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Root extracts from Mexican avocado (*Persea americana* var. *drymifolia)* inhibit the mycelial growth of the oomycete *Phytophthora cinnamomi*

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Abstract Crude root extracts from Mexican avocado trees (*Persea americana*) were screened for antioomycete activity against *Phytophthora cinnamomi*. Forty-eight accessions from Mexican avocado trees were selected with potential resistance to *P. cinnamomi* according to environmental and site descriptors. Crude root extracts from these accessions were obtained and tested *in vitro* against the oomycete *P. cinnamomi*. Seven crude root extracts inhibited mycelial growth (>50%) and only root extracts from accessions 765-01 and 773-01 showed 100% of inhibition. Extracts from accessions 765-01 and 773-01 were analysed by preparative thin-layer chromatography and six fractions were detected under UV light. In both extracts, fractions IV (R_f 0.85) and V (R_f 0.9) showed *in vitro* inhibition (100%) against mycelial growth of *P. cinnamomi.* Fraction V was subjected to GC–MS analysis and stigmastan-3,5-diene ($C_{29}H_{48}$) was identified as the major compound. *In vitro* assay showed that stigmastan-3,5-diene (100 ppm) inhibited the mycelial growth of *P. cinnamomi*. The constitutive presence of this compound in avocado roots offers possibilities to identify and to select potentially resistant plants to *P. cinnamomi.*

Keywords Antioomycete activity · Root extracts · Stigmastan-3,5-diene

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Introduction

The oomycete *Phytophthora cinnamomi* is the causal agent of avocado root rot, the most destructive and important avocado disease (*Persea americana*) of orchards worldwide (Pegg et al. 2002). *Phytophthora cinnamomi* causes massive death of fine feeder roots under wet or flooded conditions as a consequence of its short generation time and high reproductive capacity. The control of this pathogen requires a combination of many practical management procedures designed to reduce pathogen activity and increase host resistance during critical infection

periods. Complementary management practices include prevention, cultural, biological and chemical control, as well as rootstock resistance (Coffey and Guillemet 1987; Erwin and Ribeiro 1996). Some avocado rootstocks express tolerance to root rot by a rapid regeneration of active feeder roots; in others, the progress of root infection is inhibited by different factors (Phillips et al. 1987). Unfortunately, the moderate resistance expressed by existing rootstocks is not by itself adequate to provide disease control under favourable environmental conditions for root rot. The long-term and ultimate solution to Phytophthora root rot is to select or breed resistant rootstocks (Coffey 1992; Menge et al. 1992; Pegg et al. 2002).

Antimicrobial compounds from plant origin consist of a diverse array of different secondary metabolites classes, including saponins, phenolic compounds, cyclic hydroxamic acids, cyanogenic glycosides, isoflavonoids, sesquiterpenes, sulphur-containing indole derivatives, etc. (Papadopoulou et al. 1999). In avocado, biologically-active natural compounds have been isolated from different tissues. For example, secondary metabolites with antifungal (dienes) and insecticidal activity (avocadofurans) have been obtained from avocado idioblast cells, and from the exocarp and mesocarp of unripe fruits (Prusky et al. 1982; Prusky and Keen 1993; Oberlies et al. 1998; Domergue et al. 2000; Rodríguez-Saona and Trumble 2000). The compound 1,2,4-trihydroxy-n-heptadeca-16-en isolated from avocado fruit and seeds has shown antibacterial activity (Néeman et al. 1970). In addition, the estragole, found in leaf oil in Mexican avocados, has insecticidal properties (King and Knight 1987). Also, compounds with antiviral activity from avocado leaves have been reported (Wigg et al. 1996; de Almeida et al. 1998). In addition, 1,2,4-trihydroxyheptadecane and 1,2,4-trihydroxynonadecane derivatives isolated from avocado seeds have moderate activity against epimastigotes and trypomastigotes (Abe et al. 2005). It is clear that there is great compound diversity with antimicrobial properties in avocado tissues; however, root compounds biologically active against *P. cinnamomi* have not been reported.

In this work we report the identification of an antioomycete constitutive compound (stigmastan-3,5-diene) present in avocado roots (*P. americana* var. *drymifolia*). This compound shows *in vitro* activity against mycelial growth from a virulent strain of *P. cinnamomi*.

