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Short Communication

Reversed-phase liquid chromatographic isolation of lubimin and solavetivone from *Hyoscyamus muticus* "hairy" root cultures

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ABSTRACT

A high-performance liquid chromatographic reversed-phase procedure was developed to estimate the concentration of sesquiterpene phytoalexins secreted into the media by *Agrobacterium* transformed root cultures of *Hyoscyamus muticus*. An isolation procedure based on solid-phase extraction (C_{18} Sep-pak) results in very rapid analysis and gives full recovery, compared to sequential chloroform extraction. A simple isocratic procedure permits quantification of sesquiterpene production by these root cultures in response to exposure to fungal elicitors. Calibration curves for lubimin and solavetivone content were obtained by fractionating sufficient material to provide a measurable weight. The identity of these compounds was confirmed by TLC, UV, GC–MS, and NMR. This experimental system is being used to examine the regulation of sesquiterpene biosynthesis, and to develop large-scale processing techniques for the production of secondary metabolites from plant roots grown in bioreactor systems.

INTRODUCTION

A wide range of plant species have the ability to perceive attack by a pathogen and respond dynamically in an attempt to stop or mitigate damage [1]. This induced defense response involves a broad range of biochemical and physiological shifts, including the production of defense chemicals referred to as phytoalexins [2]. In addition to the fundamental importance of this response to plant pathology, fungal extracts are also being used to induce the production of

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biologically active chemicals from plant tissues grown in culture [3,4]. Development of an understanding of phytoalexin formation has been severely hindered by the lack of knowledge of the biosynthetic pathways which produce these compounds. In this regard, the formation of fungitoxic sesquiterpenes by members of the Solanaceae is an excellent model system. Due to the agronomic importance of this plant family (which includes tomato, potato, pepper, and tobacco) there has been considerable research on the sesquiterpene biosynthetic pathway [5]. A schematic of the biosynthetic pathway of sesquiterpenes is shown in Fig. 1. A recent development of particular significance is the isolation and characterization of sesquiterpene cyclase, the enzyme responsible for diverting farnesyl pyrophosphate from sterol synthesis to the sesquiterpene pathway [6]. With the aid of antibodies to this enzyme, radioactive farnesyl pyrophosphate, and cDNA probes, it is now possible to probe induction and regulation of this pathway at a molecular level. Studies of sesquiterpene formation are revealing very interesting dynamics including translational control [7], feedback repression [3], and coordinate gene regulation [8]. To carry out further studies on sesquiterpene biosynthesis, rapid and reliable methods of sesquiterpene quantification are needed to complement the rapid advances in molecular biology. The HPLC analysis described in this paper permits easy and reliable monitoring of lubimin and solavetivone production by tissue cultures of *Hyoscyamus muticus* and other solanaceous species in response to elicitation. This will facilitate physiological studies of plant–pathogen interaction as well as technological studies focused on enhancing secondary metabolite production from cultured plant tissues.

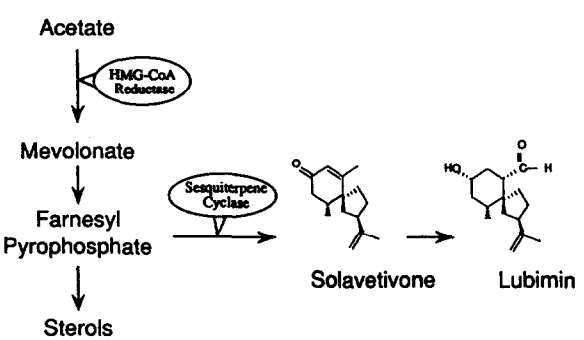

Fig. 1. Biosynthetic pathway of solanaceous sesquiterpene phytoalexins including the structure of lubimin and solavetivone: the two compounds isolated from *Hyoscyamus muticus* root cultures after exposure to extracts of pathogen *Rhizoctonia solani*.

