J. Phytopathology **154**, 616–621 (2006)

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Screening Study of Potential Lead Compounds for Natural Product-based Fungicides Against *Phytophthora* Species

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Received March 10, 2006; accepted May 29, 2006

Keywords: *Phytophthora infestans*, *Phytophthora capsici*, percentage of inhibition, rational fungicides, fungistatics

Abstract

Phytophthora spp. is one of the phytopathogenic Oomycete responsible for many important crop losses. Relevant species are *P. infestans* (causing potato late blight) and *P. capsici* (causing blight in pepper). In recent years, the use of conventional fungicides has favoured the appearance of different resistant strains. This study analyses the effect of various compounds on these two *Phytophthora* species. Those compounds were designed on the basis of known structures of natural compounds to obtain a rational control of these fungal-like species. All the analysed products showed a fungistatic activity against both strains, one of them reduced mycelial growth by over 46% at 100 p.p.m.

Introduction

The genus *Phytophthora* among the Oomycetes is responsible for many important crop losses, causing a great variety of plant diseases. Among the relevant species are *P. infestans* (causal agent of potato late blight) and *P. capsici* (causing blight in pepper).

Since the mid-1990s, new compounds with excellent activity against phytopathogenic fungi have been commercialized. However, strategies to control the fungus with classic fungicides may produce side effects, notable environmental contamination and the development of multiresistant fungal strains. Some of these fungicides, such as the dicarboximide procymidone, are persistent enough to be detected after several weeks in vegetables (Apladasarlis et al., 1994), soil (Paris-Palacios et al., 1998) and even after vinification (Sala et al., 1996).

Application of synthetic fungicides for the control of fungal diseases in major agricultural crops is a standard tool of farm production in Europe. Although, the modern fungicides have reached a considerable level of efficacy associated with increasingly reduced toxicity, some problems associated with the environmental impacts and toxicology remain. Consequently, there is a great deal of interest in developing novel, non-persistent and rational antifungal agents, especially those with activity against particularly damaging organisms such as certain *Phytophthora* species.

Over the last few years we have undertaken a research programme directed towards the rational design of fungicides to control phytopathogenic fungi infections of commercial crops. In order to find substrates with antifungal properties against Oomycetes, we have screened compounds analogous to various phytoalexins (Kokubun et al., 1994; Echeverri et al., 1997), and to clovanes derivatives which displayed antifungal activity against phytopathogenic fungi (Collado et al., 1997; Deligeorgopoulou et al., 2004).

Here, we present an evaluation of the fungicidal activity of seven natural product-based chemicals against the *Phytophthora* species *P. infestans* and *P. capsici*.

Materials and Methods

Culture and growth conditions

Phytophthora infestans and *P. capsici* strains were isolated from tomato- and pepper-infected plants and kindly supplied by Dr Rodríguez Tello (Utrera, 2001). These isolates were cultured on Petri dishes using Pea medium (Goodwin et al., 1998) for *P. infestans* and V8 medium (Lamour and Hausbeck, 2000) for *P. capsici* and then grown and maintained in an incubators at 22°C under alternating 12 h light : 12 h dark.

Synthesis of compounds

Compounds **1–7** (Fig. 1): (3′-chlorophenyl)propan-1-ol (**1**; Collado et al., 2004a), (4′-chlorophenyl)propan-1-ol (**2**; Collado et al., 2004a), 1-(4′-chlorophenyl)-2-cyclopropylethanol (**3**; Collado et al., 2004a), 1-(4′-chlorophenyl)-2-phenylethanol (**4**; Collado et al., 2004a), 2β-(2′-nitroethoxy)clovan-9α-ol (**5**; Collado et al., 2001), *N*-(9α-hydroxyclovan-2β-yl)-*N*″-*p*-bromophenylacetamidine (**6**; Collado et al., 2004b) and 2β-(2′-pseudothiouretoxy)clovan-9α-ol (**7**; Collado et al., 2001) were

