Mangotoxin: a novel antimetabolite toxin produced by *Pseudomonas syringae* inhibiting ornithine/arginine biosynthesis

Eva Arrebola, Francisco M. Cazorla, Victoria E. Durán, Eugenia Rivera, Francisco Olea, Juan C. Codina, Alejandro Pérez-García, Antonio de Vicente*

Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Campus Universitario de Teatinos s/n, 29071 Málaga, Spain

Abstract

Pseudomonas syringae pv. *syringae* is a common inhabitant of a large variety of plants and the causal agent of bacterial apical necrosis of mango. In this work, we describe production and preliminary characterization of a novel antimetabolite toxin, designated mangotoxin, by *P. syringae* pv. *syringae* strains mainly isolated from mango trees. The toxin was detected by the *Escherichia coli* growth inhibition assay, since growth inhibition was reversed by L-ornithine and not by N-acetyl ornithine, suggesting that mangotoxin could interfere with this step of ornithine/arginine biosynthesis. Cell-free culture filtrates from different mangotoxin-producing strains strongly decreased, around 75%, the activity of ornithine N-acetyltransferase (OAT; EC 2.3.1.35) from tomato leaf protein extracts, confirming this enzyme as a target of the novel toxin. Biochemical data suggest that mangotoxin is a hydrophylic oligopeptide (<3 kDa) without a complex secondary structure (sensitive to proteases, but stable at high temperatures and extreme pH), as other antimetabolite toxins, such as phaseolotoxin or tabtoxin. HPLC analyses of cell-free culture filtrates allowed us to associate mangotoxin activity to a single peak obtained from a mangotoxin-producer strain but not present in filtrates from a derivative mutant defective in the toxic activity.
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Keywords: Mangotoxin; Ornithine; Arginine; Antimetabolite toxin; *Pseudomonas syringae* pv. *syringae*; *Mangifera indica*

Resumen

Pseudomonas syringae pv. *syringae* es una bacteria aislada frecuentemente de una amplia variedad de plantas y el agente causal de la necrosis apical del mango. En este trabajo se estudia la producción y caracterización de una nueva toxina antimetabolito, denominada mangotoxina, por cepas de *P. syringae* pv. *syringae*. Esta toxina se detectó mediante el ensayo de inhibición del crecimiento de *Escherichia coli*, ya que dicha inhibición revertía con L-ornitina, pero no con N-acetil ornitina, lo que sugiere que la mangotoxina podría interferir este paso de la biosíntesis de ornitina/arginina. La actividad ornitina N-acetiltransferasa (OAT) disminuía fuertemente en extractos proteicos de hojas de tomate, alrededor del 75%, en presencia de filtrados libres de células de cepas productoras de mangotoxina, lo que confirma a dicha enzima como diana de esta toxina. Diferentes tratamientos fisico-químicos sugieren que la mangotoxina es un oligopéptido hidrofílico (<3 kDa) sin una estructura secundaria compleja (sensible a proteasas, pero estable a altas temperaturas y pH extremos), como otras toxinas antimetabolito, como tabtoxina o faseolotoxina. Mediante HPLC, la actividad de la mangotoxina se asoció a un solo pico presente en filtrados libres de células de la cepa productora, que no se detectó en los de una cepa mutante defectiva en la producción de la toxina.
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1. Introduction

Pseudomonas syringae populations exist within diverse microbial communities on nearly all of the terrestrial plant species [21]. *P. syringae* pv. *syringae* has a broader range than other pathovars, and may be present in epiphytic or pathogenic associations [21]. On mango, this bacterium elicits a disease known as bacterial apical necrosis, one of the major factors limiting mango fruit production in Southern Spain and Portugal [10].

It is well known that phytopathogenic fungi and bacteria may produce toxins in infected plants as well as in culture media [7,19,25]. Bacterial toxins have been found mainly produced by pathovars of *P. syringae*, secondary metabolites of diverse chemical structure and effective at very low concentrations [26]. Phytotoxins produced by *P. syringae* are non-host specific and cause or increase symptoms on infected plants [25], such as chlorosis or necrosis [6,16,19]. An important group of phytotoxins produced by different *P. syringae* pathovars is composed of oligopeptides inhibiting enzymes involved in the biosynthetic pathways of some amino acids [7,25,43]. The best-known antimetabolite toxins produced by *P. syringae* pathovars are tabtoxin and phaseolotoxin. Tabtoxin was originally described from *P. syringae* pv. *tabaci* as a chlorosis-inducing toxin [32], then after it was found to be produced also by strains of pathovars *coronafaciens* and *garcae* [26]. The active molecule of tabtoxin is tabtoxinine-β-lactam, an unusual dipeptide, which irreversibly inhibits glutamine synthetase (GS) [35,37]. This determines the accumulation of ammonia in the infected tissues which is responsible for the chlorosis [38]. Phaseolotoxin is a tripeptide that induces chlorotic symptoms on leaves by the competitive inhibition of ornithine carbamoyl transferase (OCT), a key enzyme in arginine biosynthesis [28,33,34]. Phaseolotoxin is produced by strains of *P. syringae* pv. *phaseolicola* [28,31], but its production has been also reported by strains of *P. syringae* pv. *actinidae* [33] and by a strain of pv. *syringae* [36].

The production of different toxins with lipodepsipeptidic structure by strains of *P. syringae* pv. *syringae* has been extensively reported [3,4,20,29]. Syringomycins are lipodepsinonapeptides produced by most of the *P. syringae* pv. *syringae* strains isolated from a wide range of host plants [7,24]. These toxins are responsible for necrotic symptoms [23,29], by pore formation and disruption of plant cell membrane functions [16,22,23]. Syringopeptins are another class of lipodepsipeptidic toxins, containing 22 or 25 amino acids [3], that induce necrotic symptoms by mechanisms similar to syringomycins [15,22,23].

