PHENOLIC CONSTITUENTS OF GLANDULAR TRICHOMES ON *SOLANUM BERTHAULTII* AND *S. POLYADENIUM*[1]

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Abstract

Phenolic compounds in Type A glandular trichomes of two insect-resistant potato species were separated and identified using a combination of high pressure liquid chromatography (HPLC), UV-visible spectrometry, and ^1H proton nuclear magnetic resonance (NMR) procedures. Type A trichomes of *Solanum berthaultii* and *S. polyadenium* contained a phenolic compound tentatively identified as the glucose ester of p-hydroxyphenyl-propionic acid. Its presence in trichomes of both species suggests that this compound may play a major role in formation of the brownish, insect entrapping exudate characteristic of trichome-mediated insect resistance in these species. Chlorogenic acid, a major phenolic constituent of Type A trichomes of *Solanum polyadenium* was not detected in trichomes of *S. berthaultii.* The exclusive presence of chlorogenic acid in trichome exudates of *S. polyadenium* may be responsible, at least in part, for the greater levels of insect entrapment by this species, compared with that of *S. berthaultii.*

Resumen

Se separaron e identificaron compuestos fenólicos en los tricomas glandulares Tipo A de dos especies de papa resistentes a insectos, utilizando una combinación de los métodos de cromatografía líquida de alta presión (HPLC), espectrometría visible UV, y la resonancia nuclear magnética del protón ^1H, (NMR). Los tricomas Tipo A de *Solanum berthaultii* y *S. polyadenium* contenían un compuesto fenólico tentativamente identificado como el ester glucosado del ácido p-hidroxifenilpropiónico. Su presencia en los tricomas de ambas especies sugiere que este compuesto puede jugar un importante rol en la formación del exudado parduzco que entrampa a los insectos y que es característico de la resistencia en estas dos especies, ejercida sobre los insectos atrapados en sus tricomas. El ácido clorogénico, un constituyente principal de los tricomas Tipo A de *Solanum polyadenium* no fue detectado en los tricomas de *Solanum berthaultii.* La presencia exclusiva del ácido clorogénico

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en los exudados de los tricomas de *Solanum polyadenium* puede ser responsable, al menos en parte, de los mayores niveles de entrampamiento encontrados en esta especie, en comparación con los observados con *S. berthaultii.*

Introduction

Wild potato species from Bolivia and Mexico bear an indument of glandular trichomes providing plants with a highly effective barrier against insect herbivory (6, 19, 20). The Type A trichomes of *Solanum berthaultii* Hawkes and *S. polyadenium* Greenm. described by Gibson (6) bear a tetralobulate head and play a major role in defense by entrapping insect pests. The mechanism of entrapment consists of the release of polyphenoloxidase (PPO) enzyme, a peroxidase (PO) enzyme, and phenolic substrates from internal storage spaces upon rupture of the trichome head by insect contact. Although the nature of the enzyme complex has been studied (2, 15), the identification of trichome phenolics has been limited by the lack of satisfactory techniques for collection of trichome exudates.

We report here procedures for collection of individual Type A trichome heads, for separation of phenolic constituents by high pressure liquid chromatography (HPLC), and the identification of several phenolic compounds.

Materials and Methods

Plant Culture—Plants of *S. berthaultii* (PI 218215, PI 473334) and *S. polyadenium* (PI 320342) were grown from botanical seed obtained from the Potato Introduction Station, Sturgeon Bay, Wisconsin. All plants were grown in a greenhouse environment using a peat-vermiculite soil mix treated with fertilizer (Osmocote). Natural light was supplemented with illumination (Metalarc) of 32,000 lux at soil level. The photoperiod was 16:8 (L:D), temperatures fluctuated between 20 and 26 C, and RH ranged from 60 and 90%. All plants used in an experiment were of the same age, had stems between 30 and 50 cm in length, and were flowering at the initiation of each test.

