

## Tracing root permeability: comparison of tracer methods

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### Abstract

Root epidermis and apoplastic barriers (endodermis and exodermis) are the critical root structures involved in setting up plant-soil interface by regulating free apoplastic movement of solutes within root tissues. Probing root apoplast permeability with “apoplastic tracers” presents one of scarce tools available for detection of “apoplastic leakage” sites and evaluation of their role in overall root uptake of water, nutrients, or pollutants. Although the tracers are used for many decades, there is still not an ideal apoplastic tracer and flawless procedure with straightforward interpretation. In this article, we present our experience with the most frequently used tracers representing various types of chemicals with different characteristics. We examine their behaviour, characteristics, and limitations. Here, we show that results gained with an apoplastic tracer assay technique are reliable but depend on many parameters – chemical properties of a selected tracer, plant species, cell wall properties, exposure time, or sample processing.

*Additional key words:* apoplast, berberine, endodermis, exodermis, ferrous ions, PAS reaction, propidium iodide, PTS.

### Introduction

Root permeability is one of the key features determining root-soil communication, resources acquisition, or resistance to pollutants with implication to plant stress tolerance or food quality. Passive non-selective transport *via* apoplast is restricted by apoplastic barriers. Among them, endodermis is the essential barrier of vascular plant roots. Its function is related to cell wall modifications and tight membrane adhesion that prevents apoplastic transport across the endodermal layer (Kroemer 1903, De Lavisson 1910, Esau 1953, Enstone *et al.* 2003, Geldner 2013). A similar barrier (exodermis) may occur in the outer cortex (Enstone *et al.* 2003). Exodermis is not an obligatory structure and its formation is under a strong environmental influence (Perumalla *et al.* 1990, Peterson and Perumalla 1990). Apoplastic barriers play a crucial role in water and nutrient uptake and are involved in stress ecophysiology (North and Nobel 1995, Armstrong *et al.* 2000, Soukup *et al.* 2007, Redjala *et al.* 2011). They should not be perceived as strictly impermeable boundaries (Hose *et al.* 2001, Ranathunge *et al.* 2005b), and their properties are modified along the root, in different root types, or in response to stress factors (Moon *et al.* 1984, Degenhardt and Gimmler 2000, Colmer 2003, Meyer *et al.* 2009, Krishnamurthy *et al.* 2014, Shiono

*et al.* 2014).

Apoplast permeability modulates root uptake characteristics substantially, but there is a limited set of methodological tools to evaluate its extent and spatial variation. Quantitative measurements of root transport parameters present the first set of available methods, *e.g.*, a root pressure probe technique (Peterson *et al.* 1993, Zimmermann and Steudle 1998, Ranathunge *et al.* 2003, 2005a,b, Bramley *et al.* 2007, Knipfer and Fricke 2010) or a vacuum perfusion technique (Knipfer and Fricke 2010). These methods allow quantifying root pressure (Wegner 2014) and water and solute flows in intact root systems or excised root segments. In addition, techniques following radial O<sub>2</sub> loss with a root-sleeving O<sub>2</sub> electrode or a methylene blue indicator dye detecting O<sub>2</sub> escaped from the root were successfully used to monitor O<sub>2</sub> permeability of barriers (Armstrong and Armstrong 2001, Soukup *et al.* 2007, Shiono *et al.* 2011). All these methods provide quantitative data for modelling of transport flows at whole plant/root levels, but their spatial resolution is rather limited.

In contrast, apoplastic tracer assays allow analysis of spatial variability in root apoplast permeability. Roots are exposed to compounds with a limited penetration cross

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Abbreviations: PAS - periodic acid – Schiff's reagent; PTS - trisodium 3-hydroxy-5,8,10-pyrenetrisulfonate.

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the plasmalema and the tracer is subsequently localised within root tissues. To our knowledge, the very first reference using an apoplastic tracer (ferrous ions) to demonstrate varying permeability of root tissues is that of De Lavison (1910). His identification of “living membranes” bordering the inner space of the central cylinder from the passive diffusion of compounds from a surrounding solution was a crucial step in root biology. Afterwards, the tracer assays successfully demonstrated the tightness of endodermal Casparian bands (Naseer *et al.* 2012, Hosmani *et al.* 2013) or matured hypodermal apoplastic barriers (Soukup *et al.* 2002, 2007, Meyer *et al.* 2009, Meyer and Peterson 2011). The tracer assays also indicated apparent apoplastic bypass sites: young root zones with immature hypodermal layers, sites of primordia or lateral roots emergence, and damaged surface layers including artificially wounded sites (Enstone and Peterson 1992, Aloni *et al.* 1998, Soukup *et al.* 2002, Ranathunge *et al.* 2005a, Faiyue *et al.* 2010). Tracers contributed to understanding water movement in roots. An interesting approach was the blockage of the root apoplastic water path with ink particles or insoluble precipitates of copper ferrocyanide, which was able to trigger a measurable decrease of the root hydraulic conductivity by 30 % or 2- to 5-times, respectively (Ranathunge *et al.* 2004, 2005a).

An apoplastic tracer approach brings the spatial resolution of permeability characteristics, which has a significant informative value irreplaceable by other techniques. Tracers penetrating the root tissues might be detected on sections, whole-mounts, or quantified after tissue elution, *e.g.*, fluorometrically (Faiyue *et al.* 2010), alternatively their concentration in collected xylem sap is measured (Krishnamurthy *et al.* 2014). Further manipulation with the tissue, *e.g.*, experimental cutting off selected laterals (Ranathunge and Schreiber 2011) or controlled damage of particular surface layers (Moon *et al.* 1984) might help further specify features and position of apoplastic barriers.