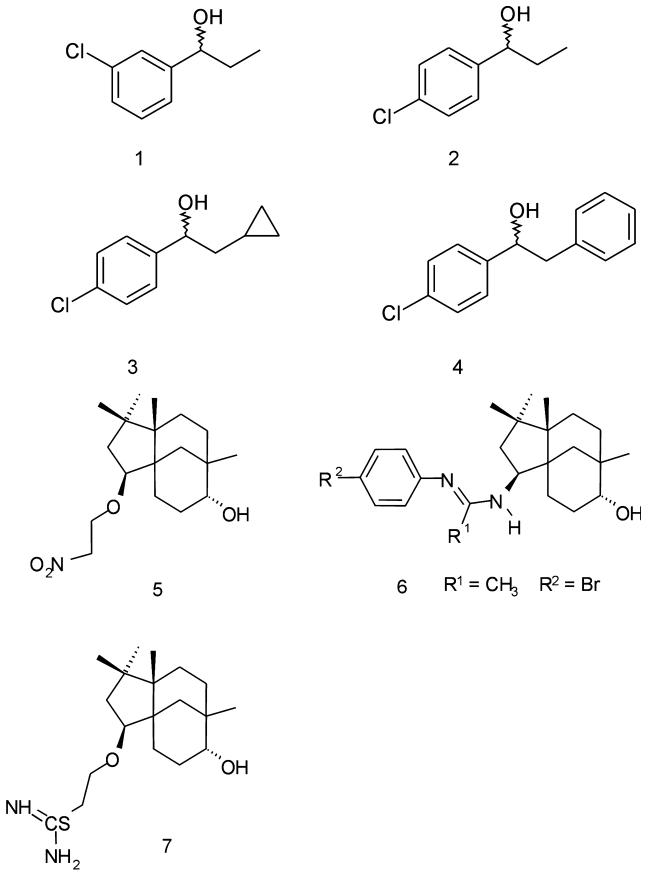

Fig. 1 Molecular structure of the assayed compounds (**1–7**): (3′-chlorophenyl)propan-1-ol (**1**), (4′-chlorophenyl)propan-1-ol (**2**), 1-(4′-chlorophenyl)-2-cyclopropylethanol (**3**), 1-(4′-chlorophenyl)-2-phenylethanol (**4**), 2β-(2′-nitroethoxy)clovan-9α-ol (**5**), *N*-(9α-hydroxyclovan-2β-yl)-*N*″-*p*-bromophenylacetamidine (**6**) and 2β-(2′-pseudothiouretoxy)clovan-9α-ol (**7**)

synthesized following procedures previously reported (Collado et al., 2001, 2004a,b). Compounds **1–4** are phytoalexin analogues obtained by I. G. Collado (Collado et al., 2004a). Compounds **5–7** were based in clovane structures.

Concentrations of 100 p.p.m. (mg/l) were used for all compounds. Also, to test whether some compounds would produce higher percentages of inhibition compounds **1** and **2** were tested at 200 p.p.m. All the products were dissolved in ethanol and added to autoclaved media cooled to 45–50°C. The concentration of solvent did not exceed 0.1%.

Bioassays of fungicide activity

Bioassays of fungicide activity were performed following as previously described (Vallejo et al., 2001) by measuring the diameter of the colonies in: (i) Petri dishes that contained medium amended with the fungicide dissolved in ethanol, (ii) Petri dishes with non-amended media and (iii) Petri dishes with medium plus ethanol, as control. The concentration of the ethanol in the agar was $<0.1\%$ (v/v) in all cases. The ethanol in the agar had no effect on the growth of the strains.

The diameters of colonies which developed at 22°C were measured periodically until the entire surface of the plate was covered by the fungus (120 h for *P. infestans* and 72 h for *P. capsici*). Percentage inhibition was calculated using the Vincent method (Vincent, 1927) and rounded to natural numbers. Tests were performed twice and all of them had three replicates.

Results

All analysed compounds were capable to prevent the growth of the two *Phytophthora* strains. After a period of time, however, the mycelia of the strains started growing, so that the compounds could be classified as fungistatics. Percentage of inhibition was calculated up to the point that colonies in the control dishes occupied the entire surface in the agar plates.

