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Studies on structure–activity relationship of sphaeropsidins A–F, phytotoxins produced by *Sphaeropsis sapinea* f. sp. *cupressi*

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Abstract

Six forms of sphaeropsidins (SA–SF), three- and tetra-cyclic unrearranged pimarane diterpenes produced by *Sphaeropsis sapinea* f. sp. *cupressi*, as well as eight derivatives obtained by chemical modification of SA–SC, were assayed for their bioactivity. The effect of each compound on plants which are host or non-host of the pathogen was investigated. Activity on some plant pathogenic fungi was also tested. Some structure–activity relationships have been identified for both phytotoxic and antifungal activity. It appears that the integrity of the tricyclic pimarane system, the preservation of the double bond C(8)–C(14), the tertiary hydroxyl group at C-9, the vinyl group at C-13, and the carboxylic group at C-10 as well as the integrity of the A-ring provide these molecules with non selective phytotoxic and antimycotic activity.

Keywords: Cypress; Cupressaceae; *Sphaeropsis sapinea* f. sp. *cupressi*; Cypress canker disease; Pimarane diterpene; Sphaeropsidin derivatives; Structure–activity relationship

1. Introduction

Sphaeropsidins A–F (SA–SF, **1**–**6**), three- and tetra-cyclic unrearranged pimarane diterpenes are secondary metabolites produced in culture by two anamorphic fungi, *S. sapinea* f. sp. *cupressi* [syn: *Diplodia pinea* (Desm.) Kickx, Petrak et Sydow f. sp. *cupressi*] and *D. mutila* (Fr.) Mont. (Evidente et al., 1996, 1997, 2002, 2003). *S. sapinea* f. sp. *cupressi* (Solel et al., 1987; Frisullo et al., 1997), the causal agent of a canker disease of cypress in the Mediterranean area, is known to produce all sphaeropsidins so far isolated. In addition, the same fungus produces two phytotoxic dimedone methyl ethers: sphaeropsidone and episphaeropsidone (Evidente et al., 1998) and two related non-toxic metabolites: chlorosphaeropsidone and epichlorosphaeropsidone (Evidente et al., 2000). *D. mutila*, the fungus most frequently isolated from the branches and twigs of declining oaks (Kowalski, 1991) produces SA (**1**) and SC (**3**) (Evidente et al., 1997). Sphaeropsidins A–C (**1**–**3**) show an interesting activity when tested against several plant pathogenic fungi, including *Seiridium cardinale* and *S. cupressi* affecting cypress (Evidente et al., 1996, 1997).

The nature and appearance of symptoms caused by *S. sapinea* f. sp. *cupressi* to its host plants suggest that phytotoxins are produced in the cankered tissues and are diffused or translocated to the adjacent and even distal parts of the infected tree.

In order to get information on the structure–activity relationship of sphaeropsidins, eight derivatives (**7**–**14**) were prepared by chemical transformation of the functionalities present in the SA–SC (**1**–**3**). The aim of present work was to identify which structural features are essential for the biological activities of these compounds, in order to better understand their mechanism of action on plants, role in pathogenesis and potential antimycotic activity.

2. Results and discussion

In this study, the phytotoxic and antifungal activity of eight sphaeropsidin derivatives was evaluated in comparison to the sphaeropsidins A (**1**), B (**2**), C (**3**), D (**4**),

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Nomenclature

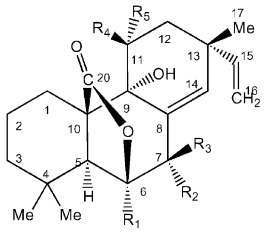
sphaeropsidin A (**1**)	6α,6β,9α-trihydroxy-7-oxopimara-8(14),15-dien-20-oic acid 6β,20-lactone
sphaeropsidin B (**2**)	6α,6β,9α,7β-tetrahydroxypimara-8(14),15-dien-20-oic acid 6β,20-lactone
sphaeropsidin C (**3**)	9α-hydroxy-7-oxopimara-8(14),15-dien-20-oic acid
sphaeropsidin D (**4**)	6α,6β,9α,11α-tetrahydroxy-7-oxopimara-8(14),15-dien-20-oic acid 6β,20-lactone
sphaeropsidin E (**5**)	7α,11β,14α-trihydroxypimara-8(9),15-diene
sphaeoropsidin F (**6**)	1β,6α,7α,9α-tetrahydroxypimara-8(14),15-diene

E (**5**) and F (**6**) (Table 1). Eight key derivatives were obtained by chemical transformations of **1**, **2** and **3**. The structural features of **1** and those of the other naturally-occurring sphaeropsidins **2**, **3**, **4**, **5** and **6** provide evidence for considering the latter as naturally modified analogues of **1**. In fact, SB (**2**) differs from **1** in the presence of a secondary hydroxyl group at C-7 instead of a ketone group, whereas SC (**3**) differs in the presence of a carboxylic and a methylene group at C-6 and the absence of an hemiketal lactone ring at C-10. Several structural modifications were observed when SD (**4**) and SE (**5**) were compared with **1**. SD (**4**) showed the hydroxylation of C-11 as the only modification already observed in **5** too. In fact, **5** showed modifications at the C-ring as the hydroxylation of both C-11 and C-14, the dehydroxylation of C-9 and the shift of the double bond from C(8)-C(14) to C(8)-C(9). Furthermore, **5** lacked the hemiketal lactone ring, while a methylene and a methyl group were present at C-6 and C-10, respectively, as well as a secondary hydroxyl group at C-7, some of which are structural modifications already observed in **2** and in **3**. Sphaeropsidin F (**6**) is in part structurally related to SC (**3**) and SE (**5**), but differed from the other sphaeropsidins due to the presence of the hydroxyl group at C-1 of the A-ring. Inspection of ^{1}H and ^{13}C NMR spectra revealed the absence of the hemiketal lactone system and, as found in **5**, the presence of an aliphatic methyl group. The presence of an olefinic group between C-8 and C-14 together with that of the tertiary hydroxyl group at C-9 further differentiated **6** from **5** (Evidente et al., 2003).

