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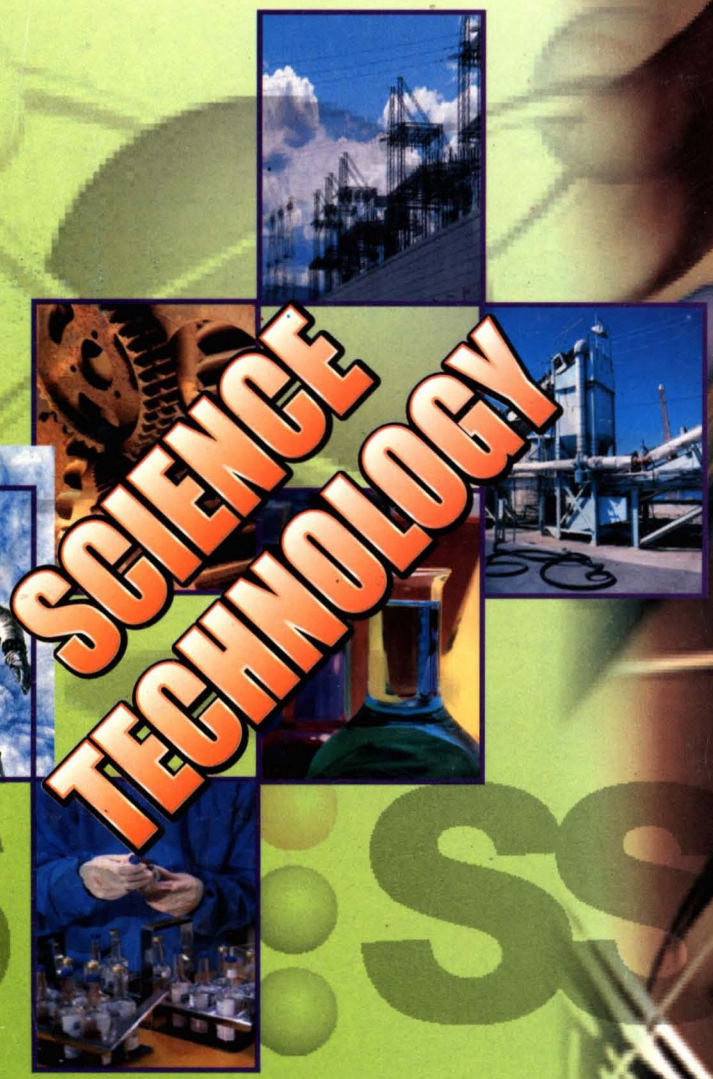

NATIONAL SEMINAR ON SCIENCE TECHNOLOGY & SOCIAL SCIENCES 2006

30-31 May 2006

Swiss Garden Resort & Spa

Kuantan, Pahang

The Effectiveness of Camalexin Against *Botrytis Cinerea*

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ABSTRACT

The antimicrobial activity and fungitoxicity of phytoalexin from Arabidopsis thaliana, camalexin against Botrytis cinerea had been studied in vitro. In a series of experiments, the activity of camalexin had been measured against spores and various types of germ tubes of B. cinerea. Activities were assessed based on the percentage of germination and mortality of germ tube besides germ tube development after challenged by camalexin. We found ungerminated conidia were more susceptible than older sporelings to the fungitoxic and inhibitory effects of camalexin. Higher concentrations of camalexin tested, produced significantly higher percentage of mortality and found to be more inhibitory compared to other lower concentrations tested for both conidia and germ tube.

Keywords: *Camalexin, Phytoalexin, Arabidopsis thaliana and Botrytis cinerea*

Introduction

Plants are constantly at the mercy of wind, rain, UV radiation, herbivores and microbial pathogens. Despite these challenges, they thrive in most places suggesting that they are much tougher than they look. In fact, plants have a variety of defensive strategies that are efficient at preventing and overcoming infection by pathogen (Snyder 2004). Pathogen invasion triggers activation of defense responses, including the synthesis of reactive oxygen species and signal molecules such as salicylic acid (SA), accumulation of antimicrobial metabolites, and expression of many defense-related genes, such as those encoding pathogenesis-related (PR) proteins (Zhou, Tootle and Glazebrook 1999). *Arabidopsis thaliana* is a well-established arsenal (Kliebenstein 2004). In recent years, *Arabidopsis* has been used in studying the impact of secondary metabolites in interaction with plant pathogens (Kliebenstein 2004). *Botrytis cinerea* is renowned for its broad host range, over 200 species can be infected (Ten Have *et al.* 1998). resulting in considerable economic losses.

