CHAPTER III

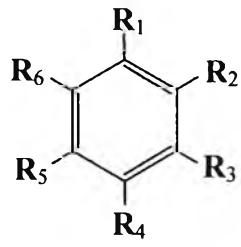
RESULTS AND DISCUSSION

The *in vitro* antifungal activity of 40 benzoic acid derivatives and related compounds and 50 cinnamic acid derivatives were thoroughly investigated against phytopathogenic fungi, such as *Alternaria porri*, *Fusarium oxysporum*, *Pestalotiopsis sp.* and *Phytophthora parasitica*, in order to define a possible structure–activity relationship. The influence of chemical structure of tested compound on their antifungal activity was also discussed. The evaluation of antifungal activity was performed by the agar medium assay according to the procedure described in Chapter II. When the potent antifungal compound has been selected, not only the antifungal activity, but also the phytotoxicity and stability should be taken into consideration. Based on that, the phytotoxicity against seed germination and stability in accelerated degradation tests on of selected compound was decided to examine.

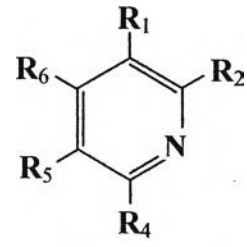
3.1 Fungal growth inhibition by benzoic acid derivatives and related compounds

The selected compounds as shown in Table 3.1 were incorporated into PDA medium. After transferring the mycelium of fungi, the tested plates were incubated at room temperature. When the mycelium of fungi reached the edges of the control plate, the antifungal percentage was calculated. Their antifungal activity was tested against four selected phytopathogenic fungi at the concentration of 5 mM. The results are displayed in Fig 3.1. However, the results obtained from the agar medium assay should be assessed critically such as the nature of dissolved test substance in agar medium, since the dissolution of active compound in agar medium has a profound effect upon fungus (Chavarin, 2002).

Table 3.1 Benzoic acid derivatives and related compounds used in this study



no.1-38



no.39 and 40

no.	compound	R_1	R_2	R_3	R_4	R_5	R_6
1	benzoic acid	COOH	H	H	H	H	H
2	salicylic acid	COOH	OH	H	H	H	H
3	3-hydroxybenzoic acid	COOH	H	OH	H	H	H
4	4-hydroxybenzoic acid	COOH	H	H	OH	H	H
5	2,4-dihydroxybenzoic acid	COOH	OH	H	OH	H	H
6	3,4-dihydroxybenzoic acid	COOH	H	OH	OH	H	H
7	3,5-dihydroxybenzoic acid	COOH	H	OH	H	OH	H
8	gallic acid	COOH	H	OH	OH	OH	H
9	2-chlorobenzoic acid	COOH	Cl	H	H	H	H
10	3-chlorobenzoic acid	COOH	H	Cl	H	H	H
11	3-(trifluoromethyl)benzoic acid	COOH	H	CF_3	H	H	H
12	*m*-toluic acid	COOH	H	CH_3	H	H	H
13	*p*-anisic acid	COOH	H	H	OCH_3	H	H
14	piperonylic	COOH	H	$O\text{-}CH_2\text{-}O$		H	H
15	2-naphthoic acid	COOH	H	CH=CH-CH=CH		H	H
16	cinnamic acid	CH=CH-COOH	H	H	H	H	H
17	3,4-methylenedioxycinnamic acid	CH=CH-COOH	H	$O\text{-}CH_2\text{-}O$		H	H
18	benzaldehyde	CHO	H	H	H	H	H
19	3-hydroxybenzaldehyde	CHO	H	OH	H	H	H
20	4-hydroxybenzaldehyde	CHO	H	H	OH	H	H

Table 3.1 (continued)

no.	compound	R_1	R_2	R_3	R_4	R_5	R_6
21	3,4-dihydroxybenzaldehyde	CHO	H	OH	OH	H	H
22	2-methoxybenzaldehyde	CHO	OCH_3	H	H	H	H
23	3-methoxybenzaldehyde	CHO	H	OCH_3	H	H	H
24	veratraldehyde	CHO	H	H	OCH_3	OCH_3	H
25	3-phenoxybenzaldehyde	CHO	H	OPh	H	H	H
26	salicylaldehyde	CHO	OH	H	H	H	H
27	cinnamaldehyde	CH=CH-COH	H	H	H	H	H
28	methyl salicylate	$COOCH_3$	OH	H	H	H	H
29	methyl 4-hydroxybenzoate	$COOCH_3$	H	H	OH	H	H
30	ethyl 4-hydroxybenzoate	$COOCH_2CH_3$	H	H	OH	H	H
31	propyl 4-hydroxybenzoate	$COOCH_2CH_2CH_3$	H	H	OH	H	H
32	butyl 4-hydroxybenzoate	$COOCH_2CH_2CH_2CH_3$	H	H	OH	H	H
33	propylgallate	$COOCH_2CH_2CH_3$	H	OH	OH	OH	H
34	cinnamyl alcohol	$CH=CH-CH_2OH$	H	H	H	H	H
35	2-hydroxyacetophenone	$COCH_3$	OH	H	H	H	H
36	allylbenzene	$CH_2CH=CH_2$	H	H	H	H	H
37	4-allylanisole	$CH_2CH=CH_2$	H	H	OCH_3	H	H
38	anethole	$CH=CHCH_3$	H	OCH_3	H	H	H
39	nicotinic acid	COOH	H	-	H	H	H
40	picolinic acid	H	COOH	-	H	H	H

