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Plants as Biofactories: Glyphosate-Induced Production of Shikimic Acid and Phenolic Antioxidants in Wounded Carrot Tissue

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ABSTRACT: The use of plants to produce chemical compounds with pharmaceutical and nutraceutical applications has intensified in recent years. In this regard, genetic engineering is the most commonly used tool to generate crop lines with enhanced concentrations of desirable chemicals. However, growing genetically modified plants is still limited because they are perceived as potential biological hazards that can create an ecological imbalance. The application of postharvest abiotic stresses on plants induces the accumulation of secondary metabolites and thus can be used as an alternative to genetic modification. The present project evaluated the feasibility of producing shikimic acid (SA) and phenolic compounds (PC) in wounded carrots (*Daucus carota*) treated with glyphosate. The spray application of a concentrated glyphosate solution on wounded carrot tissue increased the concentrations of SA and chlorogenic acid by ~1735 and ~5700%, respectively. The results presented herein demonstrate the potential of stressed carrot tissue as a biofactory of SA and PC.

KEYWORDS: *carrot, postharvest abiotic stresses, wounding, glyphosate, shikimic acid, phenolic compounds*

INTRODUCTION

In the past few years the use of plants to produce high commercial value chemical compounds has been extensively studied. Numerous crop plants, along with other prokaryotic and eukaryotic expression systems, have been genetically engineered to promote the production and accumulation of metabolites.[1−4] However, metabolic engineering is technically complex, and the extensive growth of genetically modified crops has been questioned due to potential environmental and safety issues.[5] The application of postharvest abiotic stresses in fruits and vegetables is a practical and effective technology that allows the accumulation of antioxidant metabolites.[6] For instance, carrots treated with wounding stress alone or in combination with other abiotic stresses such as UV light, hyperoxia, and phytohormones produce high levels of caffeoylquinic acids (CQAs).[7−12] The CQAs are phenolic compounds (PC) with great potential on the prevention and treatment of different degenerative diseases such as HIV,[13,14] Alzheimer's,[15] obesity,[16] and hepatitis B.[17]

Although the wound-induced biosynthesis of secondary metabolites in fruits and vegetables has been widely reported in recent years, the effect of wounding stress on the accumulation of primary metabolites has been overlooked. Wounding stress activates metabolic pathways related with the biosynthesis of primary metabolites.[18−20] A primary metabolite that may be accumulated in plants as a response to wounding stress is shikimic acid (SA). This compound has a high pharmaceutical value because it is used in the production of Oseltamivir (Tamiflu), a neuraminidase inhibitor utilized for the treatment of influenza.[21] The main natural sources of SA are plants from the *Illicium* genus such as Chinese star anise.[22,23] However, the cultivated amount of these plants is insufficient to supply the global demand for SA.[21,22] Therefore, it is necessary to explore additional sources of SA.[22]

Although the application of wounding stress activates metabolic pathways related with the biosynthesis of SA in plants, it is likely that the tissue accumulates small amounts of this metabolite because it is utilized to produce aromatic amino acids needed for secondary metabolite biosynthesis.[24] Therefore, an interesting strategy would be to decrease the rate of aromatic amino acid biosynthesis in wounded plants, allowing the accumulation of SA. The postharvest application of the herbicide glyphosate [*N*-(phosphonomethyl)glycine] in wounded tissue may be used for that purpose. Glyphosate applied in plants during different growing stages inhibits aromatic amino acid biosynthesis by blocking the action of the 5-enolpyruvylshikimate 3-phosphate synthase (EPSP synthase) enzyme.[25] Shikimic acid 3-phosphate (S3P) is the substrate of EPSP synthase, and when this enzyme is inhibited by glyphosate, S3P is less efficiently utilized and is rapidly transformed to SA by a kinase.[26] Furthermore, glyphosate-treated plants present higher concentrations of hydroxybenzoic acids, which are PC directly synthesized from SA.[27,28] However, there are no reports in the literature regarding the effect of the postharvest application of glyphosate on the accumulation of primary and secondary metabolites in wounded plants.

Glyphosate is extensively used as a nonselective herbicide, and it presents certain toxicological effects.[29] Therefore, in the present study we propose that plant tissue treated with

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glyphosate must be subjected to downstream processing using standard recovery and purification processes, to obtain pure chemical compounds produced in the stressed tissue. The technology proposed herein would be of great interest for the fresh produce industry, which is looking for additional uses of their crops especially for fruits and vegetables not meeting quality standards for human consumption.

The objectives of this research were (i) to evaluate the effect of wounding stress on the accumulation of SA in carrots, (ii) to evaluate the effect of glyphosate on the accumulation of SA in wounded carrots, and (iii) to evaluate the effect of glyphosate on the accumulation of individual PC in wounded carrots. The information generated in this research can be useful to envisage simple emerging technologies that allow the production of high commercial value chemical compounds in fruits and vegetables.

