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Antifungal monoterpene production in elicited cell suspension cultures of *Piqueria trinervia*☆

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Abstract

Cell suspension cultures of the traditional medicinal plant *Piqueria trinervia* Cav., which synthesizes monoterpene piquerol A, were established. A defense response was induced in the cultures when eight homogenized fungi isolated from wild populations of *P. trinervia* were added. Piquerol A was not produced in the elicited system, while four other substances were synthesized de novo. They were excreted into the medium and inhibited in vitro fungal growth. The most abundant substance produced in this system was a new monoterpene: 2-methylene-7,7-dimethylbicyclo (3,3,1) heptane-4,6-diol. Monoterpenes in the cell suspension culture reported here were produced via two metabolic channels: the first acted constitutively and expressed in liquid and solid cultures, the second is inducible in response to several pathogens and elicitor substances.

Keywords: *Piqueria trinervia*; Asteraceae; Plant tissue cultures; Elicitors; Secondary metabolites; Antifungal activity; Phytopathogenic fungi; Monoterpenes; Piquerinol

1. Introduction

Piqueria trinervia Cav. is a wild herbaceous Mexican plant of the Asteraceae family. It has been used since pre-Columbian times in Mexican traditional medicine against typhus, fever, malaria and tetanus (Bejar et al., 1999). Previous studies have shown that wild plants contain several monoterpenes, such as carquejile acetate, and two diastereoisomers, piquerol A (**1**) and piquerol B (Romo et al., 1970) as well as the diterpene trinervinol (**2**), (Jiménez-Estrada and González de la Parra, 1983; Soriano-García et al., 1983). With respect to the biological properties of these substances, all previous studies have focused on piquerol A (Scheme 1), which has been considered to have allelopathic (González de la Parra et al., 1981; Jiménez-Estrada et al., 1996), molusquicide (Cruz-Reyes et al., 1989), and acaricide (González de la Parra et al., 1991) properties, and which has also been shown to be active against the protozoa *Trypanosoma cruzi* (Castro et al., 1992). However, the substances responsible for the medicinal properties attributed to *P. trinervia* have not yet been isolated (Bejar et al., 1999).

Due to this plant's potential for producing important medicinal metabolites, we examined the potential of cell cultures to be stimulated by a fungal elicitor for monoterpene production. The structure of the domi-

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nant monoterpene produced in this system was determined as described below.

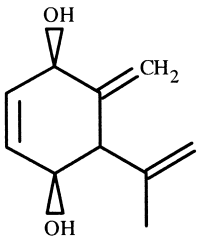
2. Results and discussion

2.1. Cell suspension culture

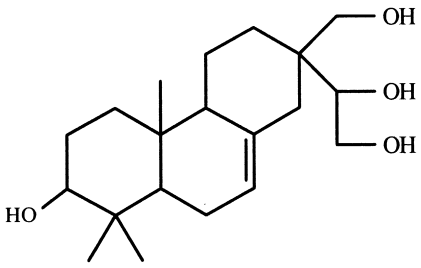
Compact, fast-growing greenish callus from explant internodes of *P. trinervia* was generated in MS-medium (Murashige and Skoog, 1962), to which 1 mg l^{-1} of naphthalene acetic acid (NAA) and 1 mg l^{-1} of benzyl amine purine (BAP) were added. Under these conditions, 90% of the explants generated a callus, 3.4 g/flask, (fr. wt.) on average, in 3 weeks. This callus accumulated piquerol A ($1.3–3.0 \times 10^3$ mg/g, dry wt.) beginning the sixth week of culture. Using 25% of the calli that showed the highest accumulation, a semi-desegregated suspension culture (small spherical calli with greenish areas) was established, that could be induced to regenerate whole plants under the proper culture conditions. The suspension culture accumulated piquerol A (**1**) during the exponential growth stage (2.1×10^{-2} mg/g, dry wt.). Its specific rate of growth was 1.84 g/day/l (dry wt.) and the duplication time was 7 days. This species, as has been reported by others (Banthorpe et al., 1986; Croteau et al., 1987; Lewisohn et al., 1994), requires a certain minimum degree of differentiation (tissue with abundant plastids) for monoterpenes to be produced. Calli with 8.7 mg/g (dry wt.) of chlorophyll produced 3.0×10^3 mg/g (dry wt.) of piquerol A, while cultures with less than 5.7 mg/g (dry wt.) of chlorophyll did not accumulate any detectable amount of this substance, since the synthesis of monoterpenes is usually associated with plastids (McGarvey and Croteau, 1995).

