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**INDUCTION OF GENTISIC ACID 5-O- $\beta$ -XYLOPYRANOSIDE IN TOMATO AND CUCUMBER PLANTS INFECTED BY DIFFERENT PATHOGENS.**

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## **Abstract**

Tomato plants infected with the citrus exocortis viroid exhibited strongly elevated levels of a **compound** identified as 2,5-dihydroxybenzoic acid (gentisic acid, GA) 5-*O*- $\beta$ -xylopyranoside. The compound accumulated early in leaves expressing mild symptoms from both citrus exocortis viroid-infected tomato, and prunus necrotic ringspot virus-infected cucumber plants, and progressively accumulated concomitant with symptom development. The work presented here demonstrates that GA, mainly associated with systemic infections in compatible plant-pathogen interactions (Bellés et al., 1999), is conjugated to xylose. Notably, this result contrasts with those previously found in other plant-pathogen interactions in which phenolics analogues of GA as benzoic or salicylic acids, are conjugated to glucose.

*Keywords:* *Cucumis*; *Lycopersicon esculentum*; Compatible interactions; Gentisic acid 5-*O*- $\beta$ -D-xyloside; Phenolics

## 1. Introduction

Gentisic acid (GA, 2,5-dihydroxybenzoic acid), a biosynthetic derivative of salicylic acid (SA, 2-hydroxybenzoic acid) has been reported to accumulate to high levels in systemic, non-necrotizing infections, as for example in citrus exocortis viroid (CEVd)-infected Rutgers tomato plants, but not in these plants infected with the necrotizing pathogen *Pseudomonas syringae*. Moreover, GA acts as a pathogenic signal additional to SA in activating defence genes in tomato (Bellés et al., 1999). For long time, a defensive role of phenolics in plant pathogen interactions has been assumed. Phenolics induced upon infection of plants by pathogens can act as antimicrobial defense and signals for induced resistance (Métraux and Raskin, 1993; Dixon, 2001). In plants, free forms of phenolic acids are, normally, less abundant than their conjugated counterparts (Harborne, 1980). In general, when induced in plants after pathogen infection, these compounds are conjugated to glucose as is the case of benzoic acid and SA in tobacco mosaic virus-infected tobacco plants (Chong et al., 2001; Malamy et al., 1992). GA is also predominantly found in a conjugated form that produced GA upon hydrolysis with  $\beta$ -glucosidase. Consequently, and based on what happens with SA in tobacco, in which this phenolic is converted into  $\beta$ -*O*-D-glucosyl SA (Enyedi et al., 1992; Malamy et al., 1992), the possibility that the conjugated form of GA could correspond to a GA  $\beta$ -*O*-D-glucoside has been entertained (Bellés et al., 1999). Related to this question, we here present results demonstrating that, unexpectedly, GA is joined to xylose, instead of glucose by a  $\beta$ -*O*-glycosidic linkage between the anomeric form of C-1 on xylose and

the hydroxyl oxygen atom on C-5 of GA. This occurs, not only in the previously studied interaction, Rutgers tomato and CEVd, but also in other compatible one between cucumber and the non necrotizing pathogen, prunus necrotic ringspot virus (PNRSV) (Sánchez-Navarro et al., 1998). **As far as we know, this is the first report on the accumulation of gentisic acid 5-O- $\beta$ -xylopyranoside in plants after pathogen infection.**

## **2. Results and discussion**

Studies that point out the importance of phenylpropanoid compounds in plant pathogen defence has been carried out in tobacco (Maher et al., 1994; Shadle et al., 2003; Niggeweg et al., 2004.), *Arabidopsis* (Hagemeier et al., 2001; Hahlbrock et al., 2003; Tan et al., 2004), and tomato plants (Roepenack-Lahaye et al., 2003). In our laboratory we are interested in elucidating the structure and, as a long-term goal, the function of phenolics induced during compatible plant-pathogen interactions. Using as our main experimental system the interaction between Rutgers tomato and CEVd, a systematic analysis by TLC of the fractions obtained after column chromatography of the methanolic extracts from control and CEVd-infected plants, was performed. TLC of appropriate fractions from CEVd-infected tissues exhibited a strong fluorescence which was not present in the equivalent fractions from control, non-infected plants. HPLC preparative analysis and UV detection allowed the purification of the compound responsible for the fluorescence. Its initial characterization was achieved by HPLC/ESI-MS in negative mode (60 mV of cone voltage) which produced a good ion fragmentation of the analyte. Figure 1A shows a total ion chromatogram for this analysis presenting a main peak (retention time 6.96 min), whose mass spectrum

