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Differential Behaviour of Mycelial Growth of Several *Botrytis cinerea* Strains on either Patchoulol- or Globulol-amended Media

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With one figure

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Abstract

Botrydial and dihidrobotrydial are two characteristic metabolites of the phytopathogenic fungus *Botrytis cinerea*, which are involved in the development of necrotic lesions on grapevine and tobacco. Patchoulol and globulol, two natural products which are analogues to precursors of botrydial and dihidrobotrydial, were tested on 10 *B. cinerea* strains which were isolated from different hosts and varied in aggressiveness on grapevine leaves. Mycelial growth of all strains was prevented when they were grown on either patchoulol- or globulol-amended malt agar media (200 $\mu g/\mu l$). Each strain displayed a specific response pattern to those products, according to the high variability previously described for this species. Furthermore, strains were different from one another with regard to their level of aggressiveness against leaves detached from sherry grapevine vineyards.

Zusammenfassung

Unterschiedliches Myzelwachstum verschiedener *Botrytis-cinerea*-Stämme auf Medien, die Patchoulol oder Globulol enthielten

Botrydial und Dihydrobotrydial sind zwei charakteristische Metaboliten des phytopathogenen Pilzes *Botrytis cinerea*; sie sind an der Entstehung nekrotischer Läsionen an Weinreben und Tabak beteiligt. Patchoulol und Globulol – natürliche Produkte, die analog zu Vorstufen von Botrydial und Dihydrobotrydial sind – wurden an 10 *B.-cinerea*-Stämmen getestet, die von verschiedenen Wirtspflanzen stammten und auf Weinblättern unterschiedlich aggressiv waren. Das Myzelwachstum aller Stämme wurde vollständig gehemmt, wenn sie auf MA-Medien gehalten wurden, denen Patchoulol oder Globulol (200 $\mu g/\mu l$) zugesetzt worden war. Jeder Stamm zeigte eine spezifische Reaktion auf diese Produkte, was der bei dieser Art bekannten hohen Variabilität entspricht. Die Stämme unterschieden sich auch bezüglich des Ausmaßes ihrer Aggressivität auf abgetrennten Blättern von Weinreben, deren Beeren zur Weinbranderzeugung genutzt werden.

Introduction

Botrytis cinerea Pers. is a phytopathogenic fungus on over 200 hosts. Grey mould, a grapevine disease caused by the fungus, results in crop losses and low quality wines from infected grapevines in many wine-producing countries. The widespread use of chemical fungicides to prevent or eliminate grey mould has resulted in both the appearance of highly resistant strains in *B. cinerea* populations, which very quickly develop resistance to benzimidazoles and dicarboximides (Bollen and Scholten, 1971; Leroux et al., 1982), and the contamination of soil and water. Therefore, new strategies for grey mould control are necessary.

Selective fungicides, that act on specific targets, do not persist in the environment and are not incorporated into the food chain, have been proposed as a more rational use of prophylactic treatments (Collado et al., 1994). Over the last 4 years, several studies on secondary metabolites of *B. cinerea* have examined their role in pathogenicity (e.g. Collado et al., 1995; Collado et al., 1996). It has been reported that botrydial, dihydrobotrydial, and some related metabolites with the botryane structure could reproduce the symptoms of grey mould on detached grapevine and tobacco leaves (Rebordinos et al., 1996; Durán-Patrón et al., 1999). The synthesis of botrydial and dihidrobotrydial can be inhibited by molecules whose structure is isometric to that of the natural substrate in the biosynthetic pathway of both metabolites (Aleu et al., 1999a). Patchoulol and globulol, two natural products from *Pogostemon cablin* and *Eucaliptus globulus*, respectively, which are analogues to precursors of botrydial and dihydrobotrydial, have been identified as good fungistatic compounds against *B. cinerea* UCA992 strain (Aleu et al., 1999b).