2.2. Elicited cell suspension culture

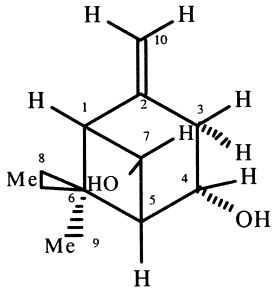
Fungal strains of low virulence associated with the plant species under study are generally used as elicitors in cell cultures (Eilert, 1987; Payne et al., 1991). Since *P. trinervia* fungi pathogens have not been previously described, it was necessary to isolate some of them directly from wild plants. Eight monosporic strains were obtained. Cultured plants of *P. trinervia* were inoculated with the isolated fungi. Only three strains were able to infect the plants, and these were reisolated from sick plants: *Alternaria alternata*, *Fusarium poae* and *Phaecilomyces elegans*. The other five strains are



Piquerol
1



Trinervinol
2



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Scheme 1.

not parasites of *P. trinervia*; four induced a hypersensitive response (HR) in the plant (*Cladosporium cladosporoides*, *Phoma macdonaldii*, *Fusarium moniliforme* and *Helmintosporium pedicellatum*) and one (*Rizoctonia solani*) was not detected in whole plants of *P. trinervia*.

The eight fungi used induced a defense response in the cultures, which was evidenced by a pronounced color change (reddish-brown) in both cells and medium. The fungal elicitors induced the production of new substances.

A crude $CHCl_3$ extract of cells and culture medium yielded 0.6 and 2.0 mg residue/g cells, respectively. TLC analysis of both extracts showed four new substances that were not found in non-elicited cultures. Piquerol A was not detected in either of the extracts. The most abundant constituent in the medium migrated to an R_f identical to that of piquerol A [0.23 (petroleum ether–EtOAc, 7:3)], but was nevertheless found to be a new monoterpene (see below), which was named piquerinol (Scheme 1). The three remaining compounds had a higher polarity, and their identities are as yet unknown. Piquerinol reached its maximum accumulation (3.17 mg/g, dry wt.) in the culture 48 h after induction, while the three unidentified substances peaked at 72 h.

All of the fungi induced a similar response, as measured by the accumulation of piquerinol at 48 h after induction: 4 mg/g (fr. wt.) of mycelium per ml of cell culture induced accumulation of 0.75–0.80 mg of piquerinol per 50 ml of culture per day. As a result of its rapid growth in the liquid medium (2.6 g/day/l, dry wt.), with a duplication time of only 13 h, *F. moniliforme* was used as an inducer in subsequent experiments.