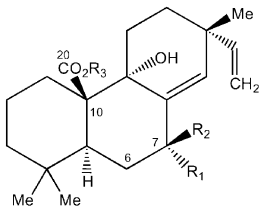
By acetylation, SA (**1**) was converted into the corresponding 6-*O*-acetylderivative (**7**), which showed the modification of the hemiketal hydroxyl group at C-6 of the B-ring. 7-*O*-Dihydrosphaeropsidin C (**8**) was obtained by $NaBH_4$ reduction of **3** (Evidente et al., 1997). The methyl ester of sphaeropsidin C (**9**) was obtained by conversion with diazomethane of **3** into its methyl ester. 6-*O*-Acetyl-14-*O*-acetyloxy-9-dehydroxy-$\Delta^{8,9}$-derivative (**10**) was obtained by reaction of SA (**1**) with the Fritz and Schenk reagent (Fritz and Schenk, 1959). This compound showed a modification of the hemiketal hydroxyl group at C-6 of the B-ring, together with the dehydroxylation of C-9 of the C-ring with the consequent shift of the double bond from C(8)-C(14) to C(8)-C(9) and the acetoxylation of the C-14. Furthermore, the catalytic hydrogenation of **1** generated the 7-*O*-15,16-tetrahydroderivative (**11**), which showed the saturation of the vinyl group at C-13 and the expected reduction of the carbonyl group at C-7 into a secondary hydroxyl group. By reaction with diazomethane, **1** was converted in the methyl ester (**12**), which showed the opening of the hemiketal lactone producing the carbonyl group at C-6 and the carboxylic group at C-10 with the latter converted into the corresponding methyl ester. Compound **12** also showed, in agreement with literature data (Ellestad et al., 1972) the formation of a cyclopropane ring between C-8 and C-14, due to the addition of the reactive methylene to the double bond previously located there. A cyclopropane ring formation was also observed in the derivative **13**, obtained from sphaeropsidin C (**3**) by reaction with diazomethane (Evidente et al., 1997). This derivative also showed the methyl esterification of the carboxylic group present in SC (**3**) at C-10 as already noted in **11**. As far as sphaeropsidin C (**3**) is concerned, the two derivatives **9** and **13** lacked the hemiketal lactone ring and displayed the presence of a methylene and a carboxylic group at C-6 and C-10 (which was converted into the corresponding methyl ester), respectively. Sphaeropsidin B (**2**), in turn, was converted by sodium periodate oxidation into the derivative **14**, which showed marked modification of the B-ring, while the other two rings (A and C) appeared practically unaltered. In particular, the derivative **14** exhibited the opening of hemiketal lactone producing a carboxylic group at C-10, and the cleavage of the C(6)–C(7) bond. The C-6 was oxidized into a carboxylic group, which formed a γ-lactone with the hydroxyl group at C-9, while the carbonyl group at C-7 appeared as a formyl group conjugated with the C(8)–C(14) double bond. These noteworthy structural modifications resulted in substantial disruption of the tricyclic pimarane system.

The structures of all derivatives (**7**–**14**) obtained by chemical modifications of sphaeropsidins A–C (**1**–**3**) were determined by extensive use of spectroscopic methods. Their spectral data as ^{1}H and ^{13}C NMR (Tables 2 and 3) and EI and FAB MS values, compared with those of sphaeropsidins A–C (**1**–**3**), were all consistent with the structures assigned.

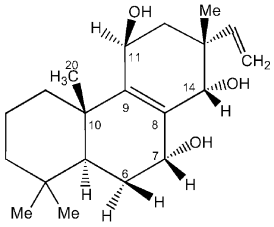
Table 1
Structure of the sphaeropsidins (**1**–**6**) and their derivatives (**7**–**14**) used in this study



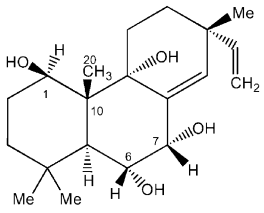
1 R_1=OH, R_2+R_3=O, R_4=R_5=H
2 R_1=OH, R_2=R_4=R_5=H, R_3=OH
4 R_1=OH, R_2+R_3=O, R_4=H, R_5=OH
7 R_1=OAc, R_2+R_3=O, R_4=R_5=H



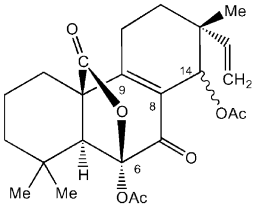
3 R_1+R_2=O, R_3=H
8 R_1=R_3=H, R_2=OH
9 R_1+R_2=O, R_3=Me



5



6



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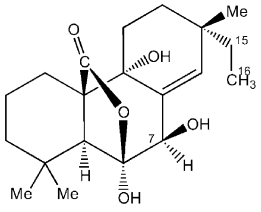
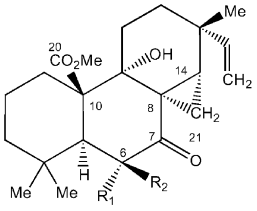
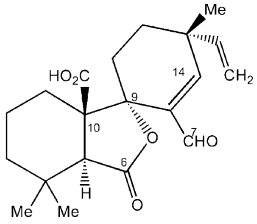


11



12 R_1+R_2=O
13 R_1=R_2=H



14

Compound	Substituents								
	C-1	C-6	C-7	C(8)–C(14)	C-9	C(6)–C(10)	C-11	C-13	C-14
1	H	OH	C=O	Double bond	OH	Hemiketal lactone	H	$CH=CH_2$	H
2	H	OH	OH	Double bond	OH	Hemiketal lactone	H	$CH=CH_2$	H
3	H	H	C=O	Double bond	OH	COOH at C-10	H	$CH=CH_2$	H
4	H	OH	C=O	Double bond	OH	Hemiketal lactone	OH	$CH=CH_2$	H
5	H	H	OH	Single bond	Double bond C(8)–C(9)	CH_3 at C-10	OH	$CH=CH_2$	OH
6	OH	OH	OH	Double bond	OH	CH_3 at C-10	H	$CH=CH_2$	H
7	H	OAc	C=O	Double bond	OH	Hemiketal lactone	H	$CH=CH_2$	H
8	H	H	OH	Double bond	OH	COOH at C-10	H	$CH=CH_2$	H
9	H	H	C=O	Double bond	OH	$COOCH_3$ at C-10	H	$CH=CH_2$	H
10	H	OAc	C=O	Single bond	Double bond C(8)–C(9)	Hemiketal lactone	H	$CH=CH_2$	OAc
11	H	OH	OH	Double bond	OH	Hemiketal lactone	H	CH_2-CH_3	H
12	H	C=O	C=O	Cyclopropane ring	OH	$COOCH_3$ at C-10	H	$CH=CH_2$	H
13	H	H	H	Cyclopropane ring	OH	$COOCH_3$ at C-10	H	$CH=CH_2$	H
14	H	γ-lactone C(6)-C(9)	CHO	Double bond	γ-lactone C(6)–C(9)	COOH at C-10	H	$CH=CH_2$	H

The stereochemistry of new stereogenic carbons of derivatives **8**, **11**, **12**, **13** and **14** was deduced from the NMR spectral data ($^3J_{H,H}$) on the basis of mechanistic grounds, and by comparison of their spectral data (IR, UV and ^{1}H NMR) with those already reported for sphaeropsidin A (**1**) and B (**2**) (Evidente et al., 1996, 1997) and partially reported for some derivatives (Ellestad et al., 1972). In particular, H-7 in **8** was located axial and its geminal hydroxyl group was located equatorial in agreement with the constants measured for the coupling between H-6 and H-6′ ($J_{6,7}$ = 10.6 and $J_{6',7}$ = 6.8 Hz). This stereochemistry was in agreement with that assigned to the same carbon (C-7) of **2**, which was also assigned on the basis of mechanistic grounds and NMR spectral data of both H-7 and H-14, when **2** was obtained by stereoselective reduction from SA (**1**). The same stereochemistry was assigned to C-7 of the tetrahydroderivative of sphaeropsidin A (**11**) by comparison of its spectral ^{1}H NMR data with those of both **2** and **8**. An α-configuration was attributed to the cyclopropane ring located between C-8 and C-14 of derivatives **12** and **13** by comparison of their spectral

