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Filter strip as a method of choice for apoplastic fluid extraction from maize roots

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ABSTRACT

Apoplastic fluid was extracted from maize (*Zea mays* L.) roots using two procedures: collection from the surface of intact plant roots by filter paper strips (AF) or vacuum infiltration and/or centrifugation from excised root segments (AWF). The content of cytoplasmic marker (glucose-6-phosphate, G-6-P) and antioxidative components (enzymes, organic acids, phenolics, sugars, ROS) were compared in the extracts. The results obtained demonstrate that AF was completely free of G-6-P, as opposed to AWF where the cytoplasmic constituent was detected even at mildest centrifugation ($200 \times g$). Isoelectric focusing of POD and SOD shows the presence of cytoplasmic isoforms in AWF, and HPLC of sugars and phenolics a much more complex composition of AWF, due to cytoplasmic contamination. Organic acid composition differed in the two extracts, much higher concentrations of malic acid being registered in AF, while oxalic acid due to intracellular contamination being present only in AWF. EPR spectroscopy of DEPMPO spin trap in the extracts showed persistent generation of hydroxyl radical adduct in AF. The results obtained argue in favor of the filter strip method for the root apoplastic fluid extraction, avoiding the problems of cytoplasmic contamination and dilution and enabling concentration measurements in minute regions of the root.

1. Introduction

The apoplast is a complex plant compartment, delimited from the symplast by the plasma membrane. It consists of the rigid cell wall fibrillar polymer network, the external surface of plasma membrane, and the liquid- and gas-filled spaces within this network, which provide interactions between the environment and the plasma membrane enclosed cytoplasm. All of these three components of the apoplast are rich in various organic molecules, enzymes and proteins attached, dissolved, or embedded in or to them.

The apoplast plays a major role in a wide range of physiological processes, including transport of water, nutrients and metabolites [1], gas exchange, growth regulation, plant-pathogen interactions, and perception and transduction of environmental signals [2]. As a thermodynamically open system, in direct contact with the environment, the root apoplast is the first to encounter varying and sometime adverse environmental conditions that are determining the response of the whole plant [3,4].

For each of the three apoplastic constituents specific isolation procedures have been developed, allowing a detailed in depth analysis of their composition, molecular organization, biochemistry and physiology. The application of perfusion and infiltration and/or centrifugation method initially developed by Söding and Klement [5,6], and further refined by numerous authors, is the most frequently used technique for the isolation of the apoplastic fluid. It is a simple, quick and inexpensive method that can yield sufficient quantities of the so called "Apoplastic Washing Fluid (AWF)". Besides the unknown dilution capacity, the infiltration of buffers or distilled water into the plant tissue alters the ionic composition,

Abbreviations: AF, apoplastic fluid; AWF, apoplastic washing fluid; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide; DEPMPO/OH, DEPMPO adduct with hydroxyl radical; EPR, spin trapping electron paramagnetic resonance; G-6-P, glucose-6-phosphate; IEF, isoelectric focusing; MDH, malate dehydrogenase; •OH, hydroxyl radical; PPB, potassium phosphate buffer; POD, peroxidase (EC 1.11.1.7); SOD, superoxide dismutase (EC 1.15.1.1).

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pH and metabolite concentration in the apoplastic fluid [7,8], and inevitably alters the chemical equilibrium existing in the apoplast [9]. Another drawback of the method is the inherent inability to accurately determine the physiological concentration of different metabolites and molecules present in the apoplastic fluid ex situ.

The major disadvantage of this technique is a risk of contamination with cytosolic or vacuolar components. The centrifugal force that is applied in the procedure raises the question of membrane rupture and cellular injury. Also, the excision of plant tissue results in inevitable contamination from injured cells at the cut surface. Furthermore, cell injury caused by mechanical stress leads to H_2O_2 production triggering biochemical and physiological 'cascades' which can disrupt physiological homeostasis [10]. This 'cascade' reaction shows that initially, a puzzling variety of responses follow from a single event. Thus, a simple signal, such as cell trauma, can generate a diversity of biochemical and physiological consequences, altering the status of the metabolites and biochemical reactions under study. The assessment of the cellular damage is usually performed by the use of specific markers characteristic for the symplast. Contamination of the apoplastic fluid has been quantified by comparing the activities of marker enzymes (e.g. malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase) or concentration of cytosolic metabolites (e.g. glucose-6-phosphate, G-6-P) in AWF with that of crude extracts [7,8,11], or as a function of the applied centrifugal force [12].

Composition of the apoplastic fluid is highly variable and depends on plant species, age, time of a day and nutritional status [11]. It should be emphasized that most of the studies on AWF have been performed on the leaf tissue. Content of the leaf apoplast has been explored in detail in terms of low molecular weight compounds such as sugars [11,13–15], amino acids [11,16] and organic acids [17–19], as well as phenolic compounds [20–22]. As opposed to the leaf apoplastic fluid, the content of the root apoplastic fluid has not been studied in detail, only a couple of reports being found in the literature [22–25].