Apoplastic tracers comprise variable chemical compounds. The first group includes small inorganic compounds detectable as precipitates or cell wall coloration under bright field optics. Among them, a periodic acid – Schiff’s reagent (PAS) reaction and  $\text{Fe}^{2+}$  are the most common. Ferrous ions applied as  $\text{FeSO}_4$  are readily mobile and penetrate tissue (De Lavison 1910, Soukup *et al.* 2002). Precipitated  $\text{Fe}^{3+}$  hydrates are subsequently detected after spontaneous oxidation of  $\text{Fe}^{2+}$  (De Lavison 1910). Addition of oxidizing compounds (*e.g.*, hydrogen peroxide) can further accelerate the precipitation (Soukup *et al.* 2002). Precipitates are histochemically localised as the insoluble pigment of Berlin Blue (also called Prussian Blue), ferric ferrocyanide  $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$ , which is formed when ferric ions react with hexaferrocyanate anions  $\text{Fe}(\text{CN})_6^{4-}$ . White precipitates of  $\text{Fe}_2[\text{Fe}(\text{CN})_6]$  might be also produced during the reaction (Pearse 1968). Alternatively,  $\text{CuSO}_4$  can be used and detected as brown insoluble crystals of copper ferrocyanide  $\text{Cu}_2[\text{Fe}(\text{CN})_6]$ , so called Hatchett’s

brown (Ranathunge *et al.* 2005a,b).

A test based on PAS reaction is the modification of a general histochemical reaction used to detect cell wall structural polysaccharides (Pearse 1968). Periodic acid ( $\text{H}_5\text{IO}_6$ ,  $M_r$  227.90) penetrates through the tissue as a tracer (Soukup *et al.* 2002) creating dialdehydes from diols of polysaccharidic rings by oxidative cleavage. Aldehydes can be afterwards localised on sections (fresh or permanent) by Schiff’s solution. The method was used before to test the piping capacity of xylem (Chaney and Kozłowski 1977, Mistrikova and Kozinka 1989). Features that might advocate usage of the method might be the relative small inorganic molecule of  $\text{H}_5\text{IO}_6$ . As a moderate acid ( $\text{pK}_a = 2 \times 10^{-2}$ ), the molecule possesses a negative charge. It does not fade, diffuse, and cannot be washed out during subsequent processing samples ensuring precise localisation.

The majority of apoplastic tracers represent large fluorescent organic molecules. To understand their behaviour in the apoplast, their properties (molecular size or charge) are important. With some generalization, we can distinguish several groups of fluorescent tracers according to affinity to apoplastic cell wall components.

Fluorescent tracers with some degree of affinity are, *e.g.*, propidium iodide, Cellufluor, or berberine. Propidium iodide ( $M_r$  668.39) contains two quaternary ammonium cations that cause affinity to the acidic compounds of cell walls. Propidium iodide is a basic fluorescent dye intercalating DNA, often used to test cell viability as it is membrane impermeable in viable cells. As an apoplastic tracer, propidium iodide was successfully used to assay endodermal Casparian bands (Naseer *et al.* 2012, Hosmani *et al.* 2013), but its low binding capacity makes it rather unsuitable for usage in combination with tissue sectioning.

Cellufluor ( $M_r$  916.98) binds tightly cellulosic and hemicellulosic components of cell walls. It is used under variable names as, *e.g.*, Calcofluor white M2R, Fluorescent Brightener 28, or similar dyes such as Fluostain I ( $M_r$  960.95), Tinopal CBS/Tinopal PRS ( $M_r$  562.56), or Uvitex CFX ( $M_r$  813.00) (Peterson *et al.* 1978, Peterson *et al.* 1981a,b, Moon *et al.* 1984, Barnabas 1996, Ochiai and Matoh 2002). Their main disadvantage is detection under UV-excited fluorescence that can be misinterpreted with cell wall auto-fluorescence, especially in commelinoid monocots – very common model species.

Berberine hemisulphate ( $M_r$  336.37) is an alkaloid with a high affinity to acidic compounds, *e.g.*, polyphenolics (Strugger 1939, Enstone and Peterson 1992, Meyer and Peterson 2011). Berberine yields a bright fluorescence under UV or blue excitation. It was first used as an apoplastic tracer by Strugger (1939) in a form of a raw extract of *Chelidonium*. The solution of berberine is usually followed with thiocyanate to induce precipitation (Enstone and Peterson 1992) and to increase its retention in cell walls.

Acidic tracers with a low affinity to a cell wall are, *e.g.*, Sulphorhodamine, Lucifer yellow, or trisodium

3-hydroxy-5,8,10-pyrenetrisulfonate (PTS). Sulphorhodamine G (Mr 552.59) is a weak acidic dye that does not bind to cell walls (Oparka 1991, Varney *et al.* 1993, Canny and Huang 1994). With some restrictions, Evans Blue, an acidic dye primarily used for detection of dead cells, can be used as an apoplastic tracer, but a high diffusibility of signal complicates the sample handling (Gierth *et al.* 1999) and makes sectioning almost impossible. Reactive dyes, such as Lucifer yellow CH (Mr 457.20; Bederska *et al.* 2012) and 4-acetoamido-4-iso-thiocyanostilbene-2,2-disulphonic acid (SITS, Mr 498.46; Skinner and Radin 1994) have a very high purchase cost and very low fluorescence, so they are used mainly for a microinjection technique as symplastic tracers.

The other acidic dyes that do not bind to components of cell walls are light green SF yellowish (Mr 749.89; Epel and Bandurski 1990, López-Pérez *et al.* 2007) or PTS (Mr 524.00, used as trisodium and tetrasodium salts), which contain even four negative charges (Skinner and Radin 1994). The PTS, also called Pyranine or Solvent Green, is a strongly fluorescent probe detectable under UV (Canny 1993, Zimmermann and Steudle 1998), blue (Faiyue *et al.* 2010), or even green excitation (Cholewa and Peterson 2001). The intensity of fluorescence depends on the degree of ionization of 8-hydroxyl groups, so pH affects emission (Faiyue *et al.* 2010). A high mobility of PTS complicates its localisation in roots even in whole-mount preparations and especially on sections although even those were occasionally used (Cholewa and Peterson 2001, Faiyue *et al.* 2010). The PTS as well as previously mentioned Sulphorhodamine G are therefore applied mostly into the rooting media to be detected in leaves (Peterson *et al.* 1981a, Zimmermann

and Steudle 1998, Krishnamurthy *et al.* 2014). Such approach was successfully used to assay the overall impact of growth conditions on root system permeability (Peterson *et al.* 1981a, Zimmermann and Steudle 1998, Krishnamurthy *et al.* 2014) and contributes to our understanding water movement in roots. However, gained results should be interpreted carefully (Varney *et al.* 1993, Canny and Huang 1994, Zimmermann and Steudle 1998). There are other not so common dyes used for tracing apoplastic transport, *e.g.*, conjugates of Dextran-Texas Red (Sivaguru *et al.* 2006).