Table 2
^{1}H NMR data of sphaeropsidin derivatives (**7**, **9**–**12** and **14**). The chemical shifts are in δ-values (ppm) from TMS

H	7[a]	9	10	11	12	14
1	2.18 *br d* (12.3)	2.30 *br d* (12.8)	2.42 *br d* (13.6)	2.12 *br d* (12.6)	2.35 *br d* (12.6)	2.36 *br d* (11.5)
1′	1.58 *m*	1.60 *m*	1.58 *m*	1.54 *m*	1.60 *m*	1.50 *m*
2	1.58 *m*	1.75 *m*	1.58 *m*	1.54 *m*	1.60 *m*	1.73 *m*
2′	1.58 *m*	1.60 *m*	1.58 *m*	1.54 *m*	1.60 *m*	1.73 *m*
3	1.86 *m*	1.88 *dt* (13.4, 3.5)	1.65 *m*	1.54 *m*	1.60 *m*	1.73 *m*
3′	1.82 *m*	1.60 *m*	1.65 *m*	1.54 *m*	1.60 *m*	1.73 *m*
5	2.85 *s*	2.40 *dd* (12.7, 6.6)	2.77 *s*	2.56 *s*	3.15 *s*	2.47 *s*
6		2.70 *dd* (18.6, 12.7)				
6′		2.53 *dd* (18.6, 6.6)				
7				4.25 *br s*		9.59 *s*[b]
11	1.33 *br d* (13.3)	1.60 *m*	2.68 *dd* (20.7, 3.8)	1.28 *br d* (12.0)	1.33 *m*	1.50 *m*
11′	1.22 *m*	1.30 *m*	2.33 *m*	1.18 *m*	1.25 *m*	1.20 *m*
12	1.84 *m*	1.75 *m*	1.90 *m*	1.92 *ddd* (14.3, 14.3, 3.8)	1.78 *ddd* (14.0, 14.0, 3.8)	2.25 *br d* (11.0)
12′	1.64 *m*	1.60 *m*	1.65 *m*	1.62 *ddd* (14.3, 3.8, 3.8)	1.65 *ddd* (14.3, 3.8, 3.8)	1.85 *m*
14	6.82 *br s*	6.69 *d* (1.6)	5.58 *s*	5.80 *br s*	2.11 *dd* (8.8, 8.8)	6.69 *s*[b]
15	5.78 *dd* (17.5, 10.6)	5.82 *dd* (18.2, 10.7)	5.83 *dd* (17.6, 10.9)	1.36 *q* (7.5) (2H)	5.81 *dd* (17.4, 10.7)	5.68 *dd* (16.5, 10.4)
16	5.04 *dd* (17.5, 1.5)	5.03 *dd* (18.2, 1.5)	5.00 *dd* (17.6, 1.5)	0.85 *t* (7.5) (3H)	5.00 *dd* (17.4, 1.5)	5.05 *dd* (10.4, 1.5)
16′	5.04 *dd* (10.6, 1.5)	5.00 *dd* (10.7, 1.5)	5.00 *dd* (10.9, 1.5)	–	5.00 *dd* (10.7, 1.5)	4.80 *dd* (16.5, 1.5)
17	1.09 *s*	0.98 *s*	0.87 *s*	0.86 *s*	0.86 *s*	0.98 *s*
18[c]	1.16 *s*	0.88 *s*	1.14 *s*	1.25 *s*	1.31 *s*	1.30 *s*
19[c]	1.09 *s*	0.70 *s*	1.11 *s*	1.13 *s*	0.97 *s*	1.19 *s*
21					1.15 *m* 1.05 *m*	
MeCO	2.24 *s*		2.25 *s*			
MeCO			1.96 *s*			
MeOCO		3.58 *s*			3.69 *s*	

[a] The signal of the HO-9 appeared as a broad singlet at δ 2.50 in agreement with the only chemical shift value (δ 2.15) reported in literature (Ellestad et al., 1972) for this derivative.
[b] In agreement with the only two chemical shifts values (δ 9.62 and 6.77) reported in literature (Ellestad et al., 1972) for this derivative.
[c] These assignments may be reversed.

data with those (IR and ^{1}H NMR) reported by the same authors for an analogous derivative prepared from **1**. Finally, an α-configuration was also assigned to the C-9 of the γ-lactone ring of **14** on the basis of mechanistic grounds and because of the agreement of its spectral data (UV, IR, ^{1}H NMR). On the contrary, the stereochemistry of C-14 remains undetermined in **10** since H-14 appeared as a singlet in its ^{1}H NMR spectrum. In fact, this proton (H-14) lacks vicinal coupling since C-14 is bonded to the two quaternary carbons C-13 and C-8, and C-8 is linked to two other quaternary carbons (C-7 and C-9).

Biological results were obtained in terms of phytotoxicity of SA–SF (**1**–**6**) and their derivatives (**7**–**14**) (Table 4) against three species of woody plants (cypress) and two herbaceous plants (tomato and mung bean). SA (**1**), SB (**2**) and SC (**3**) absorbed by severed cuttings of plants showed the greatest phytotoxic activity both on host and non-host plants. SD (**4**) was toxic only to *Cupressus macrocarpa*. SE (**5**) did not affect any cypress species. SF (**6**) was moderately toxic only to *C. sempervirens*. SA (**1**), SB (**2**), SC (**3**) and SD (**4**) also affected the herbaceous plants tested: necrotic spots on leaves and tissue browning on stem appeared on *Lycopersicon esculentum* and *Phaseolus vulgaris*. SE (**5**) and SF (**6**) did not affect any herbaceous plant tested. Compound **7** exhibited a phytotoxic activity similar to that given by **1**. Derivative **9** affected only *C. macrocarpa* and *C. arizonica* and was ineffective on the two herbaceous plants. Derivatives **8**, **10**, **11**, **12**, **13** and **14** were not toxic. SA–SF (**1**–**6**) proved to be non-selective toxins, able to cause symptoms both on host plants and non-host plants. These findings also proved that the three cypress species had a different tolerance to the toxins.

