COMMUNICATION

In situ detection of salicylate in Ocimum Basilicum plant leaves via reverse iontophoresis

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The quantitative analysis of salicylate provides useful information for the evaluation of metabolic processes in plants. We report a simple, noninvasive method to measure salicylate \textit{in situ} in \textit{Ocimum Basilicum} leaves using reverse iontophoresis in combination with cyclic voltammetry at disposable screen-printed electrodes and the concentration of salicylate in basil leaves was found to be 3 mM.

Salicylates are widely distributed phytohormones in plants, present either as the free acid (SA) (Figure 1) or as its glycosolated or methylated conjugate forms \textsuperscript{1}. Salicylates participate in plant growth \textsuperscript{1,3}, development \textsuperscript{1,3}, immunity \textsuperscript{4}, thermogenesis \textsuperscript{3,5}, flowering \textsuperscript{6}, and germination \textsuperscript{6}. It follows that the amount of salicylate present in a plant is a reflection of its metabolic and physiological activity, and is responsive, therefore, to biotic and abiotic stress \textsuperscript{7} (for example, to insect feeding \textsuperscript{7}, pathogen infection \textsuperscript{5} or environmental changes \textsuperscript{8}). The level of endogenous salicylates under non-stress conditions is highly dependent on the specific plant species of interest \textsuperscript{5}, ranging from ng to mg per gram of fresh material \textsuperscript{5}; as stated, these quantities can increase substantially increase under biotic and abiotic stress \textsuperscript{5}.

\begin{figure}[h]
\centering
\includegraphics[width=0.2\textwidth]{sa_structure.png}
\caption{Chemical structure of SA}
\end{figure}

Analysis of salicylates in leaf extracts has been reported using chromatography \textsuperscript{10,11}, fluorescence \textsuperscript{6} and electrochemical methods \textsuperscript{12} (see Table S1, ESI). While these approaches are quantitative and capable of assessing many samples \textsuperscript{13}, sample preparation typically requires plant tissue pulverisation in liquid nitrogen followed by exhaustive extraction with non-aqueous solvents and is non-trivial, expensive, and time-consuming. More relevant, of course, for the study of oxidative stress \textit{in situ} in a relevant agricultural setting would be an \textit{in vivo} determination of plant metabolites in intact leaves \textsuperscript{14,15} (see Table S2, ESI). With respect to salicylate quantification in intact plants, only a few examples have been reported using methods (Table 1) such as colorimetry \textsuperscript{13} and bioluminescence \textsuperscript{16}. The first \textit{in situ} electrochemical analysis of SA in tomato leaves was performed using a paper-based multi-walled carbon nanotube (MWCNT)/Nafion modified carbon tape electrode \textsuperscript{17}; however, the measurement required an invasive hole to be punched into the leaf so as to allow SA levels to be determined.

Table 1. Methods to measure SA in plants \textit{in situ}.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensor</th>
<th>SA quantity</th>
<th>Plant</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorimetric</td>
<td>Titanium dioxide nanoparticles</td>
<td>NA\textsuperscript{1}</td>
<td>Tobacco</td>
<td>13</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Acinetobacter sp ADP1</td>
<td>NA\textsuperscript{1}</td>
<td>Tobacco</td>
<td>16</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>MWCNTs/Nafion modified carbon tape electrode</td>
<td>4.2 ng per leaf</td>
<td>Tomato</td>
<td>17</td>
</tr>
</tbody>
</table>

NA: not available.
\textsuperscript{1} \textit{In situ} SA labelling prior to its quantification in extracts.
\textsuperscript{2} SA detection after inoculation.

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\textsuperscript{1} Electronic Supplementary Information (ESI) available: Experimental materials and procedures. Analytical methods to measure salicylates in leaf extracts. In \textit{vivo} methods to quantify plant metabolites. See DOI: 10.1039/x0xx00000x

Iontophoresis \textsuperscript{18,19} is a technique that has been primarily used to increase the transport of (primarily) ionised compounds across biological barriers, such as the skin \textsuperscript{20}. The method involves the application, via appropriately positioned (typically reversible Ag/AgCl) electrodes, of a small electric current (\textless 0.05 mA/cm\textsuperscript{2}) that promotes transmembrane transport by electromigration and/or electroosmosis.
Although most often used for transdermal drug delivery, so-called reverse iontophoresis, has been studied as a tool for extraction purposes as well, in particular for drug and glucose monitoring. In terms of applications in plants, the iontophoretic release of alkaloids from cell suspensions (followed by chromatographic analysis) has been investigated. However, this process has been never applied on a plant living tissue and constitutes a very promising non-destructive way to extract small plant metabolites.