yielded the following ions: [ $m/z$  (%): 285 (15), 153 (15), 152 (41), 109 (17), 108 (100). Negative electrospray ionization at mild conditions of low voltage (20 mV) yielded only a molecular ion of  $[M-H]^-$   $m/z = 285$  in negative ESI-MS, corresponding to a  $Mr$  of 286. **It was confirmed by HRMS which gave the molecular ion exact mass as 286.072537 (calculated 286.068868 for  $C_{12}H_{14}O_8$ ).** The  $m/z$  ion 153 is compatible with, and typical of, a dihydroxybenzoic acid, while 108  $m/z$  ion correspond to a fragment of the latter that has lost the carboxyl group. The chromatographic behaviour of the compound, both in the open column as well as in the HPLC, indicated that it possessed some polarity which could be conferred by a sugar. Moreover, the difference between the molecular weight of the compound and that of the dihydroxybenzoic acid coincided with the molecular weight of a pentose. In plants, hydroxybenzoic acids normally occur as glucosides, although glucose esters are also present (Klick and Hermann, 1988; Edwards, 1994). To more precisely elucidate the structure of the dihydroxybenzoic acid and also the nature ( $\alpha$  or  $\beta$ ) of the bond between the acid and the sugar, enzymatic hydrolysis of the purified compound with  $\beta$ -glucosidase or esterase was performed.  $\beta$ -glucosidase but not esterase, specifically released the dihydroxybenzoic acid. Its retention time (6.9 min), data on mass spectra obtained by electrospray ionization in negative mode at 20 V ( $m/z = 153$ ;  $m/z = 108$ ), and UV-absorption characteristics ( $\lambda_{max} = 327$  nm) were consistent with those of authentic GA. On the other hand, no cleavage of the *O*-glycosidic bond was detected after esterase digestion, indicating that the link between GA and the sugar was through a  $\beta$ -glucosidic bond (Table 1). The sugar joined to GA was identified by hydrolysis of the compound with HCl and analysis of the products in a pulsed electrochemical detector. As shown in Figure 2, hydrolysis of the compound produced a new peak (retention time of 7.8 min) in the chromatogram that was absent in the non hydrolysed sample. Identification of the

sugar after hydrolysis was achieved by spiking with the pentoses xylose, arabinose, or ribose. Only authentic xylose coeluted with the sugar product of the hydrolysis. These results contrast with what occurred for the analogues of GA, benzoic acid and SA in **other plant biotic or abiotic interactions**, in which these phenolics were conjugated to glucose (Bowling et al., 1994; **Coquoz et al., 1998**; Chong et al., 2001; Enyedi et al., 1992; Hammond-Kosack et al., 1996; Malamy et al., 1992). Further confirmation of the proposed identity of the dihydroxybenzoic acid was assessed by  $^1\text{H}$  and  $^{13}\text{C}$  NMR (Table 2) and **the signals for the sugar moiety in the  $^1\text{H}$  NMR spectrum were similar with those previously described for  $\beta$ -D-xylose (Hounsell, 1995)**. The proton-proton nuclear Overhauser effects (NOESY) showed a correlation between the sugar  $\text{H}_1'$  ( $\delta$  **4.96**) and the GA protons  $\text{H}_6$  ( $\delta$  **7.54**) and  $\text{H}_4$  ( $\delta$  **7.25**) but not with  $\text{H}_3$  ( $\delta$  **6.93**). These data demonstrated that the sugar is joined to GA through hydroxyl oxygen atom on C-5 and not through C-2 one. Based on the above results, we propose glycoside to be GA 5-*O*- $\beta$ -D-xylopiranoside (GAX) (Figure 3). Key ions in the ESI mass spectrum appear at  $m/z$  152 ( $\text{C}_7\text{O}_4\text{H}_4$ ), and 108 ( $\text{C}_7\text{O}_4\text{H}_4\text{-CO}_2$ ). Figure 4A shows a time course for GAX accumulation in leaves from CEVd-infected plants during the period of symptoms development. Visual symptoms, consisting of downward curling and rugosity of the leaves, develop 2-3 weeks after inoculation of cotyledons. A significant increase of GAX commenced early after appearance of symptoms, and was associated with mild symptoms in the leaves and progressively increased up to 50-fold relative to mock-inoculated plants, coinciding with the stronger symptoms of the disease. To establish whether the accumulation of this novel glycoside was restricted to CEVd-infected tomato or it was a more general phenomenon also occurring in other plant pathogen interactions, GAX was also measured in PNRSV-infected cucumber plants. Figure 4B shows a time course for its accumulation in cucumber following inoculation with