MATERIALS AND METHODS

Chemicals. Glyphosate formulation (FAENA − 482 g/L, Monsanto) was obtained from IMAISA (Monterrey, NL, Mexico). Shikimic acid (SA), chlorogenic acid (CHA), ferulic acid (FA), *p*-coumaric acid (*p*-CA), protocatechuic acid (PCA), gallic acid (GA), methanol (HPLC grade), water (HPLC grade), orthophosphoric acid, hydrochloric acid, periodic acid, sodium *m*-periodate, sodium hydroxide, and sodium sulfite were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant Material, Processing, and Storage Studies. Carrots (*Daucus carota*) were obtained from a local market (HEB, Monterrey, NL, Mexico), sorted, washed, and disinfected with chlorinated water (200 ppm, pH 6.5). To prepare the shredded carrots, whole carrots were wounded with a commercial vegetable shredder (diameter = 0.7 cm). Shredded carrots (300 g) were placed in open plastic containers with a capacity of 5.7 L (Sterilite, Townsend, MA, USA).

Three independent experiments were performed for the storage studies. In the first experiment, shredded carrots were stored for 48 h in an incubator (VWR, Radnor, PA, USA) at 25 °C. During the storage period (48 h), samples were collected every 12 h to determine the time at which the maximum accumulation of SA was obtained due to wounding stress.

In the second experiment, shredded carrots were treated with a glyphosate solution (482 g/L) by two different methods, dipping and spraying, and stored for 24 h to determine the combined effect of wounding stress and glyphosate treatments on the accumulation of SA. In the first method of glyphosate application (dipping), shredded carrots were submerged for 2 min in 500 mL of the glyphosate solution and drained for 10 min. In the second method of glyphosate application (spraying), 100 mL of the glyphosate solution was sprayed onto the shredded carrots using a commercial multifunctional pressure sprayer.

In the third experiment, solutions containing different concentrations of glyphosate (0, 100, 200, 300, 400, and 482 g/L) were sprayed onto the shredded carrots and the SA and individual PC contents were determined after 24 h of storage. The solutions with different concentrations of glyphosate were prepared by diluting the commercial formulation (FAENA, 482 g/L) with water.

Sample Preparation for Phytochemical Analysis. SA was extracted using two different procedures, depending on the method of detection and quantification to be used (spectrophotometric or chromatographic) as detailed in the following sections. For the spectrophotometric analysis, SA was extracted as described by Zelaya et al.[30] with some modifications. Carrot tissue (0.2 g) was homogenized with 0.25 N HCl aqueous solution (4 mL). Homogenizations were performed with a tissuemizer (Advanced homogenizing system, VWR). Subsequently, the homogenates were stirred for 10 min on a shaker (Glas-Col, Terre Haute, IN, USA) at 60 rpm and then centrifuged (10000*g*, 15 min, 4 °C). The clear supernatant (further referred to as SA extract) was microfiltered using nylon membranes (0.45 μm, VWR) and used for the quantification of SA by a spectrophotometric method.

For the chromatographic determination of SA and individual phenolic compounds, carrot tissue (5 g) was homogenized with methanol (20 mL). The homogenates were stored overnight (∼12 h at 4 °C) and centrifuged (10000*g*, 15 min, 4 °C). The clear supernatant (further referred to as methanol extract) was microfiltered using nylon membranes (0.45 μm) and used for the identification and quantification of SA and individual phenolic compounds by high-performance liquid chromatography with photodiode array detection (HPLC-PDA).

Spectrophotometric Determination of SA. SA extracts were analyzed according to the method reported by Zelaya et al.[30] SA extracts (250 μL) were mixed with a 0.5% periodate (w/v) and 0.5% sodium *m*-periodate (w/v) aqueous solution (250 μL). The mixtures were vortexed and incubated in the dark in a water bath (37 °C, 45 min). Subsequently, a 1 M NaOH solution (300 μL) and a 56 mM Na_2SO_3 solution (200 μL) were added to the reactions. The oxidized SA was detected at 382 nm using a microplate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT, USA). A standard curve of SA (1−1000 μM) was constructed for quantification purposes. The concentration of SA was expressed as milligrams per kilogram of carrot dry weight (DW). To calculate the concentration of SA in DW, the moisture content of the samples was determined by the air-oven method (AACC 44-15A).

Analysis of SA and Individual PC by HPLC-PDA. The qualitative and quantitative analysis of SA and individual PC in the methanol extracts was performed by HPLC-PDA. The HPLC system used was composed of two 515 binary pumps, a 717-plus autosampler, and a 996 photodiode array detector (Waters Corp., Milford, MA, USA). SA and PC were separated on a 4.6 mm × 250 mm, 5 μm, C18 reverse phase column (Luna, Phenomenex, Torrance, CA, USA). The mobile phases consisted of water (phase A) and methanol/water (60:40, v/v, phase B) adjusted to pH 2.4 with orthophosphoric acid. The gradient solvent system was 0/100, 3/70, 8/50, 35/30, 40/20, 45/0, 50/0, and 60/100 (min/% phase A) at a constant flow rate of 1 mL/min. Chromatographic data were processed with Millenium software V3.1 (Waters Corp.).

Interpretation of UV−Visible Spectra. The identification of SA and individual PC was performed by three different procedures depending on the information available for each chromatographic peak. The methods of identification were (a) identification by comparison with retention time and UV−visible absorption spectra characteristics of commercial standards, (b) identification by interpretation of UV−visible spectra characteristics and comparison with previous reports,[12,27,31−34] and (c) identification by order of elution reported in the literature.[9,11,12,27,32,34,35]

Quantification of SA and Individual PC. For the quantification of SA and individual PC, standard curves of SA, CHA, FA, *p*-CA, PCA, and GA were prepared in the range of 0.5−100 μM. The concentration of the phytochemicals was expressed as milligrams of each individual compound per kilogram of carrot DW.

