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Review

Longitudinal distribution of apoplastic antioxidative components in maize root

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Summary. The apoplast is a liquid- and gas-filled extracellular continuum which includes cell wall polymer networks and the external surface of the plasma membrane. Apoplastic constituents, such as various organic molecules, enzymes and proteins play major roles in a wide range of physiological processes. In order to investigate apoplastic fluid, two isolation procedures were compared and critically evaluated: infiltration and/or centrifugation techniques to obtain Apoplastic Washing Fluid and filter paper strips which, based on our results, enable collection of experimental data from intact plants. Different components of the antioxidative system (enzymes, phenolics, sugars, organic acids) present in apoplastic fluid were analyzed using different techniques. Three classes of non-enzymatic compounds (organic acids, sugars and phenolics) have been identified and quantified by HPLC. Detection of hydroxyl radicals was performed by EPR methods using spin-trap DEPMPO, which is capable of forming different spin-adducts with hydroxyl and superoxide anion radicals. Spectrophotometrically estimated total protein concentrations, peroxidase and superoxide dismutase specific activities, as well as their different isoforms, were visually confirmed by isoelectric focusing. These high sensitivity techniques (HPLC-ECD, EPR), as well as electrophoresis, in combination with the filter strip method provided us with the tools necessary to study the components of the antioxidative system in the apoplast of developing plant organs and their spatial-temporal changes. Such an experimental setup provided a powerful non-invasive analytical tool for studying metabolic processes occurring in the apoplast and local changes in small regions of intact root tissue.

Keywords: apoplastic fluid, EPR, HPLC, peroxidase, superoxide dismutase.

INTRODUCTION

The plant apoplast is a liquid- and gas-filled extracellular continuum which includes rigid cell wall polymer networks and the external surface of the plasma membrane. As a thermodynamically open system, it is in direct contact with the environment, thus determining the response of the whole plant to environmental conditions. The apoplast is involved, not only in the response, but also in the perception and transduction of environmental signals (Hoson 1998). In order to further clarify the role of the apoplast as a site of response to environmental signals, we must understand the metabolic processes in the apoplast as well as modifications of its content.

The major constituents of the apoplast, such as various organic molecules, enzymes and proteins, play a major role in a wide range of physiological processes in plants, including: growth regulation (González-Reyes et al. 1994; Cordoba-Pedregosa et al. 1996), plant-pathogen interactions (Vanacker et al. 1998a; Jashni et al. 2015), as well as transport of water, nutrients and metabolites (Sattelmacher et al. 1998; Sattelmacher 2001). Metabolite concentrations in apoplastic solution are very low and not constant during sampling (Lohaus et al. 2001), so their collection as well as concomitant analysis requires the use of very sensitive techniques.

The most frequently used method for collecting apoplastic fluid is a vacuum infiltration/centrifugation technique, which is in most studies performed on leaf tissue. Contents

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of the leaf apoplast have been explored in detail in terms of low molecular weight compounds such as sugars (Ho and Baker 1982; Porter et al. 1985; Lohaus et al. 2001; Nadwodnik and Lohaus 2008), amino acids (Lohaus et al. 2001; Chikov and Bakirova 2004) and organic acids (Hoffland et al. 1992; Martinoia and Rentsch 1994; Neumann and Romheld 1999), as well as phenolic compounds (Takahama et al. 1999; Fecht-Christoffers et al. 2006; Dragišić Maksimović et al. 2008). As opposed to the leaf apoplastic fluid, the content of the root apoplastic fluid has not been studied in detail; only a few reports can be found in the literature (Cordoba-Pedregosa et al. 1996; Dragišić Maksimović et al. 2008).

Recently, a novel approach for analysis of different components of the antioxidative system (enzymes, reactive oxygen species, phenolics, sugars, organic acids) present in the roots of apoplastic fluid have been estimated (Dragišić Maksimović et al. 2014). Apoplastic fluid were isolated by a filter strip technique and compared to those of infiltrated/centrifugated samples (Dragišić Maksimović et al. 2008).

