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ENHANCEMENT OF PHENYLETHANOID GLYCOSIDES BIOSYNTHESIS IN Castilleja tenuiflora Benth. SHOOT CULTURES WITH CELL WALL OLIGOSACCHARIDES FROM Fusarium oxysporum f. sp. lycopersici RACE 3

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ESTIMULACIÓN DE LA BIOSÍNTESIS DE LOS FENILETANOIDES GLICOSILADOS EN CULTIVOS DE BROTES DE Castilleja tenuiflora Benth. CON OLIGOSACÁRIDOS DE PARED CELULAR DE Fusarium oxysporum f. sp. lycopersici RAZA 3

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Abstract
Phenylethanoid glycosides (PhGs) are promising natural products for the treatment of chronic diseases because of their wide range of biological activities. Biotic stress as pathogen attack (fungi) may stimulate the synthesis of PhGs through activation of phenylalanine ammonia-lyase (PAL). Castilleja tenuiflora Benth. (Orobanchaceae) in vitro cultures are alternative sources of PhGs, however in that conditions, cultures have a diminished synthesis of these compounds. To increase the yields of PhGs, the identification of factors affecting their biosynthesis is required. Here, we show that elicitation with cell wall oligosaccharides (CWOs) from Fusarium oxysporum f. sp. lycopersici race 3 (Hyphomycetes) stimulates biosynthesis of PhGs by increasing the activity of PAL. Upon elicitation with CWOs (13 µg/mL) the production of PhGs was enhanced by 5-fold compared with untreated control. The maximum PAL activity in shoots cultured under CWOs elicitation were also increased. Elicitation did not affect the shoot growth (length and biomass) but induced chlorosis, and delayed root formation of C. tenuiflora shoots. Our results demonstrate that elicitation with CWOs increases PhGs biosynthesis in C. tenuiflora shoot culture.

Keywords: PAL, verbascoside, isoverbascoside, Castilleja tenuiflora, Fusarium oxysporum f. sp lycopersici race 3.

Resumen
Los feniletanoides glicosilados (FEGs) son sustancias naturales que pueden utilizarse para el tratamiento de enfermedades crónicas debido a su amplia gama de actividades biológicas. Un estrés biótico como el ataque de patógenos (hongos) a plantas, puede estimular la síntesis de FEGs a través de la activación de la enzima fenilalanina amoníaco-lisasa (PAL). Los brotes de Castilleja tenuiflora Benth. (Orobanchaceae) cultivados in vitro producen FEGs, sin embargo, en esas condiciones se presenta una disminución en la síntesis de estos compuestos. Para aumentar los rendimientos de los FEGs se requiere la identificación de los factores que afectan a su biosíntesis. En este trabajo se muestra que la elicitation con oligosacáridos de pared celular (OPCs) de Fusarium oxysporum f. sp. lycopersici raza 3 (Hyphomycetes) estima la biosíntesis de FEGs aumentando la actividad de la enzima PAL. La elicitation de los brotes de C. tenuiflora con OPCs (13 µg/mL) aumentó la producción de FEGs 5 veces en comparación con el control y, provocó un aumento en la actividad máxima de la enzima PAL. Por otro lado, la elicitation no afectó el crecimiento de los brotes (longitud y biomasa), pero si indujo clorosis y retardó la formación de raíces. Nuestros resultados demuestran que la elicitation con OPCs incrementa la biosíntesis de FEGs en cultivos de brotes de C. tenuiflora.

Palabras clave: PAL, verbascoside, Castilleja tenuiflora, Fusarium oxysporum f. sp lycopersici raza 3.