Phytoalexins have been defined as low molecular weight antimicrobial compounds that are both synthesized and accumulate in plants after exposure to microorganism (Paxton 1981). Phytoalexins have been characterized from 31 plant families (Grayer and Harborne 1994). To some extent the chemical class of compound is related to the plant family (Strange 2003). Despite the fact that phytoalexins have been studied extensively for many years, little direct evidence indicates whether they make important contributions to plant defenses against particular pathogens (Zhou, Tootle and Glazebrook 1999). The only phytoalexin that has been detected in *Arabidopsis* is an indole derivative called camalexin (3-thiazol-2 methyl-indole). Accumulation of camalexin was found in tissue exposed to infection by either avirulent or virulent strains of the bacterium *Pseudomonas syringae* (Tsuji *et al.* 1992; Glazebrook and Ausubel 1994), and after elicited by silver nitrate (Mert-Turk 2003). Many observations support the proposal that camalexin plays a role in the *Arabidopsis* defense system after challenged by plant pathogens.

Although resistances of *Arabidopsis* to certain pathogens have been widely investigated but most studies have been concentrated on genetics of resistance rather than mechanism. Very little is known about camalexin antimicrobial activity and its fungitoxicity *in vitro*. In this study, we looked for the antifungal activity and fungitoxic effect of different concentration of camalexin on different stages of growth of *B. cinerea in vitro*.

Materials and Methods

Plant

Arabidopsis. Thaliana

Arabidopsis. thaliana ecotype Col-5 seeds were sown with three parts of Levington commercial peat compost and one part vermiculite. Ingredients were mixed and distributed into pots. After sowing, the pots were placed in tray of water to moisturise the mixture. Seed trays were then covered with aluminium foil for seed to vernalise and incubate at 4°C for 4-5 days. Trays were then transferred to a growth room with 10 hours photo period, a light intensity of 40 W/m^2 and a temperature of 20-21°C. After cotyledon development, seedlings were transplanted to individual pots (25

cm^2). Under such conditions the plants developed large extensive rosette leaves suitable for inoculation after 6-8 weeks.

Botrytis Cinerea

Botrytis cinerea, isolated originally from tomato, was obtained from a stock culture at Imperial College, Wye Campus and maintained on Petri dish plates of Potato Dextrose Agar (PDA). The medium was produced by suspending 3.9 g of PDA in 1000 ml sterilize double distilled water (SDDW) and autoclaved to sterilize at 121°C for 15 minutes. Suspensions of conidia were prepared by flooding sporulating cultures (7-10 days old) with 1/8 strength of Potato Dextrose Broth (PDB). PDB was prepared by adding 24 g of PDB into 1000ml of SDDW and autoclaved at 121°C for 15 minutes. The resultant suspension was filtered and washed twice. Conidia were pelleted by centrifugation at 3K rpm for 3 minutes using a Denley BS400 centrifuge. The concentration of spores used was adjusted to 2.5×10^5 ml^{-1} using a haemocytometer.

Spectrophotometry

A Philips SP 8-100 UV/Vis spectrophotometer was used to estimate camalexin concentration. Bandwidth was set to 0.5 nm with wavelength speed of 5 nm per second. Absorbance was set to 1 with chart speed 2 second per cm. Wavelength was turned to 400 nm. Wavelength drive, UV lamp and recorder were turned on to record the absorbance. Hellma Precision Cells of Quartz glass (Suprasil) with a light path of 10mm were used with the spectrophotometer.

Microscopy

Microscopical observations of fungal growth were made with a Zeiss Axioplans microscope. The camera used was a Zeiss Axiocam and the camera system was Axion Vision 3.0. Spore germination *in vitro* and germ tube length was measured with an eyepiece graticule at magnification 100X.