Filter strip techniques, in combination with sensitive analytical methods (HPLC-ECD, EPR spectroscopy), as well as spectrophotometry and electrophoreses, represent a tool to estimate root apoplastic concentrations of low molecular weight compounds, as well as enzymes and proteins. This is a very important issue, since it enables detailed study of antioxidative components in the root apoplast and their longitudinal distribution.

COLLECTION OF ROOT APOPLASTIC FLUID

In order to investigate content of apoplastic fluid, different isolation procedures have been developed over time, including: the use of radioactive tracers in leaf slices (Pitman et al. 1974), use of ion-specific fluorescent indicator dyes in sunflower leaves (Hoffmann et al. 1992) and micro- electrode probes to measure ion activities through open stomata (Hanstein and Felle 2002). Results are from a very small, specific leaf site in a broadly-dispersed compartment. This led to widespread use of infiltration and/or centrifugation technique to obtain Apoplastic Washing Fluid (AWF) (Mühling and Sattelmacher 1995; Vanacker et al. 1998b; Sgherri et al. 2007). It is a simple, quick and inexpensive method that can yield sufficient quantities of so-called AWF. This method is based on excision of plant tissue, infiltration of buffer under vacuum pressure into tissue and centrifugation under high centrifugal force (Fig. 1A). Besides several problems related to dilution by infiltration of buffer, such as changes of ionic composition, pH, metabolite concentration and chemical equilibrium existing in this compartment, the major disadvantage of this technique is the risk of contamination with cytosolic or vacuolar components during plant manipulation: contamination from injured cells at the cut surface on one side, while the centrifugal force applied in the procedure raises the risk of membrane rupture and cellular injury on the other. An additional disadvantage of the infiltration/centrifugation technique is that local changes in apoplastic solutes in small regions of the tissue cannot be detected (Lohaus et al. 2001).

The suitability of the infiltration-centrifugation method for collection of apoplastic fluid from leaves was critically evaluated (Grignon and Sentenac 1991; Dietz 1997; Lohaus et al. 2001; Clarkson 2006; Dragišić Maksimović et al. 2014). The physiological significance of this approach would be greatly increased if mechanical damages could be minimized. In order to achieve this, filter paper strips were used to collect root apoplastic fluid enabling experiments with intact plants, while simultaneously providing adequate quantities of fluid for analysis. The filter paper strips were originally employed for sample application during isoelectric focusing (Sample application pieces, 10 mm × 5 mm, Serva, Heidelberg, Germany) due to their high absorptive capacity (more than 120 ml cm^{-2}) and inert composition. They were placed onto the root surface at specific morphological regions: apical zone - I; middle zone - II and basal zone (mature) - III (Fig. 1B). This sampling technique was previously applied for determination of organic acids collected from rhizosphere soil solution (Neumann et al. 1999; Marschner et al. 2002). We extended its application to the component analysis of apoplastic antioxidative system (Dragišić Maksimović et al. 2008, 2014).

Contamination of apoplastic fluid with cytosolic components

As previously noted, a significant drawback of infiltration/centrifugation technique is the risk of apoplastic fluid contamination with cytosolic or vacuolar components. In

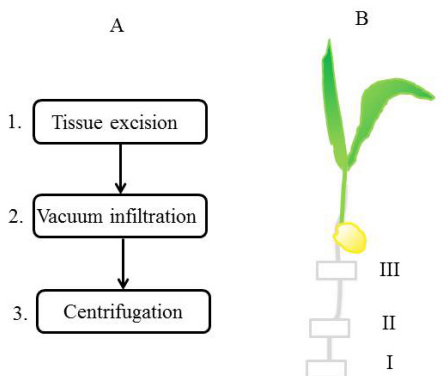

Fig. 1. Three morphological root regions: the apical zone (0-5 mm from tip), zone I; middle zone (10-15 mm from tip), zone II; basal zone (mature), 5 mm below lateral roots, zone III, used for sampling apoplastic fluid by infiltration/centrifugation (A) and filter strip (B) method.