EXPERIMENTAL

Root culture

Hyoscyamus muticus hairy root cultures were established by infection with the bacterial pathogen *Agrobacterium rhizogenes* (ATC 15834) as described previously [9] and have been maintained in culture for over 8 years. Stock cultures are maintained on liquid B5 medium [10]. Root tips are subcultured every 2 weeks into 50 ml of fresh medium in 125-ml flasks. The cultures are maintained on a gyratory shaker with a 2-in. (5-cm) stroke at 100 rpm and 25°C.

Fungal elicitor

Cultures of the fungal pathogen *Rhizoctonia solani* were maintained on SH medium [11] supplemented with 1.00 g/l myo-inositol, 2.36 g/l asparagine, 15.0 g/l pyridoxine hydrochloride, and 10 mg/l thiamine hydrochloride for 18 days on a gyratory shaker at 80 rpm and 25°C. Details on preparation of elicitor can be found elsewhere [3,4]. Briefly, the fungal mycelium is resuspended in distilled water (1 ml per 0.3 g fresh weight) and homogenized for 15 min in a blender on high speed. The homogenate is then autoclaved for 3 h to facilitate release of cell wall fragments, then centrifuged at 21000 *g* for 30 min. The final crude elicitor consists of the filter-sterilized supernatant.

Elicitation/extraction

Induction studies were carried out on root cultures grown for 14 days after inoculation with 0.2 g of root tissue in 50 ml of fresh medium. Cultures were elicited by replacing the growth media with phosphate-free media and adding 2 ml of *Rhizoctonia solani* fungal elicitor. The media was harvested 24 h after induction and extracted. Two extraction procedures were compared for isolation of lubimin and solavetivone:

chloroform partitioning and Sep-pak adsorption as shown schematically in Fig. 2.

The chloroform extraction of the media samples (*ca.* 25 ml) was carried out by partitioning twice against 25 ml of chloroform, (HPLC grade, Fisher Scientific, Pittsburgh, PA, USA). The organic layer was combined and reduced to dryness on a rotary evaporator. The residue was then dissolved in 4 ml of methanol and filtered through a 0.2-μm nylon filter syringe. The filtrate was dried under nitrogen and resuspended in acetonitrile–water (60:40, v/v) for HPLC analysis.

Isolation of lubimin and solavetivone using Sep-pak cartridges was accomplished by first passing the media through Whatman 4 filter paper, then through C_{18} Sep-pak (Waters Classic) cartridges. The cartridge was then eluted with 3 ml of methanol and filtered through 0.2-μm syringe filter. The filtrate was dried under nitrogen then resuspended in acetonitrile–water (60:40) for HPLC analysis.

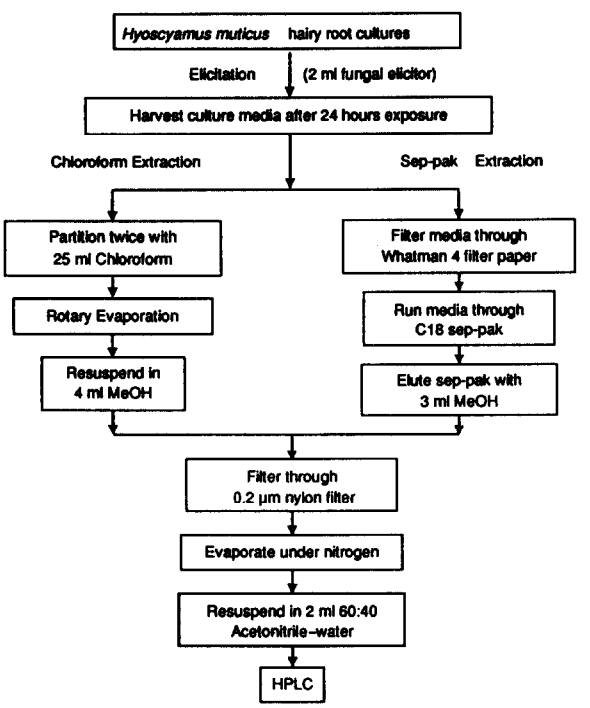

Fig. 2. Flow diagram of sample preparation for HPLC analysis of sesquiterpenes by chloroform partitioning and adsorption/elution on C_{18} cartridges.