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Ten strains of *B. cinerea*, which were isolated from different hosts and varied in aggressiveness on *Vitis vinifera* were analysed in relation to their sensitivity to patchoulol and globulol. The variation in sensitivity to fungicides could limit field performance toward *B. cinerea*. Therefore, the knowledge of the variable response of different strains to the fungistatics is important when using natural products such as patchoulol and globulol to control *B. cinerea* populations. All strains were highly variable with regard to other features (unpublished data). Differences in aggressiveness among strains were assessed using bioassays on leaves detached from sherry grapevine vineyards.

Material and Methods

Chemicals

Patchoulol and globulol were obtained from Fluka (Aldrich-Sigma, Sigma-Aldrich Quimica, S.A., Madrid, Spain). Both were dissolved in ethanol and added to autoclaved media cooled to 45–50°C. The concentration of solvents did not exceed 0.1%.

Fungal strains

The fungal strains used and their origins are listed in Table 1. They were purified by monoconidial isolation in the authors' laboratory with the exception of two strains provided by the Spanish Type Culture Collection, and two donated by Dr F. Faretra (University of Bari, Italy).

Conidia were harvested from sporulating plates by washing with 5 ml sterile water, containing 0.01% Tween 20, filtered through a 30 μm nylon filter (Nytal) and centrifuged for 5 min at $2600 \times \boldsymbol{g}$. The resulting conidial pellet was suspended in a 10% glycerol solution to a concentration of 1×10^8 conidia/ml. Conidia from each strain were stored at −80°C.

Culture and growth conditions

Conidial suspensions (10 μl) of the fungal isolates were spread on malt agar (MA) (2% malt extract, 2% agar) in Petri dishes (100 mm × 20 mm) and then grown and maintained in incubators under alternating 12 h light : 12 h dark at 21 ± 1°C.

Table 1
Hosts and geographic origin of 10 *Botrytis cinerea* strains used in this study

Isolate	Host	Origin (year of isolation)
UCA991[a]	*Vitis vinifera*	Málaga, Spain (1991)
UCA992[a]	*V. vinifera*	Jerez de la Frontera, Spain (Domecq 1992)
UCA993[a]	*V. vinifera*	Puerto de Sta. María, Spain (Osborne 1993)
UCA994[a]	*V. vinifera*	Alicante, Spain (1994)
UCA995	*V. vinifera*	León, Spain (1993)
UCA996[a]	*Cucumis sativus*	Almería, Spain (1996)
2100	*Vicia faba*	Spanish Type Culture Collection (1979)
2850	*Crosus sativa*	Spanish Type Culture Collection (1987)
SAS56	Ascospore progeny	Faretra et al. (1988)
SAS405	Ascospore progeny	Faretra et al. (1988)

[a] Strains isolated from vineyards several times treated with fungicides.

Growth test

Five millimetre diameter mycelial plugs were collected from the margin of colonies actively growing on MA medium and placed on 100 mm diameter Petri dishes of MA medium amended with either globulol or patchoulol. For monitoring tests, 200 μg/ml patchoulol or globulol was used, as that concentration on the MA medium reduced mycelial growth by over 50% (EC_{50} or ED_{50}) on most of the strains tested. The concentration of ethanol in the agar was 0.1% (v/v). Controls contained 0.1% (v/v) ethanol without fungicide. The ethanol at 0.1% (v/v) in the agar, had no effect on the growth of any strain. The diameters of colonies which developed at 21 ± 1°C were measured every 24 h for 6 days. The average diameters of colonies on fungicide-amended MA were calculated as a percentage of the colony diameters in control treatments. Percentage inhibition was calculated using the Vincent (1927) method. All tests had three replicates.

Bioassays of virulence

Leaves from *Vitis vinifera* var. Palomino, commonly used to make sherry wine, were surface disinfected with 10% (v/v) sodium hypochlorite for 3 min, washed four times with sterile water, and dried between filter papers. One leaf was placed on each Petri dish (150 mm × 20 mm) containing Whatman paper wetted with sterile water as described previously (Rebordinos et al., 1996). Three mycelial plugs (1 cm diameter) of each strain were placed on every leaf, and the dishes kept at 21–23°C under alternating 12 h light : 12 h dark. For each experiment, both intact leaves and leaves wounded with a sterile needle were used. In the last case, the agar plugs were placed just over the damaged zone. Three agar plugs without mycelia were placed on a *V. vinifera* var. Palomino leaf as a control. Bioassays had three replicates. The diameter of the necrotic lesions on each leaf was measured 7 days after inoculation. The arithmetic mean (± standard error) diameter for each strain was then calculated.