Materials and methods

Plant material

A total of 628 accessions from INIFAP (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias) avocado germplasm bank in Uruapan, Michoacán, México, were screened on a provenance basis, according to environmental and site descriptors (IPGRI 1995). The descriptors used were soil texture, soil drainage, temperature, rainfall and overall surrounding vegetation. The collected sites showed favourable environmental conditions for *P. cinnamomi* development, such as the following, (1) moist template or subtropical climates, (2) disturbed locations from human activities, (3) soils with deficient drainage, and (4) presence of other known oomycete hosts (Zentmyer 1985). According to this preliminary screening, a group of 48 genotypes with potential resistance to the pathogen was selected and used to search for antimicrobial compounds in the roots.

Phytophthora isolate

Phytophthora cinnamomi obtained from infected avocado plants was used in this study and was kindly provided by Facultad de Agrobiología (Universidad Michoacana de San Nicolás de Hidalgo, México). The isolate was routinely grown on potato dextrose agar medium (PDA, Bioxon, México) at 25±1°C.

Metabolite extraction from avocado roots

Avocado roots were collected and immediately used to extract the metabolites. Samples (1 g fresh weight) were freeze-dried with N_2 and ground to a fine powder in a cold mortar. The resulting powder was extracted with 10 ml of methanol-chloroform (2:1, HPLC grade, Sigma-Aldrich) under vigorous and continuous shaking for 10 min. Crude root extracts were then placed in a separating funnel and 10 ml of distilled water added. The mixture was vigorously shaken and allowed to settle for 15–30 min for phase separation. This procedure was repeated three times. Subsequently, the organic and aqueous phases were collected and centrifuged (10 min, 5,000 *g*) to remove plant residues. The supernatants were evaporated to dryness under vacuum at 45°C, the resultant residues dissolved in 500 µl of absolute ethanol (Sigma-

Aldrich) and stored at 4°C for further analysis. To obtain preliminary data of compounds in the extracts, each crude avocado root extract was analysed by spectrophotometry in the visible-ultraviolet spectrum in a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc.) and the λ_{max} (nm) registered.

In vitro bioassays

Crude root extracts and fractions were tested *in vitro* against *P. cinnamomi*. Bioassays were carried out using the following protocol. To obtain inoculum, a plug of 5 mm diam *P. cinnamomi* mycelium was transferred to PDA medium (Bioxon, México) and incubated in the dark at 25°C until the mycelium was approximately 25 mm from the edge of the plate. Filter paper discs (Millipore Corporation) were impregnated with 50 µl of crude root extracts or fractions and then placed in the centre of the plate (4.5 cm diam). Subsequently, a plug with mycelial fragments of the *P. cinnamomi* isolate (5 mm) was placed on the culture medium. Plates were incubated at 25°C and the radius zone of inhibition (distance between the centre of the plug and margin of the inhibited mycelium) was recorded after 8 days of culture. The fungicide mefenoxam (Ridomil Gold-4E, Novartis Greensboro, 1 mg l^{-1}) and absolute ethanol (50 µl) were used as positive and negative controls, respectively. The bioassays were performed with five replicates. The maximum growth of *P. cinnamomi* was reached after 8 days of culture on PDA plates and this condition was considered as 0% growth inhibition (Fig. 1a); 50% inhibition was considered when the radial growth of the mycelium showed <2.5 cm diam, whereas 100% inhibition corresponded to the absence of growth on the plate (Fig. 1b).

Bioactive metabolite identification from avocado roots

Crude root extracts from the avocado accessions with the highest antioomycete activity were subjected to preparative thin-layer chromatography (TLC) (Silica gel 60, Sigma-Aldrich) using methanol-chloroform (2:1, Sigma-Aldrich) as the solvent. To observe the fluorescent spots, the plaques were dried at room temperature and then irradiated with a 254/365 nm wavelength ultraviolet lamp (Cole-Parmer). The R_f for each fraction (bands) was calculated. The different fractions were recovered and then eluted with the extraction solvent (10 ml methanol-chloroform, 2:1). Each fraction was evaporated to dryness under vacuum at 45°C and dissolved in 500 µl of absolute ethanol. The fractions were analysed by spectrophotometry and then used for bioassays to identify the active fractions as indicated above.