order to assess the level of contamination, malate dehydrogenase (MDH) activity as the cytosolic marker enzyme (Husted and Schjoerring 1995), and the presence of intracellular metabolic pathway intermediate glucose-6-phosphate (G-6-P) (Burkey 1999) should be evaluated. According to Dannel and coworkers (1995), samples with MDH activity below 1.6% (relative to bulk tissue extracts) is considered to be free from cytoplasmic contamination. In previous studies, MDH activities were below the upper limit, indicating mild damage to the cells during extraction (Husted and Schjoerring 1995), whereas G-6-P was occasionally detected in AWF (Burkey 1999). Clearly, evidence of cellular damage invalidates the infiltration/centrifugation approach (Clarkson 2006). However, apoplastic fluid obtained using filter strips did not show any noticeable activity of neither MDH nor G-6-P, demonstrating that these isolates are completely free of cytoplasmic constituents (Dragišić Maksimović et al. 2008).

THE SPATIAL DISTRIBUTION OF LOW MOLECULAR WEIGHT COMPOUNDS

The overall functionality of a root system corresponds to independent physiological activities coordinated at the whole root system level and varying axially along single roots in relation to their age. This system leads to the formation of specific environments that differ significantly both chemically and biologically between root zones. The existence of a developmental gradient along the root makes it significant for investigation of biochemical processes associated with their growth. In roots, apical zone I could be considered to be a zone of intensive growth, and zones II and III correspond to sequentially older zones with decreased growth rates (Fig. 1B). It has been shown that the content of some compounds in the root apoplast may also differ depending on the distribution zone (Córdoba-Pedregosa et al. 2005; Dragišić Maksimović et al. 2008, 2014).

Three classes of non-enzymatic compounds have been identified and quantified in the root apoplast: organic acids, sugars and phenolics (Giaquinta 1977; Whetten and Sederoff 1995; Jones 1998; Dragišić Maksimović et al. 2014). The organic acid content of plants is governed primarily by their type of C fixation (e.g., CAM, C3 or C4), their nutritional status and age. The study of organic acid metabolism in the plant root is limited due to their constant exchange with soil solution, cellular compartmentation and unreliable methods for determining their actual concentration in the apoplastic space. Typically, the total concentration of organic acids in roots is around 10-20 mM (Jones 1998), while data for organic acid concentrations in root apoplast are scarce. A gradient in the specific activity of the organic acids was established along the root of *Brassica napus, Sysimbrium officinale* (Hoffland et al. 1992) and *Zea mays* (Jones 1998).

The concentration of total sugars present in the root cells of maize is ~ 90 mM and with a spatial distribution showing decreasing levels with increasing distance from the root tip (Jones and Darrah 1995). Sugars were shown to be more than adequately collected in measureable quantities from the root apoplast, in the range of 1-300 mM, depending on the technique used for apoplastic fluid isolation (Dragišić Maksimović et al. 2014).

There are numerous data describing the phenolic profiles of root homogenates, plant root exudates and leaf apoplasts of different plant species (Juszczuk et al. 2004; Dragišić Maksimović et al. 2007; Führs et al. 2009). However, there are only a few publications concerning the qualitative and quantitative analysis of specific phenolic compounds in the root apoplast during growth (Dragišić Maksimović et al. 2008, 2014). These studies revealed that phenolics also varied both spatially and temporally in the apoplast along the root, where higher concentrations were observed in the mature zone (III).