Strains were then classified into groups according to lesion diameter: group I, < 22.5; II, 22.5–45.0; and III, > 45.0 mm. Strains belonging to group I had low or no virulence, group II had an intermediate virulence and group III had high virulence.

Results

Sensitivity of strains to patchoulol

All strains were sensitive to 200 μg/ml of patchoulol. Percentage of inhibition could be calculated up to the point that colonies in the control dishes occupied the entire surface. Beyond this point, the diameter size of the colonies was measured every 24 h for a total time of 168 h to calculate the time needed to reach a four-fold increase in their size (24–25 mm in diameter). This was compared to the size-quadrupling time in control colonies not having fungicide.

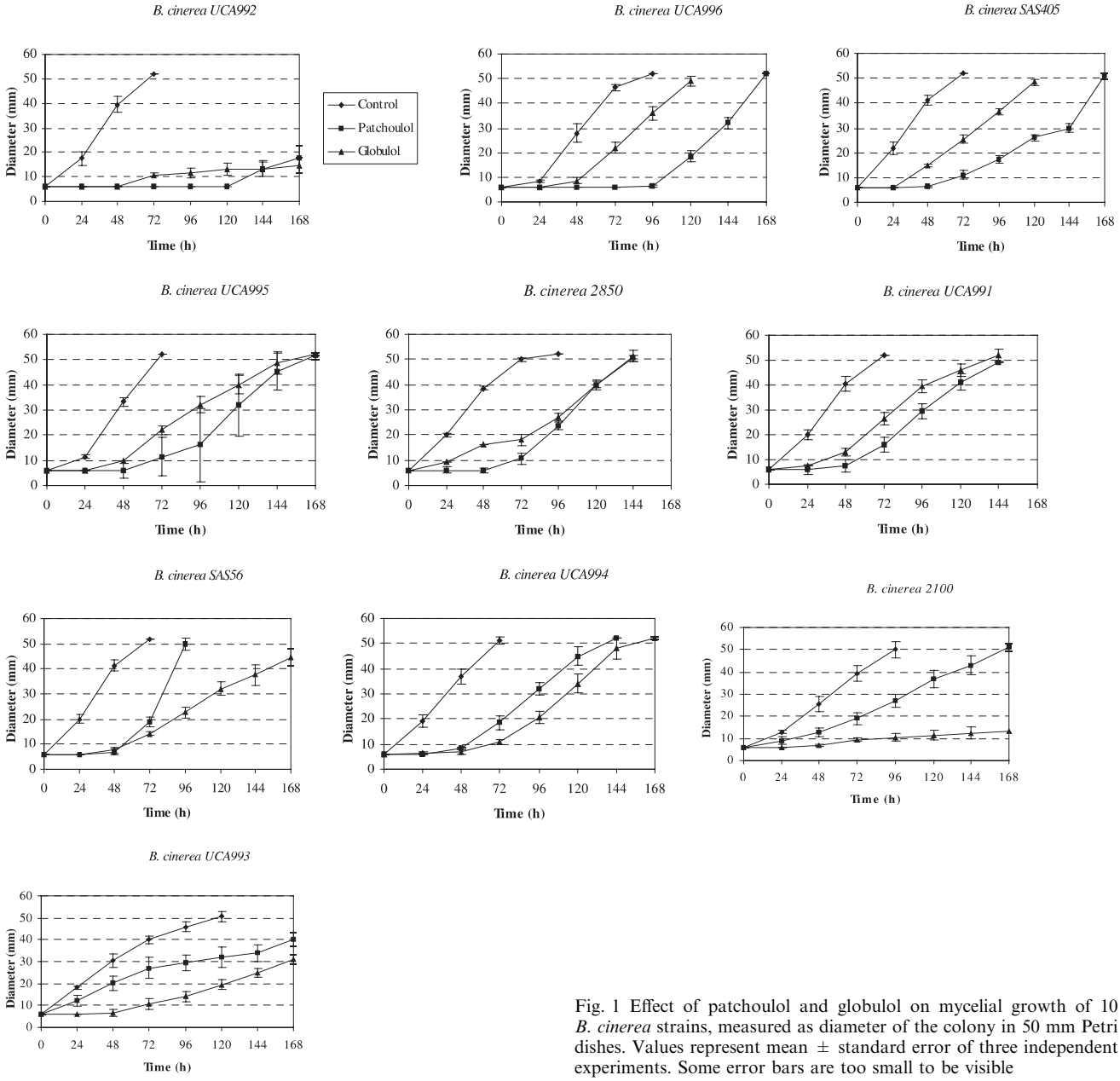

Fig. 1 Effect of patchoulol and globulol on mycelial growth of 10 *B. cinerea* strains, measured as diameter of the colony in 50 mm Petri dishes. Values represent mean ± standard error of three independent experiments. Some error bars are too small to be visible

The compound completely inhibited mycelial growth of eight of the 10 strains for at least 24 h, and the other two strains to a lesser degree for at least 72 h, as shown in Figure 1. The percentage of inhibition could be measured up to 120 h for strain UCA993, up to 96 for strains UCA996 and 2100, and up to 72 h for the remainder strains (Table 2). The most sensitive strains to patchoulol were UCA992 and UCA996, whose mycelial growth were completely inhibited for 72 and 96 h, respectively. Mycelial growth of UCA995 and 2850 was prevented for 48 h, whereas that of the strains SAS405, UCA991, SAS56, and UCA994 was prevented for only 24 h (Table 2). UCA996 later grew at the same rate as it did on MA media without fungicide, with an approximate size-quadrupling