The fraction with the highest antioomycete activity was subjected to GC–MS. The analysis was achieved in a gas chromatograph (Hewlett Packard 5890, Series II) and a mass spectrometer (Hewlett Packard 5972 series), using a 30 m-long capillary column of 0.25 mm internal diameter. The following programme was used: temperature of injector 240°C until the interface of 260°C; an initial oven temperature of 50°C

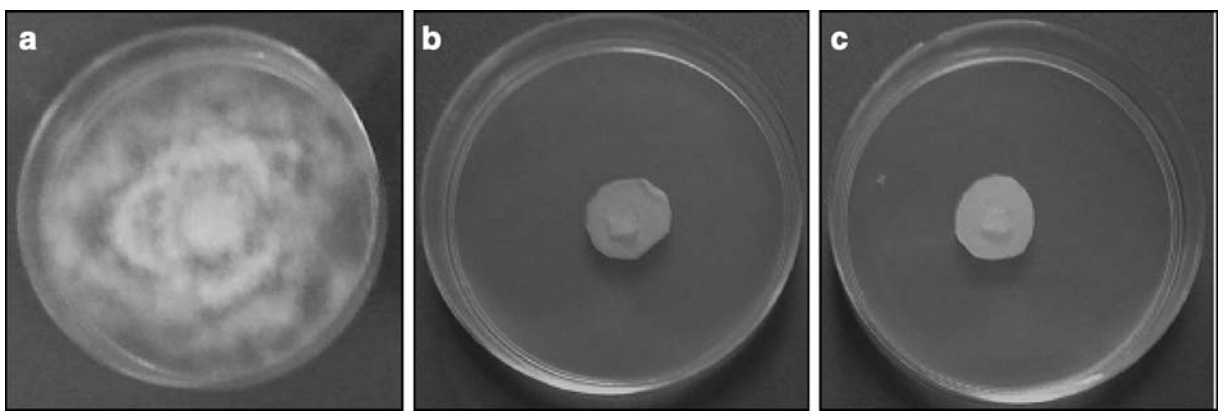

Fig. 1 Bioassay of avocado root extracts against *P. cinnamomi* mycelial growth. (**a**) Bioassay with absolute ethanol (control, considered as 0% inhibition). (**b**) Bioassay of crude root extract obtained from 765-01 avocado accession. (**c**) Bioassay with mefenoxam [1 mg l^{-1}] (positive control). This effect was considered as 100% inhibition. In all cases, the growth was registered after 8 days of culture

Table 1 Avocado genotypes used to obtain crude root extracts

Accession	Pedigree	Relevant characteristics			
		SR	CS	SS	CL
037-02	16UPN-14-02	PaM	by	Lr	sch
056-02	16QDR-13-02	PaM	wh	Wi	tsh
061-01	16QDR-18-01	PaM	wh	Wi	tsh
064-02	16BCN-02-02	PaM	mk	Lr	tsh
066-01	11CLY-01-01	PaM	mk	Lr	scsh
084-02	16ZTR-02-02	PaM	mk	Lr	scsh
139-01	16TGB-06-01	PaM	mk	Lr	tsh
203-02	16TGB-11-02	PaM	mk	Lr	tsh
209-04	15VAB-04-04	PaM	by	Lr	tsh
223-03	15MLN-07-03	PaM	by	Lr	scsh
245-01	20ETL-02-01	PaM	by	Lr	scss
277-02	24TZL-19-01	PaM	by	Lr	sch
285-02	24TQN-01-01	PaM	by	Lr	csh
292-03	28MAN-07-03	PaM	by	Lr	csh
293-04	28MAN-08-04	PaM	by	Lr	csh
310-01	21HUA-07-01	PaM	by	Lr	th
316-02	21ZAC-04-02	PaM	by	Lr	th
320-02	21AHN-02-02	PaM	mk	Lr	sch
321-03	21ZPX-01-03	PaM	mk	Lr	th
329-04	30JLG-01-04	PaM	by	Lr	th
417-01	28CMP-03-02	WxM	by	Lr	sch
482-01	07SCB-01-01	PaM	by	Lr	tsh
493-03	16SFA-12-03	PaM	by	lr	tsh
517-02	17CTC-02-02	PaM	fl	lr	scsh
518-03	17CTC-03-03	PaM	by	Lr	scsh
524-02	11CMF-21-02	PaM	by	Lr	scsh
531-01	12MTL-05-01	MxW	by	Lr	scsh
533-01	21ATX-03-01	PaM	by	Lr	tsh
582-01	16ZCR-03-01	PaM	by	Lr	sch
643-01	15TNG-08-01	PaM	by	Lr	tsh
668-01	07SLU-01-01	Ps	mk	lr	n.a.
700-03	07SCB-04-03	Pt	wh	wi	tsh
713-03	27CAR-19-03	Ps	wh	wi	ch
744-01	Duke 6	PaM	ro	brm	n.a.
745-01	Duke 7	PaM	ro	brm	n.a.
746-01	Thomas	PaM	ro	brm	n.a.
747-01	Barr Duke	PaM	ro	brm	n.a.
748-01	G755C	GxPs	ro	brm	n.a.
749-01	G755B	GxPs	ro	brm	n.a.
750-01	G755A	GxPs	ro	brm	n.a.
751-01	PT3	PaM	ro	brm	n.a.
752-01	PI-116	PaM	ro	brm	n.a.
753-01	PR-5	PaM	ro	brm	n.a.
754-01	PR-1	PaM	ro	brm	n.a.
755-01	P4	PaM	ro	brm	n.a.
757-01	P1	PaM	ro	brm	n.a.
765-01	PRMX-01	PaM	ro	brm	tsh
773-01	PRMX-02	PaM	ro	brm	tsh