The degree of phytotoxicity elicited by each compound permitted us to establish some structure–activity relationships as well as to identify the structural features responsible for the biological activity. The reduction of the carbonyl group at C-7 to a secondary hydroxyl group (**2**) and the semi-reductive opening of the hemiketal lactone ring (**3**), did not reduce the bioactivity of these sphaeropsidins. The hydroxylation of C-11 (**4**) led to partial loss of activity. For SE (**5**), the dehydroxylation of C-9, which induces the shift of the double bond

Table 3
^{13}C NMR data of sphaeropsidin derivatives (**7**, **9**–**12** and **14**). The chemical shifts are in δ-values (ppm) from TMS[a]

C	7	9	10	11	12	14
1	22.5 *t*	27.2 *t*	23.8 *t*	22.7 *t*	24.8 *t*	27.2 *t*
2	17.7 *t*	19.5 *t*	17.9 *t*	18.2 *t*	18.3 *t*	20.7 *t*
3	26.5 *t*	27.9 *t*	25.7 *t*	28.9 *t*	26.2 *t*	30.5 *t*
4	32.5 *s*	33.6 *s*	33.1 *s*	31.7 *s*	33.5 *s*	32.7 *s*
5	53.2 *d*	42.7 *d*	62.4 *d*	51.5 *d*	56.7 *d*	52.1 *d*
6	105.2 *s*	37.3 *t*	105.6 *s*	103.8 *s*	191.1 *s*	172.4 *s*
7	189.0 *s*	200.3 *s*	184.0 *s*	73.9 *d*	189.0 *s*	192.2 *d*
8	135.6 *s*	136.7 *s*	128.7 *s*	134.3 *s*	34.2 *s*	135.3 *s*
9	71.5 *s*	72.1 *s*	142.3 *s*	71.4 *s*	72.3 *s*	84.0 *s*
10	56.0 *s*	52.2 *s*	53.7 *s*	58.1 *s*	57.9 *s*	58.1 *s*
11	40.4 *t*	41.3 *t*	40.4 *t*	40.4 *t*	42.1 *t*	41.4 *t*
12	29.5 *t*	29.7 *t*	25.8 *t*	29.5 *t*	29.4 *t*	32.7 *t*
13	38.7 *s*	38.3 *s*	38.3 *s*	34.6 *s*	38.9 *s*	38.4 *s*
14	148.5 *d*	144.5 *d*	67.7 *d*	135.9 *d*	37.8 *d*	150.9 *d*
15	144.6 *d*	145.6 *d*	142.9 *d*	35.4 *t*	147.1 *d*	142.5 *d*
16	112.9 *t*	112.3 *t*	113.2 *t*	7.9 *q*	111.1 *t*	114.5 *t*
17	24.2 *q*	23.7 *q*	22.0 *q*	21.0 *q*	24.4 *q*	27.6 *q*
18[b]	32.6 *q*	31.1 *q*	32.6 *q*	31.5 *q*	32.2 *q*	31.6 *q*
19[b]	22.2 *q*	19.1 *q*	21.1 *q*	22.6 *q*	19.8 *q*	21.5 *q*
20	174.0 *s*	174.7 *s*	171.8 *s*	176.9 *s*	175.3 *s*	174.4 *s*
21					27.3 *t*	
MeCO	166.8 *s*		166.9 *s*			
MeCO			162.7 *s*			
MeCO	21.1 *q*		21.2 *q*			
MeCO			21.0 *q*			
MeOCO		51.2 *q*			52.8 *q*	

[a] The multiplicities were determined by DEPT spectra.
[b] These assignments may be reversed.

Table 4
Symptoms caused by sphaeropsidins A–F (**1**–**6**) and their derivatives (**7**–**14**) on test plants[a]

Compound	Host species			Non-host species	
	Cupressus macrocarpa	*Cupressus sempervirens*	*Cupressus arizonica*	*Lycopersicon esculentum*	*Phaseolus vulgaris*
1	necr	brown	necr	necr	necr
2	necr	necr	yel	necr	necr
3	necr	necr	yel	necr	necr
4	necr	n.s.	n.s.	necr	brown
5	n.s.	n.s.	n.s.	n.s.	n.s.
6	n.s.	yel	n.s.	n.s.	n.s.
7	necr	necr	brown	necr	necr
8	n.s.	n.s.	n.s.	n.s.	n.s.
9	yel	n.s.	yel	n.s.	n.s.
10	n.s.	n.s.	n.s.	n.s.	n.s.
11	n.s.	n.s.	n.s.	n.s.	n.s.
12	n.s.	n.s.	n.s.	n.s.	n.s.
13	n.s.	n.s.	n.s.	n.s.	n.s.
14	n.s.	n.s.	n.s.	n.s.	n.s.
Control (water)	n.s.	n.s.	n.s.	n.s.	n.s.

[a] Severed shoots of three cypress species and cuttings of herbaceous test plants were left to take up a 3 ml assay solution (100 μg ml^{-1}) for 96 and 48 h, respectively. Symptoms developed within 2, 4 and 21 days on tomato, mung bean and cypress, respectively: brown = tissue browning; necr = leaf necrosis; yel = yellowing of the whole cutting; n.s. = no symptoms.

from C(8)–C(14) to C(8)–C(9) and the hydroxylation of C-14 of the C-ring, caused the loss of bioactivity. As already demonstrated for the B- and C-rings, the modification of the A-ring appeared in **6**, changed the bioactivity of the molecule. The acetylation of the hemiketal group at C-6 of the B-ring made for **7** preserved its bioactivity. The reduction of the carbonyl group at C-7 into a secondary hydroxyl group (**8**) caused a complete loss of activity. This may be due to a synergistic effect with the other structural modifications already present

Table 5
Sensitivity to sphaeropsidins A–F (**1**–**6**) and their derivatives (**7**–**14**) assayed at 100 μg ml^{-1} on eight plant pathogenic fungi grown on PDA medium at 25 °C, in the dark[a]