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1 Introduction

Castilleja tenuiflora Benth. is a member of the Orobanchaceae family, a perennial medicinal herb with proven health benefits including anti-inflammatory (Sánchez et al., 2013), antioxidant (López-Laredo et al., 2012), anti-ulcerogenic (Sánchez et al., 2013) and cytotoxic activities (Moreno-Escobar et al., 2011). The main chemical compounds found in this species are: iridoids glycosides such as aucubin, the phenylethanoid glycosides (PhGs) verbascoside and isoverbascoside (Gómez-Aguirre et al., 2012) and flavonoids such as quercetin-type glycosides (López-Laredo et al., 2012) and apigenin (Gómez, 2011). PhGs are watersoluble natural occurring compounds, absorbable by human intestinal cells and with a wide spectrum of biological activities including anti-fungal (Funari et al., 2012), anti-inflammatory (Gyurkovska et al., 2011), antiradical (López-Laredo et al., 2012), cytotoxic (Pettit et al., 1990), and gastroprotective (Singh et al., 2010) among others.

Castilleja tenuiflora in vitro cultures exhibit pharmacological activities comparable to that of wild plants and are attractive sources of PhGs (Sánchez et al., 2013) and iridoids (Martínez-Bonfil et al., 2011) constituting a sustainable alternative to produce high-value products. Previously, we have shown that abiotic stress provoked by nitrogen limitation enhances PhGs accumulation and de novo biosynthesis of anthocyanins through phenylalanine ammonia-lyase (PAL) activity. However, that condition had a severe negative effect in biomass thus, decreasing possibilities of commercial scale (Medina, 2011). Fungal elicitation of plant cell cultures is a strategy for promoting specialized metabolism having its basis on plant defense responses. Oligosaccharides originating from fungal cell walls are potent inducers of resistance of many plants species against a wide range of pathogens since they act as primary signals in the induction of plant defense reactions. In cell suspension cultures of Cistanche deserticola (Orobanchaceae) fungal elicitors derived from Fusarium solani (Lu and Mei, 2003), chitosan (Cheng et al., 2006) or yeast (Cheng et al., 2005) presented a positive effect on the production of PhGs. Since PhGs biosynthesis appears to be influenced by both biotic and abiotic (Sánchez-Rangel et al., 2014) factors, we hypothesized that fungal elicitation may enhance biosynthesis of PhGs in C. tenuiflora. Here we tested the effects of cell wall oligosaccharides (CWOs) from Fusarium oxysporum f. sp. lycopersici race 3 in PhGs accumulation by C. tenuiflora shoot cultures and the activity of PAL and peroxidase (POD), two defense-related enzymes.

2 Materials and methods

2.1 Plant culture and experimental design

Shoots culture of C. tenuiflora was initiated and propagated in vitro as described previously (Trejo-Tapia et al., 2012). Shoots were subcultured in 300-ml shake flasks containing 50 mL B5 culture medium (Gamborg et al., 1968), 3% (w/v) sucrose and no plant growth regulators. Cultures were maintained under continuous agitation at 110 rpm on an orbital shaker in a growth room at 25 ± 2°C under a 16-h light/8-h dark photoperiod with illumination of 103 μmol/m²/s provided by 75 W cool-white fluorescent lamps. For elicitation treatments, preliminary assays were made and the elicitation time, 15 days, was defined. C. tenuiflora shoot cultures were grown in shake flasks for 15 days, then, were supplemented with CWOs from Fusarium oxysporum f. sp. lycopersici as elicitor.

2.2 Elicitation of shoots culture

The fungal strain used to obtain the CWOs for elicitation was Fusarium oxysporum f. sp. lycopersici race 3 (the strain was isolated from tomato and identified by molecular biology techniques (Ortega-García 2010)). The elicitor was prepared as described by El Modafar et al. (2001) with slight modifications. The pathogen was grown on potato dextrose broth under agitation at 150 rpm. Five-days-old mycelia from fungi was centrifuged at 13,000 × g for 40 min, the collected mycelium was harvested and rinsed thrice with sterile distilled water, then, dried at 47 °C, grinded with a mortar and pestle and suspended in deionized water (2 mg/mL). This suspension was autoclaved at 120 °C for 40 min and centrifuged at 1,000 × g for 20 min. The supernatant (CWOs) was used as elicitor and supplied to 15-day-old C. tenuiflora shoot cultures at 5 (+CWOs-L; lowest dose) and 13 (+CWOs-H; highest dose) µg/mL doses, based on the total carbohydrate content of the fungal homogenate, which was determined by the phenol/sulfuric acid method (Dubois et al., 1956) using glucose as the standard. Shoots were collected for analysis at 1-8 days after elicitation.
2.3 Chlorophyll content

The chlorophyll extraction was performed with acetone (80%) and the absorbances were measured at 663.2 and 648.8 nm. The total chlorophyll content was calculated using the equations described by Lichtenthaler (1987).

