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# An internal standard technique for improved quantitative analysis of apoplastic metabolites in tomato leaves

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ABSTRACT

The plant leaf apoplast is one of the first lines of defense against many foliar pathogens. The aqueous layer lining the airspace within leaves is enriched with secondary metabolites that can serve many roles including protection against environmental hazards, both biotic and abiotic. The constituents and their concentration change as the leaf matures or undergoes stress. To monitor and quantify changes in these metabolites during pathogen stress, we needed a more sensitive technique. We were able to modify the infiltration-centrifugation technique to use smaller samples, individual tomato leaflets, plus use an internal standard which indicated the amount of dilution in each sample. Dinotefuran, a neonicotinoid used as a systemic pesticide, proved to be resistant to the redox environment of the apoplast and had similar chemical properties allowing it to be analyzed under the same UPLC conditions as the other phenolic metabolites. Water soluble metabolites on the leaf surface were found to be a major source of contamination that could be avoided by rinsing leaves with water prior to infiltration. The improved sample efficiency and accuracy provided by this technique, along with the use of Dinotefuran as an internal standard, will provide the sensitivity needed to monitor apoplast metabolites during pathogen stress.

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## 1. Introduction

Leaves constantly respond to their environment by integrating signals received from both the symplast and apoplast. The symplast can be viewed as an intracellular continuum of the highly regulated cellular cytoplasm which is interconnected via plasmodesmata and bound by a cellular membrane. The apoplast comprises the extracellular continuum of aqueous space outside the cell membrane that includes cell walls and interstitial spaces. The apoplast surrounding the airspace within the leaf is the site of interaction between the plant and its environment – including gas exchange for growth, abiotic stresses such as ozone, or biotic stressors including pathogens [6,20,28]. The apoplast is able to accommodate a highly dynamic and complex environment that would not be tolerable in the symplast. This includes the ability of the plant to respond in some situations by producing toxic metabolites or conditions, such as phytoalexins or the oxidative burst to defend against pathogens [8,9,28].

One of the unique aspects of plants is their ability to produce a broad variety of secondary metabolites, many of which can be detected in the apoplast during different phases of growth or stress [12,15,26]. Known roles for these metabolites include phytoalexins for disease defense, lignin production for structural strength, and antioxidants to counter pro-oxidants produced during pathogen or environmental stresses [8]. Another role for some metabolites may be their bioactive influence on interactions. Many plant phenolics have been found to have a wide range of bioactive effects in animals [19,21,25], which has led to a major effort to search for phenolics with bioactive properties. There are also several examples of a bioactive role for phenolics in plants [15,18,22,31], and there is a growing awareness that phenolics can have bioactive roles in plants also.

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We have been interested in the change in the relative composition of apoplastic phenolic metabolites following pathogen stress [2,3,5]. We found that the relative composition of apoplast metabolites changed with leaf development or as a viroid moved through the symplast of the plant. The vacuum infiltration-centrifugation technique we had used for leaves provided good relative data requiring several grams of tissue.

Further investigation required a semi-quantitative technique that was relatively rapid and used smaller amounts of tissue for increased sampling. We had several concerns about contaminates from the symplast, leaf surface (including pesticides) or trichomes [29] that could be confused with the apoplast phenolics. We wanted the ability to compare phenolic profiles within a plant or leaf, so dilution of the apoplast phenolics by the infiltration fluid needed to be addressed. Addition of an internal standard would help but it could not interact with the apoplast physiology and had to be analyzed along with the apoplast phenolics [11].

Here we describe the modified technique for analyzing apoplast phenolics. In addition to describing the use of an internal standard we also describe problems that were overcome and improvements that allowed smaller tissue samples to be used. We have made modifications specific for the greenhouse tomato leaves but the basic steps could be applied to most leaves.

## 2. Materials and methods

### 2.1. Plant material

Tomato plants (*Solanum lycopersicum*) *cv* Moneymaker were grown under greenhouse conditions at 27 °C with a 14 h photoperiod supplemented by High Density Discharge lamps. Plants were seeded in Fafard Super Fine Germinating Mix (Conrad Fafard, Inc., Agawam, MA), transplanted 1 week later into 4 inch pots. Plants were transplanted again after 3 weeks into 1 gallon pots containing 50/50 Fafard Super Fine Germinating Mix and ProMix BF(Premeir Tech Horticulture, Quakertown, PA). When plants were transplanted, Scotts Osmocote Plus (15-9-12) Controlled Release Fertilizer (Scotts Company, Marysville, OH) was added to the potting mix. Plants were routinely used for experiments at approximately 6 weeks of age.

