrations of NaCl (0 - 55 mM) was lower than Ψ_{so} by an almost constant difference. According to this author, the osmotic potential of the exuded xylem sap is equal to Ψ_{sx} in the region of ion and water absorption, when the plants are in a long-term steady state with their medium. Although we found that *M* was lower than the osmotic potential of the xylem sap collected from the whole root this does not prove a rise in the osmotic potential of the xylem sap during its flow through the maternal root. Since the roots tested were in long-term steady state with their nutrient solution, significant net ion fluxes from the xylem sap to the root symplast were not probable. A more convincing explanation for the difference found is obtained when the statistical meaning of *M* and the large variation in Ψ_{sx} between individual FLRs are considered. For the studied FLRs a broad range of variation of Ψ_{sx} was indicated by the broad range of Ψ_{so} between the value which was hypotonic to Ψ_{sx} in all FLRs and the value which was hypertonic to Ψ_{sx} in all FLRs (Fig. 3). One reason for this variation is certainly the variability of age, thickness and position of the FLRs in the root system. As mentioned in the introduction, Ψ_{sx} in individual FLRs depends on the ratio between J_s and the J_v, which can both vary independently:

$$\Psi_{sx} = -RT\, J_s/J_v \qquad (1)$$

J_s depends on the energy charge, the concentration of excretable solutes in the symplast and the activity of channels and transporters. At a given osmotic driving force, J_v depends on the hydraulic conductivity L_p:

$$J_v = \sigma L_p (\Psi_{so} - \Psi_{sx}) \qquad (2)$$

The complex radial reflection coefficient of the root (σ), relates the hydraulic force of the radial osmotic difference to an equal pressure difference. The combination of Eq. 1 and Eq. 2 results in Eq. 3, showing that the absolute value

of Ψ_{sx} is an inverse function of $L_p^{1/2}$ and proportional to $J_s^{1/2}$:

$$\Psi_{sx} = - (J_s RT/\sigma L_p + \Psi_o^2/4)^{1/2} + (\Psi_o/2) \quad (3).$$

If the variation in Ψ_{sx} was mainly due to a variation of J_s, the half of the FLRs with a Ψ_{sx} below the median value *M* should contribute a larger volume to the mixed sap in the maternal root axis than the other half. Hence, the xylem sap collected from the whole root should have an osmotic potential below the median Ψ_{sx} of its FLRs. However, this was not the case. At an invariant J_s, J_v would increase and the solute concentration in the xylem sap would decrease with increasing L_p. In this case, the half of the FLRs with $\Psi_{sx} > M$ should contribute a higher volume to the mixed sap exuded by the whole root than the other half. Hence, the sap finally exuded from the whole root should have an osmotic potential higher than *M*, as was found. Since significant unloading of the ions from the xylem sap to the root symplast is not probable in roots exuding in media to which they were adapted (Klepper 1967), our results suggest that the observed variation in Ψ_{sx} was mainly due to a variability of L_p.

The external osmotic potential compensating hydraulically the xylem sap of the FLRs in 50 % of the sample was similar for different osmolytes including Dextran 8 (mean molecular mass 8 kDa) and NaCl (Table 3). To be compatible with a significant apoplastic bypath circumventing all protoplasts in the radial water flux (Steudle 1989, 2000, Steudle and Peterson 1998) this would require pores on the bypath large enough for non-selective solvent drag even to dextran 8. However, the reflection coefficient of primary cell walls for dextran 8 is close to unity (Carpita *et al*. 1979) and it is known that osmolytes of comparable Stokes' diameter (PEG 2000 and PEG 4000) do not enter the vascular system of intact roots (Lawlor 1970). The cell walls of the outer cortex of seminal maize roots behave as semipermeable membranes in solutions of dextran 8. When these roots were denatured with 70 % ethanol, rehydrated, and incubated in a dextran 8 solution with an osmotic potential of -100 kPa, they showed irreversible shrinkage (Ehwald, unpublished). If the volume flux on the proposed apoplastic bypath was significant, the median value *M* obtained with dextran 8 should be lower than that obtained with sugars and salts which might have a small reflection coefficient on the putative apoplastic bypath through the radial endodermis walls. Equal hydraulic effects of isotonic solutions prepared with polymer osmolytes and mannitol have previously been reported in a study on water transport through the living storage parenchyma of the potato tuber (Michael *et al.* 1997). The results given in Table 3 suggest that the radial reflection coefficient is close to unity for all studied osmolytes. This agrees with the results of recent studies (Fritz *et al.* 2010, Knipfer and Fricke 2010), which used different methods to prove that reflection coefficients of cereal roots for small solutes are close to unity. The same conclusion may be drawn from the absence of a significant solvent drag to NaCl, sugars and mannitol at high rates of water uptake in studies with intact root systems of different plants (Perry and Greenway 1973, Munns 1985, Tsuchiya *et al*. 1984, Ochiai and Matoh 2002, Fritz and Ehwald 2011).

Conclusions

The advantages of the Evans Blue method are its convenience and its suitability to determining the initial flow direction after the transfer of the FLRs into the test solution. The advantages of the Indian ink method consists in its suitability for observing the direction of xylem sap flow in real time and for repeatedly determining this with the same FLRs. Use of the Indian ink method proved that the ability of the FLRs to exude xylem sap at a reduced Ψ_{so} decreased due to sugar starvation during the prolonged incubation of FLRs. The osmotic potential of the xylem sap of the individual FLRs was 50 and 200 kPa below the osmotic potential of the root medium. Its median value was lower than the osmotic potential of the xylem sap released by the root system from which the FLRs were separated. This is consistent with the hypothesis that the broad variability of Ψ_{sx} was mainly due to the variability of L_p, not the variability of the radial solute flux. The almost equal hydraulic effects of isotonic solutions of dextran 8 and low-molecular mass osmolytes including NaCl on radial water transport are consistent with a radial reflection coefficient of the FLRs close to unity for the solutes tested.

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