time of 45 h. However, the growth rate of the remaining strains was lower on patchoulol-amended media than that shown by their controls: UCA992 did not increase its size four-fold within the time the experiment was performed, whereas its size-quadrupling time was approximately 45 h on non-amended MA media; SAS405, UCA995, 2850, UCA991, SAS56, and UCA994 needed about 60, 60, 52, 64, 55 and 54 h, respectively, to quadruple their size on media amended with patchoulol since they started growing, whereas their approximate size-quadrupling times on media without fungicide were 30, 38, 29, 30, 28 and 35 h, respectively.

Strains 2100 and UCA993 were not completely inhibited by patchoulol. Strain 2100 needed 88–89 h to reach a four-fold increase in its size on patchoulol-amended media, whereas the size-quadrupling time on non-fungicide-amended media was only 50 h. Strain UCA993 was

Table 2
Percentage of inhibition of 10 *Botrytis cinerea* strains by either globulol-or patchoulol-amended MA media

		Percentage of inhibition (%)									
	Time (h)	24		48		72		96		120	
Strain	Fungicide	G	P	G	P	G	P	G	P	G	P
UCA992		100	100	100	100	79.4	100				
UCA996		100	100	69.6	100	52.6	100	30.7	100		
UCA994		100	100	100	76.7	78.8	37.2				
UCA995		100	100	73.8	100	64.8	78.7				
SAS405		100	100	64.2	84.5	51.1	79				
SAS56		100	100	81.3	83	72.8	63.4				
2100		100	34.1	74.8	50.1	76.1	51.3	78.5	45.3		
UCA993		100	32.5	78	34	74.2	33.6	70	36.6	61.4	36.7
2850		100	100	59	100	63.4	8.7				
UCA991		61.1	100	67.5	32.9	49	69.2				

G, globulol; P, patchoulol; Percentage of inhibition was calculated using the Vincent method and could be calculated up to the point that colonies in the control dishes (50 mm Petri dishes) occupied the entire surface.

the least sensitive to patchoulol, which produced a maximum percentage of inhibition of 36.7% for 120 h.