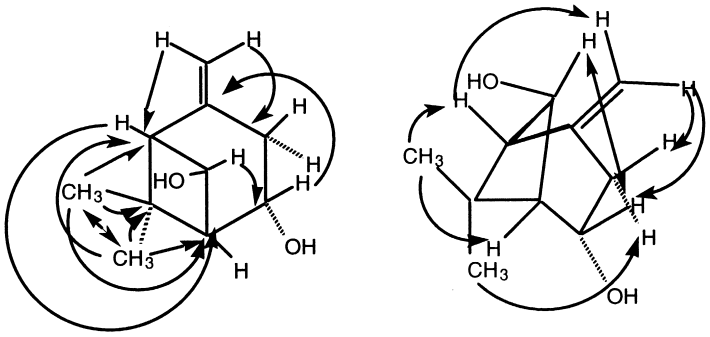
Dried mycelium from *F. moniliforme* induced a defense response in suspension culture of *P. trinervia*: 0.8 mg of dried mycelium per ml of cell culture induced accumulation of 1.6 mg of piquerinol per 50 ml of cell culture per day. A defense response could also be induced with substances of a different chemical nature: chitosan, glucan and even *P. trinervia* cell suspension homogenates. Nevertheless, the response was not as intense as that obtained with mycelium homogenates (31–50% less piquerinol was synthesized). These results suggest the presence of alternative routes in the response of this plant to pathogen attack. As in other elicitor-inducible systems (Chappell and Nable, 1987; Ren and West, 1992), the culture defense response could be induced efficiently at any time during the exponential growth stage.

2.3. Isolation and identification of: 2-methylene-7,7-dimethylbicyclo (3,3,1) heptane-4,6-diol, or piquerinol

Piquerinol (**3**) (Scheme 1), was isolated after column chromatography of the $CHCl_3$ extract of the elicited cell suspension medium. MS (CI) showed $M^+ + 1$ at m/z 169, which is in agreement with a formula of $C_{10}H_{16}O_2$. Fragments of m/z 151 and 133 indicated the subsequent loss of two water molecules. A DEPT NMR experiment indicated the presence of $2CH_2$, $2CH_3$ and 4CH groups. One exocyclic methylene was deduced from the ^1H and ^{13}C NMR signals at δ 4.70 (*t*, 2H, $J = 2.1$ Hz), δ 109.8 ($=CH_2$) and δ 147.6 (C=). An aliphatic methylene was also deduced from signals at δ 2.95 (*ddt*, 1H, $J = 2.1$, 9.6, 19.6 Hz) and δ 2.39 (*dq*, 1H, $J = 2.5$, 5.0, 19.0 Hz), which were assigned to H-3 and H-3′. The corresponding signal for C-3 appeared at δ 35.8. The ^1H NMR spectrum also showed two signals at δ 4.36 (*dt*, 1H, $J = 3.0$, 9.6 Hz) and 3.73 (*s*, 1H) that were assigned to H-4 and H-7. Chemical shifts for C-4 and C-7 (76.6 and 74.6, respectively) indicated that each of these carbons supports a hydroxyl group. The presence of the latter was evidenced by two singlets (D_2O exch.) at δ 1.90 and 1.22. H-1 and H-5 at the head of the bridge were assigned to signals at δ 2.51 (*ddt*, 1H, $J = 9.0$ Hz) and 2.26 (*dd*, 1H, $J = 3.6$, 6.0 Hz), while their corresponding carbons, C-5 and C-1, appeared at δ 54.3, and 58.8, respectively. A quaternary carbon at δ 38.9 (C-6) which supported two methyls, δ 1.58 (*s*, 3H, Me-8) and 1.05 (*s*, 3H, Me-9), was observed. The proton coupling constants between H-7 (δ 3.73) and the protons resonating at δ 2.51 (H-1) and 2.26 (H-5) are very small, with a torsion angle of about 90°. The configurations of C-7 and C-4 were established from the information obtained from NOEdiff and NOESY experiments. Structure (**3**), 2-methylene-6-6-dimethylbicyclo (3,1,1) heptane-4,7-diol or dihydroxy-β-pinene, was fully confirmed by the data obtained from HMQC and HMBC experiments (Scheme 2).

To determine if piquerinol (**3**) could also be synthesized in intact plants, wild plants of *P. trinervia* were macerated in water for 24 h. Compound **3** was isolated from this extract and verified by NMR spectroscopic analysis. Therefore, this is a constituent component of intact plants.