Data Analysis

Variant analysis (ANOVA) was used for data analysis with Genstat 7th edition software, if any significant differences found then comparison of means were checked with LSD at $\alpha = 5\%$.

Results and Discussions

Antifungal Activity of Camalexin

In order to examine the role of camalexin against *B. cinerea* it is essential to study the activity of the phytoalexin against the different types of fungal hyphae which invade *Arabidopsis* tissues. In a series of experiments, the activity of camalexin was measured against spores and various types of germ tube of *B. cinerea in vitro*. Effects on growth and also directly fungicidal activity were both examined.

Preparation of Different Concentrations of Camalexin

Purified camalexin was obtained from the established method (Chong 2005). In order to prepare high concentration of camalexin, because of poor solubility in water, the purified camalexin was dissolved in a small volume of ethanol (40 µl). The nutrient solution used, 1/8 PDB was added to the ethanolic solution to give a final volume of 2% ethanol. The actual concentrations of camalexin achieved after measured by UV spectrophotometry were 8.75, 16.25, 33.75 and 70 µg/ml.

Activity Against *B. Cinerea*

Four 10µl droplets of conidial suspension in 1/8 PDB were put on each new slide. To each droplet, 10µl of camalexin in different concentrations was added. The slides were incubated under high humidity in sandwich boxes for 18 hours. Then, lactophenol was added to each droplet to stop germ tube growth. Germination of 50 spores was checked randomly and 10 germ tubes length were measured for each droplet.

Germination

Only concentration with 16.88 and 35 µg/ml of camalexin caused significant reduction in spore germination compared to other concentrations (Table 1).

Table 1: Percentage germination of *B. cinerea* spores on glass in different concentrations of camalexin (µg/ml) after incubation for 18 hours

Concentration (µg/ml)	% germination
4.38	100±0.0
8.13	100±0.0
16.88	80±7.1
35.00	15±2.9

Germ Tube Elongation

A significant decrease in germ tube lengths was found with increasing concentration of camalexin (Fig 1). Germ tubes in the highest concentration tested, 35 µg/ml of camalexin only grew up to 1.7 µm. Even at the lowest concentration tested (4.38 µg/ml) the phytoalexin had an obvious effect on germ tube growth.

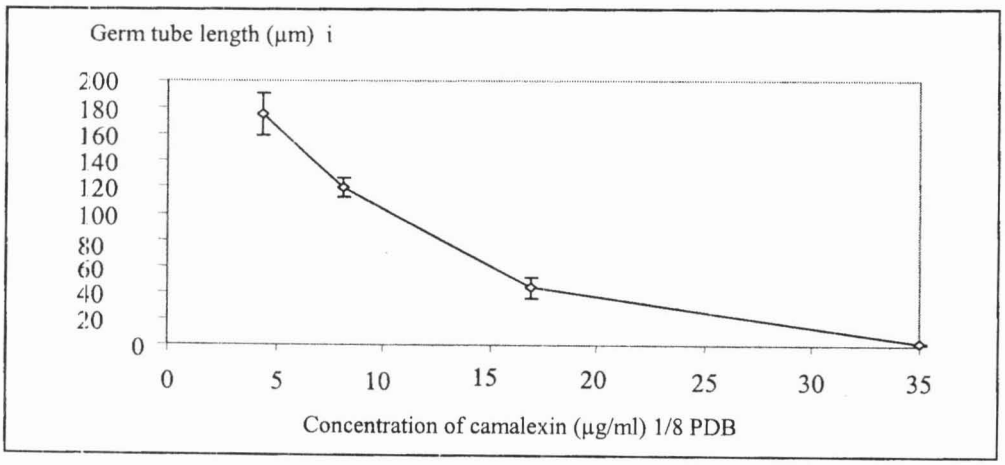

Fig 1: The development of *B. cinera* from conodia in different concentrations of camalexin in 1/8 PDB (incubated for 18 hours after addition). Daa for control is not shown as length immeasurable. All results are means from four replicates in which a total sporelings were measured.