Sensitivity of strains to globulol

Mycelial sensitivity to 200 μg/ml of globulol was above 60% for all 10 strains (Fig. 1). Most of them were completely inhibited for at least 24 h. Percentage of inhibition were calculated up to the point that colonies in the control dishes occupied the entire surface as described above and they are shown in Table 2.

Against UCA992 and UCA994, the globulol completely suppressed mycelial growth for 48 h. Mycelial growth of strains 2850, UCA996, SAS405, UCA995, SAS56, 2100 and UCA993 was totally prevented for 24 h. Globulol did not completely prevent mycelial growth of strain UCA991. After that time, the strains continued growing (Fig. 1) although the growth rate on globulol-amended media was lower than that displayed by them on media without fungicide. Strains UCA996, SAS405, UCA995, 2850, UCA991, SAS56, UCA994 and UCA993 needed about 53, 48, 58, 89, 65, 84, 55 and 120 h, respectively, to quadruple their size since they started growing, whereas their size-quadrupling time on non-amended MA media was lower (see above). Strains UCA992 and 2100 did not increase their size four-fold within 168 h.

Bioassays of virulence

Agar plugs containing a mycelial mass of each strain were assayed on wounded and intact leaves of *Vitis vinifera* var. Palomino. Necrotic lesions became visible between 24 and 72 h after infection. By measuring lesion diameters, it was found that each strain had a different level of virulence on the grapevine leaves as shown in Table 3. All strains had a similar virulence level on intact and wounded leaves except for strain 2850, which was avirulent on intact leaves but moderately virulent on wounded ones.

On intact Palomino leaves, the lowest virulence (group I) ranged from 0.0 (2850 and UCA992) to 12.2 ± 5.2 mm (UCA993). Intermediate virulence (group II) ranged from 32.2 ± 3.7 (UCA994) to 38.4 ± 5.1 mm (2100), whereas high virulence (group III) ranged from 49.5 ± 2.5 (UCA991) to 75.0 ± 10.0 mm (UCA995).

Table 3
Pathogenicity of 10 *Botrytis cinerea* strains on *Vitis vinifera* var. Palomino detached leaves

Strain	Size of lesion on intact leaves (mm)[a]	Size of lesion on wounded leaves (mm)[a]	Group
UCA992	0.0 ± 0.0	15 ± 10.0	I [b, c]
UCA993	12.2 ± 5.2	14.3 ± 1.5	I [b, c]
2850	0.0 ± 0.0	39.3 ± 5.6	I [b], II[c]
UCA994	32.2 ± 3.7	27 ± 0.8	II [b, c]
UCA996	38.3 ± 2.9	40 ± 2.4	II [b, c]
2100	38.4 ± 5.1	42.3 ± 2.2	II [b, c]
UCA991	49.5 ± 2.5	50.7 ± 1.1	III [b, c]
SAS56	54.3 ± 3.5	52.7 ± 1.4	III [b, c]
SAS405	61.6 ± 8.1	57.7 ± 5.1	III [b, c]
UCA995	75 ± 10.0	67 ± 5.1	III [b, c]

[a] Data are expressed as mean ± standard error of no fewer than three separate observations from three different experiments. Groups represent low or no virulence (I), intermediate virulence (II) and high virulence (III) on either intact [b] or wounded leaves [c].

Similarly, on wounded leaves the lowest virulence (group I) ranged from 14.3 ± 1.5 (UCA993) to 15.0 ± 10.0 mm (UCA992). Intermediate virulence (group II) ranged from 27.0 ± 0.8 (UCA994) to 42.3 ± 2.2 mm (2100). High virulence of strains (group III) ranged from 50.7 ± 1.1 (UCA991) to 67.0 ± 5.1 mm (UCA995).

