Role of Ca$^{2+}$ in the metabolism of phenolic compounds in tobacco leaves (*Nicotiana tabacum* L.)

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**Abstract**

Given the essential role played by phenol metabolism in many resistance responses to different types of stress, the aim of the present work was to determine how different application rates of calcium may influence this metabolic process. Increased calcium in the nutrient solution in which tobacco plants were grown considerably reduced the foliar concentration of phenolic compounds. Calcium clearly exerted a positive influence on the activities of enzymes (phenylalanine ammonia-lyase, polyphenol oxidase and peroxidase) involved in the metabolism of the phenolics. High dosages of calcium (5 mM) promoted more oxidation than synthesis of these compounds, thus explaining the lower concentration of the phenolics.

**Abbreviations:** BSA – bovine serum albumine; PAL – phenylalanine ammonia-lyase; POD – peroxidase; PPO – polyphenol oxidase

**Introduction**

Phenols, among the most widely distributed natural products in the plant kingdom, carry strong physiological and ecological implications (Ruiz and Romero 2001). The metabolism of phenolics involves a series of enzymes that participate in the synthesis and the oxidation of these compounds. The reaction catalysed by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), the deamination of L-phenylalanine to produce trans-cinnamate, is commonly regarded as a key step in the biosynthesis of phenolics and is affected by a number of factors (Hao et al. 1996). On the other hand, phenolics are degraded to di-quinones by peroxidases (POD, EC 1.11.1.7) and principally polyphenol oxidases (PPO, EC 1.14.18.1) (Söderhäll 1995; Thippyapong et al. 1995). Many studies have demonstrated that both enzymes increase in response to biotic and abiotic stress (Kwak et al. 1996; Ruiz et al. 1998, 1999a).

The processes which have been most thoroughly studied and which most directly involve phenolics are related to pest and disease resistance (Dübeler et al. 1997; Ruiz et al. 1999a). In addition, the metabolism of phenolics has been associated with injuries (Smith 1982), with resistance to thermal stress (Christie et al. 1994), with tolerance against exposure to UV rays and ozone (Rasmussen et al. 1991) and finally to changes in the nutritional state of nutrients such as boron (Cakmak and Romhild 1997; Ruiz et al. 1998, 1999a), nitrogen (Wojtaszek et al. 1993) and calcium (Castañeda and Pérez 1996; Penel et al. 1999).

Calcium (Ca$^{2+}$), one of the essential nutrients for plants, plays a major role in the initiation of many signal transduction processes in higher plant cells, including bud formation, polar growth, gas-exchange regulation, secretion, movements and light- and hormone-regulated growth and development (Hepler and Wayne 1985). In addition, this nutrient actively influences one of the processes most vital to plant
growth, nitrogen metabolism (Ruiz et al. 1999b; López-Lefebre et al. 2000). The role of Ca\(^{2+}\) in phenolic metabolism has been described by various authors. For example, the first work that demonstrated a direct role of Ca\(^{2+}\) in the synthesis of phenols was performed by Castañeda and Pérez (1996). In this work, the authors observe that the foliar application of 10\( \mu \)M of CaCl\(_2\) increases PAL activity, and therefore the accumulation of phenols, prompting resistance to infection by the fungus *Alternaria alternata* in citrus.

However, the literature remains controversial on the effect of Ca\(^{2+}\) on the enzymes responsible for phenol oxidation. Söderhäll (1995) and Wan and Heinrich (1997) demonstrated that increased concentrations of CaCl\(_2\) in the nutritive solution augment POD activity. Conversely, other researchers have reported that higher levels of Ca\(^{2+}\) diminished PPO and POD activities (Kawai et al. 1995; Tomasbarberan et al. 1997). Recently, Penel et al. (1999) proposed that Ca\(^{2+}\) indirectly activates POD, as this cation induces the cross-linking of polygalacturonan chains into a structure which can be recognized by isoperoxidase.

Given the essential role played by phenol metabolism in many resistance responses to different types of stress, the rapid and effective manipulation of this metabolic process could be of great help in developing plants with greater resistance against adverse conditions. Therefore, the objective of this work was to analyse how the application of different amounts of Ca\(^{2+}\) in the nutrient solution influences synthesis and oxidation of phenols.