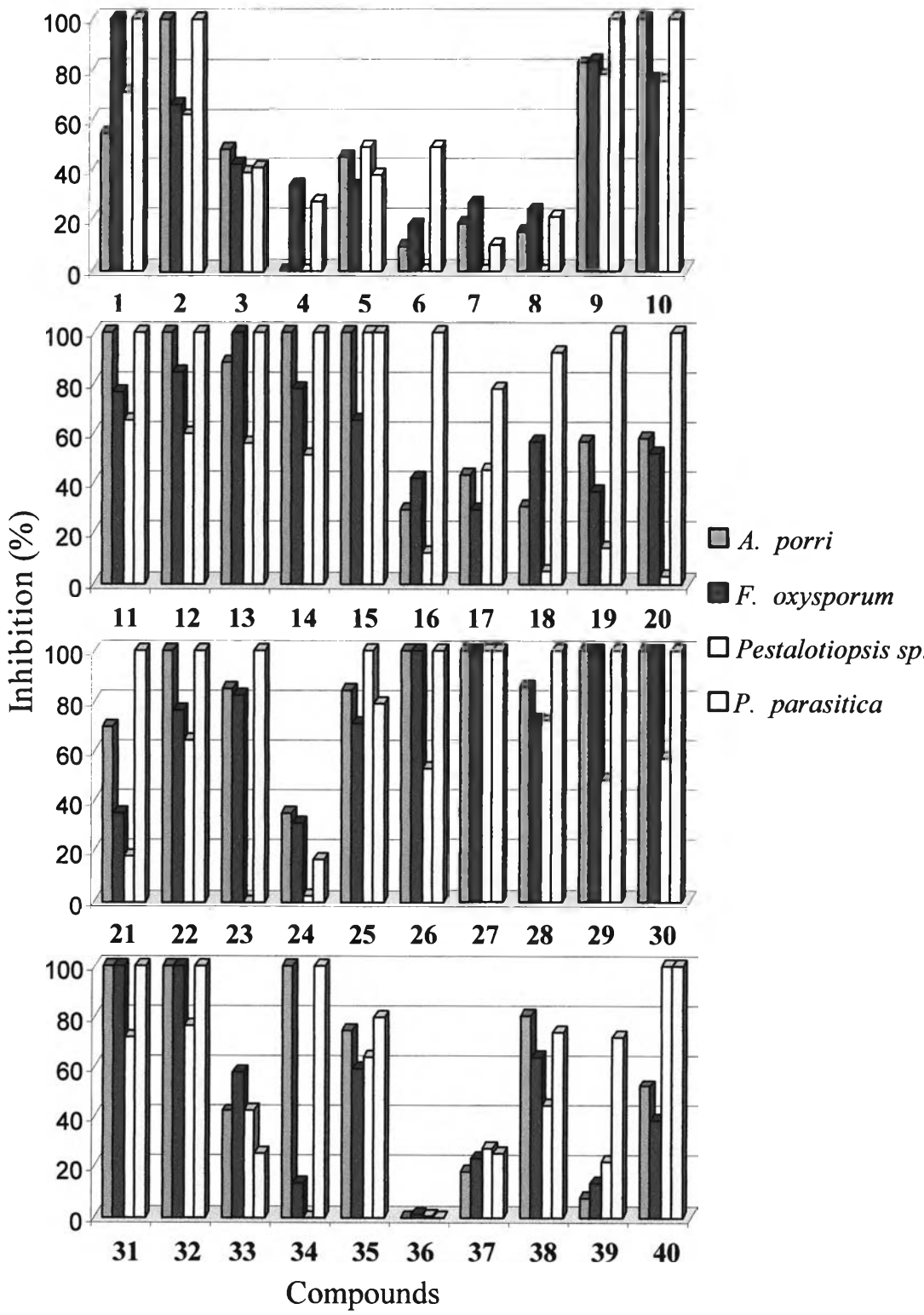


Fig. 3.1 Structure-antifungal activity of benzoic acid derivatives and related compounds assayed at 5 mM final concentration on the mycelial growth of *A. porri*, *F. oxysporum*, *Pestalotiopsis* sp. and *P. parasitica*

The mycelial growth inhibition percentages of these compounds were calculated and it was found that the most effective compound was cinnamaldehyde, which exhibited 100% of mycelial growth inhibition on all tested fungi at 5 mM (Fig. 3.1, no.27).

The screening for mycelial growth inhibition activity against *A. porri* revealed that fourteen compounds (Table 3.1, no.2, 10-12, 14-15, 22, 26-27, 29-32 and 34) exhibited complete inhibition. Twelve compounds (Table 3.1, no.1, 9, 13, 18-21, 23, 25, 28, 35, 38 and 40) exhibited medium mycelial growth inhibition more than 50%. Twelve compounds (Table 3.1, no.3, 5-8, 16-18, 24, 33, 37 and 39) exhibited slight inhibition (less than 50% inhibition) and two compounds (no.4 and 36) did not show significant inhibition activity.

The antifungal activity against *F. oxysporum* revealed that eight compounds (Table 3.1, no.1, 13, 26-27 and 29-32) exhibited complete inhibition. Sixteen compounds (Table 3.1, no.2, 9-12, 14, 15, 18, 20, 22, 23, 25, 28, 33, 35 and 38) exhibited moderate mycelial growth inhibition (more than 50% inhibition) and fourteen compounds (Table 3.1, no.3-8, 16, 17, 19, 21, 24, 34, 36, 37, 39 and 40) displayed slight inhibition (less than 50% inhibition).

The antifungal activity against *Pestalotiopsis sp.* revealed that four compounds (Table 3.1, no.15, 25, 27 and 40) exhibited complete inhibition. Fifteen compounds (Table 3.1, no.1, 2, 9-14, 22, 26, 28, 30-32 and 35) exhibited moderate mycelial growth inhibition (more than 50% inhibition). Fifteen compounds (Table 3.1, no.3, 5, 16-21, 24, 29 and 36-39) displayed slight inhibition (less than 50% inhibition) and six compounds (Table 3.1, no.4, 6-8, 23 and 34) did not show significant inhibition activity.

The antifungal activity against *P. parasitica* revealed that twenty four compounds (Table 3.1, no.1, 2, 9-16, 19-23, 26-32, 34 and 40) exhibited complete inhibition. Six compounds (Table 3.1, no.17, 18, 25, 35, 38 and 39) exhibited moderate mycelial growth inhibition (more than 50% inhibition). Nine compounds (no.3-8, 24, 33 and 37) exhibited slight inhibition (less than 50% inhibition) and one compound (no.36) did not show significant inhibition activity.

Although, *A. porri*, *F. oxysporum* and *Pestalotiopsis sp.* are three representatives of higher fungi, they are susceptible to distinct compound. The data derived from Fig.3.1 manifestly showed that *Pestalotiopsis sp.* was more resistant to antifungal agents than *A.*

porri and *F. oxysporum*. In addition, *P. parasitica* revealed more susceptible to chemicals in medium than *A. porri*, *F. oxysporum* and *Pestalotiopsis sp.*

3.2 Chemical structure-antifungal activity relationship of benzoic acid derivatives and related compounds

The results given in Fig. 3.1 also indicate a potential relationship between the chemical structure and the experimentally determined antifungal activity of the tested compounds against four phytopathogenic fungi. The functional group and the position of the functional group in aromatic ring were also shown to play an important role in the antifungal activity of tested compounds.

3.2.1 Effects of the substituent on the benzene ring

The data from Fig.3.1 pointed out that the addition of a supplementary hydroxyl group in various positions on the benzene ring of benzoic acid (Table 3.1, no.2-4) suppressed the antifungal activity on all tested fungi (except for salicylic acid (Table 3.1, no.2), which completely inhibited mycelial growth of *A. porri*). Further study upon the addition of two or three hydroxyl groups (Table 3.1, no.5-8) did not significantly improve the effectiveness. On the contrary, the addition of a chlorine in the 2 or 3 position (Table 3.1, no.9 and 10) increased the mycelial growth inhibition on *A. porri* and *Pestalotiopsis* sp. The addition of a trifluoromethyl or a methyl in the 3-position (Table 3.1, no.11 and 12) did not improve the mycelial growth inhibition on *F. oxysporum* and *Pestalotiopsis* sp. but completely inhibited mycelial growth of *A. porri*.