Discussion

The sensitivity distribution to patchoulol and globulol of the 10 *B. cinerea* strains with different levels of aggressiveness on *V. vinifera*, was based on the percentage of inhibition by those compounds. Mycelial growth was inhibited when either of these compounds was added to the MA media. Variations in sensitivity were evident between all strains tested, as demonstrated earlier for other classes of fungicides against *B. cinerea* (Grindle, 1981; Elad, 1992; Stehmann and De Waard,

1996). After a period of time, the mycelia of all strains started growing, so that the compounds could be classified as fungistatic. As reported by Aleu et al. (1999b), these compounds are clearly toxic to *B. cinerea*, but are metabolized by the fungus into non-toxic compounds. Furthermore, these authors showed that botrydial and dihydrobotrydial are not synthesized until patchoulol and globulol have been totally withdrawn from the broth by the detoxification process. Although both fungistatic products are probably blocking the same metabolic path, each strain detoxifies them at a different rate. A different capacity of *B. cinerea* strains to metabolize toxic compounds has been reported (Brunerie et al., 1988; Bock et al., 1988; Schoch et al., 1991; Sbaghi et al., 1996), which could be correlated with the high genetic variability of *B. cinerea* found by many authors (Büttner et al., 1994; Vallejo et al., 1996).

Five strains were isolated from vineyards treated several times with fungicides (Table 1), but no relationship could be established between the fact of having been previously treated and sensitivity to patchoulol or globulol.

Analogues of naturally expressed metabolites of *B. cinerea* have been suggested to be a rational alternative to the synthetic fungicides against the fungus (Aleu et al., 1999b). The variable response of different strains to some of those analogues such as patchoulol and globulol should be taken into consideration when undertaking the control of *B. cinerea* populations. Using 200 $\mu g/\mu l$ patchoulol and globulol, the mycelial growth was limited by over 50% for all strains tested except for UCA993, whose mycelial growth was never inhibited over 36.7% on patchoulol-amended MA media. Interestingly, this strain, and also 2100, grew significantly ($P < 0.05$; Student's *t*-test) slower on MA media than the remaining strains tested (data not shown), although further work needs to be carried out to determine whether the rate of growth can be correlated to sensitivity of toxic compounds.

The effect of many chemicals compounds towards *B. cinerea* have been studied for several years. Many of those studies have been carried out to investigate the control of either conidial germination or mycelial growth. Since mycelium actively growing on either dead or senescent parts of the plants has been found to be an important inoculum with which to infect healthy leaves and spread infections (Verhoeff, 1988), the control of the mycelial growth on MA media would considered to be an interesting topic for investigation. Furthermore, a relationship between mycelial morphology and virulence was found during the present study for some of the strains using in this work: UCA995 and UCA991, which produced a large amount of mycelium, produced very few conidia and did not produce sclerotia were some of the most aggressive strains, whereas 2850 and UCA992, which produced a high number of conidia along with its own growth, were found to be some of the less aggressive strains (unpublished data).

All 10 strains had different levels of virulence on the grapevine leaves used in this study. Strains 2850 and UCA992 were unable to infect intact tissue, but on wounded leaves the former produced lesions with a diameter similar to that caused by strains with an intermediate virulence. The inability of this strain to breach the plant cuticle might be due to the blocked synthesis of enzymes essential for penetrating host tissues, as shown for some host–pathogen interactions (Salinas et al., 1986; Dickman et al., 1989). However, UCA992 still had low virulence on wounded leaves, so that this strain was the least aggressive of all 10 strains tested. Interestingly, this was also the most sensitive strain to both fungistatics, but no further correlation could be established between sensitivity towards patchoulol and globulol and virulence against grapevine leaves.

In recent years, significant progress in the control of grey mould has been made. Special attention has been paid to chemical control. However, contamination problems due to the persistence of these chemicals both in soil and water result in important ecological problems. Indeed, those compounds can be incorporated into the food chain. The data confirm that analogues of naturally expressed metabolites of *B. cinerea*, which are not persistent in the ecosphere, could be a rational alternative to the synthetic fungicides against the fungus. Since those compounds produce a fungistatic effect on *B. cinerea*, they could be considered for use as part of an integrated control programme against the fungus.

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