The concentrations of these three major classes of small weight metabolites varied according to the method applied for apoplastic fluid isolation (Table 1). The concentrations of the detected compounds were one to two fold higher in apoplastic fluid collected with filter strips, indicating dilution of the AWF samples caused by the infiltration procedure. In contrast, AWF samples were qualitatively richer in terms of their constituents, indicating that AWF is a more complex matrix than apoplastic fluid obtained by filter strips (Fig. 2). For example, 11 sugar compounds were identified in AWF samples, while only 4 (galactose, glucose, fructose and sucrose) were detected in filter strip samples. These results suggest contamination of AWF samples due to intracellular leakage caused by the vigorous isolation procedure.

ENZYMES

Apoplastic enzymes and reactive oxygen species regulate the growth of higher plants in several different ways associated with cell elongation processes, but also through reactions that restrict growth. Peroxidases (POD) can oxidize various phenolic substrates in the presence of H_2O_2-producing polymeric products, such as lignin and suberin (Quiroga et al. 2000) or crosslinking wall polymers leading to cell wall stiffening and inhibition of elongation (Fry 1986). In onion root apoplasts, POD activity and electrophoretic analysis of apoplastic fluid showed differential peroxidase patterns depending on the root zone, suggesting the presence of different peroxidase proteins in the apoplast along the root axis (Córdoba-Pedregosa et al. 2005). In maize roots, free extracellular peroxidase activities increased in segments with decreased growth, which correlates with lignin synthesis during tissue maturation (Dragišić Maksimović et al. 2008).

Table 1. Concentration of representative organic acids, sugars and phenolic compounds in apoplastic fluid of maize root obtained by infiltration/centrifugation (AWF) and filter strip method (AF) detected by HPLC, from Dragišić Maksimović et al. (2014). nd- not detected.

	Compound	AWF (μM)	AF (μM)
Organic acids	Oxalic acid	5.75 ± 0.73	nd
	Citric acid	nd	11.96 ± 4.81
	Malic acid	1.13 ± 0.34	26.67 ± 8.96
	Succinic acid	287.51 ± 8.55	276.07 ± 6.17
Sugars	Glucose	365.65 ± 24.60	144.67 ± 21.95
	Fructose	263.31 ± 66.05	40.63 ± 1.17
	Sucrose	2.76 ± 0.99	3.93 ± 0.83
Phenolics	Chlorogenic acid	0.008 ± 0.003	0.45 ± 0.03
	Caffeic acid	0.006 ± 0.002	0.52 ± 0.03
	Coniferyl alcohol	1.471 ± 0.447	140.71 ± 19.85
	p-Coumaric acid	0.013 ± 0.003	0.25 ± 0.03
	Isoferulic acid	0.299 ± 0.091	4.85 ± 1.19

POD activities were significantly higher in apoplastic fluid collected by filter strips, consistent with dilution of the AWF samples due to leakage from intracellular compartments during centrifugation. Using isoelectric focusing, increased intensities of acidic POD isoforms were found toward the root base, independent of the method used for apoplastic fluid isolation (Fig. 3A).