SR species and races, *PaM P. americana*, Mexican race, *PaG P. americana*, Guatemalan race, *PaW P. americana*, West Indian race, *Ps P. schiedeana*, *Pt P. tolimanensis*, *WxM* West Indian x Mexican (natural hybrid), *MxW* Mexican x West Indian (natural hybrid), *GxPs* Guatemalan x *P. schiedeana*, (natural hybrid), *CS* collecting source, *wh* wild habitat, *fl* farm land, *by* backyard, *mk* market, *ro* research organisation, *SS* status of sample, *wi* wild, *we* weedy, *brm* breeding/research material, *lr* landrace, *ac* advanced cultivar, *CL* climate, *sch* semi-warm, humid, *tsh* temperate, semi-humid, *scsh* semi-warm, semi-humid, *scss* semi-warm, semi-dry, *csh* warm, semi-humid, *th* temperate, humid, *ch* warm, humid, *n.a.* not available

for 1 min, increasing 20°C min^{-1} until 200°C, and then maintained at this temperature for 8 min 30 s, with an overall time of 30 min. Peaks from metabolites with the highest antioomycete activity were identified by retention time (R_f) and fragmentation pattern. The mass spectra were compared with Wiley 138.L computer MS libraries.

Results and discussion

The rapid spread of *P. cinnamomi* in many countries and devastation of many food crops have stimulated the development of alternatives for its control (Pegg et al. 2002). According to environmental and site descriptors suggested by the International Plant Genetic Resources Institute (IPGRI 1995), we selected 48 accessions from the INIFAP avocado germplasm bank with potential resistance to *P. cinnamomi*. This group included the avocado clonal rootstocks Duke 6, Duke 7, Thomas, Barr-Duke, and the Martin Grande set (G755A, G755B and G755C, natural hybrids between *P. americana* var. *guatemalensis* and *P. schiedeana*) (Schieber and Zentmyer 1977), which have moderate resistance to *P. cinnamomi*. With the exception of the Martin Grande group, the accessions were derived from 'criollo' type avocados (*P. americana*

Table 2 Inhibitory effect of crude root extracts from *P. americana* on mycelial growth of *P. cinnamomi*

Accession	Race	Radial growth (cm)[a]	Inhibition (%)[a,b]
417-01	WxM	2.5±0.111	50
209-04	PaM	2.21±0.197	55
285-02	PaM	1.13±0.106	75
061-01	PaM	1.29±0.112	73
531-01	MxW	1.07±0.094	77
765-01	PaM	0*	100*
773-01	PaM	0*	100*

WxM West Indian x Mexican (natural hybrid), *MxW* Mexican x West Indian (natural hybrid), *PaM P. americana*, Mexican race

*$P \leq 0.05$ (Tukey test)

[a] Radial and inhibition of mycelial growth were registered after 8 days. Data are average of three replicates. In radial growth the ± SE values are indicated

[b] Percentage of inhibition considering the effect of mefenoxam as 100% (1 mg l^{-1})

var. *drymifolia*). The relevant characteristics from these selected accessions are shown in Table 1.