Antifungal Activity of Camalexin Directly Against Sporelings of *B. Cinerea*

Use of spores for bioassays as in the previous experiment, does not directly reproduce the in planta conditions. In leaves, spores germinate in the absence of camalexin which appears to be induced after lesion formation. It was necessary to test effects directly on germ tubes. In this experiment spores were allowed to germinate to produce sporelings before challenge with camalexin. The antifungal activity of camalexin was tested against sporelings of *B. cinerea* at different stages of growth. For each concentration of camalexin used, four 10µl droplets of conidia suspension in 1/8 PDB were put on new slides and incubated under high humidity to allow the conidia to germinate and grow for 6 hours. Then, to each droplet, 10µl of camalexin solution at different concentration was added. After 6 hours, lactophenol was added to controls to kill the germ tube for a comparison with germ tube length exposed to camalexin. The slides were incubated under high humidity in sandwich boxes for another 6 hours. Then, lactophenol was added to each droplet which contained camalexin and 12 hours control to stop germ tube growth before measurement. The experiment was repeated but this time with conidia allowed to germinate and grow for 12 hours before the addition of camalexin and further incubated for 18 hours before measurement.

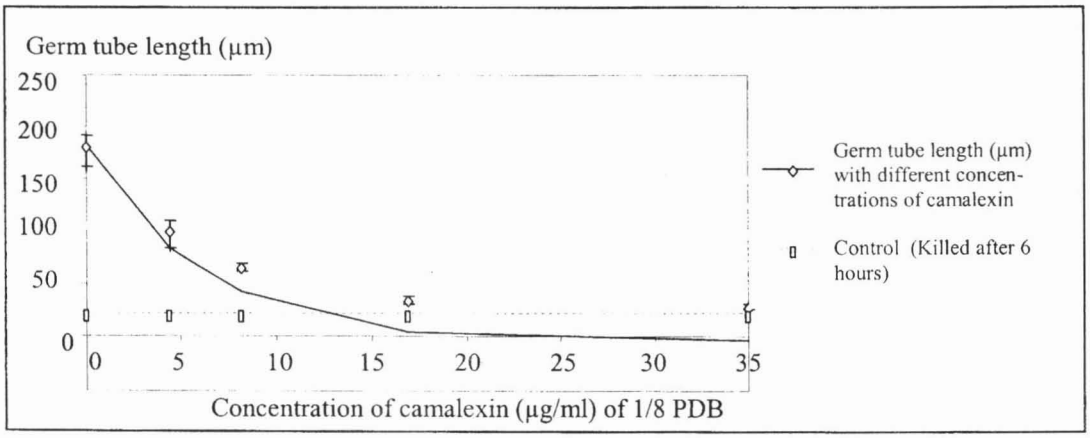

Fig 2: The development of *B. cinerea* germ tubes in different concentrations of camalexin in 1/8 PDB. Camalexin was added after 6 hours of germ tubes growth and then incubated for 6 hours. All results are means from four replicates in which a total sporelings were measured

After 6 hours of incubation, a significant decrease in germ tube length with the increasing concentration of camalexin in comparison to control was observed (Fig 2). Only concentration with 16.88 and 35 µg/ml of camalexin did not show any significant different in germ tubes length to each other. The highest concentration (35 µg/ml) tested in this experiment did not appear to stop the sporelings growth completely because sporelings in this concentration (35 µg/ml) had significantly longer germ tubes than controls.

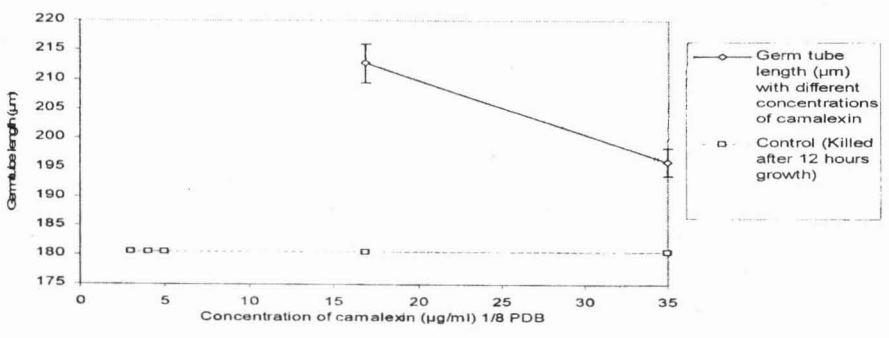

Fig 3: The development of *B. cinerea* germ tubes in different concentrations of camalexin in 1/8 PDB. Camalexin was added after 12 hours of germ tubes growth and then incubated for 18 hours. Data for other concentrations <16.88 µg/ml and controls after 18 hours were too long to measure accurately. All results are means from four replicates in which a total 40 sporelings were measured