In spite of a substantial contribution of tracer assays to understanding root functions, gained results and their interpretation are influenced by several factors, which have to be carefully considered. At first, tracer movement is not always fully restricted to the apoplastic route as endocytosis of PTS was shown in *Allium cepa* (Cholewa and Peterson 2001). A tracer may interfere with membrane integrity and its penetration depends on concentration, molecular size, and charge underlying interactions with cell wall matrix. Such interactions determine not only tracer movement within a cell wall but also its detection/localisation during subsequent sample processing.

To test reliability of the method and summarise possible sources of flaws that might help other users, we analysed root permeability of two species with contrasting tightness of the exodermal layer (*Oryza sativa* and *Zea mays*) with four principal representatives of commonly used probe categories: small inorganic tracers with a positive ( $\text{Fe}^{2+}$ ) or negative ( $\text{H}_5\text{IO}_6$ ) charge and bigger organic ones with a high (berberine hemisulphate) or low (PTS) affinity to cell wall matrix.

## Materials and methods

**Plants:** *Zea mays* L. cv. Cefran and *Oryza sativa* L. var. *japonica* cv. Nipponbare were cultivated in a growth chamber under a 16-h photoperiod, and an irradiance of 435 W m<sup>-2</sup> (photosynthetically active radiation), day/night temperatures of 22/18 °C, and a relative humidity of 50 - 75 %. The aerated hydroponics (dissolved oxygen saturation  $\geq$  50 %) contained quarter-strength Hoagland 3 solution (Hoagland and Arnon 1950) supplemented with following microelements: 11.6  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 2.3  $\mu\text{M}$   $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$ , 0.34  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ , 0.015  $\mu\text{M}$   $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ , 0.12  $\mu\text{M}$   $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ , and 5.1  $\mu\text{M}$   $\text{Fe}^{3+}$  citrate (pH 5.3 - 5.5). The solution was changed weekly. The maize plants were harvested 16 d after germination and rice plants 46 d after germination. The tissue culture of *Nicotiana tabacum* L. (line VBI-0) was cultivated *in vitro* on V4 medium (Opatrný and Opatrná 1976) solidified with 0.8 % (m/v) agar and supplemented with 3 % (m/v) sucrose, 0.1 % (m/v) casein and all necessary nutrients, vitamins, and phytohormones. The culture was kept in the dark at 25 °C in the cultivation chamber.

**Permeability test on intact root systems:** Apoplastic tracers were applied by immersion of root systems of *Z. mays* or *O. sativa* intact plants into the aqueous solution of a given tracer for periods of 30, 60, or 120 min. For berberine test (Enstone and Peterson 1992), roots were immersed into 0.05 % (m/v) berberine hemisulphate, gently rinsed with tap water, and transferred into 90 mM KSCN for the same time as the previous incubation step to trigger precipitation of berberine thiocyanate inside root tissues. Hand-sections were mounted in a 90 mM KSCN solution and observed under UV excitation. For  $\text{Fe}^{2+}$  test (De Lavison 1910, Soukup *et al.* 2002, Ranathunge *et al.* 2005a, Meyer *et al.* 2009), roots were immersed in 0.5 - 25 mM  $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ , shortly washed with tap water, and sectioned. The sections were treated with 1 % (m/v)  $\text{K}_4\text{Fe}(\text{CN})_6$  in 0.5 % (m/m) HCl for 15 min to localise ferric ions as blue insoluble  $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$  and mounted in 50 % (v/v) glycerol. For  $\text{H}_5\text{IO}_6$  test, modified PAS reaction including reducing rinse was used and it is

therefore abbreviated as PARS reaction (Pearse 1968, Soukup *et al.* 2002, Soukup 2014), roots were incubated in a 0.02 - 0.1 % (m/v)  $\text{H}_5\text{IO}_6$  solution, than shortly washed with tap water, and transferred into a reducing solution for the same time period as the previous incubation step to eliminate residues of  $\text{H}_5\text{IO}_6$ . The hand-sections were stained with Schiff's solution for 10 min and washed in  $\text{SO}_2$  water ( $3 \times 10$  min). The reducing solution, Schiff's reagent, and  $\text{SO}_2$  water were prepared exactly according to Soukup (2014). For PTS test (Cholewa and Peterson 2001, Faiyue *et al.* 2010), roots were immersed in 0.1 % (m/v) PTS. The roots were than rinsed with running tap water for 6 s. This washing step was precisely standardised for all analysed samples. The root segments were than mounted in 50 % (v/v) glycerol as whole-mounts and observed under blue or UV excitation.

**Lanoline sealed segments:** Segments (2 cm) of maize primary roots were taken at  $\frac{3}{4}$  of their total lengths from the tip. The section planes were sealed with lanoline and immersed into a given tracer solution for 30 min. Afterwards, the segments were processed similarly as incubated intact root systems to localise the tracer with the tissues.

**Tracer interference with membranes:** Tracer

interference with membrane integrity was tested on the tobacco cell culture. The tobacco callus was resuspended in 1  $\text{cm}^3$  of tap water immediately prior to incubation in tracer solutions. Following tracer solutions were tested: 0.05 % (m/v) berberine hemisulphate, 0.5 - 25 mM  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 - 0.1 % (m/v)  $\text{H}_5\text{IO}_6$ , and 0.1 % (m/v) PTS. The tracers were applied for 30 min and then viability tests were performed by a short incubation in 0.5 % (m/v) trypan blue or 15  $\mu\text{M}$  propidium iodide. Propidium iodide was observed under green excitation.