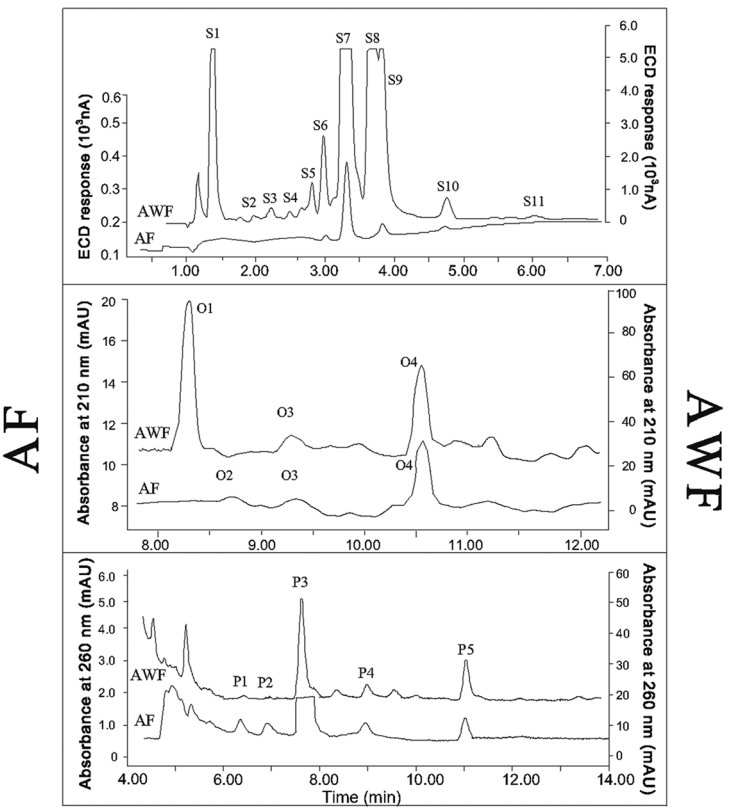

Fig. 2. Representative HPLC chromatograms of apoplastic fluids obtained by infiltration/centrifugation (AWF) and filter strip methods (AF), showing differences among three major classes of small weight metabolites; from Dragišić Maksimović et al. (2014). Inset with S marked compounds is PAD chromatograms of sugars: S1 polyols, S2 rhamnose, S3 trechalose, S4 arabinose, S5 mannose, S6 galactose, S7 glucose, S8 fructose, S9 ribose, S10 sucrose, S11 raffinose. Inset with O marked compounds represents UV chromatograms of organic acids: O1 oxalic, O2 citric, O3 malic and O4 succinic. Inset with P marked compounds represents UV chromatograms of phenolic compounds: P1 chlorogenic acid, P2 caffeic acid, P3 coniferyl alcohol, P4 *p*-Coumaric acid and P5 isoferulic acid. Note difference scaling for AWF (right ordinates) and AF (leftordinates).

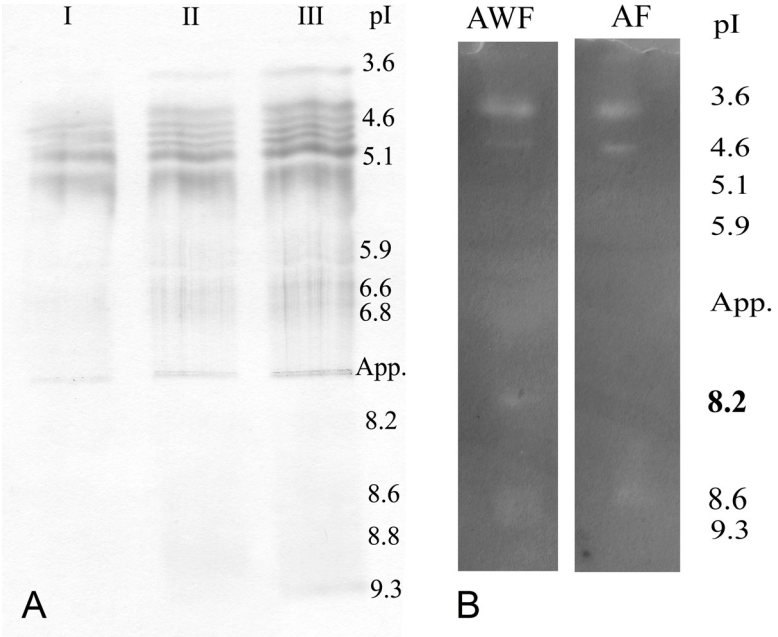

Fig. 3. Isoelectrofocusing (IEF) patterns of soluble proteins stained for peroxidase (A) and superoxide dismutase (B) activity from maize root apoplastic washing fluid obtained using different methods of extraction: AWF - buffer infiltration and centrifugation at 2000 × *g* and apoplastic fluid - AF obtained using filter strips. IEF was conducted using a pH gradient of 3–10. 10 μg protein was applied to each well. pI (marked bold) represents identification of MnSOD using KCN as inhibitor.