Differences in percentages of inhibition (Table 1) were found in *P. infestans*. Neither of the phytoalexin derivatives (**1–4**) produced an inhibition percentage over 31% at 100 p.p.m. (Fig. 2). Compounds **1** and **2** increased their fungistatic activity when assayed at 200 p.p.m. Percentage of inhibition of compound **1** increased by 33% (from 22% at 100 p.p.m. to 55% at 200 p.p.m.) and that of compound **2** increased by 27% (from 21% to 48%). The percentage of inhibition obtained by compounds **3** and **4** were 29% and 31% respectively.

Results obtained with clovane derivates (compounds **5–7**) against *P. infestans* showed that, at least, one of them produced percentage of inhibition near to 46% at 100 p.p.m. (Fig. 2). While compounds **5** and **6** produced similar results to that of the phytoalexin derivates, with 21% and 12% at 100 p.p.m., respectively, compound **7** increased this value to 46% at that

Table 1
Percentages of inhibition obtained at 100 p.p.m. for *Phytophthora* species (bold cells indicate maximum percentages of inhibition obtained)

	P. infestans [time (h)]						*P. capsici* [time (h)]					
Compounds	0	24	48	72	96	120	0	10	24	32	48	72
1	0	−1	19	**22**	21	20	0	7	17	12	4	**20**
2	0	8	20	**21**	20	13	0	**33**	16	11	10	13
3	0	8	**29**	25	26	15	0	**27**	23	19	21	0
4	0	9	**31**	29	23	19	0	59	52	60	61	**62**
5	0	17	**21**	16	7	14	0	39	38	52	52	**59**
6	0	**12**	7	2	1	3	0	**33**	15	16	16	0
7	0	23	39	**46**	43	36	0	19	50	49	**53**	43