Compd	Fungal species								L.S.D. ($P=0.05$)
	Botrytis cinerea	*Fusarium oxysporum*	*Penicillium expansum*	*Verticillium dahliae*	*Phomopsis amygdali*	*Seiridium cardinale*	*Seiridium cupressi*	*Seiridium unicorne*	
1	56.7 (48.85)	28.2 (32.08)	41.2 (39.93)	28.7 (32.39)	77.0 (61.34)	54.1 (47.35)	59.9 (50.71)	52.4 (46.38)	(9.7)
2	62.4 (52.18)	30.2 (33.34)	38.5 (38.35)	30.2 (33.34)	63.5 (52.8)	41.3 (39.99)	38.3 (38.23)	40.8 (39.70)	(11.2)
3	38.4 (38.29)	27.8 (31.82)	34.2 (35.85)	26.8 (31.18)	41.7 (40.2)	39.4 (38.88)	42.4 (40.63)	38.7 (38.47)	(8.6)
4	21.4 (27.56)	18.7 (25.62)	15.2 (22.95)	12.5 (20.70)	29.7 (33.02)	3.7 (11.09)	n.i.	56.8 (48.91)	(12.1)
5	n.i.	n.i.	n.i.	n.i.	12.5 (20.70)	7.4 (15.79)	5.0 (12.92)	18.9 (25.77)	(7.5)
6	n.i.	n.i.	n.i.	n.i.	8.2 (16.64)	5.4 (13.44)	7.2 (15.56)	8.4 (16.85)	(6.4)
7	12.5 (20.70)	20.0 (26.56)	n.i.	24.6 (29.73)	14.7 (22.55)	66.7 (54.70)	50.0 (45.00)	13.5 (21.56)	(10.5)
8	15.5 (23.19)	10.0 (18.44)	n.i.	26.1 (30.72)	17.6 (24.80)	29.6 (32.96)	25.0 (30.00)	45.9 (42.65)	(9.2)
9	10.5 (18.91)	5.0 (12.92)	n.i.	17.4 (24.65)	21.6 (27.69)	7.4 (15.79)	n.i.	24.3 (29.53)	(8.2)
10	12.5 (20.70)	3.3 (10.47)	n.i.	17.4 (24.65)	n.i.	14.8 (22.63)	20.0 (26.56)	22.5 (28.32)	(7.2)
11	7.5 (15.89)	3.5 (10.78)	n.i.	34.8 (38.53)	9.8 (18.24)	11.1 (19.46)	30.0 (33.21)	8.1 (16.54)	(7.0)
12	10.5 (18.91)	6.7 (15.00)	n.i.	24.6 (29.73)	14.7 (22.55)	3.7 (11.09)	35.0 (36.27)	29.7 (33.02)	(9.8)
13	4.5 (12.25)	8.3 (16.74)	n.i.	15.9 (23.50)	15.7 (23.34)	n.i.	n.i	21.6 (27.69)	(6.7)
14	8.3 (16.74)	13.3 (21.39)	n.i.	24.6 (29.73)	18.6 (25.55)	22.2 (28.11)	30.0 (33.21)	0.9 (5.44)	(8.7)

[a] The antifungal effect of toxins and their derivatives was evaluated by calculating the percentage of linear growth inhibition as 100 $(y-x)/y$ where y = mean colony diameter of toxin-free cultures and x = mean colony diameter of toxin-containing cultures, 1–2 weeks after inoculation. Experiments were repeated twice with five plates per species per toxic solution. The figures are the means of ten replicates. Angular transformations of percentage data are shown in parentheses. L.S.D. = least significative difference; n.i.: no growth inhibition.

in this latter derivative in respect to **1**. The dehydroxylation of C-9 of the C-ring, and the acetoxylation of C-14 together with concomitant acetylation of the hemiketal group at C-6 of the B-ring, caused the complete loss of activity for **10**. Furthermore, a complete loss of activity was observed for the saturation of the vinyl group at C-13 (**11**) or for the conversion of the C(8)-C(14) double bond into the corresponding cyclopropane ring observed in **12**, together with the opening of the hemiketal lactone ring and the consequent methyl esterification of the carboxylic group at C-10. The same structural modification was probably responsible for the lacking of toxicity of compound **13**. Finally, it is interesting to note that modification in the B-ring converted in a γ-lactone resulting in a substantial disruption of the tricyclic pimarane system (derivative **14**) strongly reduced activity, suggesting that the perhydrophenanthrene arrangement of the carbon skeleton is essential for toxicity.

The antimycotic activity of sphaeropsidins A–F (**1**–**6**) and their derivatives (**7**–**14**) was assayed on eight fungal species (Table 5). *Phomopsis amygdali* proved to be more sensitive to **1** than any other fungi tested, whereas *Fusarium oxysporum* and *Verticillium dahliae* appeared to be less sensitive to the toxins. The most sensitive fungi to **2** were *P. amygdali* and *Botrytis cinerea*. SC (**3**) appeared to be less active than **2**. SD (**4**) exhibited the highest level of antifungal activity against *S. unicorne* and a moderate or low level of fungistatic activity against the other seven fungi. SE (**5**) and SF (**6**) were ineffective or had a moderate effect on fungi tested.

The antimycotic activity of all derivatives referred to all tested fungi was lower than that shown by SA–SD (**1**–**4**). *Penicillium expansum* was not affected by any sphaeropsidin derivative. Compound **7** partly preserved its antimycotic activity; in addition, **7** was more active against *S. cardinale*. Derivative **8** caused a remarkable reduction of growth inhibition of seven fungi, with the exception of *S. unicorne* which appeared more sensitive to this compound. Compounds **9** and **10** greatly reduced antifungal activity and, in some cases they led to a complete loss of activity. Furthermore, derivative **11** caused a reduction of the activity except for *V. dahliae* whose sensitivity was greater than that shown by SA–SD (**1**–**4**) on the same fungus. Derivative **12** elicited low values of growth inhibition with the exception against *V. dahliae*, *S. cupressi* and *S. unicorne*. The same chemical modification present in **12** led in the compound **13** either to partial or even to complete loss of activity. Finally, it is interesting to note that modification in the B-ring converted in a γ-lactone resulting in a substantial disruption of the tricyclic pimarane system greatly reduced the activity of derivative **14**.

The antimycotic activity of sphaeropsidins A–F (**1**–**6**) may help the saprophytic survival of sphaeropsidin-producing fungi in their natural habitat, or when they live as parasites within plant tissues. If SA–SF were really produced *in planta* by the fungus, we could postulate that infective growth of *S. sapinea* f. sp. *cupressi* along the

stem or branches of cypress may prevent the concomitant invasion of the bark by *S. cardinale, S. cupressi* and *S. unicorne* which are well known pathogens of cypress. Actually, the fungistatic activity of **1**–**6** against the species of *Seiridium* infecting cypress can support a possible antagonistic action of *S. sapinea* f. sp. *cupressi*. This result could be applied to prevent early infections of *Seiridium* canker disease of cypress.

On the basis of the results of this study, it can be inferred that the toxicity of **1**, **2**, **3** and **4** was associated with the presence of the double bond between C-8 and C-14 and probably also with that of the tertiary hydroxyl group at C-9. The sphaeropsidins **2**, **3** and **4**, structurally related to **1**, retained these features, while SE (**5**), which lacks these structural features, did not. Moreover, **5** also differed from the other sphaeropsidins in the reduction to a methyl group (Me-20) of the carboxylic group at C-10. Furthermore, the bioactivity of SF (**6**) confirmed these results and also showed an important role of A-ring. SF (**6**), in part structurally related to SC (**3**) and SE (**5**), retained structural features important for biological activity, but differed from the other sphaerospidins due to the presence of the hydroxyl group at C-1 of the A-ring, whose modification changed the bioactivitry of the molecule.

Derivatization of sphaeropsidins produced compounds lacking phytotoxic activity and showing reduced antifungal activity. Modifications in the vinyl group at C-13 or in the tricyclic pimarane system, particularly in the C-ring, also reduced bioactivity, suggesting that both C-ring functionalities and spatial conformation are essential for activity.