In the plant, the timing of camalexin induction may be critical for resistance against invasion by *B. cinerea*. Older germ tube may more resistant to camalexin. A massive grown of longer sporelings in lower concentrations of camalexin (4.38 and 8.13 µg/ml) was observed. Higher concentrations (16.88 and 35 µg/ml) in this experiment showed a significant decrease of germ tubes length in comparison to control and respectively after 12 hours (Fig 3). Clearly larger sporelings were less sensitive to the phytoalexin.

The pattern of germ tube growth in the presence of camalexin was examined using 4.38 µg/ml of camalexin as the concentration was found to cause about 50% reduction in growth from 6 hours sporelings (see Fig 2).

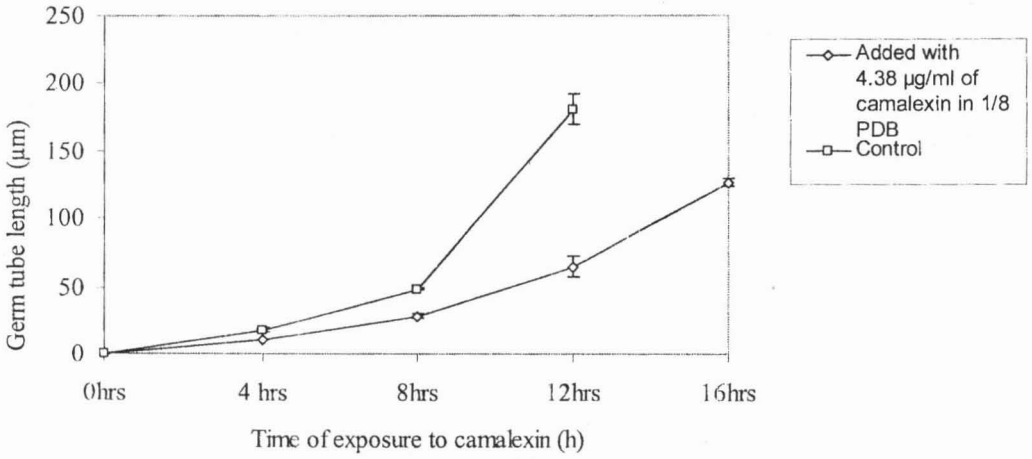

Fig. 4: The relationship between time exposure to camalexin towards sporelings of *B. cinerea*. Data for control at 16 hrs is not shown as length immeasurable. All results are means from four replicates in which a total 40 sporelings were measured

In this concentration (4.38 µg/ml) germ tube growth was found to increase significantly at every four hours interval from 0 hr to 16 hrs (Fig 4). There was no significant different between the germ tube lengths in this concentration compared with controls at 4 hour. But after 8 and 12 hours, germ tubes in this concentration were significantly shorter than controls.

Fungitoxicity of Camalexin

Camalexin proved to be a strong inhibitor of germ tube growth of *B. cinerea* at higher concentrations (16.88 and 35µg/ml). Further experiments were carried out to verify the fungitoxicity of camalexin by assessing the viability of fungal structures using aqueous trypan blue vital stain. Trypan blue stains only dead tissue and is an accurate indication of fungal cell viability in *B. cinerea* (Rossall, Mansfield and Huston 1980).

Effect of Camalexin Concentration and Stage of Fungal Growth on Fungitoxicity

A comparison was done to assess the fungitoxicity of camalexin against *B. cinerea* at various stages of growth. Experiments were set up as mentioned before to test effects against spores or germinated sporelings of different age. The replicates were incubated in sandwich boxes for 0, 6 and 12 hours before addition of different concentrations of camalexin. Then, after incubation for another 18 hours, fungal structures were stained with 0.5 % of trypan blue. Assessments were based on five categories which were: i) dead conidium, ii) dead conidium with live germ tube, iii) live conidium with dead germ tube, iv) live conidium with no germ tube and v) live conidium and germ tube. The mean percentage of cells killed was calculated from four replicates count on each treatment.