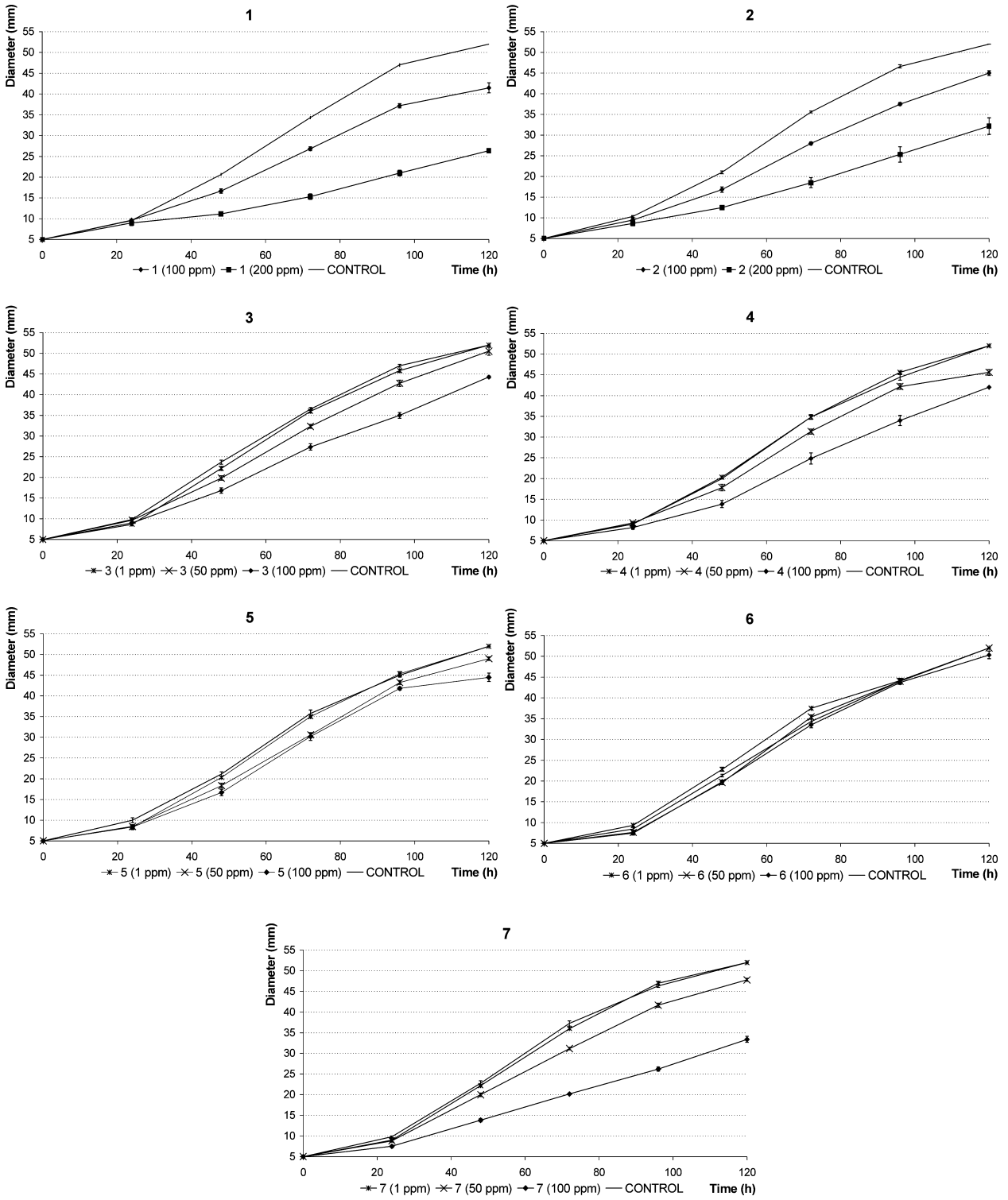

Fig. 2 Evolution of the diameter of the colony (mm/h) of *Phytophthora infestans* when growing in media amended with different compounds **1–7**. Error bars are too small to be visible

concentration, being the more effective compound against *P. infestans*.

Phytophthora capsici seemed to be less resistant than *P. infestans* to the compounds assayed (Table 1, Fig. 3). Mycelial sensitivity to compounds **1** and **3** was 20% and 27%, respectively, for *P. capsici*, lower than that obtained for *P. infestans*. However, compound **2** had a stronger effect on *P. capsici* than that produced on *P. infestans*; i.e. the percentage of inhibition was 33% in the former vs. 21% produced in the latter. Among phytoalexin derivatives, it is worth mentioning the percentage of inhibition produced by compound **4**