Statistical Analysis. Statistical analyses were performed using four replicates. Data represent the mean value of samples, and bars indicate their standard error. Analyses of variance (ANOVA) were conducted using JMP software version 5.0 (SAS Institute Inc., Cary, NC, USA) and mean separations performed using the LSD test ($p < 0.05$).

RESULTS AND DISCUSSION

Effect of Wounding Stress on the Accumulation of SA in Carrot Tissue. To determine the effect of wounding stress on the accumulation of SA in carrot tissue, shredded carrots were stored for 48 h at 25 °C. During storage, samples were collected every 12 h for SA determinations. The concentration of SA increased during the first hours of storage; maximum accumulation was observed at 24 h (Figure 1). Thereafter, SA content in the wounded tissue started to decrease. Compared with shredded carrots before storage (time 0 h samples), 24 h stored shredded carrots showed ∼173% higher SA concentration. To our knowledge, there are no previous reports in the

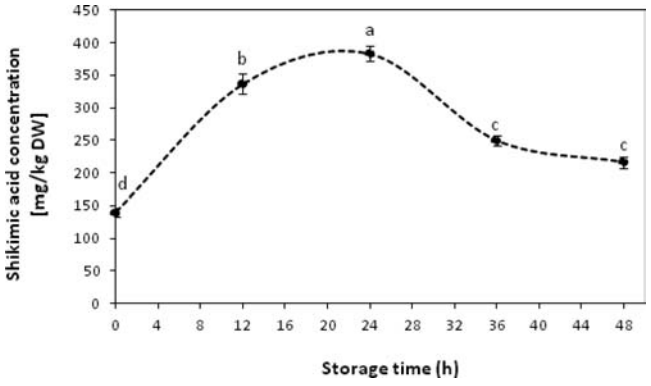

Figure 1. Accumulation of shikimic acid during the storage of shredded carrots. Samples were stored for 48 h at 25 °C. The concentration of shikimic acid was determined spectrophotometrically according to the method of Zelaya et al.[30] Values represent the mean of four replications with their standard error bars. Data points with different letters indicate statistical difference by the LSD test ($p < 0.05$).

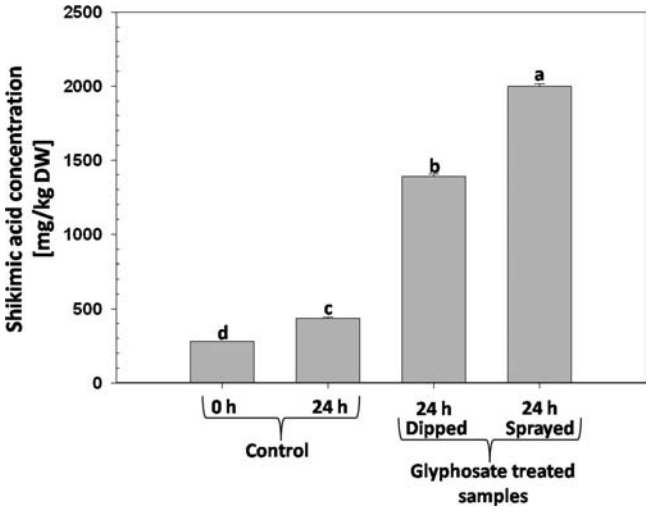

Figure 2. Shikimic acid concentration in carrot tissue treated with glyphosate (482 g/L) and stored for 24 h at 25 °C. Shredded carrots were either sprayed or dipped for 2 min with glyphosate solutions. The concentration of shikimic acid was determined spectrophotometrically according to the method of Zelaya et al.[30] Values represent the mean of four replications with their standard error bars. Different letters indicate statistical difference by the LSD test ($p < 0.05$).

literature describing the effect of postharvest abiotic stresses on the accumulation of SA in plants. When carrots are subjected to wounding stress, proteins and secondary metabolites such as phenolic compounds are synthesized during the process of acclimation.[8,11,12] SA is a primary metabolite used as substrate for the synthesis of aromatic amino acids, which are utilized for the synthesis of proteins, phenolic compounds, and other plant secondary metabolites.[24,36,37] According to Jacobo-Velázquez et al.,[11] shredded carrots stored at 20 °C reach their maximum accumulation of phenolic compounds at 48 h of storage. Therefore, it is likely that the accumulation of SA observed at 24 h of storage (Figure 1) is related with a higher rate of synthesis compared with the rate of utilization for aromatic amino acid biosynthesis, whereas at subsequent storage the utilization rate of SA increases.