The roles of compounds **1**–**3**, as well as other plant substances in the defense of *P. trinervia* against fungal attack were investigated (Table 1). These substances were tested against the eight isolated fungal strains. The most sensitive strain was *Phoma macdonaldii*. All of the compounds tested retarded its growth, while none affected *Fusarium poae*, which infects *P. trinervia* and *Rizoctonia solani* and does not exhibit any type of interaction with the plant. Piquerinol (**3**) produced fungistasis in three of the fungi; trinervinol (**2**) in two; and piquerol A (**1**) in one. The mixture of the three undetermined substances (fractions 70–99) was active in one fungus. The mother liquors retarded growth in six strains, which suggests the presence of other anti-



Scheme 2.

fungal compounds in *P. trinervia*. All of the substances tested inhibited, in low concentrations, the growth of at least one of the fungi. Therefore, it is likely that they form part of the host defense mechanisms.

Monoterpene biosynthesis has been previously reported in callus and in transformed root cultures (Banthorpe et al., 1986; Hilton et al., 1995). In addition, fungal elicitors have been used to stimulate the biosynthesis of terpenoids in this type of culture system; e.g., monoterpenes and diterpenes in Pinaceae (Croteau et al., 1987; Lewisohn et al., 1994); diterpenoid phytoalexins in rice, maize and castor beans (Bruce and West, 1982; Ren and West, 1992; Wichham and West, 1992); and sesquiterpenoids in *Solanaceae* species (Chappell and Nable, 1987; Facchini and Chappell, 1992). To the best of our knowledge, this is the first time that monoterpenes have been obtained from cell suspension cultures.

The in vitro *P. trinervia* cultures generated here are interesting because they can synthesize monoterpenes via two metabolic routes. The first route is constitutive and the monoterpene is expressed in both liquid and solid cultures, while the second route is inducible and responds to several pathogen and elicitor substances. For these reasons, these cultures could be a suitable model for studying terpenoid metabolism and plant–pathogen interactions.

3. Experimental

3.1. General

The melting points (uncorr.) were determined in a Fisher–Jones apparatus. ^{1}H and ^{13}C NMR spectra were determined with Varian Gemini-200 and Varian

Table 1
Fungal strains isolated from *P. trinervia* and inhibition of mycelial growth by the isolated compounds

Fungi	Test sample	Concentration (mg)	Inhibition of radial growth[a] (%)
Cladosporium cladosporoides (Fresen) G.A. de Vries	Mother liquors[b]	175	55.4
Phoma macdonaldii Boerema	Mother liquors	275	51.20
	Fractions 70–99	200	56.78
	Trinervinol (**1**)	250	50.04
	Piquerol (**2**)	425	49.75
	Piquerinol (**3**)	75	49.50
Alternaria alternata (Fr.:Fr.) Keills	Mother liquors	250	53.00
Rizoctonia solani KŸhn	[c]		
Fusarium poae (Peck), Wollenw	[c]		
Fusarium moniliforme Sheldon	Piquerinol (**3**)	125	50.75
Helmintosporium pedicellatum A.W. Henry	Mother liquors	325	55.80
	Piquerinol (**3**)	225	52.00
Phaecilomyces elegans (Corda) E. Manson and S.J. Hughes	Piquerol (**2**)	150	51.30
	Mother liquors	225	50.00

[a] The results are the average of five repetitions.
[b] Mother liquors from the crystallization of compounds (**1**) and (**2**).
[c] Retarded growth was not detected with any of the substances or concentrations tested.

UNITY-500 spectrometers, referenced to TMS. COSY, NOESY, HMQC, and HMBC experiments were recorded with the standard Varian Unity 500 pulse program. The BIRD sequence was optimized for short-range couplings (140 Hz, ^1JC–H). The HMBC pulse program was optimized for long-range couplings (9.0 Hz, ^3JC–H). Both HMQC and HMBC were obtained with 1024×256 points. The F2 dimension was zero-filled to achieve a 1024×512 data matrix. Analytical TLC was performed (silica gel). Plates were sprayed with a solution of $CeSO_4$ 1% in 2N H_2SO_4 and placed on a hot plate.