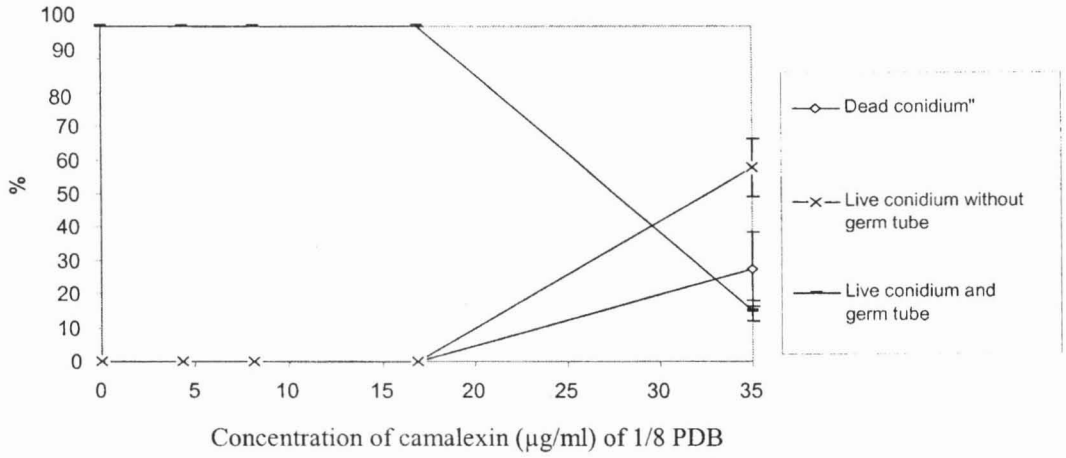

Fig. 5: Percentage cells killed of *B. cinerea* conidia after incubated for 18 hours in different concentrations of camalexin. All results are means from four replicates in which a total of 40 sporelings were measured.

Three patterns of *B. cinerea* conidia viability with direct addition of different concentrations of camalexin were observed after 18 hours (Fig 5). 27.5% with dead conidia in concentration with 35 µg/ml of camalexin and this were significantly higher compared to other concentrations and control (Fig 5). Although live conidia with dead germ tubes were also found, but the number were less than 1% and none were scored during the random count used to estimate the percentage of cells killed for Fig 5. No dead conidia with live germ tubes were found during the experiment. Camalexin at 35 µg/ml also scored only 15% of live conidia and germ tubes which were significantly lesser compared to 100% in all other concentrations and control. In the highest concentration tested, 57.5% of live conidia without germ tubes were also recorded. In summary, the highest concentration of camalexin tested did not kill 100% of the conidia of *B. cinerea* but strongly inhibiting their growth.

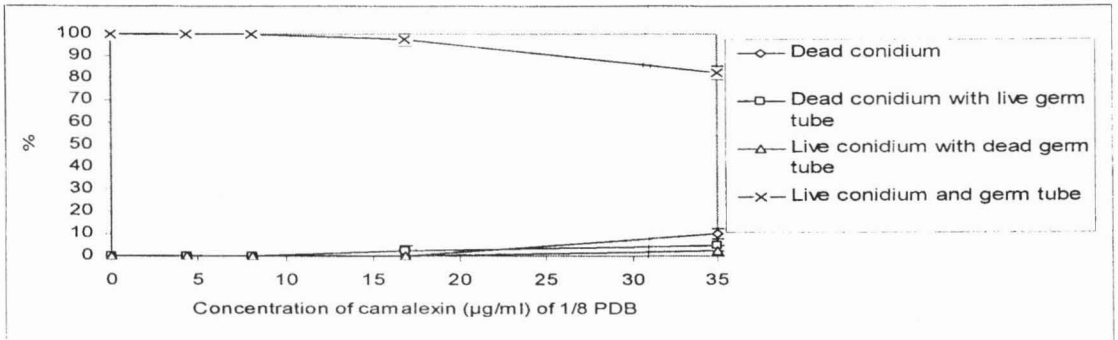

Fig 6: Pattern of *B. cinerea* sporelings mortality after incubated for 18 hours in different concentrations of camalexin (Germ tubes grown for 6 hours before treated) All results are means from four replicates in which a total of 40 sporelings were measured