The total volume of AWF (V_{inf}) was usually determined as the difference in fresh weight before and after infiltration. Due to the fact that infiltration of the apoplastic air space leads to a dilution of the apoplastic fluid, the solute concentrations in the apoplastic washing fluid were corrected by the ratio of the volume of infiltration solution (which corresponds to the volume of the apoplastic air space V_{air}) to the volume of the apoplastic water space (V_{water}). The ion concentration in the apoplast was calculated by multiplying the measured ion concentration in the apoplastic washing fluid by the dilution factor ($F_{dil} = (V_{air} + V_{water})/V_{water}$). V_{air} being determined by the silicone oil method [26] and V_{water} by the [^{14}C] sorbitol-labeled solution method [27] or using a plasma membrane impermeable dye (e.g. Indigo carmine) [28]. Blue dextran 2000 has also been recently used [29], based on the assumption that it does not enter the symplast. Thus, determination of the dilution requires additional demanding procedures for its evaluation [11] making the whole procedure complex and expensive.

In order to overcome the disadvantages of this most commonly applied infiltration and/or centrifugation technique, we used filter paper strips (hereinafter referred to filter strips) to collect root apoplastic fluid [22]. This sampling technique for organic compounds based on sorption media placed onto the root surface of plants was previously applied for determination of organic acids collected from rhizosphere soil solution [30,31]. The filter strip method, as a non-invasive technique for root apoplastic fluid collection allows experiments with intact plants. It is suitable for monitoring changes of metabolic compounds of the root apoplast, especially in hydroponically grown plants, avoiding any significant mechanical stress, implying that it is more reliable than the infiltration/centrifugation method for studying processes occurring in apoplastic compartment of the root, providing adequate quantities of the apoplastic fluid for analysis.

In the present work apoplastic fluid was collected from roots of hydroponically grown maize plants employing the two isolation techniques viz., filter strips from intact roots and infiltration and/or centrifugation from excised roots. Different components of the antioxidative system (enzymes, phenolics, ROS, sugars, organic acids) present in the apoplastic fluid were analyzed using HPLC for quantitative and qualitative determination of non-enzymatic components, electrophoretic visualization and specific activity of POD and SOD, and EPR spectroscopy of DEPMPO spin-trap for detection of oxygen-centered radicals. The results obtained by the two collection methods of the root apoplastic fluid were compared and critically evaluated.

2. Material and methods

2.1. Plant material and growth conditions

Maize (*Zea mays* L., inbred line Va35, Maize Research Institute "Zemun Polje", Serbia) seeds were surface sterilized in 10% hydrogen peroxide, washed in distilled water and germinated on double layer filter paper moistened with 0.5 mM $CaSO_4$ in darkness at 27 °C. Three days after sowing the seedlings were transferred to plastic pots containing 1/4-strength modified unbuffered nutrient solution [32] pH 5.9 and grown hydroponically with continual aeration of bathing solution around the roots under controlled conditions (light/dark 12 h/12 h; RH 70%; 25 °C) until the second leaf was fully developed. The nutrient solution was completely changed every second day in order to avoid root growth reduction and lateral root formation due to decreasing pH, as previously reported for maize root [33], and nutrient deficiencies.

2.2. Isolation of apoplastic washing fluid (AWF) by infiltration and/or centrifugation technique

Apoplastic washing fluid (AWF) was recovered from excised root segments according to a modified procedure previously described [25]. Briefly, apoplastic soluble components were obtained from maize root segments whose fresh weight was measured immediately before the extraction procedure of AWF. The following procedures were carried out at 4 °C to reduce evaporation and, thus, minimize changes in the composition of the AWF. Excised and blotted root segments were positioned with the cut ends facing down into a 10-ml plastic vessel located over a centrifuge tube and immediately after centrifugation at indicated speeds (200, 500, 1000 and 2000 × *g* denoted as CF_{200}, CF_{500}, CF_{1000} and CF_{2000}, respectively) for 15 min, while those denoted as IC_{2000} were first vacuum infiltrated in 50 mM potassium phosphate buffer (PPB) pH 5.5 and then centrifuged at 2000 × *g* for 15 min too. Excised root segments denoted as RIC_{2000} were kept in continuously aerated 1/4-strength nutrient solution to recover from wounding for 90 min in a growth chamber. Then, the segments were washed in aforementioned PPB, wiped with filter paper and vacuum infiltrated with the same buffer in a vacuum desiccator, reducing the pressure to -45 kPa, followed by slow relaxation to atmospheric pressure. Infiltrated segments were wiped and put into plastic vessel, and instantly centrifuged at 2000 × *g* for 15 min.

2.3. Collection of root apoplastic fluid (AF) by filter paper strips

For collection of fluid present in the roots apoplastic space, small paper strips were placed on the top surface of the root previously blotted with filter paper to remove any remaining liquid on the root surface after removing the plants from the hydroponic medium. The fluid was extracted from the roots using