PNRSV over the period of symptom development. At day 4 following cotyledon inoculations, when the first leaves commenced to present curling and malformations, a significant increment in the level of GAX was already evident. As the disease progressed and symptoms became progressively more severe, GAX synthesis also increased reaching about 60% the level (approximately 300  $\mu\text{g/g}$  FW) that had been observed in CEVd-infected tomato (approximately 500  $\mu\text{g/g}$  FW). **The evolution of free GA after pathogen infection was similar to that followed by GAX representing free GA less than 10 % of GAX (Fig. 4 A and B). These results are in accordance to what has been previously found in tomato (Bellés et al., 1999).** The GA 5- $\beta$ -O-xyloside found in CEVd-infected tomato and PNRSV-infected cucumber was not present in tomato leaves inoculated with *Pseudomonas syringae* producing a hypersensitive-like reaction in the leaves (data not shown). This is consistent with previous results (Bellés et al., 1999) showing that GA was not induced in tomato leaves infected with this pathogen. Conjugation of phenolics with sugars through glycosyltransferases may modulate the endogenous level of free phenolics protecting plants from phenolic toxicity (Jones and Vogt, 2001). On the other hand, assuming phenolic glycosides to be by themselves an inactive storage form of phenolics, as it is the case of SA 2-O- $\beta$ -D-glucoside (Henning et al., 1993), conjugation may also regulate the pool of active free form in those physiological situations in which elevated levels of phenolics are formed. Thus, the question arises about the enzyme which conjugates GA to xylose. Although plant glycosyltransferases usually transfer glucose (Keegstra and Raikhel, 2001; Lim and Bowles, 2004), enzymes that specifically recognize UDP-xylose as glycosyl donor have been reported (Martin et al., 1999; Rose et al., 1996). **Also, 4-methylguaiacol and vanillyl alcohol, phenolic wood components, were converted by the fungus *Cariolus versicolor* to their  $\beta$ -xylosides, indicating that**