Effect of Glyphosate Treatments on the Accumulation of SA in Wounded Carrot Tissue. To improve the potential of wounded plant tissue as a source of SA, it would be necessary to determine the synergistic effect of additional abiotic stresses on SA accumulation. A previous report showed that wounding stress up-regulates the expression of EPSP synthase in carrots,[49] suggesting that the rate of conversion of shikimate 3-phosphate (S3P) into EPSP is increased. This may be the reason for the poor accumulation of SA in wounded carrot tissue. The application of the herbicide glyphosate induces the accumulation of SA in plants by inhibiting EPSP synthase enzyme.[25,38] Therefore, in the present study it was hypothesized that the level of SA accumulated in wounded carrot increases when the tissue is treated with glyphosate. To test this hypothesis, shredded carrots were either dipped or sprayed with a glyphosate solution before storage, and the concentration of SA was determined at 24 h of storage.

The application of glyphosate (482 g/L) in the shredded carrots, as expected, induced higher accumulation of SA compared to the controls (Figure 2). Shredded carrots sprayed with glyphosate showed ∼44% higher accumulation of SA than the dipped samples. It is known that different signaling molecules mediate abiotic stress responses in plants.[39] In previous studies the extracellular ATP (eATP) accumulated at the wounding sites was proposed as a primary signal that triggers the wound response in plants by activating the production of reactive oxygen species (ROS).[11,40] ROS are signaling molecules associated with the wound-induced activation of the primary and secondary metabolism.[11,20,39,40] Therefore, it is likely that a higher accumulation of SA observed in the glyphosate-sprayed samples compared to the dipped samples is associated with a larger removal of the primary signal (eATP) in the latter process (Figure 2).

The quantification of SA by the spectrophotometric method provides a narrowed detection limit compared to HPLC-PDA.[30] Therefore, SA contents in the nonstressed as well as in the shredded carrots sprayed with 100 mL of different concentrations of glyphosate (0, 100, 200, 300, 400, and 482 g/L) were determined by HPLC-PDA (Figure 3; Tables 1 and 2). SA was identified on the basis of the retention time and the absorption maxima characteristics of the chromatographic peak by comparison with the retention time and absorption maxima characteristics of a commercial standard (Table 1). The SA content in the shredded carrots increased as the concentration of glyphosate in the spraying solutions increased (Table 2). However, SA content determined by the HPLC-PDA method was greater than that obtained by the spectrophotometric method. These results agreed with a previous paper correlating the spectrophotometric and HPLC methods for SA determination, wherein the authors determined that SA content is underestimated by ∼50% with the spectrophotometric method.[30]

On the basis of the SA content determined by the HPLC-PDA method, the concentration of SA in nonsprayed shredded carrots (control) increased by ∼77% at 24 h of storage (Table 2). When the shredded carrots were sprayed with 100 mL of water before storage, the accumulation of SA was ∼52% lower than that of the nonsprayed samples, indicating as earlier discussed the partial removal of the primary signaling molecule that triggers the activation of the primary metabolism in wounded plants.[11,40] The maximum accumulation of SA was obtained in shredded carrots sprayed with 100 mL of 482 g/L glyphosate solution. For this treatment, the concentration of SA was ∼1735% higher compared to control carrots at time 0 h.