**Anatomical analyses and microscopy:** Tracer localisation within root tissues was detected as mentioned above. Primary roots were sectioned at  $\frac{3}{4}$  of their total lengths from the tip. At least three sections from three plants in selected positions were analysed per treatment. In addition, the presence of exodermal Casparian bands was detected with berberine hemisulfate (0.1 % (m/v), 1 h) (Brundrett *et al.* 1988) counterstained with Crystal Violet (0.05 % (m/v), 10 min). Suberin lamellae were detected with Sudan Red 7B (0.01 % (m/v), 1 h, Brundrett *et al.* 1991). The sections were mounted in 65 % (v/v) glycerol. The documentation system involved *Olympus BX51* (*Olympus*, Tokyo, Japan) and a microscope (*UV Olympus U-MWU*, *U-MWB*, and *U-MWG* filter blocks) with digital camera *Apogee U4000* (*Apogee Imaging Systems*, Roseville, CA, USA).

## Results and discussion

An important source of doubts related with apoplastic tracer assays relies on the fact that different apoplastic tracers do not always gain similar results for the same analysed plant material. To summarise possible sources of flaws and analyse their significance, we compared four principal representatives of commonly used probe categories: small inorganic tracers with a positive ( $\text{Fe}^{2+}$ ) or negative ( $\text{H}_5\text{IO}_6$ ) charge and bigger organic molecules with a high (berberine hemisulphate) or low (PTS) affinity to cell wall matrix. We have applied selected tracers on *Z. mays* with uniseriate exodermis (Fig. 1A,B) and *O. sativa* with exodermis and underlying sclerenchyma (Fig. 1C,D). We have analysed their movement within root tissues and checked the reliability of gained results. In *Z. mays*,  $\text{Fe}^{2+}$ ,  $\text{H}_5\text{IO}_6$ , and berberine pervaded established exodermis with Casparian bands and suberin lamellae in 30 min assay (Fig. 1E,I,M), reaching a deeper cortical layer in 60 min assay with  $\text{Fe}^{2+}$  moving slightly faster compared to other tracers (Fig. 1F,J,N). In *O. sativa*, no tracer pervaded exodermis in 30 min assay (data not shown). In 60 min assay,  $\text{H}_5\text{IO}_6$  was the only tracer passing across rice exodermis (Fig. 1G,K,Q). A proper detection of PTS in the sections was not possible in both species, which corresponds to its high solubility, low affinity to cell wall matrix, and strong autofluorescence background of commelinoid cell walls interfering with the proper localisation of this probe.

These results clearly indicate that  $\text{Fe}^{2+}$ ,  $\text{H}_5\text{IO}_6$ , and berberine are capable of proving the species-specific differences in tightness of the exodermal/hypodermal layer in intact transpiring plants, which corresponds to root anatomical structure. The observed differences in results gained by the different probes on the same material (a slightly faster movement of  $\text{Fe}^{2+}$  compared to berberine and  $\text{H}_5\text{IO}_6$  in maize or the same for  $\text{H}_5\text{IO}_6$  in rice) can be partially explained by a general variability of root structure but indicates also interference with plant tissue, *e.g.*, some toxicity and membrane disruption. Periodic acid is an oxidant and its adverse effect on cell membranes should be expected. Some toxicity was demonstrated also for  $\text{Fe}^{2+}$  (De Lavison 1910, Soukup *et al.* 2002, Ranathunge *et al.* 2005a, Meyer *et al.* 2009). Application of  $\text{Fe}^{2+}$  to older main root segments of *Iris germanica* changes the pattern of subsequent berberine penetration. While berberine does not pass behind the multiple exodermal layers in  $\text{Fe}^{2+}$  untreated root segments, it reaches the middle cortex in segments previously treated with 0.5 mM  $\text{FeSO}_4$  (Meyer *et al.* 2009).

The extent of toxicity of the selected tracers was tested using the tobacco cell culture (VBI-0 line). The membrane continuity tests with trypan blue (0.5 % (m/v)) or propidium iodide (15  $\mu\text{M}$ ) did not show any significant membrane disruption caused by 30 min incubation in 10 - 25 mM  $\text{FeSO}_4$ , 0.02 %  $\text{H}_5\text{IO}_6$ , 0.05 % berberine, or

0.1 % PTS solutions (Fig. 2A,B,E-G). In contrast, 0.1 %  $H_5IO_6$  decreased membrane integrity (Fig. 2C,D). Interestingly, 0.1 %  $H_5IO_6$  does not get over the surface of *Phragmites* roots even after 4 h (Soukup *et al.* 2002, 2007). A stronger exodermal barrier of this wetland species could probably prevent plasma membrane exposition and its disruption. So generally,  $H_5IO_6$  seems to be a valuable estimation of root surface permeability,

but the tracer concentration and application time have to be chosen carefully according to toxicity tests for a particular plant material (Ranathunge *et al.* 2005a, Meyer *et al.* 2009).

Further explanation of not fully comparable results gained by different probes lies in their diffusion rate within “unmodified” cell walls. In non-exodermal roots of *Pisum sativum* and *Vicia faba*, most of the berberine