**Materials and methods**

**Plant material and culture conditions**

Seeds of *Nicotiana tabacum* L. cv Tennessee 86 were sown in March 1998. The seedlings were grown in an experimental greenhouse in southern Spain (Granada) for 45 days and then transferred to a cultivation chamber under controlled environmental conditions with relative humidity of 60–80%, temperature 30/20 °C (day/night), and 16/8 h photoperiod at a PPFD of 350 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) (measured at the top of the plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). The plants were grown in individual pots (25 cm upper diameter, 17 cm lower diameter, 25 cm in height) of 8 l volume, filled with vermiculite. For 1 month (from day 45 until day 75 after sowing), before the experimental treatments, the plant received a modified Hoagland medium (López-Lefebre et al. 2000). The nutrient solution (pH 5.5–6.0) was renewed every 3 days and the vermiculite rinsed with Millipore-filtered water.

At 75 days after sowing, we applied different levels of Ca\(^{2+}\), the initial level being 1.25 mM for the Ca1 treatment, 2.5 mM for the Ca2 treatment, and 5.0 mM for the Ca3 treatment. The application rates of Ca\(^{2+}\) in the present work were similar to those used in other works performed by our research group (Ruiz and Romero 1998; Ruiz et al. 1999b; López-Lefebre et al. 2000), and these rates, in addition to influencing the physiology of the plant, do not provoke symptoms of Ca\(^{2+}\) deficiency or toxicity and therefore are ideal for these types of studies. The experimental design was a randomized complete block with three treatments, arranged in individual pots with six plants per treatment, each one replicated three times.

**Plant sampling**

The plants were sampled twice, beginning at the 14-leaf stage just before the onset of flowering, using three plants per sampling. At the first sampling, day 105 after sowing, leaves were picked from nodes 10 and 11. At the second sampling, 2 weeks later, leaves from nodes 12 and 13 were picked. The leaves were rinsed three times with distilled water after disinfecting with non-ionic detergent at 1%, then blotted on filter paper. The leaves from nodes 10 (first sampling), and 12 (second sampling) were used fresh for the analysis of enzymatic activities and phenolic compounds. The leaves from node 11 (first sampling), and 13 (second sampling) were dried in a forced air oven at 70 °C for 24 h and were used for the analysis of Ca\(^{2+}\) content.

**Enzymatic analysis**

The extraction of PAL was carried out following the method proposed by Lister et al. (1996) and its activity was assayed by an adaptation of the methods of Zucker (1965) and McCallum and Walker (1990), determined from the yield of cinnamic acid, estimated from absorbance at A\(_{290}\) in the presence and absence of phenylalanine.
Table 1  Effect of CaCl₂·2H₂O treatments on the foliar concentration of Ca²⁺ and the metabolism of the phenolics compounds in the leaves of tobacco plants. Data are means ± S.E. (n = 6). The least significant difference (LSD) is given for each plant.

<table>
<thead>
<tr>
<th>Treatments (mM)</th>
<th>Total Ca²⁺</th>
<th>Total phenolics</th>
<th>PAL</th>
<th>PPO</th>
<th>POD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>502.7 ± 54.4</td>
<td>2467 ± 176</td>
<td>1.10 ± 0.21</td>
<td>3.52 ± 0.65</td>
<td>2.57 ± 0.43</td>
</tr>
<tr>
<td>2.50</td>
<td>675.2 ± 62.9</td>
<td>2028 ± 158</td>
<td>1.54 ± 0.27</td>
<td>5.06 ± 0.80</td>
<td>3.35 ± 0.63</td>
</tr>
<tr>
<td>5.00</td>
<td>833.7 ± 67.1</td>
<td>1715 ± 166</td>
<td>1.73 ± 0.36</td>
<td>6.55 ± 0.79</td>
<td>3.92 ± 0.74</td>
</tr>
</tbody>
</table>

Significance *** *** ** *** *  
LSD at 5% 157 239 0.33 1.15 0.74  

Total Ca²⁺: μmol g⁻¹ DW; Total phenolics: μg caffeic acid g⁻¹ FW; PAL: μmol cinnamic acid produced mg⁻¹ protein min⁻¹; PPO: μmol caffeic acid oxidised mg⁻¹ protein min⁻¹; POD: μmol guaiacol oxidised mg⁻¹ protein min⁻¹.

The extraction method used for the determination of PPO was that proposed by Thypyapong et al. (1995) and its activity assayed as described by Nicoli et al. (1991), measured by the change in ΔA₅₇₀₀ of the assay mixture (30 °C) based on the enzymatic oxidation of caffeic acid.