SODs also vary depending on the metabolic activity in each part of the root. Isoelectric focusing of both isolates differentiated anionic Cu/ZnSOD isoforms at a pI ranging from 3.6 to 5.1 and cationic isoforms at a pI from 8.6 to 9.3. However, MnSOD isoforms characteristic for the mitochondrial matrix (Fridovich 1986) were also unequivocally detected exclusively in AWF samples at pI 8.2 (Fig. 3B), again suggesting the presence of intracellular contamination.

REACTIVE OXYGEN SPECIES

Cell wall loosening during plant growth is considered to be a process involving reactive oxygen species (ROS) (Rodrìguez et al. 2002). For this process, ROS must be present in the apoplast, and apoplastic ROS accumulation has been shown in many plant tissues (Schopfer 1994). Evidence for •OH radical attack on cell wall polysaccharides support the hypothesis that ROS participate in wall softening during maturation (Fry et al. 2001). Spin trapping electron paramagnetic resonance (EPR) spectroscopy enables detection and quantitative estimation of *in vivo* •OH production in plant research (Müller et al. 2009; Dragišić Maksimović et al. 2012). Additionally, it is possible to localize the •OH production site in the root growth zone by varying the position of the intact root inside the resonator cavity of the EPR spectrometer (Renew et al. 2005). When the growing zone of the root was positioned at the sensitivity optimum, a much larger •OH signal was measured than for the non-growing zone. This technique has also been successfully used to detect apoplastic •OH. In fact, signal localization to the growing zone during seed germination was even possible (Müller et al. 2009). EPR spectroscopy performed with AWF from maize root (regardless of centrifugation speed and DEPMPO spin-trap concentration) gave no signal, while the DEPMPO/OH complex formed on filter strips (AF) was more stable even when diluted DEPMPO was used (Fig. 4). Thus, the filter strip technique in combination with EPR spectroscopy provides a tool to study *in vivo* production of •OH and other free radical species in apoplastic fluid at high resolution, even in small tissue samples, without interaction with cellular or fibrillar cell wall components.

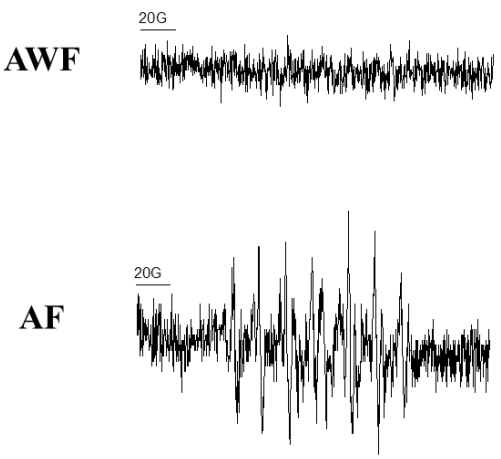

Fig. 4. EPR spectra of DEPMPO/OH adducts from apoplastic fluid collected with different AWF - buffer infiltration and centrifugation at 2000 × *g* and apoplastic fluid - AF obtained using filter strips.

In conclusion, there are several demonstrated advantages to using filter strip methods for apoplastic fluid collection from intact plant roots, including: the ability to provide small amounts of uncontaminated samples; much higher specific enzyme activities and metabolite concentrations; and gentler handling with intact plants where symplastic contamination due to tissue damages is avoided.

High sensitivity HPLC-ECD, EPR and electrophoretic techniques in combination with filter strips provide an applicable tool to study components of the antioxidative system in the apoplast of developing plant organs, as well as their spatial-temporal changes. It enables distinct concentration measurements of a wide-range of root metabolites in the apoplast, without pre-concentration or purification steps. Such an experimental setup provides a powerful, non-invasive analytical tool for studying metabolic processes occurring in the apoplast and local changes in restricted regions of the intact root tissue.

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