Our group is interested in dissecting the molecular bases of pathogenicity of *P. syringae* pv. *syringae* towards mango, with particular emphasis on virulence factors such as toxin production [9,10]. In this paper, we describe detection, isolation and biochemical characterization of a novel antimetabolite toxin, called mangotoxin, produced by *P. syringae* pv. *syringae* strains mainly isolated from mango. We also provide evidence that the target enzyme of mangotoxin is ornithine N-acetyltransferase (OAT; EC 2.3.1.35). The possible role of mangotoxin in the pathogenic and epiphytic life styles of *P. syringae* pv. *syringae* is discussed.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains of *P. syringae* pv. *syringae* used in this work are listed in Table 1. *P. syringae* strains were grown in King's medium B (KMB) at 27 °C. Nearly all the strains used in this work were obtained in previous studies; they were mainly isolated from mango trees in different years (from 1990 to 2000) and from different geographical locations in the South of the Iberian Peninsula [9,10]. The rest of *P. syringae* pv. *syringae* strains used were isolated from other plants or obtained from different laboratories (Table 1). Stable mutants impaired in the production of mangotoxin were constructed using Tn5-derived minitransposons and the wild type *P. syringae* pv. *syringae* strain UMAF0158, by mating experiments with *E. coli* S17-λpir (mini-Tn5 km2) on membrane filters (0.45 μm, Millipore Corporation, Bedford, USA) onto LB agar at 27 °C for 2 h [13]. Mutants were isolated on KMB with kanamycin and nitrofurantoin as selective agents.

2.2. Detection of P. syringae toxins

The syringomycin complex production by *P. syringae* strains was determined by growth inhibition tests on potato-dextrose agar (PDA) against *Geotrichum candidum* [20] and *Rhodotorula pilimanae* [23].

The antimetabolite toxin production was assayed by the indicator technique previously described by Gasson [17] with minor modifications [9], involving growth inhibition of *Escherichia coli* on Pseudomonas minimal medium (PMS) [17]. Briefly, a double layer of indicator microorganism was made using strain CECT831 of *E. coli*. After solidification, strains of *P. syringae* pv. *syringae* to be tested were stabbed and plates were incubated at 22 °C for 24 h and at 37 °C for an additional 24 h period. To assess the biochemical step that is the putative target of the toxin, the same plate bioassay was carried out, but adding to the double layer 100 μl of a 6 mM solution of the corresponding amino acid or intermediate.

Different *P. syringae* strains (Table 2) were grown on liquid PMS for 5 days at 22 °C with shaking. After centrifugation at 4000 g for 10 min, supernatants were filtered through 0.2 μm nitrocellulose membranes (Millipore Corporation, Bedford, USA). The cell-free culture filtrates were stored at 4 °C and protected from light for no longer than 1 week. The toxic activity of filtrates was evaluated by the *E. coli* growth inhibition bioassay previously described. Briefly, wells of 7-mm diameter were made into the PMS agar plates, and then filtrate samples were placed in each well mixed with sterile melted media. After, a double layer with *E. coli* was added and plates were then incubated at 37 °C for 24 h. Inhibition zones were measured from the border of the well to the perimeter of visible growth of *E. coli*.

2.3. Ornithine-acetyltransferase enzymatic assay

Because OAT is not commercially available and protein yields in crude extracts from mango leaves were very low, OAT activity experiments were carried

Table 1
Toxin production by *P. syringae* strains isolated from mango and other plants

Strain	Isolated from/in		Syringomycin production	Antimetabolite toxin production							Antimetabolite produced
	Plant	Location		*E. coli* inhibition in minimal medium added							
				−	Gln	Glu	NAG	NAO	Orn	Arg	
P.s.s. UMAF0100	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin[a]
P.s.s. UMAF0118	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0122	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0114	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0048	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0130	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0158	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0523	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0171	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0115	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0166	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0176	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0139	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0511	Mango	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0081	Mango	Málaga, Spain	−	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF0517	Mango	Málaga, Spain	−	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF3028	Mango	Málaga, Spain	+	−							
P.s.s. UMAF3041	Mango	Málaga, Spain	+	−							
P.s.s. UMAF0158-3αE10	Tn-5 mutant of UMAF0158		+	−							
P.s.s. UMAF0158-2βH4	Tn-5 mutant of UMAF0158		+	−							
P.s.s. UMAF1003	Mango	Huelva, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF1065	Mango	Huelva, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF1094	Mango	Huelva, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF1118	Mango	Huelva, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF1051	Mango	Huelva, Spain	−	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF1214	Mango	Huelva, Spain	nd	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF1216	Mango	Huelva, Spain	nd	−							
P.s.s. UMAF2901	Mango	Granada, Spain	nd	−							
P.s.s. UMAF2802	Mango	Canary Islands, Spain	nd	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF2007	Mango	Almansil, Portugal	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF2010	Mango	Mesines, Portugal	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF2025	Mango	Almansil, Portugal	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF2023	Mango	Almansil, Portugal	−	−							
P.s.s. 1559-1[b]	Mango	Huelva, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. 1559-9[b]	Mango	Huelva, Spain	nd	−							
P.s.s. Ps-10[c]	Mango	Israel	nd	+	+	+	+	+	−	−	Mangotoxin
P.s.s. Ps-35[c]	Mango	Israel	nd	+	+	+	+	+	−	−	Mangotoxin
P.s.s. Ps-6[c]	Mango	Israel	nd	−							
P.s.s. UMAF 4002	Tomato	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF 6016	Chesnut	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF 6582	Peach	Málaga, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. UMAF 6000	Orange	Málaga, Spain	+	−							
P.s.s. UMAF 6021	Almond	Málaga, Spain	−	−							
P.s.s. EPS17A[d]	Pear	Gerona, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. EPSMV3[d]	Pear	Gerona, Spain	+	−							
P.s.s. 1507-7[b]	Crataegus	Valencia, Spain	+	+	+	+	+	+	−	−	Mangotoxin
P.s.s. 1444-5[b]	Laurel	Madrid, Spain	+	−							
P.s.s. 2242[e]	Bean	Zaire	+	−							
P.s.s. 2676[e]	Bean	Lesotho	+	−							
P.s.s. CFBP 3388[f]	Vetch	Tourte and Manceau (1995) [36]	nd	+	+	+	+	+	+	−	Phaseolotoxin
P.s.t. UMAF 4007	Tomato	Málaga, Spain	−	+	+	+	+	−	−	−	Unknown
P.s.t. UMAF 6018	Tomato	Málaga, Spain	−	+	+	+	+	−	−	−	Unknown
P.s.t. UMAF 4004	Tomato	Málaga, Spain	−	+	+	+	+	−	−	−	Unknown

Table 1 (*continued*)