Trichome Collection—Leaves excised from mature flowering plants were placed on moist filter paper and the head contents of 500 Type A trichomes were collected using a glass capillary at magnification of 30X. Light contact of the capillary on the membrane surrounding the tetralobulate head spilled its contents into the capillary's solvent (aqueous potassium cyanide; 2.9 mM, pH 10.0). Cyanide inhibits polyphenoloxidase activity preventing conversion of trichome phenolics into quinones (12). Degradation of the phenolics was not observed even during the extended periods of trichome exudate collection. The contents of the glass capillary, about 8 μl representing solvent and 500 trichome heads were transferred to a syringe for injection into the HPLC system.

HPLC Procedures—The C18, 5 μm particle size, reversed phase column, 250 x 4.0 mm (SGE, Australia) was coupled to a Waters Associates HPLC system. A binary solvent gradient (Waters #9) [14% to 85% of solvent B into solvent A, 25 minutes, flow rate: 1.5 ml/min] was used. The solvent systems were as follows: (A), distilled water with 0.4% KH_2PO_4 (pH 4.5), (B), 25% acetonitrile in aqueous 0.4% KH_2PO_4 (pH 5.0). Phenolics were detected with an LKB Model 2238 single wavelength instrument at 279 nm or 225 nm; signals were integrated and recorded using a Hewlett Packard Model 3390A integrator.

Measurement of Phenolic Oxidation Activity—The phenolic oxidation activity of Type A trichome exudate was determined using the method of Ryan, *et al.* (16). Three leaflets were placed in a 20 ml glass tube containing 3 ml of buffer solution (70 mM sodium phosphate, 1% Triton X-100, 0.075% p-phenylenediamine). The tube was agitated in a Vortex for 30 seconds, then held at 37 C in a water bath for 15 minutes. Color development of the reaction mixture was measured as percentage transmittance at 440 nm.

Identification of Phenolics—A dimethylsulfoxide (DMSO) leaf extract modified after Banwart, *et al.* (1) was made to obtain the phenolic compounds of interest in quantities sufficient for proton NMR. Freshly excised leaves were quickly frozen at -80 C and ground with mortar and pestle in DMSO in a 2:1 (w/w) ratio. The slurry was filtered through cheesecloth and subsequently through a 5 μm Millipore® filter. Four HPLC runs of 60 μl samples were made and identical fractions were combined. The solvent gradient system was slightly modified to optimize fraction collection, i.e., solvent B: 10% acetonitrile in aqueous 0.4% KH_2PO_4, flow rate: 2.0 ml/min. Other parameters were the same as described earlier. Cysteine (15 mM) was added to the fractions to prevent oxidation of the phenolics. The fractions were lyophilized and then redissolved in acetonitrile/methanol (6:4) for removal of phosphate and cysteine. The phenolics were further redissolved in DMSO-d_6 for ^1H proton NMR analysis (Varian XL-400).

Results

HPLC provided excellent separation of polar compounds in Type A glandular trichome exudate of *S. berthaultii* and *S. polyadenium*. The contents of only 500 tetralobulate heads were sufficient to produce well-defined chromatograms (Figure 1). Five HPLC fractions [*S. polyadenium*, (A) 5.32, (B) 9.98, (C) 28.61 minutes; *S. berthaultii*, (D) 5.26, (E) 5.31 minutes] (see Figure 1) were selected for further analysis. All of these fractions displayed a positive reaction when treated with the phenol-specific reagent, Folin-Ciocalteu (Sigma Chemical Co.).

Fraction B—The UV-Vis (200-400 nm) absorption spectrum (*S. polyadenium*, fraction B, 9.98 minutes) was identical with that of n-chlorogenic acid (7). The absorption maxima in water/acetonitrile of this fraction and