**UDP-glycosyltransferase was involved in the xylosylation of ligning model compounds (Kondo et al., 1993).** It has been reported that leaves from tomato plants infected with citrus exocortis viroid (CEVd) exhibited elevated levels of a conjugated form GA (Bellés et al., 1999). Based on the fact that the major part of conjugated GA was hydrolyzed by almond  $\beta$ -glucosidase, it was hypothesised (Bellés et al., 1999) that this conjugated form could be a  $\beta$ -O-D glycosyl GA but the nature of the sugar moiety was not investigated.  $\beta$ -glucosidase specifically hydrolyses  $\beta$ -glucosidic bonds, but its specificity for the sugar, however, is much lower. The work presented here demonstrates that in CEVd-infected tomato leaves, GA is conjugated to xylose instead of glucose, as it is the case of the two GA O- $\beta$ -glucosides described in *Cotoneaster orbicularis* (El-Mousallamy et al., 2000), and buckwheat (Schulz et al., 1993), in which glucose is joined to GA by a  $\beta$ -1,2- and a  $\beta$ -1,5-glycosidic linkage, respectively. On the other hand, the xylosides of gentisic and caffeic acids has been recently characterized in *Erythrina indica* (Nassar et al., 2003), and *Rubus* species (Hussein et al., 2003), respectively. **It is interesting to note that these glycosides are not associated with plant responses to biotic or abiotic stresses. Flavonoid xylosides involved in plant responses to abiotic stress (Lancaster et al., 2000) or pathogens has been reported (Elliger and Halloin, 1994).** The induction of GAX in CEVd-infected tomato and PNRSV-infected cucumber is similar to that observed for the compound 3'-O- $\beta$ -D-ribofuranosyl adenosine which accumulates in tomato and *Arabidopsis* upon infection with virulent pathogens (Bednarek et al., 2004). The accumulation to high levels of this xyloside in compatible interactions which involve very different pathogens (viroid and virus) and plants (tomato and cucumber), in addition to its novelty, constitute an interesting observation that in turn give rises to new important questions. What is the significance of the accumulation of GA joined to xylose rather than glucose as in the

case of SA? What is the possible role of the presently described xyloside in plant defence? In recent years, the implication of other glycosides in plant defence has started to be investigated. For example, engineered alfalfa plants accumulating resveratrol glucoside showed increased resistance to *Phoma medicaginis* (Hipskind and Paiva, 2000), and enhancing the levels of coumarin glucoside (scopolin) or tyrosol glucoside was correlated with the induction of defence responses in tobacco (Baumert et al., 2001) and potato (Landtag et al., 2002).

### **3. Experimental**

#### *3.1. Plant material and inoculations*

Cultivation and inoculation procedures of tomato (*Lycopersicon esculentum* L. cv. Rutgers) plants were as previously described (Bellés et al., 1991). *Cucumis sativus* L. cv. Wisconsin SMR-58 plants were grown from seeds in 10-cm-diameter pots containing a mixture of peat and vermiculite (1:1) at 22 °C /18 °C (day/night) and relative humidity from 50% to 70%. Plants were irrigated with Hoagland solution. Infection of cucumber plants with PNRSV (isolate NCM1; Aparicio et al., 1999) was performed with a viral extract obtained from leaves of PNRSV-infected cucumber plants homogenized in 30 mM sodium phosphate buffer (pH 8) and supplemented with 0.1% 2-mercaptoethanol (3 ml of buffer per g of fresh weight of PNRSV-infected cucumber). Seven days after sowing, cotyledons were dusted with carborundum, and buffer (mock-inoculated control) or viral extract was applied by gently rubbing the upper face of the two cotyledons. The cotyledons were washed with distilled water 5 min later.

### *3.2. Extraction and preliminary fractionation*

Frozen control or CEVd-infected tomato leaves showing strong symptoms of the disease (100 g of each) were homogenized with a Heidolph homogenizer (Diox 900) in methanol (500 ml). The extracts were maintained at 40 °C with constant agitation in magnetic stirrer hotplates during 2 hours, then sonicated for 15 min, and centrifuged at 14,000 g for 15 min to remove cellular debris. The supernatant (approx. 400 ml) was dried in vacuo. The residue (2 g) was dissolved in 20 ml of methanol, and submitted to column chromatography silicagel using a stepwise gradient of 25 ml each from dichlorometane to methanol as described by González et al., (2003). Aliquots were pooled in 6 fractions according to their similarity by TLC and dried under vacuum.