2.4 Phenylalanine ammonia-lyase (PAL) activity

PAL was extracted and its activity was determined according to the methods described by Yan et al. (2006). Fresh samples (0.2 g) were frozen in liquid nitrogen and ground in a mortar with 40 mg of polyvinyl polypyrrolidone. The powder was mixed with 3 mL of a solution comprising 100 mM sodium phosphate buffer (pH 6.0), 2 mM EDTA, and 4 mM DTT. The mixture was centrifuged at 13,500 × g at 4°C for 15 min, and the supernatant was employed for the enzymatic assay. The protein concentration was determined using the method of Bradford (1976). The reaction mixture comprising 550 µL of 50 mM Tris-HCl (pH 8.8) and 200 µL of extract (2-16 µg of protein) was preincubated at 40°C for 60 min and the reaction was initiated by addition of 250 µL of 20 mM L-phenylalanine (pH 8.8). After 1 h of incubation at 40°C, the reaction was stopped by addition of 50 µL of 5 N HCl. The blank reaction for every sample was carried out with Tris-HCl and extract but without phenylalanine. The absorbance change at 290 nm was evaluated after 1 h. The enzymatic activity was reported as µmol cinnamic acid h⁻¹ mg⁻¹ protein.

2.5 Guaiacol peroxidase (POD) activity

POD activity was measured according to the method of Stasolla and Yeung (2007). The reaction mixture (1 mL) contained 100 mM sodium phosphate buffer (pH 6.0), 0.3% hydrogen peroxide, 100 mM guaiacol, 2 mM EDTA, and 4 mM DTT, and enzymatic extract (2-16 µg of protein). The change in absorbance at 470 nm caused by guaiacol oxidation was followed at 30-s intervals for 6 min. The POD activity was expressed as mM tetraguaiacol min⁻¹ mg⁻¹ protein using the extinction coefficient of tetraguaiacol (26.6 mM⁻¹cm⁻¹).

2.6 Evaluation of total phenolic compounds (TPC)

Fresh plant material (0.2 g) was ground and extracted with methanol (1 mL) for 24 h with agitation. The extract was filtered through Whatman paper No. 1, and concentrated in a rotary evaporator (Büchi-490; Büchi, Switzerland) under reduced pressure (210 mbar) at 40°C for 50 min. The residue was suspended in 10 mL of water and freeze-dried. Phenolic compounds in the methanol extract were estimated using the Folin-Ciocalteau reagent. Results were expressed as mg of gallic acid equivalents (GAE) per g of dry weight (DW) (Cardenas-Sandoval et al., 2012). The samples were analyzed in triplicate.

2.7 Quantification of phenylethanoid glycosides (PhGs) by HPLC

PhGs were quantified by HPLC using a Waters 2695 separation module HPLC system equipped with a Waters 996 photodiode array detector and Empower Pro software (Waters Corporation, USA) as described previously (Gómez-Aguirre et al., 2012). Compounds were separated on a LiChrospher 100 RP-18 column (4 mm × 250 mm i.d., 5-µm particle size) (Merck, Darmstadt, Germany) connected to a guard column. The mobile phase consisted of water (pH 3.5) containing 5% TFA (solvent A) and acetonitrile (solvent B). The gradient system was as follows: 0-2 min, 100-0% B; 2-10 min, 77-23% B; 12-13 min, 70-30% B; 14-15 min, 100% B; 16-17 min, 0% B. The flow rate was maintained at 1 mL min⁻¹ and the injection volume was 20 µL. The absorbance was measured at 330 nm. The PhGs peaks were identified by comparison of the retention times and UV spectra with those of reference compounds isolated and purified from the roots of C. tenuiflora (Gómez-Aguirre et al., 2012).
Table 1. Growth parameters of *C. tenuiflora* shoots under control conditions or elicitor-treated cultures with two doses of CWOs from *F. oxysporum*