Concerning the phytotoxic and antifungal behaviour of SA–SE (**1**–**6**) and their derivatives (**7**–**14**), it can be speculated that the integrity of the tricyclic pimarane system, the preservation of the double bond from C(8) to C(14), the tertiary hydroxyl group at C-9, the vinyl group at C-13, the carboxylic group at C-10 and the integrity of the A-ring endow these molecules with toxicity to host and non-host plants and activity against several plant pathogenic fungi.

When begin to study a phytotoxin we are primarily interested in determining what its role is in plant disease production. Toxin production by plant pathogenic fungi has a critical role in understanding the ecology and epidemiology of some plants diseases. However, once the structure and how it acts have been elucidated or even before, we frequently find that the toxin has utility in other areas. Toxins of plant pathogenic fungi, their derivatives or related compounds are eliciting widespread interest as agrochemicals. Certain ophiobolins, a class of sesterterpenes produced by species of *Cochliobolus* and other fungi, show interesting antifungal activity and can be used as lead molecules to develop new fungicides (Kim et al., 1999). All these considerations have lead our investigation by choosing to assay phytotoxic and antimycotic activities of sphaeropsidins and their derivatives.

3. Experimental

3.1. General

The optical rotation were measured in $CHCl_3$ soln., (unless otherwise noted) on Jasco P-1010 polarimeter. IR and UV spectra were determined as neat and in MeCN soln. (unless otherwise noted), respectively, on a Perkin-Elmer IR FT-1720X spectrometer and a Perkin-Elmer Lamda 3B spectrophotometer; ^1H and ^{13}C NMR spectra were recorded at 500, 300 or 250 MHz and at 75 or 62.5 MHz, respectively, in $CDCl_3$, on Bruker spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra (Breitmaier and Woelter, 1987) using Bruker microprograms. EI MS and HR EI MS were taken at 70 eV on a Fisons Trio-2000 and a Fisons ProSpec spectrometers, respectively; FAB MS were recorded in glycerol/thioglycerol using Cs as bombarding atoms on a VG ZAB 2SE spectrometer. Analytical and preparative TLC was performed on silica gel plates (Merck, Kieselgel 60 F_{254} 0.25 and 0.50 mm, respectively). The spots were visualized by exposure to UV radiation and/or by spraying with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in MeOH, followed by heating at 110 °C for 10 min. Column chromatography: silica gel (Merck, Kieselgel 60, 0.063–0.20 mm). Solvent systems: (A) $CHCl_3$–*iso*-PrOH (19:1), (B) $CHCl_3$–*iso*-PrOH (99:1), (C) $CHCl_3$–*iso*-PrOH (85:15), (D) petrol–Me_2CO (3:1).

3.2. Production, extraction and purification of sphaeropsidins A–F (1–6)

Sphaeropsidins A–F (**1**–**6**) were purified from the culture filtrates of *S. sapinea* f. sp. *cupressi*. The strain used, its cultural characteristics as well as the purification procedures of phytotoxins have already been described in detail (Evidente et al., 1996, 1997, 2002, 2003).

3.3. 6-O-Acetylsphaeropsidin A (7)

SA (**1**) (100 mg) was acetylated with dry pyridine (2 ml) and Ac_2O (2 ml) in presence of *p*-toluensulfonic acid as catalyst and at room temp. overnight. The reaction was stopped with MeOH and the resulting mixture was dried by evaporation under N_2 stream of the azeotrope formed adding C_6H_6. The crude residue was purified by prep. TLC (eluent A) to give 90 mg of the 6-*O*-acetylsphaeropsidin A (**7**): $[\alpha]_D^{25}$ +128.5° (*c* 0.3); UV (MeOH) λ_{max} (log ϵ): 240 (sh), 220 (4.81), 210 (4.88) nm; IR ν_{max} cm^{-1} 3479, 1790, 1729, 1633, 1287, 1257 [Ellestad

et al., 1972: IR (KBr) ν_{max} cm^{-1} 1785]; ^1H and ^{13}C NMR, see Tables 2 and 3, respectively; HR EIMS *m/z* (rel. int.): 388.1916 ($C_{22}H_{28}O_6$, calc. 388.1886, 1) $[M]^+$, 387 $[M-H]^+$ (1), 370 $[M-H_2O]^+$ (2), 355 $[M-H_2O-Me]^+$ (2), 346 $[M-CH_2CO]^+$ (12), 345 $[M-CH_3CO]^+$ (38), 328 $[M-AcOH]^+$ (47), 327 $[M-H-AcOH]^+$ (88), 310 $[M-H_2O-AcOH]^+$ (5), 300 $[M-AcOH-CO]^+$ (18), 299 $[M-H-AcOH-CO]^+$ (25), 281 $[M-H-AcOH-CO-H_2O]^+$ (19), 43 (100).

3.4. 7-O-Dihydrosphaeropsidin C (8)

Compound **8** was obtained by $NaBH_4$ reaction of sphaeropsidin C (**3**) as previousely reported (Evidente et al., 1997).

3.5. Methyl ester of sphaeropsidin C (9)

SC (**3**) (34 mg) dissolved in MeOH (10 ml) was converted into its methyl ester and the corresponding 8,14-methylene derivatives (**9** and **13**) by reaction with an ethereal soln. of diazomethane as previously described (Evidente et al., 1997). The residue obtained by the work-up reaction was purified by prep. TLC (eluent D) to give 20 and 13 mg, respectively, of the methyl ester of SC (**9**) and the derivative **13** cited below: $[\alpha]_D^{25}$ +18.7° (*c* 0.4); UV (MeOH) λ_{max} log (ϵ): 242 (3.78) nm; IR ν_{max} cm^{-1} 3422, 1723, 1678, 1612, 1201; ^1H and ^{13}C NMR, see Tables 2 and 3, respectively; HR EIMS *m/z* (rel. int.): 346.2146 ($C_{21}H_{30}O_4$, calc. 346.2118, 1) $[M]^+$, 328 $[M-H_2O]^+$ (3), 315 $[M-CH_3O]^+$ (2), 314 $[M-CH_3OH]^+$ (1), 300 $[M-H_2O-CO]^+$ (0.5), 297 $[M-H_2O-CH_3O]^+$ (0.7), 296 $[M-H_2O-CH_3OH]^+$ (0.7), 287 $[M-CH_3O-CO]^+$ (2), 286 $[M-CH_3OH-CO]^+$ (2), 281 $[M-H_2O-CH_3OH-Me]^+$ (2.5), 271 $[M-CH_3OH-CO-Me]^+$ (2), 269 $[M-CH_3O-CO-H_2O]^+$ (2.5), 178 (100); FABMS (+) *m/z* (rel. int.): 347 $[MH]^+$ (36), 329 $[MH-H_2O]^+$ (100).