### *3.3. Purification and MS analysis of GA 5-O- $\beta$ -xyloside*

Fraction 4 (98 mg) was used as source to purify the compound by HPLC (Waters 600E) under isocratic conditions using a mixture of methanol and 1% acetic acid (30:70) at a flow rate of 1 ml/min. Samples (100  $\mu$ l) were injected into a SymmetryPrep C18 7  $\mu$ m (19 x 150 mm) column and the unknown compound was photometrically detected (280 nm) with a Waters 460 tunable absorbance detector. Several runs were performed and the total amount of collected compound was approximately 4 mg. Identification of the glycoside was by HPLC in combination with electrospray ionization (ESI) mass spectrometry (Willoughby et al., 2002). Samples (10  $\mu$ g) from purified compound were injected into a reverse-phase X-Terra MS C18 5  $\mu$ m (3.9 x 150 mm) column. A 20-min

linear gradient of 1 % (v/v) acetic acid in water to 100% methanol at a flow rate of 1 ml/min (Yalpani et al., 1992) was applied with a Waters 1525 HPLC binary pump. A post-column split delivered approximately 25% of the flow to a Waters ZMD mass spectrometer and the rest to a Waters 996 photodiode array detector (maxplot between 240 nm and 400 nm). The source parameters of the mass spectrometer for ESI in negative mode were the following: capillary voltage 2,500 V, cone voltage 20 V or 60 V, extractor 5 V, RF Lens 0.5 V, source block temperature 100 °C and desolvation gas temperature 300 °C. The desolvation and cone gas used was nitrogen at a flow of 300 l and 50 l per min, respectively. Other mass spectrometer conditions were: low mass resolution 13.5, high mass resolution 13.5, ion energy 0.5, multiplier 650. ESI data acquisition was in the conditions of a full scan range from mass-to-charge ratio ( $m/z$ ) 100 to 700 at 1 s per scan. **HRMS have been performed with a VG Auto Spec Fisons spectrometer.**

#### *3.4. GA 5-O- $\beta$ -xyloside measurements*

Leaf material (0.5 g) from CEVd- and PNRSV-infected leaves and the corresponding controls were ground in mortar using liquid nitrogen and then, homogenized in 1.5 ml of methanol. The extracts were vortexed vigorously, sonicated for 15 min, and centrifuged at 14,000  $g$  for 15 min in 2-ml Eppendorf tubes to remove cellular debris. The supernatant (1.5 ml) was dried under nitrogen at 40 °C, and the residue dissolved in 200  $\mu$ l of methanol. Samples were filtered through a 0.45  $\mu$ m Spartan 13/0.45RC filters (Schleicher & Schuell), and then 40  $\mu$ l assayed on an HPLC system (Waters 600E) on a reverse-phase Symmetry 5  $\mu$ m C18 (4.6 x 150 mm) column at 30 °C. A 20-min linear gradient of 1 % (v/v) acetic acid to 100 % methanol at a flow rate of 1 ml/min was

applied and glycoside was detected with a Waters 470 scanning fluorescence detector (excitation wavelength 313 nm; emission wavelength 405 nm) and quantified with the Waters Millennium<sup>32</sup> software, using authentic standards. Data were corrected for losses in the extraction procedure, and recovery of GA 5-*O*- $\beta$ -xyloside, based on SA recovery, ranged between 50-80%.

### 3.5. Enzymatic digestions

Hydrolysis with  $\beta$ -glucosidase (14.3 U/mg, Fluka) or esterase (41 U/mg, Sigma) was performed adding 10 units enzyme to 100  $\mu$ g of GA 5-*O*- $\beta$ -xyloside dissolved in 200  $\mu$ l of 50 mM sodium acetate (pH 4.5), for  $\beta$ -glucosidase or 50 mM Tris-HCl (pH 8), for esterase, and incubating the mixtures for 2 h at 37 °C. The reaction was stopped by adding 1 mL methanol and centrifuged at 12,000 *g* for 10 min to remove enzymes. The supernatants were dried in a flow of nitrogen and dissolved in 100  $\mu$ l of methanol for HPLC/MS analysis for hydrolysis products as above.

### 3.6. Sugar analysis

100  $\mu$ g of GA 5-*O*- $\beta$ -xyloside were hydrolysed at 80 °C for 1 h in 500  $\mu$ l 0.1 M HCl. After hydrolysis, 20  $\mu$ l samples were injected into a RCX-10 anion exchange 7  $\mu$ m (4.6 x 250 mm) column (Hamilton). Compounds were isocratically eluted at room temperature with 30 mM NaOH at a flow rate of 1 ml/min with a Waters 515 HPLC binary pump. Xylose was detected in a Waters 464 pulsed electrochemical detector with a range of 10  $\mu$ A and the following settings (E1 = -80 mV, T1 = 0.26 s, E2 = 570 mV, T2 = 0.26 s, E3 = -730 mV, T3 = 0.36 s).