<table>
<thead>
<tr>
<th></th>
<th>Fresh biomass (g per flask)</th>
<th>Shoot length (cm)</th>
<th>Chlorophyll (µg/g DW)</th>
<th>Tissue water content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.76 ± 0.01^A</td>
<td>3.36 ± 0.17^B</td>
<td>1.05 ± 0.01^A</td>
<td>93.36^A</td>
</tr>
<tr>
<td>+CWOs-L</td>
<td>11.82 ± 0.01^A</td>
<td>3.49 ± 0.01^B</td>
<td>0.79 ± 0.01^B</td>
<td>93.20^A</td>
</tr>
<tr>
<td>+CWOs-H</td>
<td>11.70 ± 0.01^A</td>
<td>4.25 ± 0.22^A</td>
<td>0.78 ± 0.01^B</td>
<td>93.08^A</td>
</tr>
</tbody>
</table>

Elicitor were added after 15 days of growth and shoots were cultivated for further 8 days. Data represent means ± standard errors (*n* = 9). Within each column, different letters indicate significant difference according to Tukey’s test (*α* =0.05).

2.8 Statistical analysis

Statistical analyses were performed by one-way ANOVA and the all-pairwise multiple comparison procedure of Tukey was employed. The significance level for all statistical analyses was 5%. Differences of *P* < 0.05 were considered significant. The software SigmaPlot for Windows version 11.0 software (Systat Software Inc., San Jose, CA, USA) was used to perform the statistical analyses.

3 Results

3.1 Effects of fungal elicitor on growth and enzymes activities

The effects of two doses, 5 and 13 µg/mL, of elicitor, on growth and enzyme activities were tested in shoots of *C. tenuiflora*. Neither of the doses tested altered biomass nor tissue water content. Elicitor-treated shoots with the highest dose (+CWOs-H, 13 µg/mL) were significantly longer than untreated shoots or shoots treated with the lowest dose (+CWOs-L, 5 µg/mL). Elicited shoots presented yellowish of their leaves (chlorosis) and total chlorophyll content was significantly reduced in these shoots compared with untreated shoots (Table 1). Elicitor application delayed root formation compared to the control. Maximum POD activity was higher in +CWOs-H shoots compared with untreated shoots or +CWOs-L shoots, from days 1 to 8 after elicitation (Fig. 1A).

PAL activity observed in untreated shoots and +FE-L shoots was 1.5-1.7 µmol CA/h/mg protein and decreased at days 2 and 3 (0.83 µmol CA/h/mg protein) (Fig. 1B). Meanwhile, the maximum PAL activity in +FE-H elicited shoots was reached 4 days after elicitation (2.67 µmol CA/h/mg protein). PAL activity observed at this point was significantly higher in +FE-H shoots (*p* <0.05) than in untreated shoots and +FE-L shoots (1.67 µmol CA/h/mg protein).

Fig. 1. Time course of POD (A) and PAL (B) activity in *C. tenuiflora* control and elicitor-treated cultures with two doses of CWOs from *F. oxysporum*. Elicitor was added after 15 days of growth and shoots were cultivated for further 8 days. Data represents means ± standard errors (*n* = 5). Within each time point assayed, (*) indicates significant difference according to Tukey’s test (*α*=0.05).

3.2 Contents of TPC and PhGs

Time-course accumulation of total phenolic content in *C. tenuiflora* after elicitation with the two doses of CWOs is shown in Fig. 2. In this case, the effect turned out to be different depending on the CWOs dose. TPC on untreated shoots ranged from 21.6-49.5 mg GAE/g DW; in shoots elicited with 5 µg CWOs/mL were 30.8-
Fig. 2. Time course of total phenolic compounds content in *C. tenuiflora* control and elicitor-treated cultures with two doses of CWOs from *F. oxysporum*. Elicitor was added after 15 days of growth and shoots were cultivated for further 8 days. Data represents means ± standard errors (*n* = 9). Within each time point assayed, (*) indicates significant difference according to Tukey’s test (*α*=0.05).