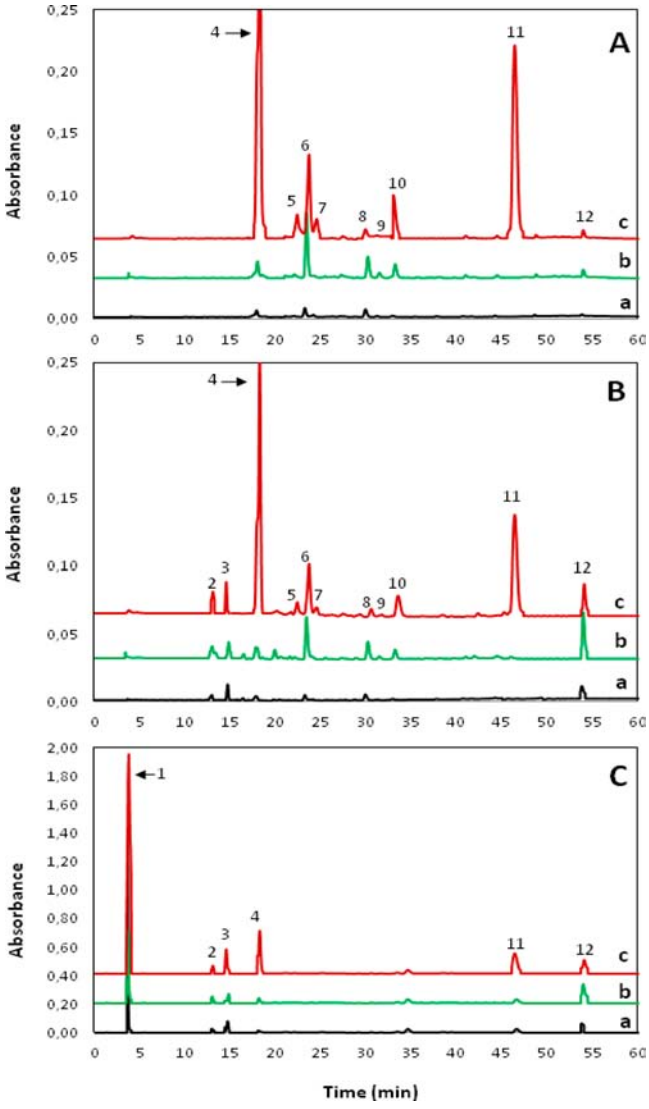

Figure 3. Typical HPLC-PDA chromatograms shown at 320 nm (A), 280 nm (B), and 215 nm (C) from methanol extracts of shredded carrot tissue untreated (a, b) and treated (c) with glyphosate: (a) shredded carrot before storage; (b) shredded carrot stored for 24 h at 25 °C; (c) shredded carrot sprayed with glyphosate and stored for 24 h at 25 °C. Tentative identification of the chromatographic peaks was performed as indicated in Table 1. Peaks: (1) shikimic acid; (2) protocatechuic acid; (3) gallic acid derivative; (4) chlorogenic acid; (5) 3,5-dicaffeoylquinic acid; (6) *p*-coumaric acid derivative A; (7) 4,5-dicaffeoylquinic acid; (8) *p*-coumaric acid; (9) ferulic acid; (10) *p*-coumaric acid derivative B; (11) ferulic acid derivative; (12) isocoumarin.

This is the first report that proposes a new postharvest technology to induce the production of SA in horticultural crops. This technology has several advantages compared with the processes commonly used for the production of SA. For instance, carrot is produced worldwide, whereas the productions of Chinese star anise and *Liquidambar styraciflua* are insufficient to meet the worldwide demand for SA for Tamiflu production.[21] Although carrot is mainly used for human consumption, produce not meeting quality standards could be stressed by wounding and glyphosate used for the production of SA. The technology proposed herein for the production of SA is technically less complex than the biotechnological process currently utilized.[23]

Effect of Glyphosate Treatments on the Accumulation of Individual PC in Wounded Carrots. The accumulation of hydroxybenzoic acids such as protocatechuic acid (PCA), gallic acid (GA), vanillic acid, and syringic acid in glyphosate-treated pigweed (*Amaranthus retroflexius* L.), ryegrass (*Lolium prenne* L.), soybean (*Glycine max* L.), and lupine (*Lupinus albus* L.) during different growing stages has been previously reported.[27,41,42] Likewise, wounding stress applied alone or in combination with other postharvest abiotic stresses (i.e., hyperoxia, UV- light, and phytohormones) in carrots induces the accumulation of hydroxycinnamic acids, mainly caffeoylquinic acids (CQAs) such as chlorogenic acid (CHA) and its derivatives 3,5-dicaffeoylquinic acid (3,5-diCQA) and 4,5-dicaffeoylquinic acid (4,5-diCQA).[7−9,11,12]

Previous papers indicate that the biosynthesis of hydroxycinnamic acids in glyphosate-treated plants is inhibited due to the blockage effect that the herbicide exerts on EPSP synthase and thus on the conversion of SA to L-phenylalanine.[42,43] Therefore, in the present study it was considered relevant to evaluate by HPLC-PDA the effect of glyphosate treatments on the accumulation of individual PC in wounded carrot tissue.

The tentative identification of each chromatographic peak obtained from methanol extracts of the nonstressed and stressed carrots is shown in Table 1. The identification of PC included protocatechuic acid (PCA), gallic acid derivative (GAD), CHA, 3,5-diCQA, *p*-coumaric acid derivative A (*p*-CADA), 4,5-diCQA, *p*-coumaric acid (*p*-CA), ferulic acid (FA), *p*-CA derivative B (*p*-CADB), ferulic acid derivative (FAD), and isocoumarin (IC) (Figure 3; Table 1). The 3,5-diCQA, 4,5-diCQA, and FAD were identified only in the glyphosate-treated stored samples (Table 2).

Wounding stress induced the accumulation of PCA, CHA, *p*-CADA, *p*-CA, FA, *p*-CADB, and IC (Table 2). The individual phenolic compound that showed the higher percentage of increase was *p*-CADA, followed by *p*-CADB, IC, *p*-CA, CHA, FA, and PCA. The wound-induced accumulation of these PC in carrot tissue has been previously reported.[7−9,11,12] However, these papers indicated that the phenolic that increased in higher concentration by wounding stress is CHA. The difference between the results presented herein and those from previous studies is related with the time at which the quantification of individual PC was performed. The maximum accumulation of PC, mainly CQAs, in shredded carrots stored under similar conditions as the present study is obtained at 48 h of storage.[11] The CQAs accumulated at 48 h of storage are subsequently used in vegetal tissue for the synthesis of lignin, which is composed of monolignol residues synthesized from hydroxycinnamic acids precursors.[44] *p*-CA is precursor for the synthesis of CQAs;[45] therefore, the high accumulation of *p*-CA and its derivatives observed at 24 h indicates that the lignification process was at early stages.