Strain	Isolated from/in		Syringomycin production	Antimetabolite toxin production							
	Plant	Location		*E. coli* inhibition in minimal medium added							Antimetabolite produced
				−	Gln	Glu	NAG	NAO	Orn	Arg	
P.s.t. UMAF 4512	Tomato	Málaga, Spain	−	−							
P.s.t. UMAF 5003	Tomato	Almería, Spain	−	+	+	+	+	−	−	−	Unknown
P.s.t. UMAF 5010	Tomato	Almería, Spain	−	+	+	+	+	−	−	−	Unknown
P.s.t. UMAF 5004	Tomato	Almería, Spain	−	+	+	+	+	−	−	−	Unknown
P.s.t. UMAF 5009	Tomato	Almería, Spain	−	−							
P.s.t. DCT6D1[g]	Tomato	Moore et al. (1989) [27]	−	+	+	+	+	−	−	−	Unknown
P.s.t. DC3000[g]	Tomato	Moore et al. (1989) [27]	−	−							
P.s.t. PT23[h]	Tomato	Bender and Cooksey (1986) [8]	−	−							
P.s. *syringae* CECT 127			+	+	+	+	+	+	−	−	Mangotoxin
P.s. *syringae* CECT 4429 (NCPPB 281)			+	+	+	+	+	+	−	−	Mangotoxin
P.s. *syringae* NCPPB 1239			+	+	+	+	+	+	−	−	Mangotoxin
P.s. *coronafaciens* CECT 4389 (NCPPB 1357)			−	+	−	+	+	+	+	+	Tabtoxin
P.s. *phaseolicola* CECT 4390 (NCPPB 1103)			−	+	+	+	+	+	+	−	Phaseolotoxin
P.s. *tomato* CECT 4393 (NCPPB 1106)			−	+	+	+	+	−	−	−	Unknown

NAG, N-acetyl glutamate; NAO, N-acetyl ornithine; Orn, Ornithine; Glu, Glutamate; Gln, Glutamine; Arg, Arginine; P.s.s., *Pseudomonas syringae* pv. *syringae*; P.s.t., *Pseudomonas syringae* pv. *tomato*; CECT, Spanish Type Culture Collection; NCPPB, National Collection Plant Pathogenic Bacteria; nd, not determined.

[a] The name of Mangotoxin is proposed for the antimetabolite toxin described in this work.
[b] Strains were kindly gifted by Dr López from I.V.I.A. (Valencia, Spain);
[c] Strains were kindly gifted by Dr Lichter from Volcani Center (Bet-Dagan, Israel);
[d] Strains were kindly gifted by Dr Montesinos from Univ. of Girona (Spain);
[e] Strains were kindly gifted by Dr Vivian from Univ. West England (Bristol,UK);
[f] Strains were kindly gifted by Dr Manceau from I.N.R.A. (Angers, France);
[g] Strains were kindly gifted by Dr Cuppels from A.C.R.C. (London, Canada);
[h] Strains were kindly gifted by Dr Murillo from Univ. Pública of Navarra (Pamplona, Spain).

out using crude protein extracts from tomato leaves. Ornithine N-acetyltransferase (OAT) activity was measured from crude protein extracts of tomato leaves (cv. Hellfrucht-Früstamm) as increase of ornithine (Orn) amounts, using a modification of the ninhydrin procedure [14]. The development of this modification permits the specific detection of ornithine at acidic pH, because under these conditions N-acetyl-L-ornithine gives very little ninhydrin reaction [39]. In addition, this method is rather specific for ornithine, when glutamate concentrations are lower than 6 mM [14]. Extraction buffer ((Tris-HCl 50 mM pH 7.5; $CoCl_2$ $6H_2O$ 0.2 mM; glutathione 1 mM; phenylmethylsulfonyl fluoride (PMSF) 0.1 mM) was added to 3–5 g of washed tissue in a ratio tissue:buffer 1:1 [14]. Plant tissue was homogenized with an Ultra-Turrax device (IKA, Staufen, Germany) and the mixture was filtered through two layers of cheesecloth and then centrifuged. The supernatant was used as crude extract for determination of OAT activity in the presence of bacterial culture filtrates.

In the standard assay, 25 µl of 6 mM N-acetyl-L-ornithine (NAO) and 25 µl of 6 mM L-glutamate were added to 50 µl of crude protein extract of tomato leaves, and 25 µl of the culture filtrate to be tested were then added. After 1 h of incubation at 37 °C the reaction was stopped by adding three volumes of ninhydrin reagent (a mixture 1:2 of 0.4 M citric acid and 1% ninhydrin in α-methoxyethanol) and heated at 100 °C for 1.5 min. Two volumes of 0.7 M NaOH were then added and the absorbance at 470 nm recorded after 20 min of incubation at room temperature. The amount of Orn was determined colorimetrically by using as reference a standard curve prepared for the purpose [14,40]. A 0.2 mM solution of *p*-chloromercuribenzoic acid (PCMB), a chemical inhibitor of OAT [14], and sterile liquid PMS were used as controls.

2.4. Biochemical characterization of toxic activity from culture filtrates

Cell-free filtrates from antimetabolite toxin-producers *P. syringae* pv. *syringae* strains UMAF0158, UMAF1003

Table 2
E. coli growth inhibition tests and Ornithine N-acetyl-transferase (OAT) activity of tomato leaves and its specific inhibition by cell-free culture filtrates of Pseudomonas syringae pv. syringae in liquid minimal medium (PMS broth)

Culture filtrates of:	E. coli growth inhibition on:			OAT activity[a] (nkat/gfw)	OAT activity (%)[b]
	PMS[c]	PMS + NAO	PMS + Orn		
PMS[d]				6.75 ± 1.36	100
PMS + PCMB[e]				1.21 ± 0.81	17.9
P.s. syringae UMAF0081	+	+	−	1.69 ± 1.15	25.0
P.s. syringae UMAF0158	+	+	−	1.14 ± 0.98	16.9
P.s. syringae UMAF1003	+	+	−	1.45 ± 1.08	21.5
P.s. syringae UMAF2010	+	+	−	1.14 ± 0.77	16.9
P.s. syringae UMAF0122	+	+	(−)[f]	3.69 ± 1.35	54.7
P.s. syringae 1507-7	+	+	−	2.08 ± 1.17	30.8
P.s. syringae EPSMV3	−			6.04 ± 1.06	89.5
P.s. syringae UMAF0158-3αE10	−			6.45 ± 1.12	95.6
P.s. syringae UMAF0158-2βH4	−			5.80 ± 1.08	85.9
P.s. syringae CECT 4429	+	+	−	3.74 ± 0.89	55.4
P.s. coronafaciens CECT 4389	+	+	+	5.83 ± 0.86	86.4
P.s. phaseolicola CECT 4390	+	+	+	5.57 ± 0.97	82.5
P.s. tomato UMAF4007	+	−	−	5.72 ± 1.39	84.7