51.4 mg GAE/g DW. In shoots elicited with the highest dose, the content of phenolic compounds increased from days 1 (60.2 mg GAE/g DW) to 8 of elicitation (91.8 mg GAE/g DW), reaching the maximum at day 4 (97.1 mg GAE/g DW) (Fig. 2).

Out of the two elicitor doses tested, the highest yield in TPC and the changes in enzymes activities were found in shoots treated with 13 µg FE/mL (+FE-H), thus, further experiments were done only with shoots treated with this dose of elicitor. According to HPLC analyses *C. tenuiflora* shoots accumulated the PhGs verbascoside (1, *t_R* = 7.9 min, *λ_max* = 200, 330 nm) and isoverbascoside (2, *t_R* = 7.5 min, *λ_max* = 200, 330 nm) and, a yet unidentified phenylethanoid glycoside (3, *t_R* = 6.3 min, *λ_max* = 200, 330 nm) (Fig. 3). The HPLC profile suggested that verbascoside was the main PhGs in untreated shoots and fungal elicitated.

Fig. 3. Chromatographic HPLC profiles of methanol extracts from *C. tenuiflora* control and elicitor-treated cultures with two doses of CWOs from *F. oxysporum*. Elicitor was added after 15 days of growth and shoots were cultivated for further 8 days. Verbascoside (1, *t_R* = 7.9 min, *λ_max* = 200, 330 nm), Isoverbascoside (2, *t_R* = 7.5 min, *λ_max* = 200, 330 nm) and unidentified phenylethanoid glycoside (3, *t_R* = 6.3 min, *λ_max* = 200, 330 nm).
Table 2. Phenylethanoids glycospides concentration in C. tenuiflora shoots under control conditions or elicitor-treated cultures with 13 µg/mL (+CWOs-H) from F. oxysporum

<table>
<thead>
<tr>
<th>Elicitor</th>
<th>Verbascoside (mg/g DW)</th>
<th>Isoverbascoside (mg/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.19 ± 0.2B</td>
<td>14.11 ± 0.1B</td>
</tr>
<tr>
<td>+CWOs-H</td>
<td>95.66 ± 0.8A</td>
<td>54.29 ± 0.3A</td>
</tr>
</tbody>
</table>

Elicitor was added after 15 days of growth and shoots were cultivated for further 8 days. Data represents means ± standard errors (n = 5). Within each column, different letters indicate significant difference according to Tukey’s test (α=0.05).

Eight days after elicitation, the specific concentration of verbascoside in fungal elicitated shoots was 95.66 mg/g DW compared with 19.19 mg/G DW in untreated shoots (Table 2). Isoverbascoside was also enhanced by fungal elicitation, but to a lesser extent (14.11 vs 54.29 mg/g DW).

4 Discussion

Elicitation with CWOs from F. oxysporum did not modify C. tenuiflora growth measured as biomass. However, elicitation provoked that leaves turned yellow (chlorosis), total chlorophylls decreased, shoots elongated and root formation was delayed. This response was similar to reported by (Boari and Vurro, 2004) where, development of shoots from Orobanche ramosa (Orobanchaceae) is severely affected by F. oxysporum and F. solani. Similarly, F. oxysporum has been used in growth control of Striga hermonthica (Del.) (Orobanchaceae) since it can cause plant death (Ndambi et al., 2011). In contrast, growth of cell suspension cultures of Cistanche deserticola (Orobanchaceae) was not affected by elicitation with F. solani (carbohydrate equivalents)(40 mg/L) (Lu and Mei, 2003). Oligosaccharides (galactoglucomannans) inhibited growth, morphology and adventitious root formation of Karwinskia humboldtiana (Rhamnaceae) (Kollárová et al., 2007). That effect depended on oligosaccharides concentration. Similarly, hypocotyl elongation of Vigna radiata (L.) Wilczek (Fabaceae) was negatively affected by oligosaccharides (Kollárová et al., 2009). Those changes were explained based on the effect of oligosaccharides on auxins metabolism and thus, influence in plant growth regulation. In other species, exposure to oligosaccharides provoked oxidative stress condition (Orozco-Cárdenas et al., 2001) and chlorosis (De Wit et al., 1984).