Several avocado metabolites are known to have antibacterial, antifungal, and insecticidal activity (Néeman et al. 1970; King and Knight 1987; Rodríguez-Saona and Trumble 2000). However, compounds biologically active from avocado roots (*P. americana* var. *drymifolia*) have not been evaluated recently. In order to identify active compounds in roots from Mexican avocados, 48 accessions chosen in this study were used to obtain organic and aqueous crude extracts from roots, and then used to test their activity *in vitro* against *P. cinnamomi* mycelium. According to the bioassay results, only organic root extracts inhibited mycelial growth after 8 days of culture. Aqueous extracts showed an inhibition of <50% of mycelial growth, and were not included in this study (data not shown). Organic crude root extracts from seven accessions (14%) inhibited *P. cinnamomi* mycelial growth ranging from 50% to 100% (Table 2). These accessions were identified as the *P. americana* Mexican race with the exception of 417-01 and 531-01 accessions, which correspond to the hybrids (Table 1). Crude root extracts from 417-01 and 209-04 accessions showed 50% inhibition of mycelial growth after 8 days. Crude root extracts from 285-02, 061-01 and 531-01 accessions showed 75% inhibition. Crude root extracts from 765-01 and 773-01 accessions and mefenoxam (positive control) showed 100% inhibition of mycelial growth (Fig. 1). Mefenoxam is a fungicide with activity against several species of *Phytophthora* (Thomidis and Michailidis 2002).

In order to identify the avocado root metabolites able to inhibit mycelial growth of *P. cinnamomi*, organic crude root extracts from 765-01 and 773-01 avocado accessions were analysed by preparative TLC. The crude root extracts separated by TLC showed six fractions according to their relative mobility, and their λ_{max} were registered, which helped to differentiate between fractions (Table 3). These fractions were recovered and then tested *in vitro* to assess their antioomycete activity (Table 3). In both avocado accessions, fractions IV (R_f 0.85, λ_{max} 245 nm) and V (R_f 0.9, λ_{max} 245 nm) showed 100% inhibition of mycelial growth of *P. cinnamomi* at 100 ppm concentration, whereas fraction III (R_f 0.75) from the 773-01 and 765-01 avocado accessions showed 50% and 100% inhibition, respectively. When the other fractions were tested against *P. cinnamomi* no inhibition was observed.

With the purpose of determining the chemical nature of the active metabolite for both accessions, fraction V with the highest antioomycete activity (R_f 0.9, λ_{max} 245 nm) from these materials, was subjected to GC–

Table 3 Characteristics of fractions from *P. americana* Mexican race crude root extracts separated by preparative TLC chromatography

Fractions	Relative mobility (R_f)	λ_{max} (nm)	Mycelial growth inhibition (%)[a]	
			Accession	
			765-01	773-01
I	0.3	214	0	0
II	0.6	218	0	0
III	0.75	236	100	50
IV	0.85	245	100	100
V	0.9	245	100	100
VI	0.95	215	0	0

[a] Aliquots of each fraction (100 ppm) were incorporated in filter paper discs. Mycelial growth inhibition was registered after 8 days. Values are the average of three replicates