NAO, N-acetyl ornithine; Orn, Ornithine.
[a] Mean and standard deviation values obtained from five independent replicate experiments. nkat/gfw, nanokatals/g of fresh weight.
[b] Percentage of OAT activity related to the activity obtained with PMS broth.
[c] Solid minimal medium (PMS agar).
[d] Liquid minimal medium (PMS broth); (negative inhibition control).
[e] PCMB, p-chloromercuribenzoic acid is an specificic inhibitor of OAT activity (positive inhibition control).
[f] Inhibition zone is turbid.

and UMAF2010 (Table 1) were subjected to the following treatments: (1) Heat treatments at 50, 75, 100 and 121 °C for 15 min, and at 100 °C for 60 min. (2) Extreme pH (2 and 12) for 10 min, and then, the original pH was reestablished (pH 6.5–7). (3) Overnight treatment with proteases (pronase 2 units and proteinase K 2.5 units). (4) Extractions with different organic solvents in a ratio 1:1 for 1 h [acetonitrile, ethylacetate, and methanol:chloroform 2:1 (v/v)], followed by separation of aqueous and organic phases by centrifugation at 2500 g for 20 min and concentration of the latter by evaporation. After treatments, the presence/absence of the toxic activity was tested by using the bioassay against E. coli in presence of Orn or NAO, as previously described. Finally, the molecular size was estimated using centrifugal filter devices (10 and 3 kDa) (Centricon®, Millipore Corporation, Bedford, USA).

2.5. Chromatographic analyses

Cell-free filtrates from cultures of three P. syringae pv. syringae mangotoxin-producing strains (UMAF0158, UMAF1003 and UMAF2010), a P. syringae pv. coronafaciens tabtoxin-producing strain (CECT4389), a P. syringae pv. phaseolicola phaseolotoxin-producing strain (CECT4490), and a Tn5 mutant of the wild-type strain P. syringae pv. syringae UMAF0158 (UMAF0158-3αE10), defective in mangotoxin production were obtained. The crude cell-free filtrates were extracted with an equal volume of methanol:chloroform 2:1 (v/v). The aqueous phases were concentrated by evaporation in vacuo and fractionated on Silica gel 60 F254 TLC plates (Merck, Darmstadt, Germany) and developed in a solvent mixture of methanol:water 70:30 (v/v). Thin layer chromatograms were visualized under u.v. light at 254 nm. TLC plates were then covered with a thin layer of PMS medium amended with 2,3,5 triphenyltetrazolium chloride (TTC) [30], an aliquot of an overnight culture of E. coli CECT831 and the corresponding amino acids to check the specific activities. After incubation at 37 °C for 24 h, growth inhibition areas of the indicator microorganim were observed as haloes with no reddish color, that revealed absence of respiration as a consequence of indicator microorganism growth inhibition.

For partial purification of mangotoxin, 50 ml of cell-free supernatant fluids were extracted with an equal volume of methanol:chloroform 2:1 (v/v) and the aqueous phases recovered. After concentration by evaporation in vacuo of aqueous phase, concentrated samples were dissolved in 10 ml of 20% acetonitrile and 500 μl of sample were injected and fractionated by HPLC with an Alltech Hypersyl ODS 5 μm 250 × 4.6 mm column (Alltech Associates, Inc., Deerfield, USA) and a 20% isocratic gradient of acetonitrile in water, with a flow rate of 0.250 ml/min. UV detection was

performed with a Pharmacia RSD 2140 diode array detector (Pharmacia, Uppsala, Sweden) with wave-length scanning from 190 to 400 nm. Only one peak showing toxic activity was recovered. It was collected and fractionated by TLC on silica plates. The spot corresponding to mangotoxin was scratched from the plate, extracted in 20% acetonitrile, and concentrated by evaporation in vacuo. To localize the toxic spot, a duplicate TLC plate was developed and revealed by the assay of *E. coli* growth inhibition in a double layer of PMS amended TTC, as previously described. This concentrated extract was again fractionated by HPLC, and 250-µl fractions were tested for the antimicrobial activity against *E. coli* in PMS plates, as previously described.

3. Results

3.1. Production of an antimetabolite toxin by P. syringae pv. syringae strains

To get insight into the virulence factors that allow *P. syringae* pv. *syringae* to cause disease in mango, we analyzed toxin production by a collection of mango strains. The production of lipodepsinonapeptide toxins, as syringomycin, by *P. syringae* strains is shown in Table 1. The results obtained indicated a generalized production of syringomycins by nearly all the *P. syringae* pv. *syringae* strains tested. As expected, strains of other pathovars tested did not produce syringomycins. The analysis of the production of antimetabolite toxins showed the presence of toxic activity inhibiting growth of *E. coli* in most (87.6%) of the strains of *P. syringae* pv. *syringae* isolated from mango. Some strains of *P. syringae* pv. *syringae* from plants different from mango, including strains from Type Culture Collections, also produced antimetabolite toxins (31.3%).