TPC was used as a first evidence of the effect of elicitation on secondary metabolism; changes were observed since the first 24 h with the highest TPC at fourth day. Based on this result, the influence of fungal elicitation on enzymes activities and HPLC profile was analyzed. Fungal elicitation provoked changes in the activities of POD and PAL compared with the control. POD is a pathogen-related enzymes associated with the plant defense pathway that is challenged by elicitors (Dutsaadee and Nunta, 2008) and used as an indicative of an oxidative stress condition (Arencibia et al., 2012). Fungal elicitation with CWOs from F. oxysporum provoked an evident increase (20-fold) in C. tenuiflora POD activity compared with non-elicited shoots. A similar response was recorded in Lupinus luteus (Fabaceae) embryos inoculated with F. oxysporum spore suspension, where POD activity increased between 0 to 96 h after inoculation (Morkunas and Gmerek, 2007). PAL is a key branch enzyme on the biosynthesis of phenolic compounds such as phenolic acids, flavonoids or anthocyanins. The increase in its activity is an indicator of plant resistance. In C. tenuiflora, PAL activity increased in response to elicitor with a differential effect depending of dose. With the highest dose, increase was obvious at 2 days with a maximum at 4 days (1.7-fold compared with the control). While with the lowest dose, the increase was evident at 3 days. PAL activity agrees with the reports on Taxus chinensis suspension cultures (Yuan et al., 2002) and C. deserticola (Lu and Mei, 2003) elicited with F. oxysporum and F. solani, respectively. Elicitation with oligosaccharides isolated from F. oxysporum induced PAL activity in Rubus fruticosus cells (Nita-Lazar et al., 2004). Plant cell response to pathogen attack or other stress condition involves events at biochemical and molecular level occurring at different times (Zhao et al., 2005). Reactive Oxygen Species (ROS) production constitutes one of the earliest events while secondary metabolism accumulation occurs later. This may explain that maximum POD activity preceded maximum PAL activity. Other authors explain that POD activity is link to lignin biosynthesis, which is also associated to PAL activity. Thus, POD activation indirectly participates in PAL activation as one of the control points for synthesis of phenolic compounds (Arencibia et al., 2012; Jouili et al., 2011).

Fungal elicitation clearly enhanced PhGs accumulation. Shoots elicited with the highest FE
dose accumulated 5-fold verbascoside than non-elicited shoots. PhGs enhancement agrees with that observed in cell suspension cultures of Cistanche deserticola elicited with F. solani oligosaccharides (Lu and Mei, 2003), chitosan (Cheng et al., 2006) or a yeast-derived elicitor (Cheng et al., 2005). PhGs role in plants has been little explored since researches on their pharmacological activities have received more attention. Verbascoside influences fungi morphology and decreases sporulation of Fusarium culmorum, Bipolaris sorokiniana and Botrytis cinerea (Nikonorova et al., 2009). Also inhibit growth of Penicillium digitatum (Oyourou et al., 2013). The effects of fungal elicitation on PhGs biosynthesis have not been tested in previous studies. Verbascoside and isoverbascoside were significantly increased after fungal elicitation, suggesting antioxidant protective roles of these compounds against the oxidative stress caused by F. oxysporum. This possibility is under further investigation.

Conclusions

Fungal elicitation with WCOs from F. oxysporum increases PhGs accumulation in C. tenuiflora via the stimulation of POD and PAL and, without reduction in biomass.

Acknowledgements

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Notation

| CWOs DW | cell wall oligosaccharides dry weight |
| FE      | fungal elicitation                   |
| GAE     | gallic acid equivalents              |
| PhGs    | phenylethanoid glycosides            |
| PAL     | phenylalanine ammonia-lyase          |
| POD     | guaiacol peroxidase                  |
| ROS     | reactive oxygen species              |
| TPC     | total phenolic compounds             |

References