3.6. 6-O-Acetyl-14-O-acetyloxy-9-dehydroxy-$\Delta^{8,9}$-sphaeropsidin A (10)

A soln. of SA (**1**) (200 mg) in EtOAc (7 ml) was treated with Fritz and Schenk reagent (7 ml) at 0 °C under stirring. After 2.5 h the soln. was poured into ice-water, its pH adjusted to 7 with a saturated soln. of $NaHCO_3$, and the mixture extracted with EtOAc (3×20 ml). The combined extracts were dried (Na_2SO_4) and evaporated under reduced pressure to give a residue which was purified by prep. TLC (eluent B) to give 95 mg of the 6-*O*-acetyl-14-*O*-acetyloxy-9-dehydroxy-$\Delta^{8,9}$-sphaeropsidin A (**10**): $[\alpha]_D^{25}$ +38.1° (*c* 0.4); UV λ_{max} (log ϵ): 260 (3.63), 225 (3.65) nm; IR ν_{max} cm^{-1} 1793, 1740, 1708, 1618, 1229; ^1H and ^{13}C NMR, see Tables 2 and 3, respectively; HR EIMS *m/z* (rel. int.): 430.9466 ($C_{24}H_{30}O_7$, calc. 430.9439, 0.6) $[M]^+$, 388 $[M-CH_2CO]^+$ (2), 370 $[M-AcOH]^+$ (2), 346 $[M-2\times CH_2CO]^+$ (3), 328 $[M-AcOH-CH_2CO]^+$ (9), 310 $[M-2\times AcOH]^+$ (10), 300 $[M-AcOH-CH_2CO-CO]^+$ (14), 284 $[M-AcOH-CH_2CO-CO_2]^+$ (24), 43 (100); FABMS (+) *m/z* (rel. int.): 431 $[MH]^+$ (50), 371 $[MH-AcOH]^+$ (100), 329 $[MH-AcOH-CH_2CO]^+$ (60).

3.7. 7-O-15,16-Tetrahydrosphaeropsidin A (11)

SA (**1**) (200 mg), dissolved in MeOH (6 ml), was added to a presaturated suspension of PtO_2 in MeOH (6 ml). Hydrogenation was carried out at room temp. and atmospheric pressure with continuous stirring. After 18 h, the reaction was stopped by filtration and the clear soln. evaporated under a N_2 stream. The residue was purified by prep. TLC (eluent A) to give 91 mg of the 7-*O*-15,16-tetrahydrosphaeropsidin A (**11**): $[\alpha]_D^{25}$ +67.5° (*c* 0.6, MeOH); UV λ_{max} <220 nm; 260 (3.63), 225 (3.65); IR ν_{max} cm^{-1} 3359, 1729, 1630, 1296; ^1H and ^{13}C NMR, see Tables 2 and 3, respectively; HR EIMS *m/z* (rel. int.): 350.2121 ($C_{20}H_{30}O_5$, calc. 350.2093, 3) $[M]^+$, 332 $[M-H_2O]^+$ (22), 317 $[M-H_2O-Me]^+$ (2), 314 $[M-2\times H_2O]^+$ (43), 299 $[M-2\times H_2O-Me]^+$ (19), 288 $[M-H_2O-CO_2]^+$ (68), 273 $[M-H_2O-Me-CO_2]^+$ (100), 255 $[M-2\times H_2O-Me-CO_2]^+$ (19); FAB MS (+) *m/z* (rel. int.): 351 $[MH]^+$ (95), 333 $[MH-H_2O]^+$ (100), 315 $[MH-2\times H_2O]^+$ (42).

3.8. 8,14-Methylensphaeropsidin A methyl ester (12)

An ethereal soln. of CH_2N_2 was added to a soln. of **1** (150 mg) in MeOH (15 ml) to obtain a persistent yellow colour. The reaction was carried out at room temp. under stirring and was stopped after 18 h by evaporation under a N_2 stream. The crude residue was purified by prep. TLC (eluent A) to give 105 mg of the 8,14-methylensphaeropsidin A methyl ester (**12**): $[\alpha]_D^{25}$ −15.2° (*c* 0.3); UV λ_{max} <220 nm; IR ν_{max} cm^{-1} 3446, 1727, 1696, 1654, 1636, 1233; ^1H and ^{13}C NMR, see Tables 2 and 3, respectively; HR EIMS *m/z* (rel. int.): 374.9571 ($C_{22}H_{30}O_5$, calc. 374.9541, 26) $[M]^+$, 356 $[M-H_2O]^+$ (22), 342 $[M-CH_3OH]^+$ (19), 330 $[M-CO_2]^+$ (1), 328 $[M-H_2O-CO]^+$ (6), 324 $[M-CH_3OH-H_2O]^+$ (14), 315 $[M-CO_2-Me]^+$ (22), 297 $[M-CO_2-Me-H_2O]^+$ (50), 169 (100).

3.9. 8,14-Methylenesphaeropsidin C methyl ester (13)

Compound **13** was obtained from SC (**3**) by esterification with CH_2N_2, as previously reported (Evidente et al., 1997).

3.10. Oxidized derivative of sphaeropsidin B (14)

SB (**2**) (150 mg), dissolved in MeOH (100 ml), was oxidized with an aqueous soln. (300 ml) of $NaIO_4$ (950 mg) under stirring at room temp. After 2 h, the reaction was stopped by adding cold Me_2CO (300 ml) and ethylene glycole (75 ml). The mixture was filtered, evaporated under reduced pressure and the corresponding aqueous

phase was extracted with CH_2Cl_2 (4×150 ml). The organic extracts were combined, dried (Na_2SO_4) and evaporated under vacuum to give a crude residue which was purified by prep. TLC (eluent C) to give 80 mg of the oxidized derivative of sphaeropsidin B (**14**): $[\alpha]_D^{25}$ −110.7° (*c* 0.2, MeOH); UV (MeOH) λ_{max} log (ϵ): 230 (3.88) nm; IR ν_{max} cm^{-1} 3455, 1785, 1722, 1697, 1615, 1236 [Ellestad et al., 1972: UV (MeOH) λ_{max} 233 (ϵ 8700); IR (KBr) ν_{max} cm^{-1} 1775, 1725 and 1685]; ^1H and ^{13}C NMR, see Tables 2 and 3, respectively; HR EIMS *m/z* (rel. int.) 346.1903 ($C_{20}H_{26}O_5$, calc. 346.1780, 4); $[M]^+$, 331 $[M\text{-}Me]^+$ (1), 328 $[M\text{-}H_2O]^+$ (1), 313 $[M\text{-}Me\text{-}H_2O]^+$ (25), 310 $[M\text{-}2\times H_2O]^+$ (0.5), 302 $[M\text{-}CO_2]^+$ (1.5), 301 $[M\text{-}COOH]^+$ (2.5), 300 $[M\text{-}H_2O\text{-}CO]^+$ (2), 287 $[M\text{-}CO_2\text{-}Me]^+$ (1), 285 $[M\text{-}Me\text{-}CO\text{-}H_2O]^+$ (1.5), 270 $[M\text{-}CO\text{-}H_2O\text{-}2\times Me]^+$ (1), 269 $[M\text{-}CO_2\text{-}Me\text{-}H_2O]^+$ (4), 254 $[M\text{-}CO_2\text{-}H_2O\text{-}2\times Me]^+$ (1.5), 241 $[M\text{-}Me\text{-}CO\text{-}H_2O\text{-}CO_2]^+$ (2), 239 $[M\text{-}CO_2\text{-}H_2O\text{-}3\times Me]^+$ (2), 95 (100); FAB MS (+) *m/z* (rel. int.): 347 $[MH]^+$ (95), 329 $[MH\text{-}H_2O]^+$ (100).