The method used for the determination of POD was a modified version of that proposed by Kalir et al. (1984) and Badini et al. (1990) and its activity was determined by following the change of ΔA₄₈₅ due to guaiacol oxidation (Kalir et al. 1984; Ruiz et al. 1998). To test whether the reaction was due to peroxidase, control assays contained catalase from bovine liver (EC 1.11.1.6) (Fluka).

In all cases, the samples extracts were boiled and assayed to determine whether the reactions were enzymatic. Protein in the samples extracts were estimated by the method of Bradford (1976) using BSA as a standard.

Extraction and quantification of total phenols
Leaves were ground to a fine powder with a pestle and extracted at a ratio of 100 mg fresh weight to 1 ml methanol. Total phenolic content was assayed quantitatively with Folin-Ciocalteau reagent (Singleton and Rossi 1965; Singleton et al. 1985). The results obtained were expressed as μg of caffeic acid g⁻¹ fresh weight (FW).

Extraction and quantification of total Ca²⁺
Total Ca²⁺ was analysed by atomic-absorption spectrophotometry (Hocking and Pate 1977), after digestion of dry and milled material with 12 N H₂SO₄ and H₂O₂. The content of Ca²⁺ was expressed as μmol g⁻¹ dry weight (DW).

Statistical analysis
Standard analysis of variance techniques were used to assess the significance of treatment means. The data shown are mean values ± S.E. Differences between treatment means were compared using the LSD at the 0.05 probability level. Levels of significance are represented by * at P < 0.05, ** at P < 0.01, *** at P < 0.001, and ns: not significant.

Results and discussion
As confirmed in previous work, the highest foliar accumulation of Ca²⁺, in treatment Ca3 (Table 1), was due to the fact that, after Ca²⁺ absorption by the root cells, this cation is transported through the xylem towards the shoot, accumulating in zones where transpiration is greatest (Bharti et al. 1996; Ruiz and Romero 1998; Ruiz et al. 1999b).

Foliar PAL activity (Table 1), was affected significantly by the different Ca²⁺ treatments applied, the highest activities being registered in the Ca3 treatment, which also showed the highest foliar levels of total Ca²⁺, thus presenting a positive and significant relationship (r = 0.941***) between these two parameters. With reference to the response of PAL activity to Ca²⁺ application, Castañeda and Pérez (1996), using lemon seedlings injured or treated with fungal elicitors, suggested that the increase in this activity was not directly caused by the Ca²⁺, but that this cation participates in the cell response, triggering a series of transduction signals. Our results show that even in uninfected tobacco leaves, the application of different dosages of Ca²⁺ stimulated PAL activity.

On the contrary, the results showed a fall in the concentration of the phenolics with increasing Ca²⁺ level (Table 1), the highest concentration appearing at Ca1 and the lowest at Ca3. Although the highest PAL activity occurred in the Ca3 treatment, this treatment contained the lowest concentration of total phenolics.
This behaviour appears to explain the negative relationship between these parameters ($r = -0.989***$).

The activity of the enzymes POD and PPO was affected positively by the application of $\text{Ca}^{2+}$. Like PAL activity, the PPO and POD activities increased in the Ca3 treatment, by 85 and 52%, respectively as compared to the lowest activities occurring in the Ca1 treatment (Table 1).

In agreement with other reports, in our experiment the enzymes responsible for phenolic oxidation, PPO and POD, were very positively affected by the presence of $\text{Ca}^{2+}$ ($\text{Ca}^{2+}$ concentration–PPO, $r = 0.987***$; $\text{Ca}^{2+}$ concentration–POD, $r = 0.981***$). This activation of PPO by $\text{Ca}^{2+}$ has been described elsewhere, and it has been reported that $\text{Ca}^{2+}$ acts on PPO, which normally is found in its latent form, modifying the conformational state of this enzyme and thus boosting its activity (Soderhall 1995). The enzyme POD also reportedly reacts positively to $\text{Ca}^{2+}$, and Penel et al. (1999), recently demonstrated that this cation is necessary for the action of this enzyme to induce the cross-linking of polygalacturonan chains into a structure which could be recognized by the peroxidase.

In our experiment, we found an inversely proportional relationship between the activity of these enzymes and phenolic accumulation (total phenolics–PPO, $r = -0.992***$; total phenolics–POD, $r = -0.999***$). Thus, we conclude that in response to $\text{Ca}^{2+}$ application, the metabolism of phenolic compounds is affected by a stimulation in the rate of oxidation as compared to that of synthesis.

References