3.2. Biological material

P. trinervia seeds and wild plants were collected from the Sierra del Ajusco, Mexico. Vouchers were deposited in the National Herbarium of Mexico (MEXU). The seeds were germinated in solid MS-medium (Murashige and Skoog, 1962) without growth regulators, and 2-month-old seedlings were used for explants. The fungi used as elicitors were isolated from infected leaves, roots and flowers of wild *P. trinervia*. The fungi were identified at the Laboratory of Plant Pathogenic Fungi, Colegio de Postgraduados en Agricultura, Texcoco, Mexico. Fungal duplication time, growth specific celerity, and pathogenicity (in greenhouse plants of *P. trinervia*) were determined by the usual procedures.

3.3. Callus induction

P. trinervia callus cultures were grown from internode explants on MS-medium using 1 mg l^{-1} of NAA combined with 1 mg l^{-1} of BAP. Cultures were established in a flask (125 ml) containing 25 ml of solid medium. Five explants were used per flask. The flasks were kept in a photoperiod with 16 h of light, with a light intensity of 0.5 W m^{-2} in incubation cameras at 25 ± 1°C.

3.4. Cell suspension culture

P. trinervia cell suspension cultures were started with a callus (6 weeks). A callus (8 g) was added to an Erlenmeyer flask (250 ml) containing 50 ml of the previously described medium with 20 g l^{-1} of sucrose. The flasks were kept in a gyratory shaker (100 rpm) under the conditions described above.

3.5. Fungal elicitors

The fungi were grown on potato dextrose agar (PDA) plates at 25 ± 1°C. To establish the liquid culture conditions, two portions of 1 cm^2 of mycelium were added to Erlenmeyer flasks (125 ml) containing 25 ml of a liquid medium. These cultures were kept in a gyratory shaker (150 rpm) at 25 ± 1°C in continuous light for 6–8 days. The mycelium was harvested and homogenized (Polytron, 10 min) with half of the original medium. Five-milliliter portions were poured into glass tubes and autoclaved for 20 min (121°C) to obtain the elicitor preparation.

3.6. Elicited cell suspension cultures

The *P. trinervia* cell suspension cultures were elicited at the middle of the exponential growth stage (10 days of culture). One tube containing a fungal elicitor preparation was added to each flask. The stimulated cultures were maintained for three additional days under the experimental conditions described above.

3.7. Isolation of 2-methylene-7,7-dimethylbicyclo (3,3,1) heptane-4,6-diol

P. trinervia elicited cell suspension cultures were harvested after 3 days. The cultures were filtered with a stainless steel sieve (200 mesh). The filtrate was extracted three times with $CHCl_3$. The solvent was eliminated in a rotary evaporator. The extract (300 mg) was fractionated by CC (silica gel 60 Merck, 35–70 mesh). The column was first eluted with a mixture of petroleum ether and EtOAc (8:2) and afterwards with a mixture of petroleum ether–EtOAc–CH_2Cl_2 (5:3:2). Fractions 47–69, eluted with this mixture, contained piquerinol (3) as a yellow solid, which was recrystallized from CH_2Cl_2. Further fractions (70–99) eluted with the same mobile phase contained a mixture of three unidentified substances ($R_f = 0.37$, 0.17 and 0.11 TLC silica gel with EtOAc–MeOH 9:1).

Shoots and leaves of *P. trinervia* wild plants (300 g) were macerated with 500 ml of water for 24 h. The aq. soln. was filtered and extracted three times with CH_2Cl_2. Fractional crystallization first gave trinervinol (**2**) and later piquerol A (**1**). The remaining oily mother liquors (150 mg) were subjected to successive TLC prep. (two plates, silica gel 60 F_{254}, 2 mm) using petroleum ether–EtOAc (7:3) as a mobile phase. A band at $R_f = 0.28$ was revealed with UV light (207 nm) and with spray reagent ($CeSO_4$). A gummy, colorless oil (15 mg) with a pleasant odor was recovered from the plates with CH_2Cl_2. According to its spectral data, it was identified as compound **3**.