The aim of this work was to apply iontophoresis coupled with electroanalysis at disposable carbon nanotube screenprinted electrodes to determine the amount of salicylate in intact Ocimum Basilicum leaves. Such an approach, to our knowledge, has never been performed on a living plant. Electrochemical analysis using cyclic voltammetry is well suited for this application, the method being cheap, technically straightforward and having good sensitivity. Although electrochemical detection per se has been used before with several plant species, the need for a complex pre-treatment protocol to release the target analyte from the sample is clearly disadvantageous as mentioned above. In contrast, the in situ method described here to determine salicylate in the leaves of an intact, living plant is facile and non-destructive (Figure 2). The procedure employs a glass iontophoresis cell clipped onto an undamaged and untreated leaf. Then a period of current passage was used to extract materials. Subsequently, cyclic voltammetry on multi-walled carbon nanotube screen printed electrodes (MWCNT-SPE) were used to determine salicylate via its oxidation to the carboxyhydroquinone.

To verify that the oxidation peak was indeed due to salicylate, the reverse iontophoresis experiment was repeated under identical conditions except that the cathode chamber contained a 10 mM salicylate solution in pH 7.0 phosphate buffer (as opposed to just buffer alone). At the end of 8-hours of current passage, cyclic voltammetry was performed on the anode solution and the results (Figure 3, right panel) shows that the +0.7 V (vs. Ag) oxidation peak was significantly greater. The interpretation of this finding is that salicylate extraction from the leaf is now being augmented by the analyte, which is delivered from the cathode chamber through the leaf into the lower compartment, and then extracted back (through the leaf again) into the anode chamber.

![Figure 2](image-url)  
**Figure 2.** Experimental set-up for the iontophoretic extraction of salicylate from an Ocimum Basilicum leaf; (A) real-life image, (B) schematic representation.

Iontophoresis at 0.5 mA applied to the basil leaf for 8 hours, followed by cyclic voltammetry on the solutions present in the electrode chambers, clearly showed that, at pH 7, where salicylic acid (pK_a = 2.97 for the carboxylic acid functional group) is essentially fully and negatively ionised, is extracted to the anode (Figure 3, left panel). The cyclic voltammograms on the iontophoresically-extracted solution in the anode has an obvious oxidation peak at about +0.7 V (vs. Ag), which was absent when no iontophoretic current was applied (i.e., any passive diffusion contribution can be considered negligible).
Finally, to estimate quantitatively the concentration of salicylate in the leaf, a further series of 8-hour iontophoresis experiments was conducted with different salicylate concentrations (1, 3, 7 and 10 mM) in the cathode chamber, essentially an ‘internal’ standard addition. Once again, at the end of current passage, cyclic voltammetry was used to detect the analyte in the anode compartment of the iontophoresis cell. The results in Figure 5 show that the salicylate peak current ($I_p / \mu A$) increases linearly with the added salicylate concentration in the cathode compartment ([SA] mM) with a relationship of $I_p = (0.201 \pm 0.023) + (0.065 \pm 0.005) \times [SA]$.

![Figure 5](Image)

**Figure 5.** Peak oxidation current (mean ± SD; n = 3) of salicylate measured in the anode compartment solution as a function of the salicylate concentration added to the cathode chamber.

Compared to the value of $I_p$ when no salicylate was introduced into the cathode (i.e., about 0.2 μA), it can be seen that the peak current was approximately doubled when the cathode concentration of salicylate was ca. (3.1 ± 0.12) mM. It follows, therefore, that the salicylate concentration in the leaf is approximately 3.0 mM. Although this deduction assumes that the inner and outer surfaces of the leaf offer comparable resistances to salicylate transport and recognising that there is an additional resistance offered to the salicylate that penetrates from the cathodal chamber. The concentration is likely to reflect the composition of fluid (sap) in the leaf and is significantly different from the composition determined by analysis of the total leaf content, reported in the literature (see Table S1, ESI) as wt/wt.

The successful extraction and proof-of-concept monitoring of endogenous salicylate from the leaves of *Ocimum Basilicum in situ* has been achieved by reverse iontophoresis coupled with cyclic voltammetry. The results of these experiments demonstrate that the concentration of salicylate in the leaf is ca. (3.1 ± 0.12) mM. The method is simple, non-destructive, relatively fast and does not require independent calibration of any sort. The approach has potential, therefore, as a monitor of plant metabolism, its growth and developmental processes, and its response to a variety of external stressors.

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### Notes and references