Interestingly, these strains of *P. syringae* pv. *syringae* produced neither tabtoxin nor phaseolotoxin, based on the *E. coli* growth inhibition assay. These strains produced a toxin whose effect remained in presence of glutamate or glutamine, but was reversed by ornithine or citrulline, the amino acid pairs used to detect the production of tabtoxin or phaseolotoxin, respectively (Table 1). Consequently, these results revealed that the toxin produced inhibited a different step in the biosynthetic pathway of arginine (Fig. 1). To determine the specific step affected by the antimetabolite toxin produced by the *P. syringae* pv. *syringae* strains, the reversion of the growth inhibition of *E. coli* in PMS plates supplemented with different intermediate amino acids of arginine biosynthesis was undertaken (Table 1, Fig. 1). The antimetabolite toxic activity produced by many *P. syringae* pv. *syringae* strains did not reverse the *E. coli* growth inhibition when N-acetyl glutamate or NAO were added, but it was completely reversed after adding Orn (Table 1), suggesting as a toxin target the step that produces Orn from NAO. Therefore, the target enzyme of mangotoxin could be
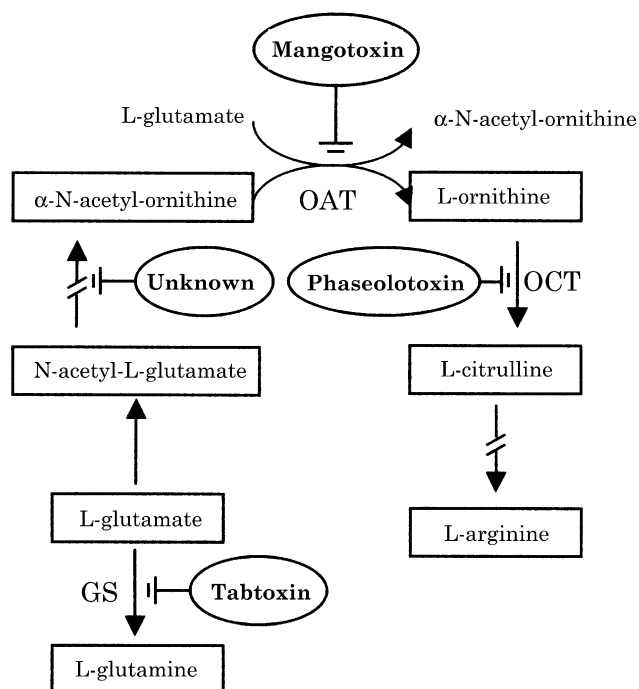

Fig. 1. Schematic representation of glutamine and arginine biosynthetic pathways and the corresponding target enzymes inhibited by antimetabolite toxins produced by different *Pseudomonas syringae* pathovars. Unknown refers to the uncharacterized toxic activity from some strains of *P. syringae* pv. *tomato*. GS: Glutamine synthetase. OCT, Ornithine carbamoyl-transferase; OAT, Ornithine N-acetyl-transferase.

either N-acetylornithinase (AO, EC 3.5.1.16) in *E. coli* and other *Enterobacteriaceae* or ornithine N-acetyltransferase (OAT, EC 2.3.1.35) in most of these organisms (Fig. 1). These enzymes have not been previously described as targets of antimetabolite toxin. This toxin not previously reported was named mangotoxin, in reference to the plant source (mango) of most of the producer strains. So far, its production has only been detected from *P. syringae* pv. *syringae* strains, although a small number of strains from pathovars other than *syringae* were tested.

Furthermore, some strains of *P. syringae* pv. *tomato* were also positive for production of antimetabolite toxins whose activity was reversed by NAO, but not by N-acetylgluta-mate (Table 1). These toxins may inhibit an earlier and undetermined step of the arginine biosynthetic pathway, although the observed behavior may instead be due to inability to import N-acetylglutamate.

3.2. Inhibition of OAT by mangotoxin

To confirm OAT as a target enzyme for mangotoxin, determinations of OAT activity from plant extracts in the presence of cell-free filtrates from cultures of *P. syringae* strains were performed (Table 2). The results obtained showed a strong decrease of OAT activity when cell-free filtrates from *P. syringae* pv. *syringae* strains that produce mangotoxin were assayed. The decrease was similar to that observed with PCMB, a specific inhibitor of OAT [14].

In contrast, a minimal reduction of OAT activity was observed when filtrates from mangotoxin non-producing strains (*P. syringae* pv. *syringae* EPSMV3 or two mutants of *P. syringae* pv. *syringae* UMAF0158 impaired in the production of mangotoxin) were used. Levels of OAT activity were close to those observed with controls with sterilized PMS medium. Similar results were observed when cell-free filtrates from *P. syringae* strains that produce antimetabolite toxins other than mangotoxin (pvs. *corona-faciens*, *phaseolicola* and *tomato*) were used.

The antimetabolite activity of mangotoxin on OAT from tomato plants and its growth inhibitory activity toward *E. coli* were confirmed by assays using different dilutions of cell-free filtrates from some *P. syringae* pv. *syringae* strains. These experiments showed that both inhibitory activities were proportional to the amount of toxic filtrates added in the bioassays, so, a reduction of halo diameters of *E. coli* growth and an increase in OAT activity were observed when the toxic filtrates were diluted (Fig. 2). A possible interference of lipodepsinonapeptidic toxins with the mangotoxin activity was ruled out, because syringomy-cins production was not detected in *P. syringae* cultures on PMS, an observation previously reported by other authors [18,24].

3.3. Biochemical characterization of mangotoxin from cell-free filtrates

In order to get some preliminary data about the chemical nature of mangotoxin, toxic cell-free filtrates from *P. syringae* pv. *syringae* strains were subjected to different treatments (Table 3). The specific toxic activity present in cell-free filtrates of three *P. syringae* pv. *syringae* strains showed a clear resistance to high temperature (100 °C) and to extreme pH values (2 and 12). In contrast, treatments with proteinase K and pronase eliminated the toxic activity. Furthermore, ultrafiltration tests showed that the antimicrobial activity was always present in the filtrable fraction, suggesting that the active molecule was of a molecular size lower than 3 kDa. Finally, the toxic activity was always found in the aqueous phase when extractions with different organic solvents were performed (Table 3). Taken together, these results indicated that mangotoxin is secreted to the medium as a small (<3 kDa) hydrophilic molecule with a peptidic nature.