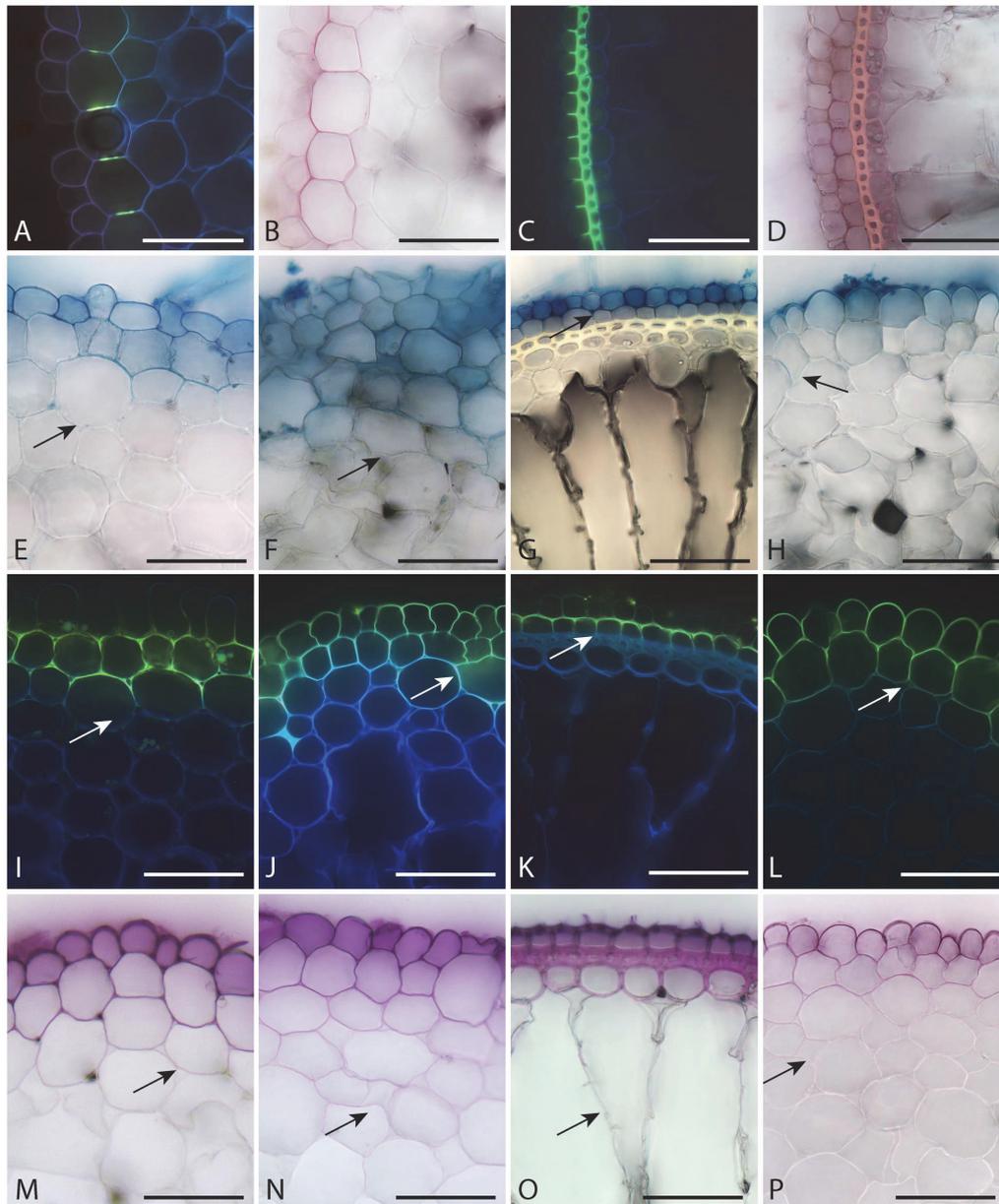


Fig. 1. Cross sections of main roots treated with variable apoplastic tracers. A, C - Uniseriate exodermis with Casparian bands (CBs) in *Zea mays* (A) and exodermis plus underlying sclerenchyma in *Oryza sativa* (C) detected with berberine hemisulphate (0.1 %, m/v, 1 h) and Crystal Violet counterstaining (0.05 %, m/v, 10 min). B, D - Suberin lamellae in exodermis of maize (B) and rice (D) stained with Sudan Red 7 B (0.01 %, m/v, 1 h). E-H -  $Fe^{2+}$  test. I-L - Berberine test. M-P - Periodic acid (PARS) test. E-G, I-K, M-O - Roots of intact plants incubated in the tracer solutions. In maize, all the probes passed through the exodermis in 30 min (E,I,M) and reached almost a half of the cortex in 60 min (F,J,N). In rice,  $Fe^{2+}$  and berberine did not pass through the exodermis even in 60 min test (G,K), but  $H_5IO_6$  did (O). H,L,P - Lanoline sealed segments of maize main roots incubated in the tracer solutions for 30 min. The arrows indicate the position reached by the tracer. Bright field (B,D, E-H, M-P) and U-MWU filter block (A,C, I-L). All the sections were taken in the basal root zone - at  $\frac{3}{4}$  of the total length (from the tip). The scale bars = 50  $\mu m$ .