The 4-methoxy derivative (Table 3.1, no.13) showed a similar activity to benzoic acid. The 3,4-methylenedioxy derivative (Table 3.1, no.14) had a low inhibitory effect on mycelial growth of *F. oxysporum* and *Pestalotiopsis* sp. Interestingly, 2-naphthoic acid (Table 3.1, no.15) completely inhibited mycelial growth of *A. porri, Pestalotiopsis* sp. and *P. parasitica.* This intriguing point was certainly called for further investigation.

3.2.2 Effects of chain length and functional group

Increasing the length of carbon chain and introduction of a double bond in the aliphatic carbon chain bearing the carboxylic acid function as in cinnamoyl moiety (Table 3.1, no.16 and 17) reduced the efficiency of the molecule as antifungal agent. Modification of carboxylic acid function to an aldehyde (Table 3.1, no.19-21, 26 and 27) increased the biological activity of the original structure, for example, the antifungal activity against *A. porri*, *F. oxysporum* and *Pestalotiopsis sp.* of cinnamic acid (Table 3.1, no.16) was less than 50% inhibition, while that on all tested fungi of cinnamaldehyde (Table 3.1, no.27) was 100% inhibition. In contrast, allylbenzene and 4-allylanisole (Table 3.1, no.36 and 37) exhibited poor antifungal activity. Interestingly, lipophilic compounds, such as esters of 4-hydroxybenzoic acid (Table 3.1, no.29-32) dramatically increased the biological activity of 4-hydroxybenzoic acid by completely suppressed mycelial development of *A. porri*, *F. oxysporum* and *P. parasitica*. This finding, however, was different from that reported in 2002 that methyl esters of salicylic acid and 3-benzoic acid did not show biological activity against *Eutypa lata* (Amborabe *et al.*, 2002).

3.3 Further study on antifungal activity of cinnamaldehyde

Further evaluation of antifungal activity of cinnamaldehyde on all tested fungi was determined by agar medium assay and their IC_{50}'s were calculated. Cinnamaldehyde was diluted to the final concentration of 0.5, 1, 2.5 and 5 mM. A mycelial disc inoculated in the center of each Petri dish, and the fungi were allowed to grow. The results are presented in Table 3.2.

Table 3.2 Effects of cinnamaldehyde as a function of concentration on the mycelial growth of phytopathogenic fungi on a solid culture medium

Fungi tested	Growth inhibition (%)				IC_{50} [a]
	0.5 mM	1.0 mM	2.5 mM	5 mM	(mM)
A. porri	14	40	100	100	1.09
F. oxysporum	0	4	100	100	1.46
Pestalotiopsis sp.	0	0	93	100	2.02
P. parasitica	90	100	100	100	0.36

[a] Inhibition percentage of each concentration was calculated as IC_{50} using Probit analysis program

Cinnamaldehyde exhibited high antifungal activity. It completely suppressed *A. porri, F. oxysporum* and *P. parasitica* at 2.5 mM and nearly completely suppressed *Pestalotiopsis sp.* at the same concentration. The IC_{50} values of cinnamaldehyde against *A. porri, F. oxysporum, Pestalotiopsis sp.* and *P. parasitica* were 1.09, 1.46, 2.02 and 0.36 mM, respectively.

Cinnamaldehyde has been reported to have antifungal property against several phytopathogenic fungi. Dipping tulip bulbs in an aqueous solution of 3.9 mM cinnamaldehyde gave a 40-fold reduction of the fungal population but treatment of tulip bulbs with cinnamaldehyde had no effect on the total stalk length or the flowering capacity of tulip bulbs (Smid *et al.*, 1995). In addition, 30 ppm cinnamaldehyde completely inhibited mycelial growth and germination of conidia of *Botryodiplodia theobromae*, *Colletotrichum gloeosporioides* and *Gliocephalotrichum microchlamydosporum* which cause the postharvest diseases, stem-end rot, anthracnose and brown spot, respectively (Sivakumar *et al.*, 2002). Moreover, treatment of tomatoes with an aqueous solution of 13 mM cinnamaldehyde reduced the number of bacteria and fungi by one order of magnitude within 10 and 30 min, respectively. With tomatoes that had been treated for 30 min with cinnamaldehyde, visible mould growth was delayed by seven days during storage under modified atmosphere conditions at 18°C (Smid *et al.*, 1995).

3.4 Further study on antifungal activity of esters of 4-hydroxybenzoic acid

In continuing search for antifungal agents, methyl, ethyl, propyl, butyl, hexyl, octyl and dodecyl 4-hydroxybenzoates were tested against *A. porri*, *Pestalotiopsis* sp. and *P. parasitica*, in order to investigate the structure –activity relationship on the length of alkyl chain. The esters (hexyl, octyl and dodecyl 4-hydroxybenzoate) were synthesized by refluxing 4-hydroxybenzoic acid with corresponding alcohols as described in Chapter II. In addition, the antifungal effect of esters of 4-hydroxybenzoic acid was clearly observed when applied at a concentration of 1 mM. The results are presented in Fig 3.2.

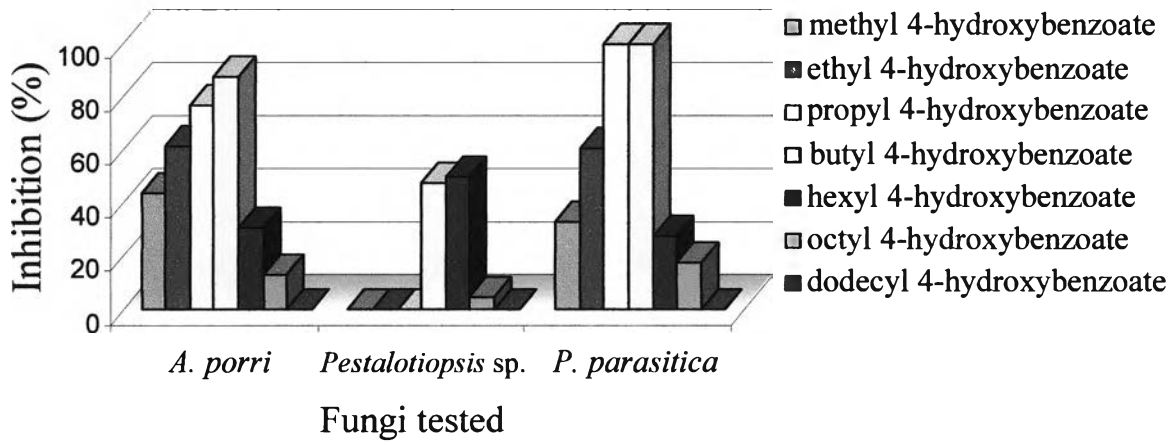


Fig. 3.2 Effects of esters of 4-hydroxybenzoic acid assayed at 1 mM final concentration on the mycelial growth of phytopathogenic fungi on a solid culture medium

In terms of structure activity relationship, among seven tested 4-hydroxybenzoates, butyl 4-hydroxybenzoate showed generally good activity against all tested fungi. The inhibition percentage of butyl 4-hydroxybenzoate was 87% for *A. porri*, 47% for *Pestalotiopsis* sp. and 100% for *P. parasitica*, while the inhibition percentage of dodecyl 4-hydroxybenzoic acid was 0% for all tested fungi.