3.4. Partial purification of mangotoxin

Taking into account the biochemical data previously mentioned, we proceeded to attempt the purification of mangotoxin by chromatographic techniques. Aqueous phase from cell-free culture filtrates of *P. syringae* strains, after methanol/chloroform extractions were recovered, concentrated, and fractionated on silica TLC plates. TLC plates were developed and several dark spots were visualized under u.v. light at 254 nm (Fig. 3A). The spots
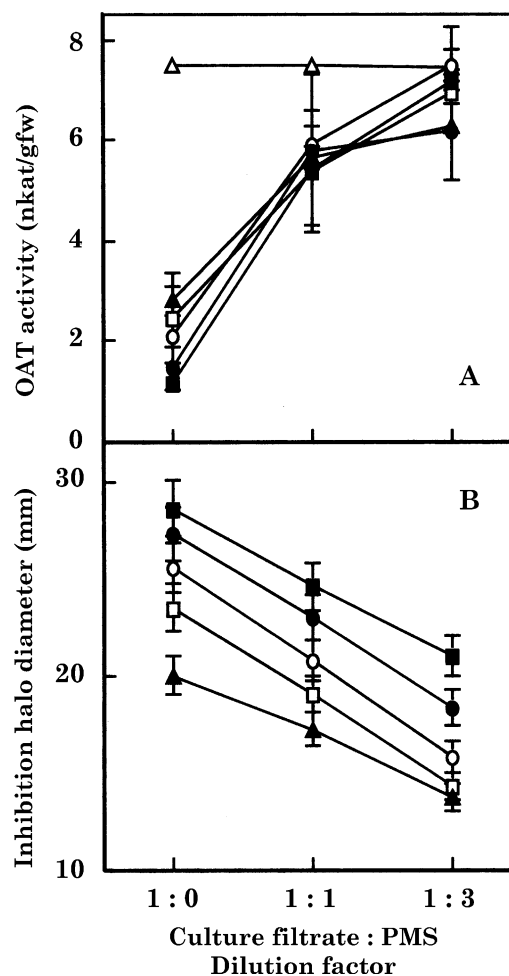

Fig. 2. Toxic activity of cell-free filtrates from cultures in liquid minimal medium (PMS broth) of *Pseudomonas syringae* pv. *syringae* strains that produce mangotoxin. (A) Inhibition of ornithine N-acetyl-transferase (OAT) activity in crude extracts of tomato leaves by the cell-free filtrates and their dilutions. OAT activity with sterilized PMS (negative inhibition control $\triangle$) was 7.54 ± 0.58 nkat/gfw. (B) Inhibition zones of *Escherichia coli* growth produced by the filtrates and their dilutions. *P. syringae* pv. *syringae* strains UMAF0158($\blacksquare$), UMAF0081 ($\blacktriangle$), UMAF1003 ($\bigcirc$), 1507-7 ($\square$) and UMAF2010 ($\bullet$) were used. The cell-free filtrate dilutions were made with sterilized PMS. Mean and standard deviation values of three replicate experiments are shown.

corresponding to the antimetabolite toxins were identified by using the *E. coli* growth inhibition bioassay in a thin layer of PMS amended TTC. The toxic activity from *P. syringae* pv. *syringae*, corresponding to mangotoxin, was associated to a single growth-inhibiting spot with an *Rf*-value of 0.62. This spot was not detected in filtrates from the non-producer mutant (Fig. 3B). The mangotoxin spot was specifically detected, since *E. coli* growth inhibition was reversed with Orn, whereas the toxicity of tabtoxin and phaseolotoxin remained (Fig. 3C).

Additional steps for purification of the mangotoxin were carried out. Cell-free filtrates from a *P. syringae* pv. *syringae* strain that produce mangotoxin were fractionated by HPLC and TLC. The toxic spot corresponding to mangotoxin was recovered from TLC plates and, after

Table 3
Characterization of the inhibitory activity of cell-free culture filtrates of three mangotoxin-producing *Pseudomonas syringae* pv. *syringae* strains

Culture filtrate on PMS	P.s.s. UMAF0158			P.s.s. UMAF1003			P.s.s. UMAF2010		
	PMS[a]	+NAO	+Orn	PMS	+NAO	+Orn	PMS	+NAO	+Orn
Heat treatments:									
Untreated	23 ± 1.0	23 ± 1.0	0[b]	13 ± 0.6	13 ± 0.6	0	18 ± 0.5	18 ± 0.5	0
50°C-15 min	23 ± 0.5	22 ± 0.7	0	12 ± 0.5	12 ± 0.5	0	19 ± 0.8	19 ± 2.0	0
75°C-15 min	22 ± 0.9	24 ± 1.4	0	13 ± 0.5	13 ± 0.7	0	19 ± 0.6	21 ± 2.5	0
100°C-15 min	21 ± 0.9	22 ± 2.0	0	12 ± 1.0	13 ± 0.7	0	13 ± 2.3	15 ± 1.4	0
100°C-60 min	16 ± 0.9	17 ± 0.7	0	0	0	0	0	0	0
121°C-15 min	0	0	0	0	0	0	0	0	0
pH tolerance:									
Untreated	18 ± 0.7	19 ± 1.0	0	18 ± 1.4	18 ± 0.7	0	18 ± 0.5	17 ± 0.7	0
Acid pH = 2	19 ± 0.3	20 ± 0.7	0	19 ± 0.7	19 ± 0.3	0	15 ± 0.7	14 ± 0.3	0
Basic pH = 12	20 ± 1.4	20 ± 1.0	0	15 ± 0.7	16 ± 1.0	0	14 ± 0.7	14 ± 0.3	0
Enzymatic treatments:									
Untreated	22 ± 2.0	20 ± 2.5	0	10 ± 0.7	11 ± 2.0	0	16 ± 2.0	15 ± 2.5	0
Pronase	0	0	0	0	0	0	0	0	0
Proteinase K	0	0	0	0	0	0	0	0	0
Ultrafiltration:									
Untreated	21 ± 0.6	21 ± 0.5	0	11 ± 0.8	11 ± 0.8	0	18 ± 2.0	18 ± 1.9	0
> 10 kDa fraction	0	0	0	0	0	0	0	0	0
< 10 kDa fraction	19 ± 0.6	19 ± 0.1	0	14 ± 0.5	15 ± 1.3	0	16 ± 1.0	16 ± 1.4	0
> 3 kDa fraction	0	0	0	0	0	0	0	0	0
< 3 kDa fraction	23 ± 1.9	24 ± 0.5	0	12 ± 1.2	11 ± 1.4	0	14 ± 0.9	15 ± 0.6	0
Aqueous phase after extraction with organic solvents:[c]									
Untreated	29 ± 0.0	29 ± 0.1	0	23 ± 0.8	22 ± 0.8	0	30 ± 0.1	30 ± 0.0	0
Acetonitrile	27 ± 0.1	25 + 0.5	0	21 ± 1.3	19 ± 1.0	0	28 ± 0.5	28 ± 0.2	0
Ethyl Acetate	31 ± 0.1	29 ± 0.1	0	26 ± 0.4	28 ± 0.2	0	23 ± 1.3	23 ± 1.1	0
Methanol-Chloroform	24 ± 0.9	25 ± 0.6	0	27 ± 0.6	25 ± 0.7	0	30 ± 0.1	29 ± 0.3	0