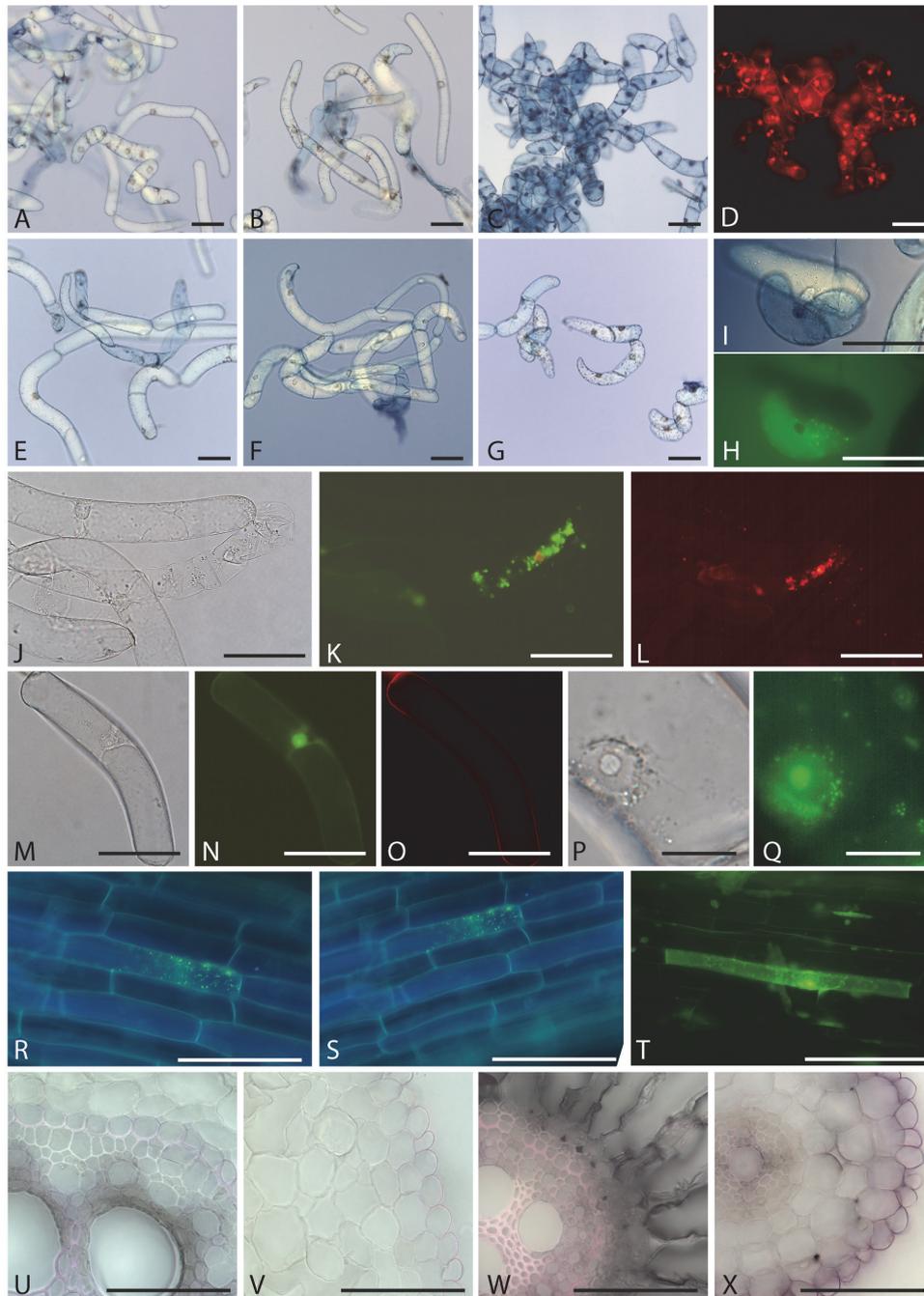


Fig. 2. Toxicity tests and tracer cellular uptake. *A-H* - Toxicity tests on a tobacco cell culture (VBI-0) using tryptan blue (TB; 0.5 %, m/v, *A-C*, *E-H*) or propidium iodide (PI; 15  $\mu$ M, *D*). Accumulation of TB indicates only some non-viable cells after 30 min incubation in distilled water (*A*), 0.02 % (m/v)  $H_5IO_6$  (*B*), 25 mM  $Fe^{2+}$  (*E*), 0.05 % (m/v) berberine (*F*), and 0.1 % (m/v) trisodium 3-hydroxy-5,8,10-pyrenetrisulfonate (PTS) (*G*). *C, D* - The complete loss of viability triggered by 0.1 %  $H_5IO_6$  – all cells accumulate TB (*C*) or PI (*D*). *H-L* - PTS was preferentially accumulated in non-viable tobacco cells after 30 min incubation in a 0.1 % PTS solution. The PTS (*I, K*) accumulated in cells with TB (*H*) or PI (*L*) staining, those cells frequently show a visible loss of integrity (*J*). *M-Q* - Berberine accumulated preferentially in viable cells (*N*) showing a negative nuclear signal of PI (*O*). A detail of berberine localisation in the nuclear region and cytoplasm (*P, Q*). *R, S* - A detail of berberine localisation in the maize rhizodermis (*R*) including emerging root hairs (*S*) in 30 min 0.05 % (m/v) berberine test. *T* - Localization of PTS in the maize rhizodermis in 30 min (0.1 %, m/v, PTS test). *U-W* - False positive staining (purple) by Schiff's reagent without preceding  $H_5IO_6$  treatment in the endodermis and stele of maize (*U*) and rice (*W*) and in the rhizodermis and exodermis of maize (*V*). *X* - Weak positive staining (purple) of the middle cortex in a tiny lateral root of maize after 4 h of incubation in a 0.02 % (m/v)  $H_5IO_6$  solution and subsequent detection by Schiff's reagent. Bright field (*A-C*, *E-H*, *M, P*, *U-X*), *U-MWG* filter block (*D, L, O*), *U-MWB* filter block (*I, K, T*), and *U-MWU* filter block (*N, Q*, *R-S*). The scale bars = 100  $\mu$ m, except *P, Q* which are 20  $\mu$ m.

precipitates are observed just in the surface area even after 60 min (Wilson and Peterson 1983). The authors claim that the presence of ferrulic acid and phenolic compounds in cortical cell walls bind berberine and restrict its further penetration. A controlled damage of peripheral tissues of the older part of the primary root of species with exodermis also allowed precipitated

berberine to move only several layers from the damage, and berberine did not reach endodermis after 60 min. This indicates that the diffusion rate within primary cell walls of cortical tissues should be taken into account and related to differences in composition and thickness of cell walls or their porosity. Noticeable differences in berberine penetration rate through the cortex were