### 3.7. NMR analysis

1-D ( $^1\text{H}$ ), ( $^{13}\text{C}$ ) and 2-D (NOESY), (COSY) NMR spectra was recorded at 298 K on a Bruker spectrometer at a frequency of 300 MHz in  $\text{D}_2\text{O}$  solvent; chemical shifts are reported in  $\delta$  (parts per million) values and couplings constants in Hz, using TMS as internal standard.

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## FIGURE LEGENDS

Figure 1. Total ion current HPLC/ESI-MS chromatogram in negative ion mode of GA 5-*O*- $\beta$ -xyloside (A). Mass spectra of GA 5-*O*- $\beta$ -xyloside (peak of retention time 6.96 min) in electrospray ionization negative ion mode at a cone voltage of 20 V (B) or 60 V (C).

Figure 2. HPLC profiles of GA 5-*O*- $\beta$ -xyloside before (A), after its hydrolysis (B), and after its hydrolysis coinjected with authentic xylose. Arrow indicates xylose peak. **Sugars from the hydrolysed mixture were detected in a Waters 464 pulsed electrochemical detector.**

Figure 3. Structure of GA 5-*O*- $\beta$ -xyloside and scheme of fragmentation of the compound obtained by electrospray ionization in negative ion mode at a cone voltage of 60 V.

Figure 4. Time courses for the accumulation of GA 5-*O*- $\beta$ -xyloside **and free GA in CEVd-infected Rutgers tomato (A), and PNRSV-infected cucumber plants (B).** **Open bars, GAX in control plants; dark bars, GAX in CEVd- or PNRSV-infected plants; cross-hatched bars, free GA in CEVd- or PNRSV-infected plants.** Results are the mean  $\pm$  SE of 3 replicates.

Table 1. Fragment ions of GA 5-*O*- $\beta$ -xyloside obtained by electrospray ionization in negative ion mode at a cone voltage of 20 V, before and after its hydrolysis with  $\beta$ -glucosidase or esterase.

<i>Treatment</i>	<i>m/z</i>	<i>fragment ion</i>	<i>UV spectral data <math>\lambda_{max}</math> (nm)</i>
no hydrolysis	285	M-H	315
$\beta$ -glucosidase	153	gentisic acid -H	327
	108	gentisic acid -CO <sub>2</sub> H	
esterase	285	M-H	315

Table 2.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data of gentisic acid 5-*O*- $\beta$ -xyloside.

<i>Site</i>	$\delta_{\text{H}}$	<i>multiplicity</i> <sup>a</sup>	$J_{\text{HH}}(\text{Hz})$	$\delta_{\text{C}}$
<i>gentisic acid</i>				
1				114.47
2				156.28
3	6.93	( <i>d</i> )	9.0	118.33
4	7.25	( <i>dd</i> )	9.0, 3.0	125.92
5				149.37
6	7.54	( <i>d</i> )	3.0	118.43
7				172.65
<i><math>\beta</math>-xylose</i>				
1'	4.96	( <i>brd</i> )	7.8	102.30
2'	3.50-3.56 <sup>b</sup>	( <i>m</i> )	-	73.22
3'	3.50-3.56	( <i>m</i> )	-	75.87
4'	3.65-3.76	( <i>m</i> )	-	69.44
5'ax	3.44 <sup>b</sup>	( <i>dd</i> )	11.3, 10.5	
5'eq	3.99	( <i>dd</i> )	11.3, 5.4	65.62

<sup>a</sup> (*d*)=doublet, (*dd*)=doublet of doublet, (*brd*)=broad doublet, (*m*)=multiplet

<sup>b</sup> data assigned by 2D-COSY correlation.