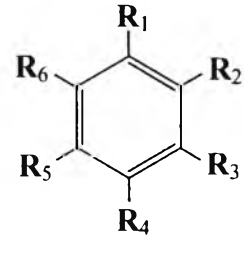
The length of alkyl group in 4-hydroxybenzoate was associated with their antifungal activity, similar to those found in the esters of gallic acid, which found that the potency of antifungal activity of the gallates against microorganisms depend on their alkyl chain length (Fujita and Kubo, 2002). On the basis of the results obtained, it appears that the fungicidal activity of 4-hydroxybenzoate was distinctly increased for every additional

CH_2 group. The antifungal activity disappeared after the alkyl length reached the maximum, and the dodecyl group in dodecyl 4-hydroxybenzoic acid is beyond this point. On the other hand, propyl group in propyl 4-hydroxybenzoate is not long enough to induce the best activity.

3.5 Fungal growth inhibition by cinnamic acid derivatives

From the results given in Fig.3.1, cinnamic acid exhibited slight mycelial growth inhibition. Thus, it was interesting to test whether the antifungal activity could be improved if the initial cinnamic acid structure was modified. Therefore, the effect of cinnamic acid derivatives (Table 3.3) on mycelial growth was studied by screening test at the concentration of 5 mM with the agar medium assay using PDA as the growth medium for all tested fungi and the results are displayed in Fig 3.3.

Table 3.3 Cinnamic acid derivatives used in this study



no.	compound	R_1	R_2	R_3	R_4	R_5	R_6
41	cinnamic acid	CH=CH-COOH	H	H	H	H	H
42	3-fluorocinnamic acid	CH=CH-COOH	H	F	H	H	H
43	4-fluorocinnamic acid	CH=CH-COOH	H	H	F	H	H
44	2-chlorocinnamic acid	CH=CH-COOH	Cl	H	H	H	H
45	3-chlorocinnamic acid	CH=CH-COOH	H	Cl	H	H	H
46	4-chlorocinnamic acid	CH=CH-COOH	H	H	Cl	H	H
47	2-bromocinnamic acid	CH=CH-COOH	Br	H	H	H	H
48	3-bromocinnamic acid	CH=CH-COOH	H	Br	H	H	H
49	4-bromocinnamic acid	CH=CH-COOH	H	H	Br	H	H
50	2,4-dichlorocinnamic acid *	CH=CH-COOH	Cl	H	Cl	H	H

Table 3.3 (continued)

no.	compound	R_1	R_2	R_3	R_4	R_5	R_6
51	2,6-dichlorocinnamic acid	CH=CH-COOH	Cl	H	H	H	Cl
52	3,4-dichlorocinnamic acid *	CH=CH-COOH	H	Cl	Cl	H	H
53	2-chloro-6-fluorocinnamic acid	CH=CH-COOH	Cl	H	H	H	F
54	2-methoxycinnamic acid	CH=CH-COOH	OCH_3	H	H	H	H
55	3-methoxycinnamic acid	CH=CH-COOH	H	OCH_3	H	H	H
56	4-methoxycinnamic acid	CH=CH-COOH	H	H	OCH_3	H	H
57	4-butyloxycinnamic acid *	CH=CH-COOH	H	H	OC_4H_9	H	H
58	4-hexyloxycinnamic acid *	CH=CH-COOH	H	H	OC_6H_{13}	H	H
59	4-octyloxycinnamic acid *	CH=CH-COOH	H	H	OC_8H_{17}	H	H
60	4-benzyloxycinnamic acid *	CH=CH-COOH	H	OH	OBn	H	H
61	4-phenoxycinnamic acid	CH=CH-COOH	H	H	OPh	H	H
62	3-methoxy-4-butyloxycinnamic acid	CH=CH-COOH	H	OCH_3	OC_4H_9	H	H
63	3-methoxy-4-hexyloxycinnamic acid	CH=CH-COOH	H	OCH_3	OC_6H_{13}	H	H
64	3-methoxy-4-octyloxycinnamic acid	CH=CH-COOH	H	OCH_3	OC_8H_{17}	H	H
65	3-methoxy-4-benzyloxycinnamic acid	CH=CH-COOH	H	OCH_3	OBn	H	H
66	2,3-dimethoxycinnamic acid	CH=CH-COOH	OCH_3	OCH_3	H	H	H
67	2,4-dimethoxycinnamic acid	CH=CH-COOH	OCH_3	H	OCH_3	H	H
68	2,5-dimethoxycinnamic acid	CH=CH-COOH	OCH_3	H	OH	OCH_3	H
69	3,4-dimethoxycinnamic acid	CH=CH-COOH	H	OCH_3	OCH_3	H	H
70	3,5-dimethoxycinnamic acid	CH=CH-COOH	H	OCH_3	H	OCH_3	H
71	3,4,5-trimethoxycinnamic acid	CH=CH-COOH	H	OCH_3	OCH_3	OCH_3	H

Table 3.3 (continued)

no.	compound	R_1	R_2	R_3	R_4	R_5	R_6
72	3,4-methylenedioxycinnamic acid	CH=CH-COOH	H	O-CH_2-O		H	H
73	2-nitrocinnamic acid	CH=CH-COOH	NO_2	H	H	H	H
74	3-nitrocinnamic acid	CH=CH-COOH	H	NO_2	H	H	H
75	4-nitrocinnamic acid	CH=CH-COOH	H	H	NO_2	H	H
76	2-chloro-5-nitrocinnamic acid	CH=CH-COOH	Cl	H	H	NO_2	H
77	3-nitro-4-chlorocinnamic acid	CH=CH-COOH	H	NO_2	Cl	H	H
78	2-nitro-5-chlorocinnamic acid	CH=CH-COOH	NO_2	H	H	Cl	H
79	4-methylcinnamic acid	CH=CH-COOH	H	H	CH_3	H	H
80	4-*sec*-propylcinnamic acid	CH=CH-COOH	H	H	$CH(CH_3)_2$	H	H
81	4-*tert*-butylcinnamic acid	CH=CH-COOH	H	H	$C(CH_3)_3$	H	H
82	4-trifluoromethylcinnamic acid	CH=CH-COOH	H	H	CF_3	H	H
83	4-cyanocinnamic acid	CH=CH-COOH	H	H	CN	H	H
84	*alpha*-methylcinnamic acid	CH=C(CH_3)-COOH	H	H	H	H	H
85	*alpha*-methyl-*trans*-cinnamaldehyde	CH=C(CH_3)-COH	H	H	H	H	H
86	cinnamonitrile	CH=CH-CN	H	H	H	H	H
87	2-methoxycinnamaldehyde	CH=CH-COH	OCH_3	H	H	H	H
88	cinnamamide	CH=CH-$CONH_2$	H	H	H	H	H
89	methyl *trans*-cinnamate	CH=CH-CO_2CH_3	H	H	H	H	H
90	*trans*-1-cinnamyl piperazine	CH=CH-C-$C_4H_9N_2$	H	H	H	H	H