The application of glyphosate in wounded carrots, before storage, induced higher accumulation of certain hydroxycinnamic acids (CHA, 3,5-diCQA, 4,5-diCQA, *p*-CADB, and FAD) after 24 h of storage compared with wounding alone, whereas the concentration of the hydroxybenzoic acid PCA decreased (Table 2). The phenolic compound that showed the highest percentage of increase by the effect of the application of glyphosate in wounded carrots was CHA. At 24 h of storage, the content of CHA in wounded carrots sprayed with 100 mL of 482 g/L glyphosate was ∼1980% higher compared with the control samples at 24 h and ∼5700% higher compared with the control samples at time 0 h (Table 2). Moreover, the

Table 1. Tentative Identification of Shikimic Acid and Phenolic Compounds in Carrot Tissue Obtained by HPLC-PDA

peak[a] (retention time)	λ_{max}[b] (nm)	tentative identification[c]	previously reported in carrot[d]	method of identification[e]
1 (3.7)	215	SA		A, B
2 (13.0)	215, 253, 290	PCA	iv	A, B, C
3 (14.8)	217, 271	GAD		A
4 (18.1)	217, 242, 320	CHA	i, ii, iii, iv, v, vi	A, B, C
5 (22.2)	217, 238, 325	3,5-diCQA	ii, iii, iv, v, vi	A, B, C
6 (23.2)	228, 313	*p*-CADA		A
7 (24.1)	215, 240, 326	4,5-diCQA	i, ii, iii, iv, v, vi	A, B, C
8 (29.9)	226, 310	*p*-CA	i	A, B, C
9 (31.4)	217, 237, 323	FA	i, iii, iv, v, vi	A, B
10 (33.2)	225, 313	*p*-CADB		A
11 (45.8)	218, 239, 328	FAD		A, B
12 (53.9)	215, 268, 301	IC	iii, iv, vi	B, C

[a]Number of peak assigned according to the order of elution from the C18 stationary phase (Figure 3). [b]Wavelengths of maximum absorption in the UV−vis spectra of each chromatographic peak. [c]Abbreviations: shikimic acid (SA); protocatechuic acid (PCA); gallic acid derivative (GAD); chlorogenic acid (CHA); 3,5-dicaffeoylquinic acid (3,5-diCQA); *p*-coumaric acid derivative A (*p*-CADA); 4,5- dicaffeoylquinic acid (4,5-diCQA); *p*-coumaric acid (*p*-CA); ferulic acid (FA); *p*-coumaric acid derivative B (*p*-CADB); ferulic acid derivative (FAD); isocoumarin (IC). [d]Previously reported b: (i),[48] (ii),[35] (iii),[8] (iv),[9] (v),[11] and (vi).[12] [e]Method applied for the identification of the chromatographic peak: (A) identification by comparison with the retention time and wavelengths of maximum absorption in the UV−vis spectra of commercial standards; (B) identification by spectral interpretation of the wavelengths of maximum absorption in the UV−vis spectra and comparison with wavelengths of maximum absorption reported in the literature;[12,27,31−34] (C) identification by the order of chromatographic elution reported in the literature.[9,11,12,27,32,34,35]

application of glyphosate induced the synthesis and accumulation of 3,5-diCQA and 4,5-diCQA, whereas in treatments in which only wounding was applied these compounds were not detected. This is the first study that evaluates the effect of glyphosate treatments on the accumulation of PC in wounded plant tissue. Previous investigations, regarding the effect of glyphosate treatments on the phenylpropanoid metabolism of whole plants such as pigweed, ryegrass, soybean, and lupine, reported a contrary behavior.[27,42] The authors observed that the biosynthesis of hydroxycinnamic acids is blocked, whereas that of hydroxybenzoic acids is favored. The observations from those authors are supported by the fact that hydroxybenzoic acids are directly synthesized from SA accumulated in glyphosate-treated plants.[27,28] Likewise, the biosynthesis of hydroxycinnamic acids requires L-phenylalanine, the biosynthesis of which in whole plants is inhibited by glyphosate.[27,28]

Although the biosynthetic pathway of CQAs in plants is not fully understood, it has been proposed that SA and quinic acid (QA) are biosynthetic precursors.[45,46] SA is required at two different stages of CQA biosynthesis, which include the production of L-phenylalanine (biosynthetic step from the SA pathway) and the conversion of *p*-coumaroyl-CoA to CQAs.[45] When glyphosate is applied in whole plants, the biosynthesis of L-phenylalanine stops or is drastically reduced due to inhibition of EPSP synthase key enzyme.[25] However, when plants are wounded, the whole metabolic machinery (primary and secondary) is highly activated.[20] Therefore, it is likely that in the critical point of EPSP synthase enzyme, which is the bottleneck for the biosynthesis of L-phenylalanine in glyphosate-treated plants, two scenarios are taking place simultaneously in wounded tissue treated with glyphosate: EPSP enzyme is being inhibited by glyphosate, but at the same time the gene expression and biosynthesis of the enzyme are increased due to wounding. Thus, it is likely that the two stresses are counteracting at that point with kinetics of biosynthesis and kinetics of inhibition brought up to higher rate levels than normal but favoring inhibition and thus accumulation of SA and QA. Furthermore, because the whole metabolic machinery is highly active due to wounding with biosynthesis of EPSP enzyme taking place and with partial inhibition of its activity happening at the same time, then the outcome is that there is enough EPSP enzymatic activity to allow the biosynthesis of L-phenylalanine for CQA production. PAL is still active and stimulated by both stresses,[11,42,47] and it seems that higher than normal levels of QA and SA would favor the higher biosynthesis levels of CQAs observed. Further work is needed to better understand the physiological mechanism by which wounded carrot tissue treated with glyphosate accumulates high levels of CQAs.