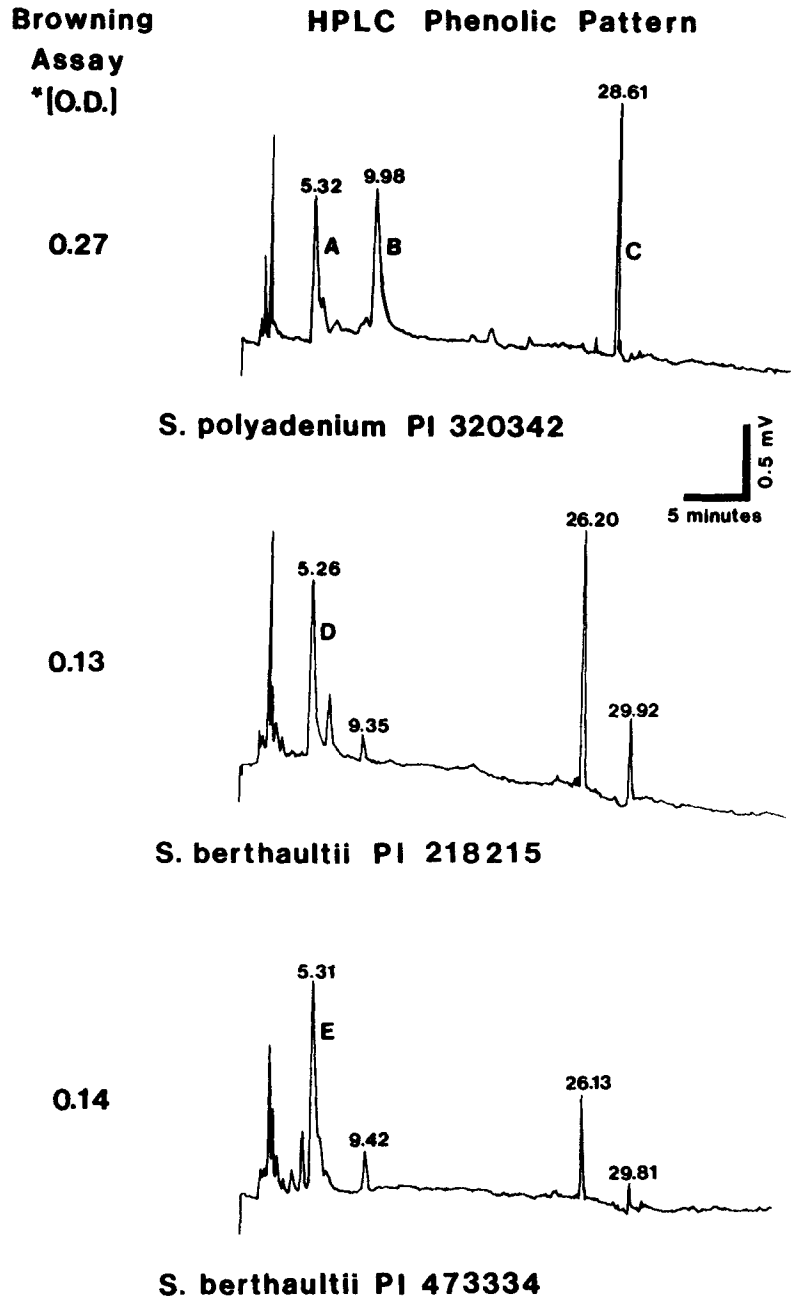

FIG. 1. Phenolic compounds of Type A glandular trichomes and potential to form brownish insect-trapping exudate. O.D.=optical density from total browning assay (16); HPLC=retention time (minutes) and recorder response at 279 nm.

n-chlorogenic acid were 215 and 322 nm. An authentic sample of n-chlorogenic acid eluted at 9.98 minutes by HPLC providing further evidence for the identity of fraction B. Finally, ^{1}H proton analysis of fraction B extracted from foliage of *S. polyadenium* and verified to have an identical UV-Vis spectrum, revealed a resonance pattern identical to that published for n-chlorogenic acid (7).

Fractions A,D,E—Type A glandular trichomes for both species contained a phenolic compound eluting at about 5.30 minutes by HPLC. The UV-V is spectrum of each fraction (absorption maxima at 223 and 282 nm in aqueous acetonitrile) was identical to that of the fraction obtained by preparative HPLC of *S. berthaultii* (PI 473334) foliage. The ^{1}H proton NMR spectrum of the foliar fraction had two doublets with a chemical shift of 6.72 and 7.04 ppm, indicating the presence of two types of protons on a para-substituted aromatic ring. No other resonance peaks were found in this region, which together with the presence of a cluster of resonance peaks with a chemical shift of 2.65-3.0 ppm indicated methylene groups at the phenyl 1 position. Based on this evidence, we propose a tentative identification of Fraction D as p-hydroxyphenylpropionic acid. Proton NMR and UV-Vis spectra for an authentic standard of p-hydroxy-phenylpropionic acid were extremely similar to that of fraction D. However, Fraction D eluted earlier than p-hydroxyphenylpropionic acid on HPLC. This finding is consistent with the likelihood that this phenolic acid in glandular trichomes is esterified, because of a broad cluster of resonance peaks with a chemical shift at 3.5 ppm most likely to be β-D-glucose.