Toxic activity of different fractions or after different treatments was tested by the *E. coli* growth inhibition assay in minimal medium (PMS) and reversion with ornithine. Toxic activity is expressed as diameter (in mm) of inhibition zone. Mean and standard deviation values were obtained from three replicate experiments. NAO, N-acetyl ornithine; Orn, Ornithine.
[a] Solid minimal medium (PMS agar).
[b] No inhibition halo was observed.
[c] Toxic activity always was only detected in aqueous phase.

checking the antimicrobial activity in the extract; it was newly purified by HPLC analysis. The toxic activity eluted from the HPLC column after 10 min associated with a single peak, was detected only in the filtrates from the wild type strain, but not present in filtrates from its derivative non-producer mutant (Fig. 4). Unfortunately, this purified fraction was very unstable and lost its toxic activity in a few hours, when it was recovered in a medium without salts.

4. Discussion

The results of this study confirm that production of phytotoxins is widespread among *P. syringae* strains of some pathovars, which are able to produce more than one toxin [7]. Chlorosis-inducing phytotoxins are produced by some pathovars of *P. syringae*, and the most common of these toxins are the so-called antimetabolite toxins, tabtoxin or phaseolotoxin. They are not required for pathogenicity in *P. syringae* pv. *tabaci* and *P. syringae* pv. *phaseolicola*, instead they function as virulence factors. Their production results in the increase of disease severity [6,25,33]. In contrast, the necrosis inducing lipodepsipeptidic toxins, such as syringomycins or syringopeptins, are restricted to strains of *P. syringae* pv. *syringae* and related pathovars [4,15,19]. Despite the structural differences, different lipodepsinonapeptides exhibit similar biological activity toward the plasma membrane of host cell, and this is associated with formation of characteristic necrotic lesions [7,22,23].

Nearly all the *P. syringae* pv. *syringae* strains isolated from mango produced syringomycins, as previously described for this pathovar [20]. In addition, most of these strains (87.6%) produced in vitro an antimetabolite toxin, regardless of the production of syringomycins [9]. The *E. coli* growth in presence of *P. syringae* pv. *syringae* was
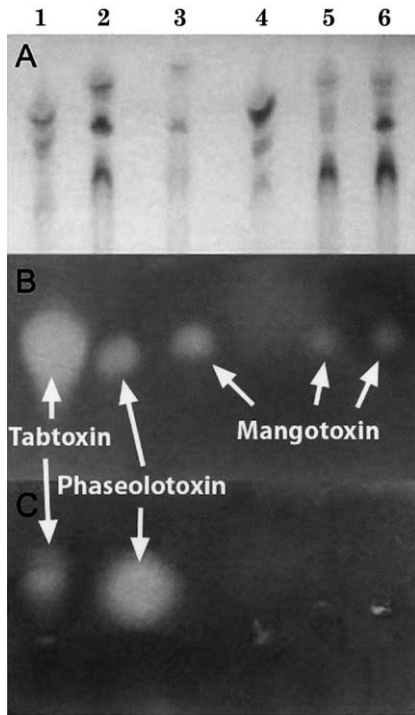
Fig. 3. TLC analysis of cell-free culture filtrates of *P. syringae* pv. *coronafaciens* CECT4389 (tabtoxin-producing, lane 1), *P. syringae* pv. *phaseolicola* CECT4490 (phaseolotoxin-producing, lane 2), and *P. syringae* pv. *syringae* UMAF0158, UMAF1003, UMAF2010 (mango-toxin-producing, lanes 3, 5 and 6), and UMAF0158-3αE10 Tn5-mutant tox⁻ (non-mangotoxin-producing, lane 4) were separated by TLC on silica plates and chromatograms visualized under u.v. light of 254 nm (A) and their corresponding toxic activities located on TLC plates by an *E. coli* growth inhibition assay on a thin layer of PMS agar over the TLC plate (B), or PMS supplemented with ornithine (C).

restored by presence in the medium of Orn, but not by NAO. These results suggest that the enzymatic step of the ornithine/arginine byosinthetic pathway which converts NAO to Orn was inhibited by the antimetabolite toxin produced by *P. syringae* pv. *syringae* strains. Therefore, this toxin seems to act on the same biosynthetic pathway as phaseolotoxin [28,34], but on a different step. The novel antimetabolite toxin was named mangotoxin, referring to the host where the first and most of the producing strains were isolated from. Mangotoxin appears to be produced only by certain *P. syringae* pv. *syringae* strains, isolated from mango trees and also from other plants, such as pear, chesnut, peach, tomato or wild plants. Its production has not been detected in any other pathovars of *P. syringae*, although the number of the strains tested is small.