High-performance liquid chromatography

The liquid chromatographic system consisted of a Waters Associates Model 600E multisolvent delivery system with a Model 730 data module with WISP Model 712 auto-injector, and a Model 990 photodiode array detector. The system is interfaced with a personal computer for data acquisition and control. The column used for HPLC was a 300 × 3.9 mm Waters μ-Bondapak C_{18} reversed-phase column. All solvents and mobile phases were of HPLC grade, and water was purified on a Millipore Milli-Q system.

The separation was undertaken isocratically with a solvent composition of acetonitrile–water (60:40) at a flow rate of 2 ml/min. A wavelength range of 195–260 nm was chosen to cover the individual λ_{max} for both lubimin and solavetivone.

Lubimin and solavetivone were purified by HPLC using repeated fraction collection, concentration and reinjection. The purified samples used for GC–MS, NMR, and calibration curves were subject to 10 cycles of fractionation and repeated chromatography at the conditions described above. To facilitate quantitative serial dilution for the calibration curves, a known quantity of acenaphthene was added to the purified samples during reconstitution. The calibration curve was constructed by plotting the peak area (absorbance × retention time) of lubimin and solavetivone against their respective weights which were calculated from initial purified sample weight and extent of dilution based on acenaphthene level.

UV spectroscopy

UV spectra of the samples in methanol (HPLC grade) were performed on Beckman, DU 7.

Thin-layer chromatography

The samples were run on silica TLC glass plates (20 × 20 cm) precoated with 0.3 μm thick silica gel without activation. Samples dissolved in $CHCl_3$ were spotted and developed with MeOH–$CHCl_3$ (1:19) The chromatograms were then air dried and sprayed with 5% phosphomolybdic acid solution in alcohol [12]. The spots were visualized by heating the plate at 110°C for calculation of R_F values. Both the

crude extract from the media and the purified samples were spotted to establish unambiguous R_F values for each compound.

Gas chromatography and mass spectrometry

Gas chromatography was performed on a Hewlett Packard (HP) Model 5890 Series 11 chromatograph equipped with flame ionization detectors and split injectors. Helium was used as a carrier gas. The column used was a wall-coated open tubular (WCOT) fused-silica capillary column, B-Dex 120, 30 m × 0.25 mm I.D., 0.25 μm film thickness (Supelco, Bellefonte, PA). The analysis was done isothermally at 180°C with an injector temperature of 280°C, column pressure, 18 p.s.i. (1 p.s.i. = 6894.76 Pa), and a 1:80 split ratio. GC–MS was carried out isothermally at 160°C on a Hewlett Packard 5971A mass selective detector (electron impact 70 keV) in the mass range 50–450 with a PTE-5 column, 30 m × 0.25 mm I.D., 0.25 μm film thickness, 5% phenyl, 95% methyl polysiloxane (Supelco).

NMR spectroscopy

NMR spectroscopy was performed on a Bruker WM360 Spectrometer. Solvent ([$^2H_6$]dimethyl sulfoxide) was obtained from Isotech, OH, USA.

RESULTS AND DISCUSSION

The sesquiterpenes, lubimin and solavetivone were extracted from the media of *Hyoscyamus muticus* hairy root culture by two procedures: chloroform partitioning and C_{18} adsorption cartridges (Sep-paks, Waters Assoc.) Chloroform extracts showed a greater number of background impurity peaks some of which interfered with lubimin and solavetivone, samples eluted from C_{18} cartridges provided better resolution for these sesquiterpenes. To detect and quantify lubimin and solavetivone at the highest possible resolution, different λ_{max} (200 nm and 245 nm, respectively) were chosen for multiple wavelength HPLC detection based on UV spectral data. Lubimin and solavetivone elute at retention times of 3.04 min and 5.61 min, respectively, as shown in the HPLC chromatogram of a crude media extract (Fig. 3).