3.8. Characterization of 2-methylene-7,7-dimethylbicyclo (3,3,1) heptane-4,6-diol

Yellow prisms, mp 118–120°C. $[\alpha]_D^{24} + 8.35°$ ($CHCl_3$, *c* 0.30). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3357 (OH) and 1644 (C=C). ^1H NMR, (500 MHz, $CDCl_3$) ppm δ: 4.70 (*t*, 2H, $J =$ 2.1 Hz, H-10, H-10′), 4.36 (*dt*, 1H, $J = 3.0$, 9.6 Hz, H-

4), 3.73 (*s*, 1H, H-7), 2.95 (*ddt*, 1H, $J = 2.1$, 9.6, 19.6 Hz, H-3), 2.51 (*ddt*, 1H, $J = 9.0$ Hz, H-1), 2.39 (*dq*, 1H, $J = 2.5$, 19.0, 5.0 Hz, H-3), 2.26(*d*, *d*, 1H, $J = 3.6$, 6.0 Hz, H-5), 1.90 (*bs*, 1H, D_2O exch.), 1.58 (*s*, 3H, Me-8), 1.22 (*s*, 1H, D_2O), 1.05 (*s*, 3H, Me-9). ^{13}C NMR (125 MHz, $CDCl_3$): δ 147.64 (C-2), 109.8 (C-10), 76.6 (C-4), 74.6 (C-7), 58.8 (C-1), 54.3 (C-5), 38.9 (C-6), 35.8 (C-3), 28.7 (C-8), 25.4 (C-9). CIMS 70 eV, *m/z* (rel. int.): 169 ($M^+ + 1$), 151, 133, 123, 107, 95, 81. EIMS 70 eV, *m/z* (rel. int.): 150 ($M^+ - 18$), 135, 121, 107, 101, 92, 85 (100), 73, 55, 41, 39. (Found: C, 71.56; H, 9.19. C_{10} H_{16} O_2 requires: C, 71.39; H, 9.59 %).

3.9. Biological activity

The antifungal properties of piquerol A (**1**), trinervinol (**2**), piquerinol (**3**), fractions (70–99) eluted from CC, and the mother liquors from the crystallization of compounds **1** and **2** were tested against the eight fungal strains. Each substance was dissolved in 2 ml of EtOH, passed through *Millipore* filters (45 μm) and then added to a previously sterilized PDA medium. The substances were tested at the following concentrations: 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425 and 450 mg l^{-1}. Two different controls were conducted: the first contained only PDA, and the second had PDA with 2 ml l^{-1} of ethanol. The dishes were incubated in darkness at $25 \pm 1°C$. The percentage of growth inhibition was calculated after 48 h. Five replicates were run per treatment. The results were analyzed by ANOVA ($p > 0.05$) and Tukey's test.

3.10. Quantification of monoterpenes

The percentage of piquerol (**1**) and piquerinol (**3**) was determined by high performance thin layer chromatography (HPTLC) densitometry using a CAMAG II apparatus. The UV wavelength was fixed at 210 nm for piquerol and 207 nm for piquerinol (**3**). The plates (silica gel SIL $60F_{254}$) were developed with petroleum ether–EtOAc–CH_2Cl_2 (5:3:2) in a horizontal chamber. The R_f values of piquerol and piquerinol were 0.17 and 0.28, respectively. The concentrations of **1** and **3** in samples were calculated according to standards of known concentrations.

3.11. Quantification of chlorophyll

The total chlorophyll concentration in an 80% aq. acetone extract of *P. trinervia* callus was calculated according to the spectrophotometric procedure of Porra et al. (1989).

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