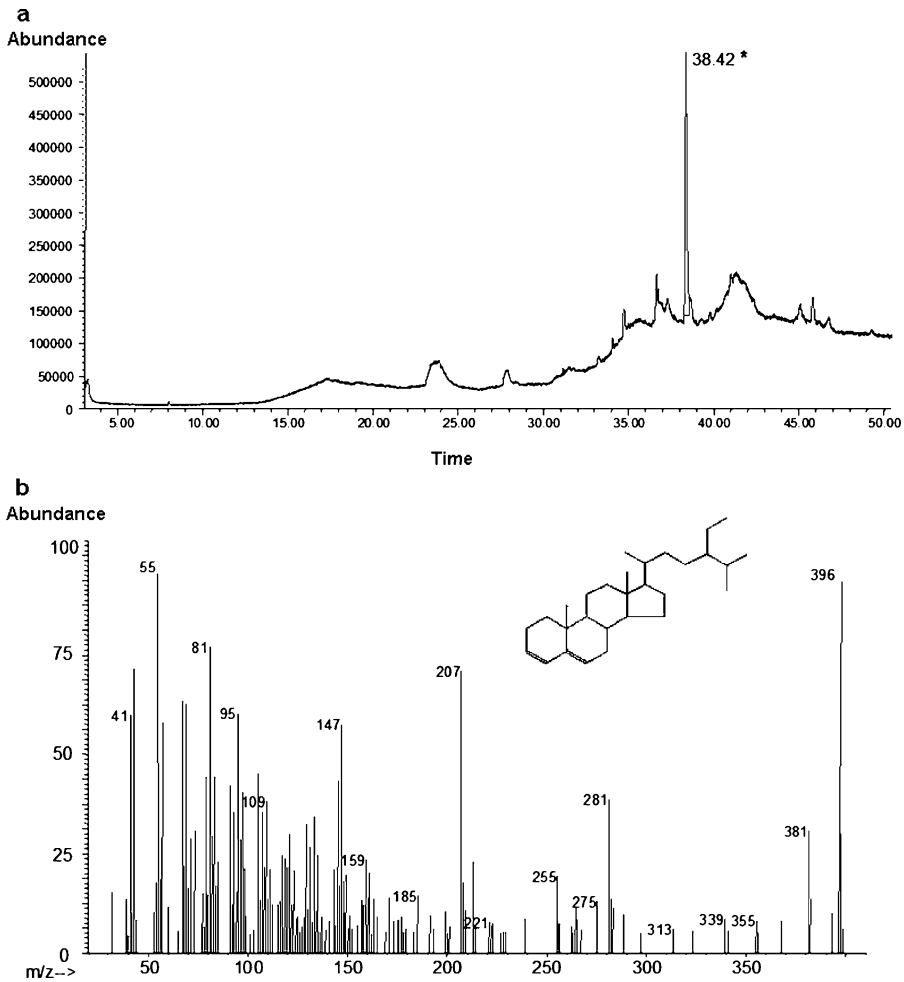

Fig. 2 Identification of compounds isolated from the organic phase of root extracts from *P. americana*. **a** Ion chromatogram and **b** mass spectra for *m/z* 396 of the principal compound with antioomycete activity purified from the organic extracts in roots from 765-01 and 773-01 avocado accessions. The *asterisk* on the ion chromatogram indicates the peak from which the spectrum was taken

MS analysis. The metabolite fragmentation pattern detected by GC–MS (retention time of 38.42) represented roughly ~95% of the extract (Fig. 2a). The chemical characteristics of the principal compound in both materials were: molecular ion [M (% relative intensity)] at *m/z* 396 (92), base peak at *m/z* 41 (62) and major fragment ions at *m/z* 381 (32) and 355 (10) (Fig. 2b). Based on GC–MS and TLC data, this compound was identified as stigmastan-3,5-diene ($C_{29}H_{48}$). This metabolite was soluble in most organic solvents, such as methanol, ethanol, chloroform and ethyl acetate, but had poor solubility in water. Sitosterol and stigmasterol, the possible intermediates of stigmastan-3,5-diene, have been described as chemical defences against some bacterial and fungal pathogens (Kiprono et al. 2000; El-Shazly et al. 2002; Mitova et al. 2003), and as inhibitors of DNA polymerase (Prakash Chaturvedula et al. 2003; Li et al. 2004). Additionally, Wang and Maas (1997), reported that strawberry roots resistant to *Phytophthora fragariae* have higher levels of sitosterol. Other compounds with structural similarity to stigmastan-3,5-diene have been reported as antimicrobials (Zhao et al. 2005), or with antiviral activity (Wachsman et al. 2002). Also, there is a patented antibacterial compound which includes stigmastan-3,5-diene and a pharmaceutical carrier (http://www.freepatentsonline.com/EP1461055.html).

To our knowledge, this is the first report on the *in vitro* biological activity of stigmastan-3,5-diene extracted from avocado roots with inhibitory activity against *P. cinnamomi* mycelial growth. The constitutive presence of this compound in avocado roots offers possibilities for the identification and selection of potentially resistant plants to *P. cinnamomi.*

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