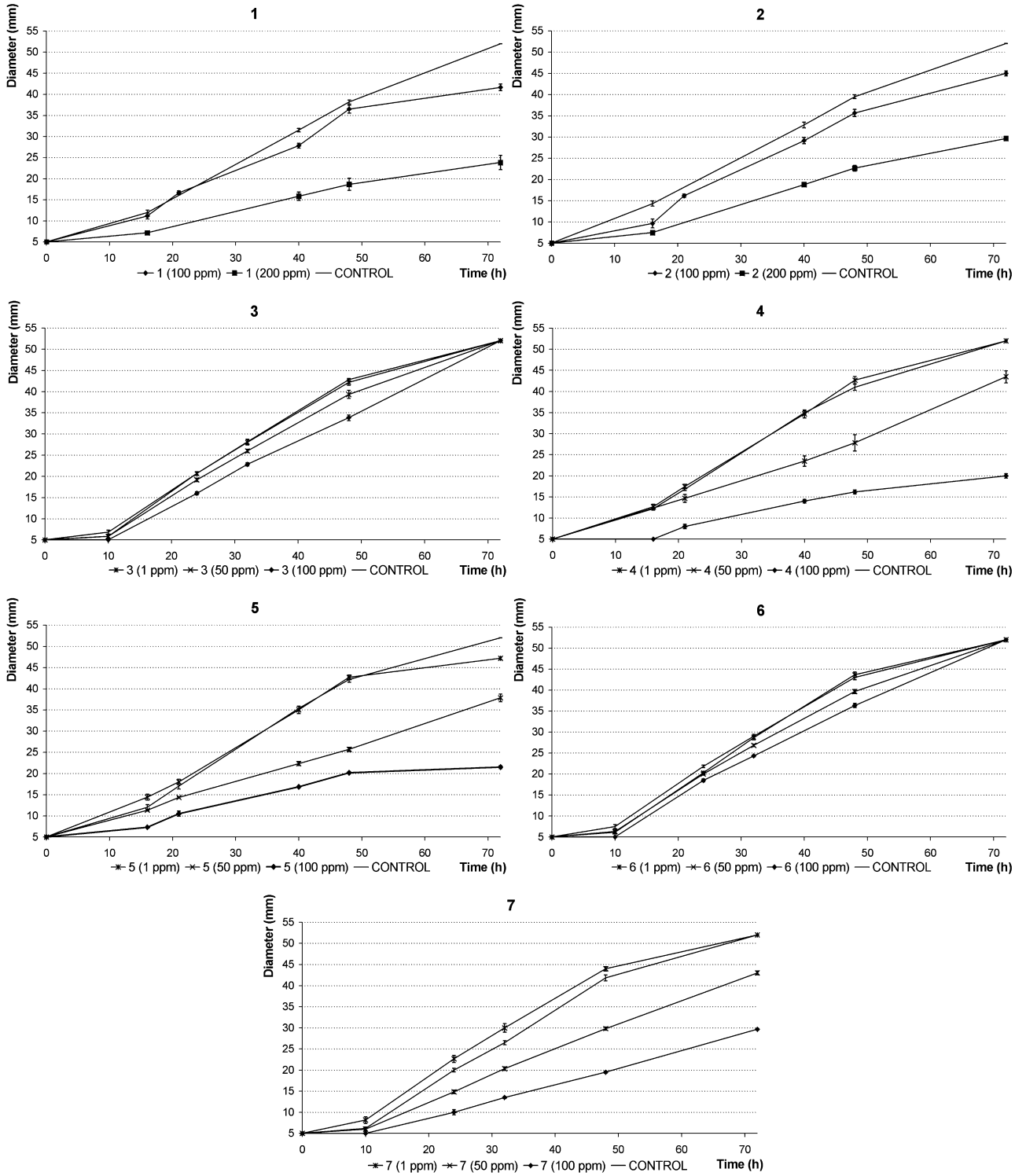

Fig. 3 Evolution of the diameter of the colony (mm/h) of *Phytophthora capsici* when growing in media amended with different compounds **1–7**. Error bars are too small to be visible

in *P. capsici*, which reached a value of 62%, the highest one obtained in this study for all compounds assayed and both strains analysed. When increasing the concentration of compounds **1** and **2** up to 200 p.p.m., the percentages of inhibition increased in a 34% (from 20% to 54%) and a 15% (from 33% to 48%) respectively.

Results obtained with clovane derivates showed that these compounds have a higher effect against mycelial growth of *P. capsici* than that produced on *P. infestans*. Indeed, it is noteworthy that the percentages of inhibition displayed by *P. capsici* when cultured in compounds **5**- and **6**-amended media were 59% and 33%, respectively, i.e. an approximately threefold

increase with respect to the percentage of inhibition displayed by *P. infestans* when growing in media amended with those compounds. Compound **7** produced a slightly higher percentage of inhibition against *P. capsici* (53%) than that obtained against *P. infestans* (46%).

Discussion

Oomycetes, such as *P. infestans* and *P. capsici*, cause important and devastating agricultural diseases of recognized importance, being considered high-risk pathogens (Brent and Hollomon, 1998). In an effort to control these Oomycetes, a high percentage of pesticide expenditure has been spent along the two last centuries (Staub and Hubele, 1981; Copping and Hewitt, 1998). Lost of effectiveness and development of fungicide resistance have compromised control strategies and driven towards the continuous search for new fungicides (Griffiths et al., 2003). However, persistence of these chemicals compounds both in soil and water result in important ecological problems and their possible incorporation into the food chain. The use of rational fungicides which are not persistent in the ecosystem, could be a rational alternative to the synthetic fungicides against the fungus. The mechanism by which rational fungicides act consists in confusing the enzymatic system of fungus as they are analogues of naturally expressed metabolites. These compounds are detected by the fungus as own metabolites, so that they might be processed and transformed. Then, toxic functional groups are released into the medium and eventually kill the fungus.