* slightly soluble in medium containing 0.4% DMSO

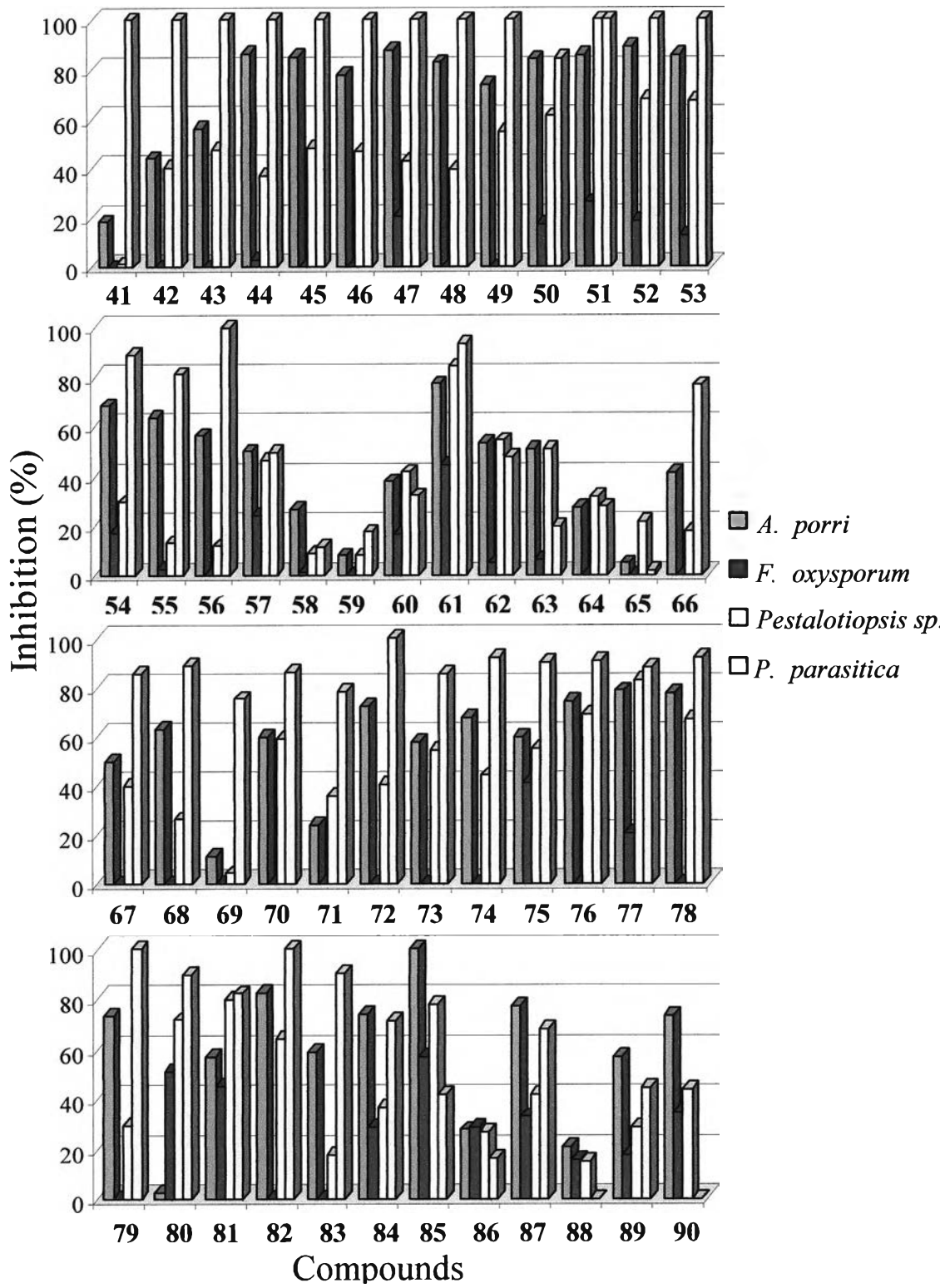


Fig. 3.3 Structure-antifungal activity of cinnamic acid derivatives assayed at 1 mM final concentration on the mycelial growth of *A. porri, F. oxysporum, Pestalotiopsis* sp. and *P. parasitica*

The results of the screening test indicate that only one compound (Table 3.3, no.85) exhibited complete inhibition and twenty compounds (Table 3.3, no.44-53, 61, 72, 76-79, 82, 84, 87 and 90) exhibited strong mycelial growth inhibition (more than 70% inhibition) in the case of *A. porri*, while eighteen compounds (Table 3.3, no.42, 43, 54-57, 62, 63, 66-68, 70, 73-75, 81, 83 and 89) exhibited moderate inhibition and eleven compounds (Table 3.3, no.41, 58-60, 64, 65, 69, 71, 80, 86 and 88) exhibited slight mycelial growth suppression (less than 40% inhibition).

The screening for mycelial growth inhibition activity against *F. oxysporum* revealed that five compounds (Table 3.3, no.61, 75, 80, 81 and 85) exhibited moderate inhibition, while twenty compounds (Table 3.3, no.44, 47, 50-55, 57, 60, 62, 63, 77, 84 and 86-90) exhibited slight inhibition and twenty five compounds (Table 3.3 no.41-43, 45, 46, 48, 49, 56, 59, 64-74, 76, 78, 79, 82 and 83) did not show significant inhibition activity.

The antifungal activity against *Pestalotiopsis* sp. revealed that only one compound (Table 3.3, no.51) exhibited complete inhibition. Five compounds (Table 3.3, no.61, 77, 80, 81 and 85) exhibited strong mycelial growth inhibition. Twenty four compounds (Table 3.3, no.42, 43, 45-47, 49, 50, 52, 53, 57, 60, 62, 63, 70, 72-76, 78, 80, 81, 87 and 90) exhibited moderate inhibition and twenty compounds (Table 3.3, no.41, 44, 48, 54-56, 58, 59, 64-69, 71, 79, 83, 84, 86, 88 and 89) exhibited slight inhibition.

The antifungal activity against *P. parasitica* revealed that sixteen compounds (Table 3.3, no.41-49, 51-53, 56, 72, 79 and 82) exhibited complete inhibition. Twenty one compounds (Table 3.3, no.50, 54, 55, 61, 66-71, 73-78, 80, 81, 83, 84 and 87) exhibited strong mycelial growth inhibition. Four compounds (Table 3.3, no. 57, 62, 85 and 89) exhibited moderate inhibition. Seven compounds (Table 3.3, no.58-60, 63-65 and 86) exhibited slight inhibition and two compounds (Table 3.3 no.88 and 90) did not show significant inhibition activity.