3.11. Phytotoxin bioassays

Sphaeropsidins A–F (**1**–**6**) and their derivatives (**7**–**14**) were assayed for phytotoxicity using severed twigs of three cypress species (*Cupressus sempervirens* var. *pyramidalis*, *C. macrocarpa* var. *lambertiana* and *C. arizonica*). The apical parts of twigs, approximately 12 cm long, were used for the experiments. The cuttings were taken from young cypress seedlings (3-year old) grown in the greenhouse at 25–27 °C and 60–70% relative humidity (RH). The phytotoxicity of sphaeropsidins (**1**–**6**) and their derivatives (**7**–**14**) was also tested on herbaceous non-host plants (tomato: *Lycopersicon esculentum* cv. Marmande; mung bean: *Phaseolus vulgaris* var. *aureus*). Tomato and mung bean seedlings were grown in a growth chamber at 25 °C and 70–80% RH, exposed to a luminous flux of 400 μE m^{-2} s^{-1} with a 12-h photoperiod. Cuttings were taken from 21-day old seedlings. During the assay the severed twigs of cypress trees and the cuttings of tomato or mung bean plants were maintained in a growth chamber at relatively low values of RH (60%), temperature (23 °C) and light (150 μE m^{-2} s^{-1}). The pure substances were assayed at 10–100 μg ml^{-1}. Toxicity of these solutions was evaluated by placing the test plant parts (excised cypress twigs for 96 h, tomato and mung bean cuttings for 48 h) in the assay soln. and then transferring them to distilled water. Symptoms developed within 2, 4 and 21 days on tomato, mung bean and cypress, respectively. Solutions of SA (**1**), SB (**2**) and SC (**3**) (100 μg ml^{-1}) were prepared with sterile distilled water. Using a hypodermic syringe, 3 ml of each soln. was injected into the stem bark of 3-year old *C. sempervirens*, *C. macrocarpa* and *C. arizonica* seedlings, at a distance of 30 cm from the apex. Symptoms on the stem were assessed over a period of two months.

3.12. Antifungal activity

The assays were carried out with *Seiridium cardinale*, *S. cupressi*, and *S. unicorne* three causal agents of canker disease on cypress, and five other plant pathogenic fungi: *Botrytis cinerea*, *Fusarium oxysporum*, *Penicillium expansum*, *Phomopsis amygdali* and *Verticillium dahliae*, as test microorganisms. A dilution series was prepared in the range 20–100 μg ml^{-1} toxic substance. The test was carried out by growing each fungal species in Petri dishes containing 20 ml of potato-dextrose agar (PDA) amended with concentrations of 20, 40, 60, 80 and 100 μg ml^{-1} of each toxic substance. The plates (five per fungal species) were seeded with two small pieces of a 10-day old colony mat and incubated at 25 °C for 1–2 weeks, depending on the fungal species. The isolates were tested at least twice. The antifungal effect of toxins and they derivatives was evaluated by calculating the percentage of linear growth inhibition as 100 $(y-x)/y$ where y = mean colony diameter of toxin-free cultures and x = mean colony diameter of toxin-containing cultures.

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References

Breitmaier, E., Voelter, W., 1987. Carbon-13 NMR Spectroscopy. VCH, Weinheim.

Ellestad, G.A., Kunstmann, M.P., Mirando, P., Morton, G.O., 1972. Structures of fungal diterpene antibiotics LL-S491β and -γ. Journal of American Chemical Society 94, 6206–6208.

Evidente, A., Sparapano, L., Andolfi, A., Bruno, G., Giordano, F., Motta, A., 2000. Chlorosphaeropsidone and epichlorosphaeropsidone, two new chlorinated dimedone methyl ethers isolated from liquid cultures of *Sphaeropsis sapinea* f. sp. *cupressi*. Phytopathologia Mediterranea 39, 299–309.

Evidente, A., Sparapano, L., Andolfi, A., Bruno, G., Motta, A., 2003. Sphaeropsidin F, a new pimarane diterpene produced *in vitro* by the cypress pathogen *Sphaeropsis sapinea* f. sp. *cupressi*. Australian Journal of Chemistry 56, 615–619.

Evidente, A., Sparapano, L., Bruno, G., Motta, A., 2002. Sphaeropsidins D and E, two other pimarane diterpenes produced *in vitro* by the plant pathogenic fungus *Sphaeropsis sapinea* f. sp. *cupressi*. Phytochemistry 59, 817–823.

Evidente, A., Sparapano, L., Fierro, O., Bruno, G., Giordano, F., Motta, A., 1997. Sphaeropsidins B and C, phytotoxic pimarane diterpenes from *Sphaeropsis sapinea* f. sp. *cupressi* and *Diplodia mutila*. Phytochemistry 45, 705–713.

Evidente, A., Sparapano, L., Fierro, O., Bruno, G., Giordano, F., Motta, A., 1998. Sphaeropsidone and episphaeropsidone, phytotoxic dimedone methyl ethers produced by *Sphaeropsis sapinea* f. sp. *cupressi* grown in liquid culture. Phytochemistry 48, 1139–1143.

Evidente, A., Sparapano, L., Motta, A., Giordano, F., Fierro, O., Frisullo, S., 1996. A phytotoxic pimarane diterpene of *Sphaeropsis sapinea* f. sp. *cupressi*, the pathogen of a canker disease of cypress. Phytochemistry 42, 1541–1546.

Frisullo, S., Bruno, G., Lops, F., Sparapano, L., 1997. Un nuovo agente di cancro del cipresso in Italia. Petria 7, 141–158.

Fritz, J.S., Schenk, G.H., 1959. Acid-catalyzed acetylation of organic hydroxyl groups. Analytical Chemistry 31, 1808–1812.

Kim, H.J., Kim, J.C., Kim, H.G., Cho, K.Y., 1999. Antibiotic and phytotoxic activities of ophiobolins from *Helmintosporium* species. The Plant Pathology Journal 15, 14–20.

Kowalski, T., 1991. Oak decline: I. Fungi associated with various disease symptoms on overground portions of middle-aged and old oak (*Quercus robur* L.). European Journal of Forest Pathology 21, 136–151.

Solel, Z., Madar, Z., Kinchi, M., Golan, Y., 1987. Diplodia canker of cypress. Canadian Journal of Plant Pathology 9, 115–118.