Sesquiterpene analogs were not commercially available as quantitative internal standards; therefore, it was necessary to established calibration curves based on purification of sufficient material to obtain a measurable weight. Acenaphthene (λ_{max} = 220 nm) was used for determining the extent of dilution of purified lubimin and solavetivone samples since it is eluted at 6.7 min under the isocratic conditions of this separation. The calibration curves were obtained for lubimin and solavetivone over the ranges 1.26-10 μg and 0.5-4 μg, respectively. The response was linear over these ranges for both sesquiterpenes at the stated operating conditions. Regression analysis shows that the conversions factors for peak area to sesquiterpene mass at their respective λ_{max} are nearly the same.

Lubimin and solavetivone were further identified by TLC, GC–MS and NMR. GC–MS of lubimin and solavetivone showed single peaks in GC with retention times of 3.99 and 4.67 min, respectively. The mass spectra of these compounds match corresponding compounds iden-

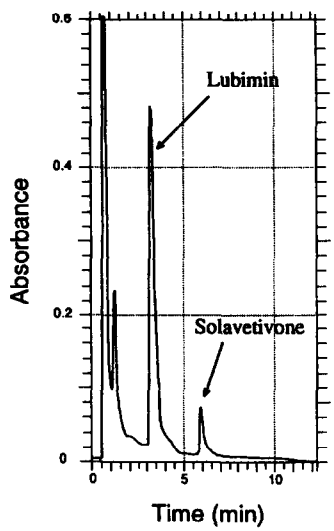

Fig. 3. HPLC chromatogram of sesquiterpenes extracted from the media of elicited root cultures using adsorption on hydrophobic C_{18} cartridges. Chromatogram was obtained from a 50-μl injection of a 25-fold media concentrate with UV detection at 200 nm. Dual wavelength detection at 245 nm provides a 2.1-fold enhancement in absorbance for solavetivone. Chromatographic conditions are given in Experimental.

tified by a mass spectral library search (NBS49K.L). The mass spectra of lubimin displayed molecular ion peak (M^+) at 236 which corresponds to the molecular formula, $C_{15}H_{24}O_2$. The mass spectra of the solavetivone showed a molecular ion peak (M^+) at 218, which corresponds to the molecular formula $C_{15}H_{22}O$. The proton NMR spectra of lubimin and solavetivone were found to display the anticipated characteristic peaks for these compounds as previously reported [13,14]. Lubimin and solavetivone showed single spots on TLC with R_F values of 0.43 and 0.65, respectively, which is also consistent with previous reports for these compounds [12].

A comparison of the chloroform extraction and Sep-pak procedures clearly shows that the Sep-pak procedure is more convenient, time saving, and cost effective. The recovery of sesquiterpenes was statistically indistinguishable (95% *t*-test) for replicated extractions performed with both procedures. Both procedures showed comparable reproducibility: the standard deviation of replicated extractions was about 1% of the response for lubimin, and 5% for solavetivone (with Sep-pak extraction displaying slightly better reproducibility due to fewer interfering peaks). The Sep-pak extraction procedure for HPLC sample preparation takes about three min. In contrast, chloroform extraction takes at least 30 min and often takes much longer due to emulsion formation caused by surface active agents in the culture medium. This means that using Sep-paks, 100 samples can easily be processed in less than a day which would require more than a week using chloroform extraction. There is a tremendous savings in solvents as well: 100 samples would require 5 l of chloroform (*ca.* US$ 95) in comparison to 500 ml of methanol (*ca.* US$ 5) which does not include disposal costs which are escalating due to environmental concerns for chlorinated hydrocarbons. This cost savings would be offset by the cost of Sep-pak cartridges (*ca.* US$ 150/100); however, since the media extracts are very "clean" (in comparison to cellular extracts for example), we found that the cartridge could be used repeatedly, as many as 50 times without degradation of recovery. Even without such repeated use, the time savings clearly makes extraction by adsorption and elution on adsorbent resins a far superior procedure for HPLC sample preparation. The speed of sample preparation will facilitate rapid evaluation of the dynamic response of induced sesquiterpene formation in response to challenge by fungal elicitors.

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