3.6 Chemical structure-antifungal activity relationship of cinnamic acid derivatives

In order to determine the most efficient compounds in this chemical family expressing antifungal properties, a study dealing with a structure-activity relationship of various molecular arrangements was carried out. Following this screening, involving the

determination of the antifungal efficiency of 43 compounds, some structure features have emerged which affect the antifungal properties against the tested fungi.

3.6.1 Effects of the substituent on the benzene ring

According to the results obtained in Fig 3.3, it was disclosed that the substitution of various groups on the benzene ring was also shown to play a vital role in the antifungal activity on all tested fungi of cinnamic acid derivatives. Chlorinated derivatives also showed strong activity and may be suitable compounds for an application to plants. In particular, 2,4-dichlorocinnamic acid, 2,6-dichlorocinnamic acid and 3,4-dichlorocinnamic acid (Table 3.3, no.50-52) could be put forward as candidates. 2,6-Dichlorocinnamic acid indeed induced a 86% growth inhibition on *A. porri* and completely suppressed mycelial development of *Pestalotiopsis* sp. and *P. parasitica*. In addition, the addition of second chlorine atom was found to increase the biological activity compared with other chlorocinnamic acid derivatives (Table 3.3, no.44-46).

The substitution of other halogen atom, such as fluorine and bromine atom on the benzene ring also increased the biological activity of original structure (Table 3.3, no.42, 43, 47-49, 53). In agreement with this, (Narasimhan *et al.*, 2004) have shown that dibromo cinnamic acid exhibited strong antibacterial activity against Gram positive and Gram negative bacteria and good antifungal activities on *Candida albicans* and *Aspergillus niger*. A methoxy in 2-position (Table 3.3, no.54) showed more activity on *A. porri*, *F. oxysporum* and *Pestalotiopsis* sp. than a methoxy in 3 and 4-positions (Table 3.3, no.55, 56). Octyl group (Table 3.3, no.59) in the forth position of the aromatic ring strongly decreased the antifungal property of a compound in comparison with other oxygen-containing compounds as methoxyl, butyloxyl, hexyloxyl, benzyloxyl and phenoxyl group (Table 3.3, no.56-58, 60, 61) at the same position. A decreasing inhibitory action of 3-methoxycinnamic acid on *A. porri* and *Pestalotiopsis* sp., from 54% to 5% and 55 to 22%, respectively followed the addition at the 4-position by oxygen containing group in the series butyloxyl > hexyloxyl > octyloxyl > benzyloxyl (Table 3.3 no.62-65).

The addition of a second methoxyl group reduced the biological activity of 2-methoxyl derivative on *A. porri*, *F. oxysporum* and *P. parasitica* (Table 3.3, no.66-68).

Similarly, the addition of a second methoxyl group also reduced the biological activity of 3-methoxyl derivative on *A. porri*. Compared to nitro derivatives (Table 3.3, no.73-75), the derivatives bearing a nitro group and a chlorine atom (Table 3.3, no. 76-78) exhibited more inhibitory activity against *A. porri* and *Pestalotiopsis* sp. Interestingly, 4-trifluoromethylcinnamic acid (Table 3.3 no.82) exhibited the strongest activity against *A. porri* amongst the fifteen cinnamic derivatives, which obtained by substitution of various groups in 4-position on the benzene ring.

3.6.2 Effects of other functional groups

From the results of the modifications on aliphatic chain in cinnamoyl moiety (Table 3.3 no.84-90), it is evident that *alpha*-methyl-*trans*-cinnamaldehyde (Table 3.3, no.85) completely suppressed mycelial development of *A. porri* and exhibited good antifungal properties on *Pestalotiopsis* sp. and *F. oxysporum*. On the other hand, it is surprising that modification of the carboxylic acid function reduced the biological activity on *P. parasitica* of the original structure. Indeed, both cinnamamide (Table 3.3, no.88) and *trans*-1-cinnamyl piperazine (Table 3.3, no.90) did not show biological activity.

3.7 Further study on biological activity and stability of 2,6-dichlorocinnamic acid

Following the general antifungal screening tests, it was obviously indicated with sufficiently encouraging effect that 2,6-dichlorocinnamic acid was a potent candidate as a new emerged antifungal agent. Thus, this compound was selected for an extensive evaluation of its biological effects and stability.

3.7.1 Effect of 2,6-dichlorocinnamic acid on the mycelial growth of *A. porri*, *Pestalotiopsis* sp. and *P. parasitica*

Further evaluation of antifungal activity of 2,6-dichlorocinnamic acid was determined by agar medium assay and the IC_{50} was calculated. Each tested compound was diluted to the final concentration of 0.1, 0.5 and 1 mM. The results are tabulated in Tables 3.4 and 3.5.

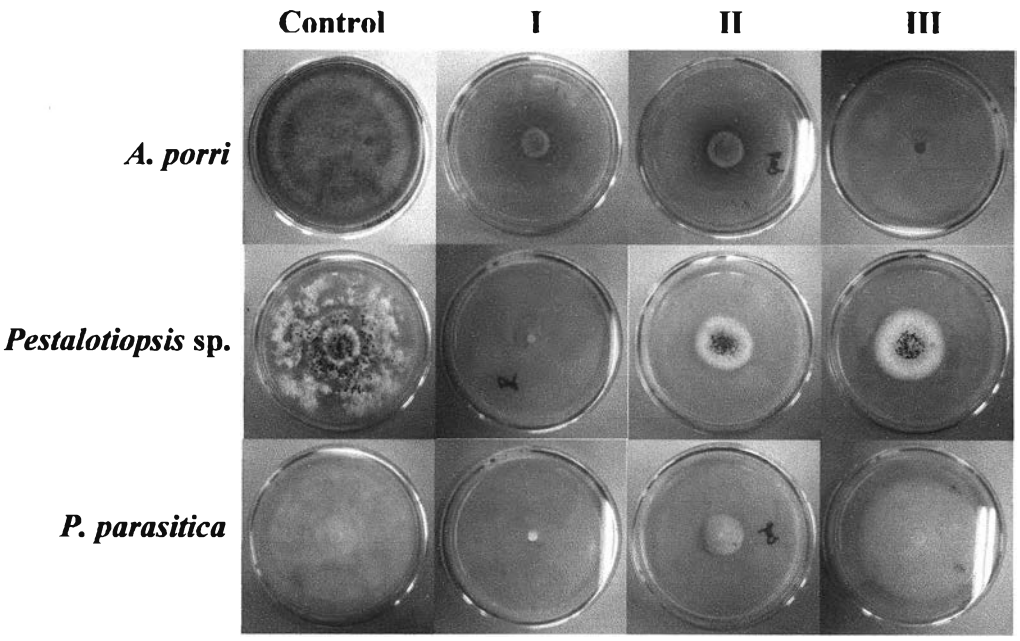


Fig 3.4 The mycelial growth inhibition of 2,6-dichlorocinnamic acid (**I**), captan (**II**), Iprodione (**III**) at 1 mM

Table 3.4 Effects of 2,6-dichlorocinnamic acid and two standard fungicides as a function of concentration on the mycelial growth of *A. porri*, *Pestalotiopsis* sp. and *P. parasitica* on a solid culture medium.