According to the results presented herein and previous studies, a hypothetical mechanism is proposed (Figure 4). Upon the application of wounding stress in carrots, eATPs are produced at the site of injury. Those eATPs serve as primary signal for the production of ROS.[40] ROS are proposed as the signaling molecules that induce the activation of the primary and secondary metabolism in wounded carrots.[11,20] When shredded carrots are treated with glyphosate, the wound-induced activation of EPSP synthase is only partially inhibited and thus higher levels of SA and QA are accumulated in the tissue. Likewise, the application of glyphosate in shredded carrots induces higher wound-induced activation of PAL,[42,47] transforming more efficiently L-phenylalanine to CQAs precursors. Because the wounded carrot tissue treated with glyphosate contains high levels of CQAs precursors (*p*-CA and its derivatives, SA, and QA), a higher accumulation of CQAs is observed.

In the present study the effect of wounding and glyphosate treatments on the accumulation of SA and PC in carrot tissue was established. Wounding stress induced the accumulation of SA in carrot tissue, and the application of glyphosate treatments on wounded carrot tissue before storage induced an even higher accumulation of SA. Two different methods of glyphosate applications were evaluated, dipping and spraying, the latter being more efficient in inducing the production of SA. The application of glyphosate also increased the concentration of high levels of PC, specifically hydroxycinnamic acids, which have potential applications for the prevention of different degenerative diseases. The spray application of a concentrated glyphosate solution (482 g/L) on wounded carrot tissue resulted in a SA concentration of ∼4755.3 mg/kg DW (24 h

Table 2. Concentration of Shikimic Acid and Individual Phenolic Compounds in Shredded Carrots Sprayed with 100 mL of a Solution Containing Different Concentrations of Glyphosate and Stored for 24 h at 25 °C

		SA and individual PC concentrations[a] (mg/kg DW)							
			stored samples 24 h at 25 °C						
				glyphosate concentration					
peak	compound[b]	control, t 0 h	control	0 g/L	100 g/L	200 g/L	300 g/L	400 g/L	482 g/L
1	SA	259.1 ± 4.7 g	458.8 ± 6.3 f	323.1 ± 7.1 fg	789.8 ± 62.3 e	1250.9 ± 72.0 d	2538.3 ± 56.9 c	4306.3 ± 79.1 b	4755.3 ± 117.9 a
2	PCA	111.2 ± 1.1 b	206.5 ± 10.1 a	85.7 ± 4.0 c	58.0 ± 2.6 de	40.1 ± 3.1 f	25.3 ± 0.7 g	45.8 ± 1.8 ef	58.8 ± 1.1 d
3	GAD	286.5 ± 4.8 a	205.1 ± 1.4 c	167.2 ± 3.3 e	198.4 ± 1.6 c	196.2 ± 2.1 c	220.4 ± 7.9 c	229.4 ± 8.4 c	254.1 ± 8.9 a
4	CHA	18.4 ± 0.3 e	50.1 ± 4.3 e	46.1 ± 3.4 e	21.9 ± 2.8 e	147.5 ± 5.6 d	346.7 ± 11.8 c	745.0 ± 27.1 b	1044.2 ± 29.0 a
5	3,5-diCQA	ND	ND	ND	2.8 ± 0.0 d	13.0 ± 0.2 c	14.9 ± 0.4 bc	17.8 ± 0.6 b	29.4 ± 3.8 a
6	*p*-CADA	24.5 ± 1.2 d	204.2 ± 13.5 a	212.4 ± 19.6 a	25.3 ± 1.0 d	38.6 ± 0.9 cd	50.1 ± 1.2 bc	68.4 ± 2.6 b	195.3 ± 11.8 a
7	4,5-diCQA	ND	ND	ND	3.0 ± 0.0 d	6.0 ± 0.0 c	6.7 ± 0.0 c	10.0 ± 0.2 b	14.8 ± 0.6 a
8	*p*-CA	27.3 ± 0.7 e	78.6 ± 1.2 a	46.4 ± 1.6 b	36.7 ± 1.9 c	32.1 ± 1.2 d	19.9 ± 0.9 f	11.4 ± 0.4 g	5.7 ± 0.1 h
9	FA	5.1 ± 0.0 b	13.4 ± 0.9 a	4.8 ± 0.0 b	6.5 ± 0.0 c	7.1 ± 0.1 c	4.6 ± 0.0 c	3.1 ± 0.0 d	3.0 ± 0.0 d
10	*p*-CADB	7.9 ± 0.3 f	52.1 ± 1.8 d	47.4 ± 1.6 de	14.2 ± 0.4 f	43.7 ± 2.2 e	64.4 ± 3.8 c	90.7 ± 4.5 b	135.8 ± 5.6 a
11	FAD	ND	ND	ND	212.4 ± 7.5 e	319.4 ± 9.6 d	437.9 ± 13.2 c	548.5 ± 16.3 b	659.1 ± 15.7 a
12	IC	36.8 ± 0.8 d	178.4 ± 2.8 a	126.8 ± 1.3 b	22.4 ± 0.3 f	17.9 ± 0.2 g	16.9 ± 0.3 g	27.6 ± 0.6 e	70.6 ± 1.1 c