The compounds used in this study have displayed fungistatic activity against the phytopathogenic fungi *B. cinerea* and *Colletotrichum* spp. and they are under patent protection (Collado et al., 2001, 2004a,b).

In the present study, important differences between the response of *P. infestans* and *P. capsici* to the compounds assayed have been observed. Differences in sensitivity to a given compound may be due to a differential mechanism utilized for the transformation of such compound, which indicates the existence of biochemical and metabolic differences between both species. In most cases, a higher percentage of inhibition in *P. capsici* than in *P. infestans* was obtained. It may indicate that these substances act against indispensable enzymes on *P. capsici* whereas *P. infestans*: (i) lacks these enzymes, (ii) has metabolic alternative routes that permit to avoid the enzymatic blockage of those enzymes, or (iii) has a higher ability of detoxifying these substances. This metabolic diversity of *P. infestans* may explain the problems of resistances presents in nature (Fry and Goodwin, 1997).

When the percentages of inhibition obtained for both species at 100 p.p.m. were compared, two different types of responses were observed (Fig. 4):

Type (I)

Compounds that produce similar effect in *P. infestans* and in *P. capsici*; i.e. the difference between the percentages of inhibition produced in both species is ≤12% (compounds **1**, **2**, **3** and **7**).

Type (II)

Compounds that produce different effect in *P. infestans* and in *P. capsici*; i.e. the difference between the percentages of inhibition produced in both species is ≥21% (compounds **4**, **5** and **6**). These differential responses seem to be independent of the chemical structure (phytoalexin or clovane derivates) of the assayed compounds.

It is interesting to note that commercial fungicides doses against *Phytophthora* spp. are much higher 2000/3000 p.p.m.) (De Liñan, 2001) than those used in this study. Also, the percentages of inhibition produced in both *Phythophthora* species, demonstrate the effectiveness of all used compounds as fungistatics. These compounds could be used at a therapeutic scale as part of an integrated control programme against the fungus.

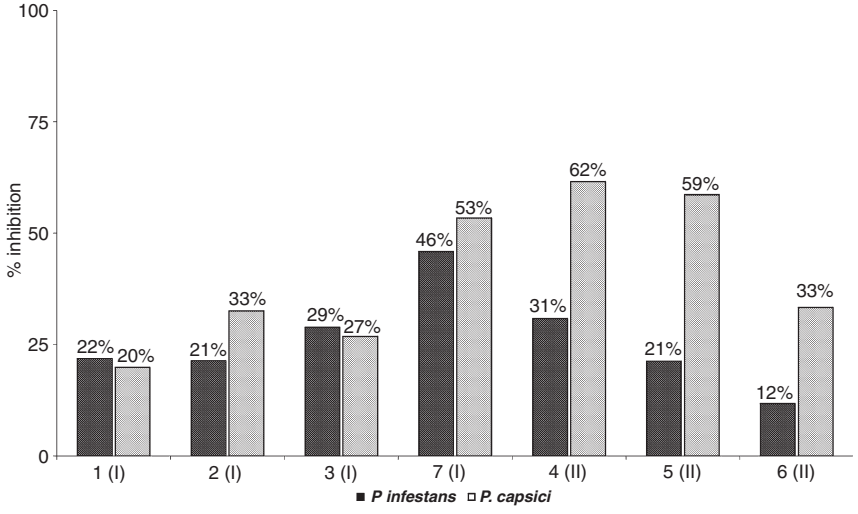

Fig. 4 Percentages of inhibition obtained at 100 p.p.m. indicating two types of response: type (I) and type (II). Percentages of inhibition are displayed for each bar

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