Our results demonstrate that OAT is a specific target enzyme for mangotoxin. OAT is a key enzyme in the biosynthetic pathway of ornithine and arginine, which transfers the acetyl group of NAO onto glutamate to yield N-acetylglutamate and Orn, in most of the bacteria and eukaryotic organisms [11,12]. Mangotoxin could act as an antimetabolite inhibiting this enzyme and provoking an accumulation of NAO and glutamate and a deficiency in the intracellular pools of Orn and arginine (Fig. 1). However, in certain bacteria, as *Enterobacteriaceae*, NAO is converted into Orn by a simple deacetylation catalyzed by AO [12], that apparently may be also inhibited by mangotoxin, because it inhibits the *E. coli* growth. In this sense, PCMB is also an inhibitor of both enzymes AO and OAT [14,40]. Similarly, besides OCT, ornithine decarboxylase (ODC) has been reported as an additional target for phaseolotoxin [2]. The results presented show conclusively and for the first time, that the target of mangotoxin is a step of the arginine
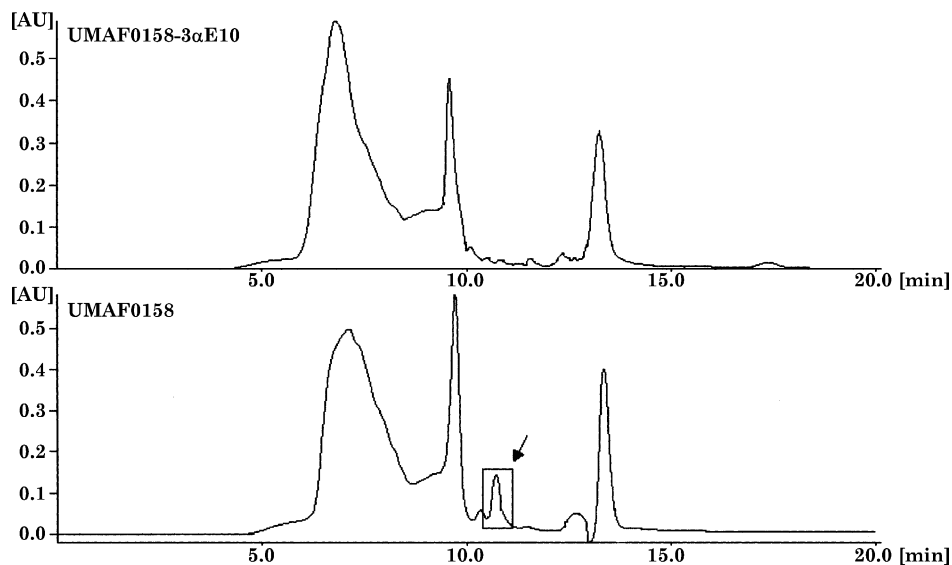

Fig. 4. HPLC separation of mangotoxin present on the aqueous phase after extraction with methanol/chloroform from cell-free filtrates of 5-day-old cultures of the wild type *P. syringae* pv. *syringae* strain UMAF0158 and its Tn5-derivative mutant impaired in mangotoxin production (*P. syringae* pv. *syringae* UMAF0158-3αE10). All the eluted fractions coming from different peaks were tested for the specific toxic activity by the *E. coli* growth inhibition assay. Only one peak (enclosed by a box), present in the wild type filtrate but not in the mutant one, showed a toxic activity. The toxic activity was reversed by addition of ornithine, but not after adding N-acetyl ornithine. AU absorbance units determined at the wavelength showing the maximum absorbance level (250–260 nm).

biosynthetic pathway and specifically, the OAT enzyme. The metabolic step affected by mangotoxin is different from those affected by tabtoxin, inhibiting GS [35,37], phaseolotoxin, inhibiting OCT [28,33], or the non-characterized antimetabolite toxins produced by *P. syringae* strains of pvs. *tomato*, *apii* [9,41] and *syringae* [7,16,43] (Fig. 1). Possibly, mangotoxin could act by inhibiting its target enzymes as a structural analogue of NAO, as shown for phaseolotoxin [28] and tabtoxin [35].

P. syringae pv. *tomato* strains produce a distinct antimetabolite toxin, which inhibits ornithine/arginine biosynthesis in a previous step to that in which mangotoxin does (Fig. 1), possibly in one of the three enzymatic steps that produce NAO from N-acetyl glutamate. The target enzyme could be either acetylglutamate kinase, or acetyl-glutamyl phosphate reductase or acetylornithine aminotransferase. The specific target enzyme of this antimetabolite toxin from *P. syringae* pv. *tomato* remains to be determined, since the intermediary metabolites needed are not commercially available. A similar antimetabolite toxic activity was reported [9,41].

The preliminary characterization of mangotoxin suggests that it is probably a small oligopeptide, as are the best known antimetabolite toxins, tabtoxin or phaseolotoxin [7,34,35]. Mangotoxin is secreted to the media as an hydrophilic molecule, lower than 3 kDa and sensitive to proteases, but resisting extreme levels of pH and high temperatures. Further evidence was derived from chromatographic analyses. TLC experiments revealed that mangotoxin, tabtoxin and phaseolotoxin show similar behaviour on silica plates. Finally, mangotoxin purification was partially achieved by HPLC. Mangotoxin activity was associated with a single active peak eluted from filtrates of the wild type mangotoxin-producing *P syringae* pv. *syringae* UMAF0158, but it was not present in filtrates from the defective mutant *P. syringae* pv. *syringae* UMAF0158-3αE10. Unfortunately, the purified fraction was very unstable, remaining active only for a few hours. Additional experiments will be necessary to establish the chemical structure of mangotoxin and to learn more about its mechanism of action.

In general, antimetabolite toxins act on amino acid biosynthesis, which results in the induction of amino acid deficiencies in plant tissues and the concomitant accumulation of nitrogen-containing intermediates, that can be metabolized by bacteria as nitrogen source, and their phytotoxic action is usually associated with chlorotic symptoms [6,33,38]. In our view, mangotoxin could have two major impacts on plant biology. First, as inhibitor of arginine biosynthesis, which may prevent protein synthesis by plant cells. Second, as inhibitor of ornithine biosynthesis, a precursor of the polyamines, affecting putrescine, spermidine and spermine biosynthesis, essential plant growth factors, especially in quickly dividing cells, involved in regulation of different plant processes such as flowering [42]. Unfortunately, we

have not yet direct evidence about a role of mangotoxin as a virulence determinant of *P. syringae* pv. *syringae* to cause symptoms on its host plants. Nevertheless, our group is currently obtaining results that suggest a role of mangotoxin production in virulence, showing a delay and reduction in the induced symptoms, when cells of mangotoxin-defective mutants were inoculated on tomato leaves, in comparison with those caused by the mangotoxin-producing wild type strain [1].

Furthermore, most antimetabolite toxins show antimicrobial activity [41], and they may contribute to bacterial competitive ability and epiphytic fitness [5]. The ability of *P. syringae* pv. *syringae* strains to produce different toxins with different chemical natures, as well as different modes of action, could provide these bacteria with selective advantages for competition with other microorganisms in the phyllosphere to colonize and cause symptoms in their host plants.

Work is in progress in our laboratory to elucidate the genetic bases of mangotoxin biosynthesis by *P. syringae* pv. *syringae*, and its potential role in the epiphytic and pathogenic stages of bacteria in the host plant.

Acknowledgements

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