Compound	Fungi tested	Growth inhibition (%)			IC_{50} [a] (mM)
		0.1 mM	0.5 mM	1 mM	
2,6-Dichlorocinnamic acid	*A. porri*	40.59	83.60	87.83	0.14
	Pestalotiopsis sp.	28.60	74.49	100.00	0.28
	P. parasitica	81.30	89.72	100.00	0.07
captan	*A. porri*	5.93	37.37	73.97	0.70
	Pestalotiopsis sp.	9.63	67.62	77.03	0.52
	P. parasitica	14.71	48.08	81.26	0.56
iprodione	*A. porri*	80.28	100.00	100.00	0.03
	Pestalotiopsis sp.	47.15	47.97	73.89	0.32
	P. parasitica	2.35	15.98	25.21	1.49

[a] Inhibition percentage of each concentration was calculated as IC_{50} using Probit analysis program

2,6-Dichlorocinnamic acid undoubtedly possessed fungicidal properties, resulting from considerable IC_{50} observed in *A. porri*, *Pestalotiopsis* sp. and *P. parasitica.* The conventional chemical fungicide captan and iprodione were used as the reference compounds. The IC_{50} of 2,6-dichlorocinnamic acid was 0.14 mM for *A. porri*, 0.28 mM for *Pestalotiopsis* sp. and 0.07 mM for *P. parasitica.* Compared to captan and iprodione, 2,6-dichlorocinnamic acid is much more efficient and therefore can be a superior antifungal agent, particularly to control *Pestalotiopsis* sp. and *P. parasitica.*

3.7.2 Effect of 2,6-dichlorocinnamic acid on the mycelial growth *Pythium* sp. and *Colletotrichum gloeosporioides.*

Antracnose (*C. gloeosporioides*) and Damping-off (*Pythium* sp.) are the major diseases of a wide variety of hosts in Thailand (Nongnoot, 2004). Further evaluation of

antifungal activity of 2,6-dichlorocinnamic acid on both fungi was determined by agar medium assay and the IC_{50} was calculated.

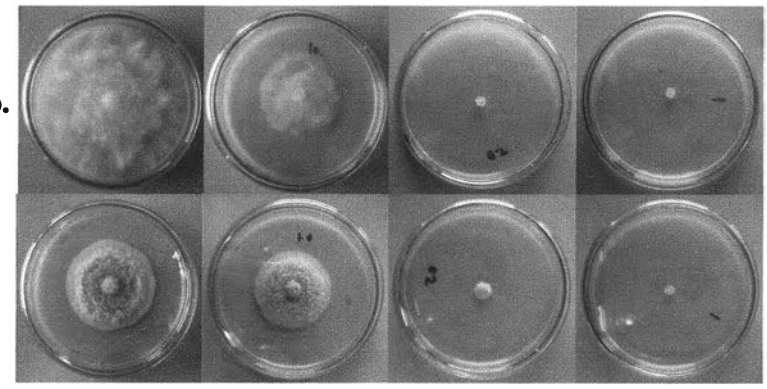


Fig 3.5 The mycelial growth inhibition of 2,6-dichlorocinnamic acid on *Pythium* sp. and *C. gloeosporioides*

Table 3.5 Effects of 2,6-dichlorocinnamic acid as a function of concentration on the mycelial growth of *Pythium* sp. and *C. gloeosporioides* on a solid culture medium.

Fungi tested	Growth inhibition (%)			IC_{50} [a] (mM)
	0.1 mM	0.5 mM	1 mM	
Pythium sp.	31.11	100	100	0.12
C. gloeosporioides	18.79	78.79	100	0.33

[a] Inhibition percentage of each concentration was calculated as IC_{50} using Probit analysis program

2,6-Dichlorocinnamic acid at 1 mM completely inhibited radial mycelial growth of both fungi. On the other hand, 2,6-dichlorocinnamic acid showed antifungal activity against *Pythium* sp. and *C. gloeosporioides* with IC_{50} of 0.12 and 0.33, respectively.

According to the results obtained in Tables 3.4 and 3.5, it is clear that 2,6-dichlorocinnamic acid has inhibitory effects against *A. porri*, *Pestalotiopsis* sp., *P. parasitica*, *Pythium* sp. and *C. gloeosporioides in vitro*.

3.7.3 Effect of 2,6-dichlorocinnamic acid on spore germination of *Pestalotiopsis* sp.

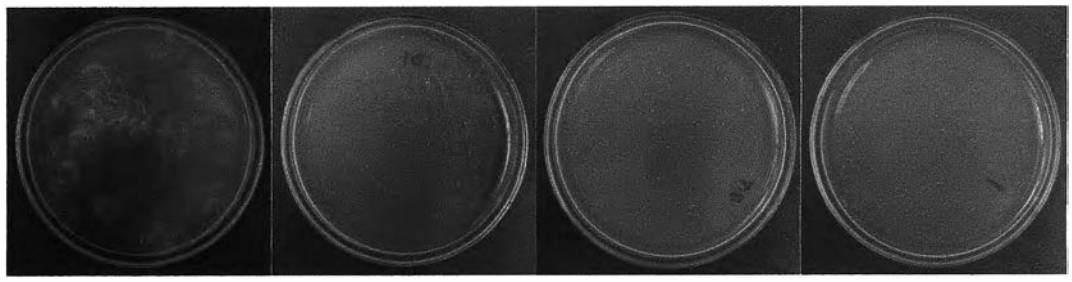
The antifungal activity on mycelial growth and spore germination greatly affects the costs and benefits of fungal disease control. If 2,6-dichlorocinnamic acid was active for inhibition of both mycelial growth and spore germination, it would then be able to control all the subsequent steps in the disease cycle.

Pestalotiopsis sp. are parasitic or endophytic on living leaves and twigs but are often isolated from dead plant matter and even soil, because the spore infest in plant debris or contaminate in environment can stay alive as resting spore when the condition optimized, resting spore will germinate germ tubes, attack host and cause plant disease (Chavarin, 2002). Therefore, 2,6-dichlorocinnamic acid was further evaluated for spore germination of *Pestalotiopsis* sp.