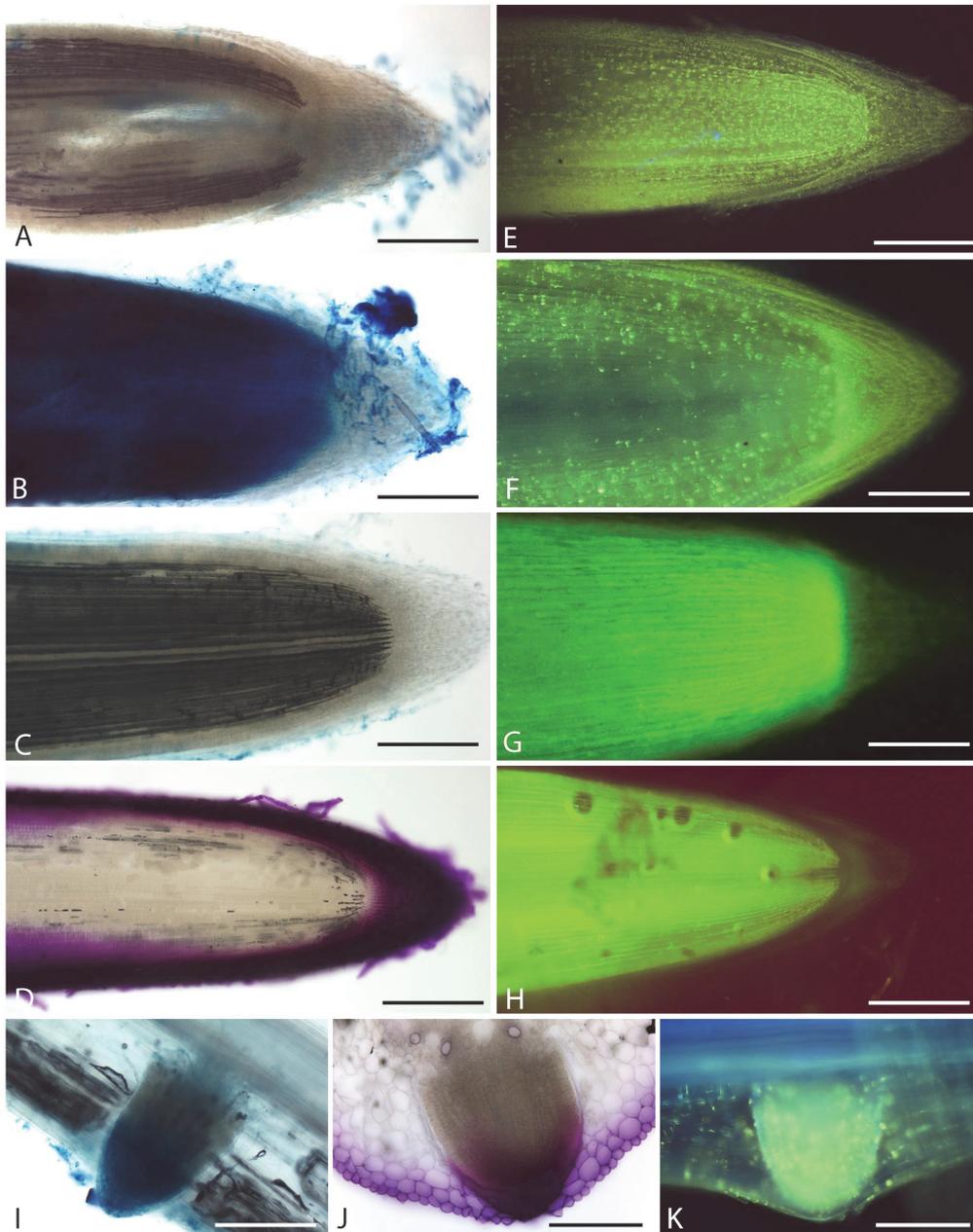


Fig. 3. Tracer tests on main root apices and emerging primordia. *A-C* -  $\text{Fe}^{2+}$  ions did not penetrate main root tips of maize in 30 min (*A*) and rice in 60 min (*C*) incubation but reached inner tissues (not the root cap) of maize in 60 min incubation in a 25 mM  $\text{Fe}^{2+}$  solution (*B*). *D* -  $\text{H}_3\text{IO}_6$  reached deeper tissues including the whole root cap of rice during 30 min incubation in a 0.1 %  $\text{H}_3\text{IO}_6$  solution. *E, F* - Berberine precipitates are present in the whole root apex in maize (*E*) and rice (*F*) after 60 min incubation in a 0.05 % berberine solution and subsequent precipitation with KSCN. *G, H* - PTS stayed mainly in inner root tip tissues, but not in the root cap of maize (*G*) and rice (*H*) after 30 - 120 min incubation in a 0.1 % PTS solution and subsequent 6 s wash with tap water. *I-K* - Permeability of emerging primordia using 25 mM  $\text{Fe}^{2+}$  (*J*), 0.1 % (m/v)  $\text{H}_3\text{IO}_6$  (*J*), and 0.05 % (m/v) berberine (*K*). Bright field (*A-D*), *U-MWB* filter block (*G-H*), and *U-MWU* filter block (*E-F, K*). The scale bars = 200  $\mu\text{m}$ .

recorded also between dicots and monocots (Aloni *et al.* 1998).

Tissue specific permeability independent of the presence of apoplastic barriers occurs also in root apices and emerging primordia. An apical part in the vicinity of the meristem is not fully permeable for tracers (Enstone and Peterson 1992) although lignification and suberization of cell walls take part later in some distance from the tip. Smaller intermicrofibrillar spaces within cell walls and different composition of matrix polysaccharides contrary to the older parts or mucigel secretion are among feasible explanations (Enstone and Peterson 1992). A previous work on *Phragmites* (Soukup *et al.* 2002) showed a different behaviour of some tracers in the root apex. The depth of penetration depends on the position relative to the meristem, but generally,  $\text{Fe}^{2+}$  ions are restricted to the surface layers, however,  $\text{H}_5\text{IO}_6$  and precipitated berberine stain the whole tip in 60 min assay (Soukup *et al.* 2002). Ions of  $\text{Fe}^{2+}$  were also obviously restricted in the root apex, especially in the root cap in *Z. mays* (Fig. 3A,B) and *O. sativa* (Fig. 3C). This is consistent with results from  $\text{CuSO}_4$  application to the root apex of *Z. mays* (Ranathunge *et al.* 2005b). No staining the stele in the very apex and root cap were also showed for *Iris germanica* in 0.5 mM  $\text{Fe}^{2+}$  test (Meyer *et al.* 2009). We can speculate about a strong affinity of ions to the surface of root tips where cell walls are rich of pectins.

In contrast, berberine precipitates are repeatedly detected in the root apex of *Z. mays*, *Pisum sativum*, *Vicia faba*, *Allium cepa*, or *Iris germanica* (Enstone and Peterson 1992, Meyer *et al.* 2009). These precipitates were well retained in the apex (Fig. 3E,F), probably because of a smaller cell size and less porous cell walls. Similarly,  $\text{H}_5\text{IO}_6$  was able to penetrate the root cap (Fig. 3D). Similar differences were found in emerging primordia that were fully-penetrated with berberine (Fig. 3K) but not with  $\text{H}_5\text{IO}_6$  or  $\text{Fe}^{2+}$  (Fig. 3I,J).