[a]Concentrations are reported as chlorogenic acid equivalents for peaks 5, 7, and 12; as gallic acid equivalents for peak 3; as *p*-coumaric acid equivalents for peaks 6 and 10; and as ferulic acid equivalents for peak 11. Compounds were quantified at 215 nm (peak 1), at 280 nm (peaks 2, 3, and 12), and at 320 nm (peaks 4−11). Values represent the mean of four replications ± standard error of the mean. Different letters in the same row indicate statistical difference by the LSD test ($p < 0.05$). ND = not detected. Compounds were identified and quantified by HPLC-PDA. [b]Abbreviations: shikimic acid (SA); protocatechuic acid (PCA); gallic acid derivative (GAD); chlorogenic acid (CHA); 3,5-dicaffeoylquinic acid (3,5-diCQA); *p*-coumaric acid derivative A (*p*-CADA); 4,5-dicaffeoylquinic acid (4,5-diCQA); *p*-coumaric acid (*p*-CA); ferulic acid (FA); *p*-coumaric acid derivative B (*p*-CADB); ferulic acid derivative (FAD); isocoumarin (IC).

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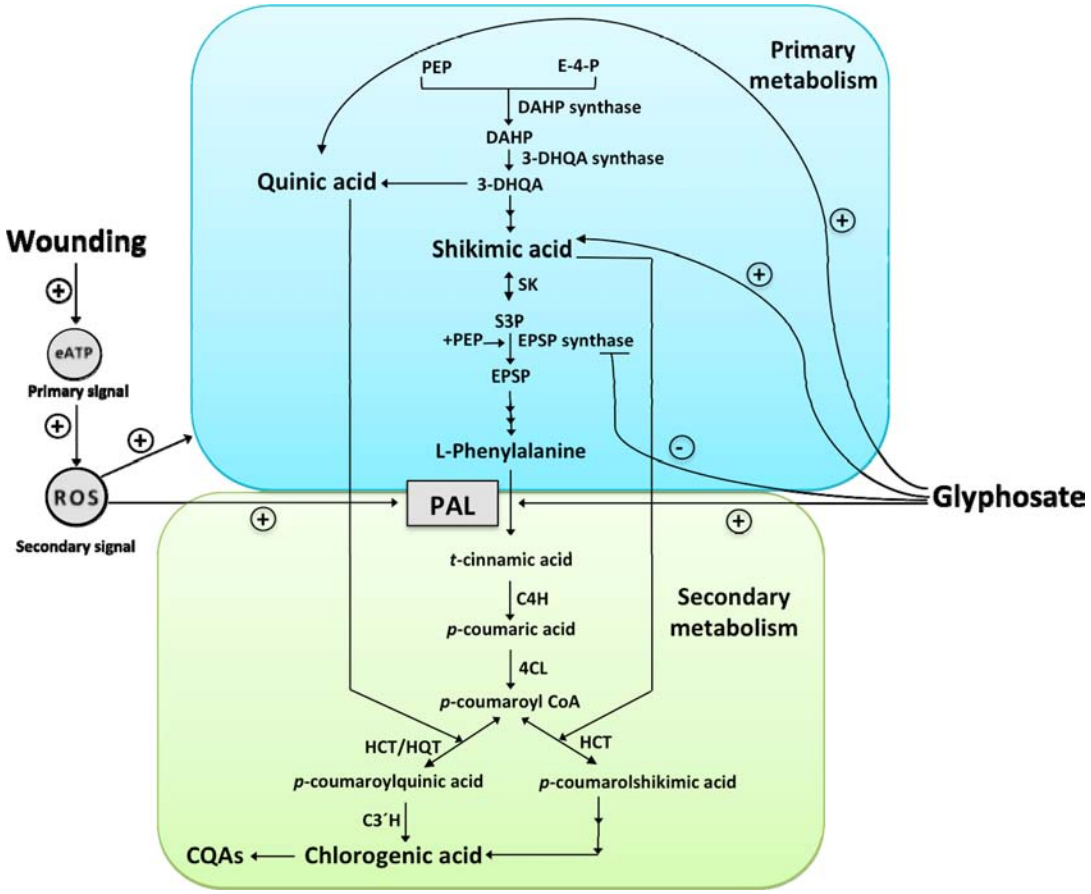

Figure 4. Hypothetical model explaining the mechanism by which wounded carrot tissue treated with glyphosate produces high levels of shikimic acid and phenolic compounds. Phosphoenolpyruvic acid (PEP); erythrose 4-phospate (E-4-P); 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP); 3-dehydroquinic acid (3-DHQA); shikimate kinase (SK); shikimic acid 3-phosphate (S3P); 5-enolpyruvylshikimate 3-phosphate (EPSP); phenylalanine ammonia-lyase (PAL); cinnamic acid 4-hydroxylase (C4H); 4-coumarate:CoA ligase AMP forming (4CL); *p*-coumarate 3′-hydroxylase (C3′H); hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase (HCT); hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (HQT); reactive oxygen species (ROS); eATP (extracellular ATP).

storage time). This represents an increment of ∼1735% in SA concentration when compared to time 0 h carrots (controls). These results demonstrate the potential of stressed carrot tissue as a biofactory of SA and valuable PC. The stressed tissue can be subsequently subjected to downstream processing to recover and purify the SA and PC with potential application in the pharmaceutical and dietary supplement industries. These findings could be extrapolated to other horticultural crops.

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Notes

The authors declare no competing financial interest.

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