To test spore germination, 100 µl of the suspensions (5×10^3 spore per ml) were streaked aseptically on PDA plates, supplemented with different 2,6-dichlorocinnamic acid concentration (0, 0.1, 0.5, 1 mM). The plates were incubated at room temperature for 48 h. Following incubation, fungal colonies originated from germinated spores were enumerated in order to evaluate the percentage of inhibition of spore germination. The results are tabulated in Table 3.6.

Table 3.6 Effect of 2,6-dichlorocinnamic acid on spore germination of *Pestalotiopsis* sp.

concentration (mM)	%inhibition of spore germination
0	0
0.1	100
0.5	100
1	100



Control **0.1 mM** **0.5 mM** **1 mM**

Fig 3.6 Effect of 2,6-dichlorocinnamic acid at different concentrations on spore germination of *Pestalotiopsis* sp.

According to the results obtained in Tables 3.6, 2,6-dichlorocinnamic acid completely inhibited spore germination at a concentration of 0.1 mM. From this study it appears that 2,6-dichlorocinnamic acid treatment inhibited *in vitro* both spore germination and mycelial growth of *Pestalotiopsis* sp.

3.7.4 Phytotoxicity of 2,6-dichlorocinnamic acid

For phytotoxicity bioassays (according to the procedure described in Chapter II), Chinese mustard (*Brassica juncea* (Linn.) Czern. et Coss.) seeds were used. The effects of 2,6-dichlorocinnamic acid on relative root growth were analysed and the results are displayed in Fig 3.7.

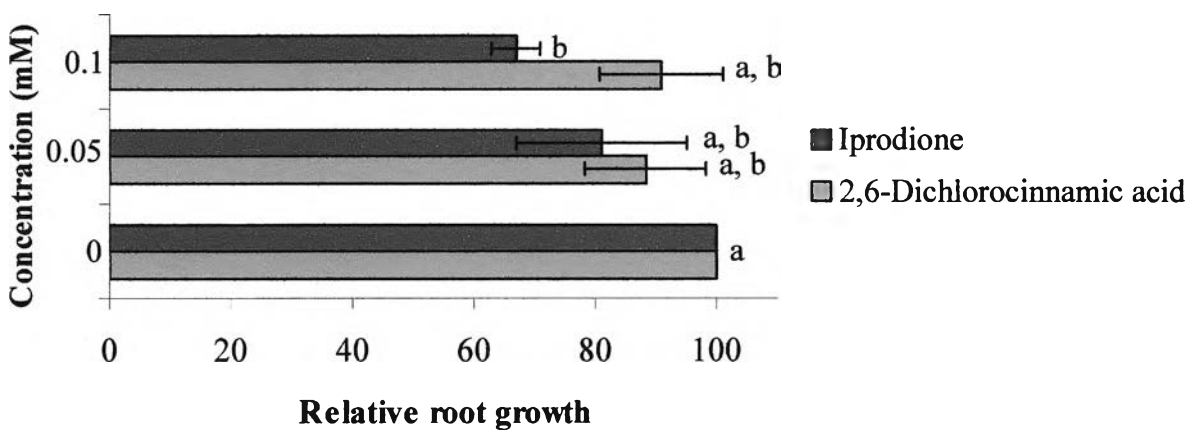


Fig 3.7 Relative root growth for Chinese mustard. Different letters (a, b) are significantly different at the level of $P<0.05$ according to the Scheffe test.

In all the cases, the percentage of germinated seeds with respect to the control was 100%. However, seed germination has been regarded as a less sensitive method than root elongation when used as a bioassay for the evaluation of phytotoxicity (Smid *et al.*, 1995).

As shown in Fig 3.7, conducting the growth in the solutions of 2,6-dichlorocinnamic acid (0.05 and 0.1 mM) for four days did not significantly alter the total root length of the seedling, while the relative root growth obtained with the solution of Iprodione (0.1 mM) was lower than those obtained in the control. Therefore, it can be concluded that under the experimental conditions, 2,6-dichlorocinnamic acid has potent antifungal effects but no evident phytotoxic effects.

3.7.5 Stability of 2,6-dichlorocinnamic acid

2,6-Dichlorocinnamic acid was evaluated under oven conditions at 80 °C, daylight and 256 wavelength UV light for a period of 8 h. Samples were removed at 0, 2, 4 and 8 h from the tested conditions. DMSO solutions of the each collected sample were prepared at concentration of 0.5 mM. Absorbance was measured with UV–Vis spectrophotometer at 279 nm and the results are displayed in Fig 3.8.

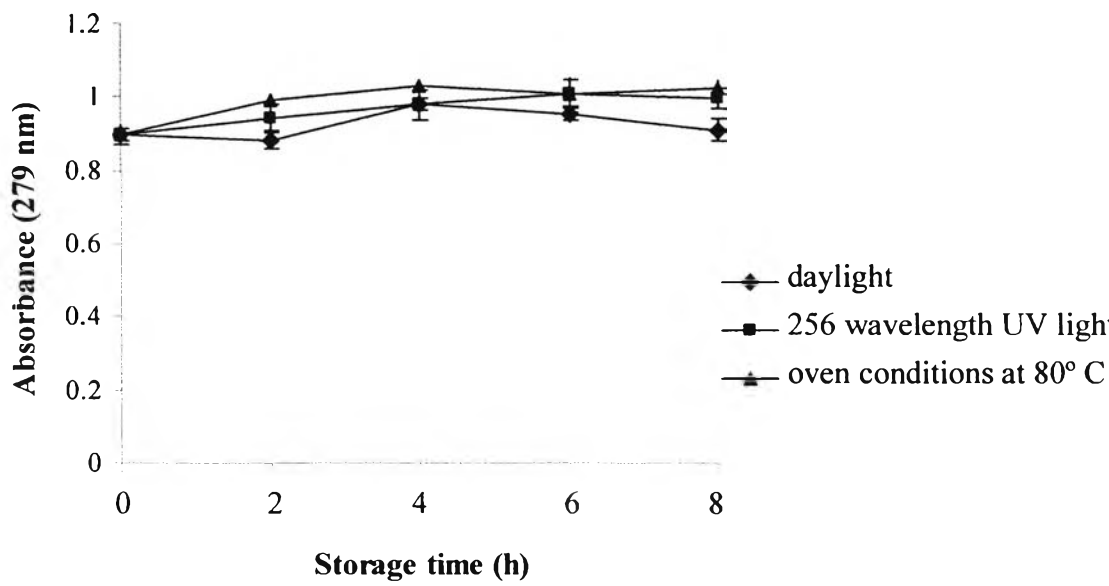


Fig 3.8 Effect of oven conditions at 80° C, daylight and 256 wavelength UV light on the stability of 2,6-dichlorocinnamic acid

The results of stability tests (Fig 3.8) clearly showed that at each condition, the A_{279} did not decrease with time. It can be concluded that 2,6-dichlorocinnamic acid had a higher thermal and light stability. The bioactivity and stability of 2,6-dichlorocinnamic acid suggest that it may present interest for use as fungicide.