The PTS, a highly mobile probe, seems to have a low rate of penetration into the root apex in some studies (Peterson and Edgington 1975, Cholewa and Peterson 2001). In our hands, the various combinations of exposure time and following washing in maize/rice had no effect on the results. The PTS was obviously washed out very rapidly from older tissues so we could not properly distinguish between stained tissues and background (data not shown). Contrarily to that, PTS was retained in the very tip but not root cap of *Z. mays* and *O. sativa* (Fig. 3G,H). A similar staining pattern was recorded for *Allium cepa* (Peterson and Edgington 1975), and *Agave* roots (North and Nobel 1995).

Root tissues contain substances that may interfere with probe detection causing a false positive signal. It highlights the necessity of a proper negative control. In the case of  $\text{H}_5\text{IO}_6$  permeability test, the central cylinder containing polyaromatic substances (lignin) might show positive staining with Schiff's reagent without any preceding  $\text{H}_5\text{IO}_6$  application and in spite of proper handling of sections. Such a false positive response that

changes with root ontogeny may occur around the late metaxylem vessels, in pericycle, and endodermis (Fig. 2U,W). Some positive staining might occur also in the rhizodermis and exodermis (Fig. 2V).

A possible false negative tracer location is commonly related to washing out highly mobile probes (*e.g.*, PTS). Similarly, berberine precipitates could be leached out during handling sections, particularly in root segments with higher tissue porosity and thin cell walls. Detection of berberine seems to be related to tissue properties and the size of the precipitate, which depends on its concentration and the period of exposure (Enstone and Peterson 1992, Meyer *et al.* 2009). Some underestimation of tissue permeability may also be related to usage of a less concentrated  $\text{H}_5\text{IO}_6$ . We found that 0.02 % (m/v)  $\text{H}_5\text{IO}_6$  almost failed to reach the middle cortex even in maize tiny lateral roots without differentiated exodermis in spite of a prolonged incubation (up to 4 h, Fig. 2X). Such a solution is less harmful to membranes, but the possibility of  $\text{H}_5\text{IO}_6$  exhaustion on its way through the tissue should be taken into account.

From its broad definition, an apoplastic tracer should mimic the apoplastic transport route within root tissues, but this is not always fully accomplished, and tracers are partially taken up by cells. The fluid-phase endocytosis of PTS was demonstrated in *Allium cepa* (Cholewa and Peterson 2001). Berberine was observed in the protoplast of *Phragmites australis* after an extended time of treatment duration (Soukup *et al.* 2002) as well as in some rhizodermal cells of *Z. mays* in our 30 min assay (Fig. 2R,S). This does not necessarily mean plasma membrane destruction because plant alkaloids may present substrates of multidrug resistance pumps as was described for bacterial membranes (Severina *et al.* 2001). Using the tobacco cell culture, we observed berberine accumulation more frequently in cells that subsequently did not accumulate trypan blue or propidium iodide in the viability tests (Fig. 2 M-O, P,Q). The intracellular localisation of PTS was also observed in some rhizodermal cells in 30 min assay (Fig. 2T). Based on tobacco cells subjected to a similar PTS test, such accumulation seems rather be present in non-viable cells with some loss of membrane integrity (Fig. 2H-L).

A tracer application method is another factor influencing the pattern of gained results (Aloni *et al.* 1998). Tracers can be applied to the root system without shoots or only to excised root segments. Sticky wax or wool grease (lanolin) are successfully used to dip blotted cut ends of segments and seal them before tracer application (Aloni *et al.* 1998, Soukup *et al.* 2002). In this case, penetration is driven almost exclusively by diffusion. In contrast, the incubation of the whole root system of intact plants in the aqueous solution of a selected tracer allows transpiration to drive mass-flow of water, which may enhance tracer penetration into and through plants as was described with berberine (Aloni *et al.* 1998). To check the effect of different application procedures, we compared the permeability of lanoline-sealed primary root segments with the permeability of

incubated intact *Z. mays* plants. In 30 min assay, 0.1 %  $\text{H}_3\text{IO}_6$  pervaded the exodermis in the lanoline-sealed segments (Fig. 1P) reaching a very similar position as in the incubated intact plants (Fig. 1M). In contrast, 25 mM  $\text{Fe}^{2+}$  and 0.05 % berberine was stopped by the exodermis (Fig. 1H, L) and penetrated into the cortex only in the sites of damaged surface layers in the lanoline-sealed

segments, whereas it pervaded the exodermis in the incubated intact plants (Fig. 1E, J). The berberine and  $\text{Fe}^{2+}$  tests thus seem more sensitive compared to 0.1 %  $\text{H}_3\text{IO}_6$  to highlight differences in tracer movement within root tissues of transpiring plants and non-transpiring (lanoline-sealed) segments.

## Conclusions

Taking together available data, the tracer approach is a valuable tool for analysing species-specific differences in root permeability related to anatomical structure of the outer cortex (dicots/monocots, non/creating the constitutively developed exodermis, or stressed/unstressed plants). Some sources of method inaccuracy, however, exist and have to be taken into account. At first, the tracer mimics apoplastic transport route only partially, in relation to its chemical characteristics. An advantage can be seen in using relatively small inorganic tracers ( $\text{Fe}^{2+}$ ,  $\text{H}_3\text{IO}_6$ ) that copy the movement of solutes better than high molecular-mass organic tracers. The tracer concentration and duration of exposure should be tested

for a particular plant material as toxicity, interference with the membrane, or cellular uptake may affect the results. A proper subsequent visualisation and localisation of the tracer is a further critical step. A high autofluorescence background, especially in grasses, may interfere with the detection of Cellufluor and similar tracers under UV-excited fluorescence. The PTS or precipitates of berberine can be partially washed out during sample processing. Periodic acid may give a false positive response particularly in the stele or endodermis. We therefore highly recommend combining at least two different tracers and always preparing proper negative controls to get unbiased results.

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