Fraction C—Fraction C (*S. polyadenium*, 28.61 minutes) was obtained by HPLC from trichome collections. The UV-Vis spectrum in water/acetonitrile (254, 261 sh, 290 sh, 325 sh, 353) was characteristic of a flavonoid, possibly a quercetin glycoside. The late eluting phenolics of *S. berthaultii* (26.13, 26.20 minutes) have not been identified but their long retention times also suggest a flavonoid glycoside nature.

The phenolic oxidation activity of Type A trichomes from *S. polyadenium* was nearly twice as great as that of the trichomes of the two *S. berthaultii* accessions studied (Figure 1).

Discussion

Phenolic compounds in Type A glandular trichomes of *S. berthaultii* and *S. polyadenium* were readily separated using HPLC procedures. Although methanol and aqueous starting solutions acidified with phosphoric or acetic acid have been successfully used by others to separate simple phenolics such as hydroxycinnamic and hydrobenzoic acids (3-5, 11, 21), the aqueous monobasic potassium phosphate/acetonitrile gradient system we employed provided good separation of the small quantities of phenolic compounds present in trichome exudates.

Relatively few phenolics are present in the Type A trichomes of *S. berthaultii* and *S. polyadenium*. The likely presence of p-hydroxyphenylpropionic acid glucose ester (retention time: 5.30 minutes) in trichomes of both species suggests that this compound may play a major role in formation of the brownish, insect entrapping exudate characteristic of trichome-mediated insect resistance in *S. berthaultii* and *S. polyadenium*. Additional support for this hypothesis was provided by Ryan, *et al.* (15) who reported a high affinity of *S. berthaultii* Type A trichome PPO and PO for the p-hydroxyphenyl moiety.

The plant phenolases collectively referred to as "polyphenoloxidases" are generally capable of oxidizing both monohydric and ortho-dihydric phenols (9). This dualistic nature has important implications for our finding that, in addition to p-hydroxyphenylpropionic acid ester, the other major simple phenolic present in *S. polyadenium* glandular trichomes is n-chlorogenic acid. This compound was not detected in trichome exudates of either of the two *S. berthaultii* accessions studied, an unusual finding because chlorogenic acid has been reported from tissues of plants across a wide range of taxa (8), occurs in the foliage of the cultivated potato, *S. tuberosum* (10), and constitutes more than 90% of the total tuber phenolic content (11).

As early as 1978, Tingey and Gibson (17), reported much greater levels of tarsal and labial encasement by glandular trichomes of *S. polyadenium* than from those of *S. berthaultii*. Bouthyette, *et al.* (2) reported a high affinity of trichome PPO for chlorogenic acid. In the present study, phenolic oxidation activity of *S. polyadenium* trichomes was 2-fold greater than that of *S. berthaultii* trichomes. This combination of observations strongly suggests that the exclusive presence of chlorogenic acid in trichome exudates of *S. polyadenium* may be responsible for the greater levels of trichome phenolic oxidation activity and subsequent insect entrapment by this species, compared with *S. berthaultii*.

Glandular pubescent potato species, notably *S. berthaultii* are currently being used as sources of insect resistance in potato breeding. Resistant plants have been identified in segregating populations of *S. tuberosum* x *S. berthaultii* hybrids using insect performance criteria (18) as well as data on glandular trichome density and droplet size (13, 14). More recently, chemical procedures have been developed to supplement these selection criteria because of the importance of phenolics and phenolases in the expression of resistance by entrapment in trichome exudate (16). The findings reported here improve the understanding of insect resistance in glandular pubescent potatoes and will help optimize the development and selection of resistant germplasm.

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