### 2.2. Apoplast fluid extraction and preparation

Plants were watered 30 min prior to experimentation to help ensure that all plants were of similar turgor. Leaflets were cut from the leaf and weighed immediately, then dipped in deionized water to remove any surface/trichome contaminates. Vacuum infiltration of the leaflets was carried out in a 250 ml side-arm flask containing an internal standard, 50 μM Dinotefuran, (Sigma, St. Louis MO: see structure Fig. 3A) in deionized water. The vacuum was released and reapplied successively 4–8 times in an attempt to reach 90–100% infiltration, which was estimated by the dark green color of the saturated tissue. Following infiltration the leaflets were again dipped in deionized water to remove excess Dinotefuran from the leaf surface. Leaflets were dried lightly with kimwipes, and post-infiltration weight was taken. To collect apoplast wash fluid (AWF), the leaflets for each replicate were stacked individually between sheets of parafilm (Pechiney Packaging, Chicago, IL) and rolled around a 2 ml pipet tip (for support, pipet tip up, leaflet tip down) and inserted into a 30 ml syringe (with tip cut at an angle to allow drainage). The syringe was inserted into a 50 ml polycarbonate centrifuge tube. The tissue was centrifuged at 1800 rpm (600*g*) for 15 min at 24 °C using a Sorvall SH3000 swinging bucket rotor. The AWF that collected in the centrifuge tube was weighed and placed in

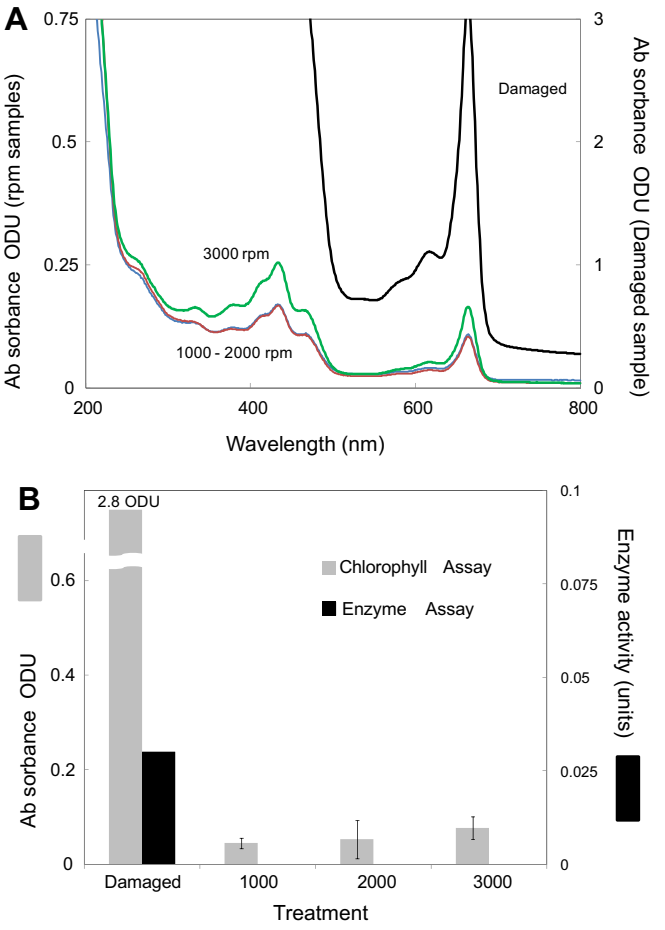


**Fig. 1.** Cytoplasmic leakage using chlorophyll and glucose-6-phosphate dehydrogenase (Glc6PDH) as markers. (A) Scan of chlorophyll leakage from manually damaged tissue or subjected to different centrifuged speeds (1000, 2000, 3000 rpm = 207, 828, 1864*g*) as described in Materials and methods. (B) Comparison of chlorophyll and Glc6PDH activity in apoplast samples. Chlorophyll is represented as ODU (664 nm minus 700 nm) normalized for 1 ml of apoplast fluid. Glc6PDH activity is expressed as activity units. See Materials and methods for further details.

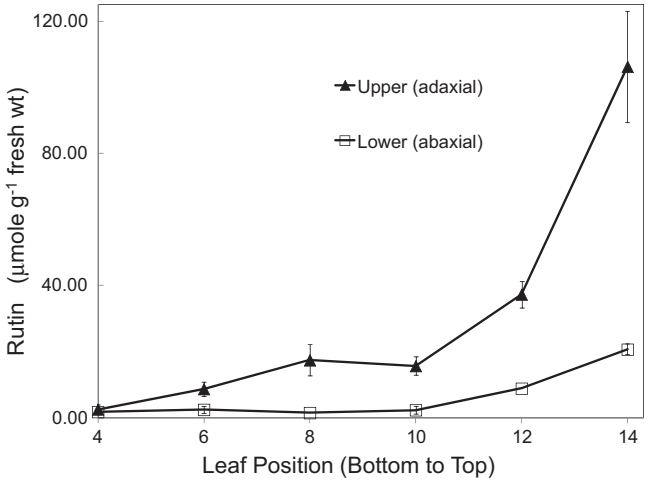


**Fig. 2.** Detection of rutin on the external upper vs lower surfaces of tomato leaves. Leaf position originates from the bottom of the plant, higher numbers indicating younger leaves. See Materials and methods for further details.