Result for camalexin added 6 hours after germination (Fig 6) showed that in 35 µg/ml of camalexin, 10% of conidia was dead. This 10% of mortality represented conidia which were killed by camalexin (35 µg/ml) after they had failed to germinate after incubation for 6 hours. No live conidia without germ tubes were observed in this experiment. At the highest concentration of camalexin tested, 82.5% of sporelings had live conidia and germ tubes, a figure significantly less than 100% recorded in all other concentrations and control. In summary, camalexin has less inhibitory and fungitoxicity effect on 6 hours sporelings of *B. cinerea* than on conidia. Significantly, in sporelings germ tube were more sensitive than conidia.

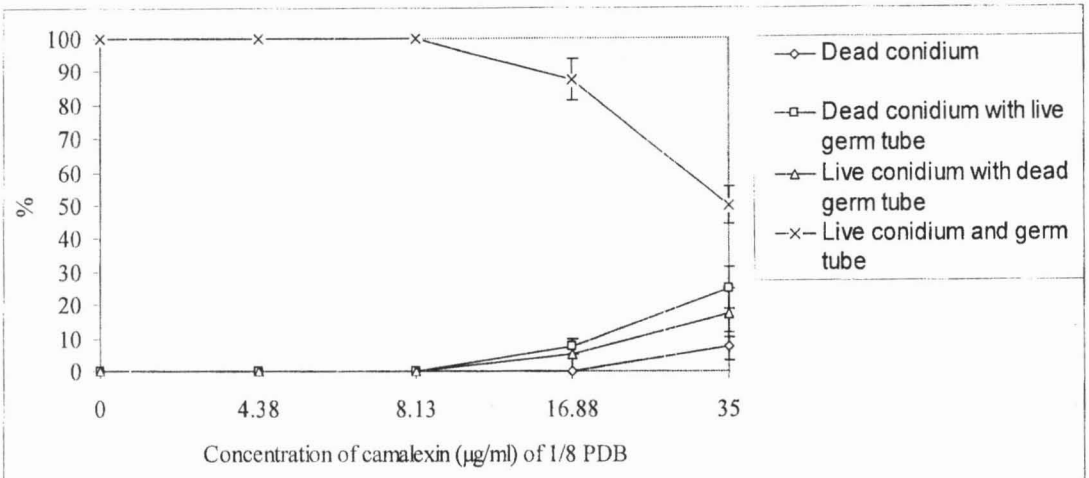

Fig 7: Pattern of *B. cinerea* sporelings mortality after incubated in different concentrations of camalexin for 18 hours (Germ tubes grown for 12 hours before treated) All results are means from four replicates in which a total of 40 sporelings were measured

If camalexin was added 12 hours after germination (Fig 7), 7.5% of dead conidia were found in concentration with 35 µg/ml of camalexin. This was significantly higher than in other concentrations or controls. Conidia which failed to germinate after 12 hours were killed by this high concentration. No live conidia without germ tubes were found in this experiment. At a concentration of 35 µg/ml of camalexin 25% of sporelings had dead conidia but live germ tubes. At 16.88 µg/ml, 7.5% of sporelings were in this category. These values were significantly higher than in other concentrations and controls. Besides that, in these two concentrations 5% (in 16.88 µg/ml) and 12.5% (in 35 µg/ml) of live conidia with dead germ tubes were found, again significantly higher death than in controls. Again, in these two concentrations 87.5% (in 16.88 µg/ml) and 50% (in 35 µg/ml) of live conidia with germ tubes were found, a figure significantly less than 100% recorded in all other concentrations and control.

The results of fungitoxicity are summarized in Fig 8 which records the percentage of each type of fungal structure killed. Conidia were more sensitive to camalexin, but surprisingly there was little difference between 6 and 12 hours old sporelings.

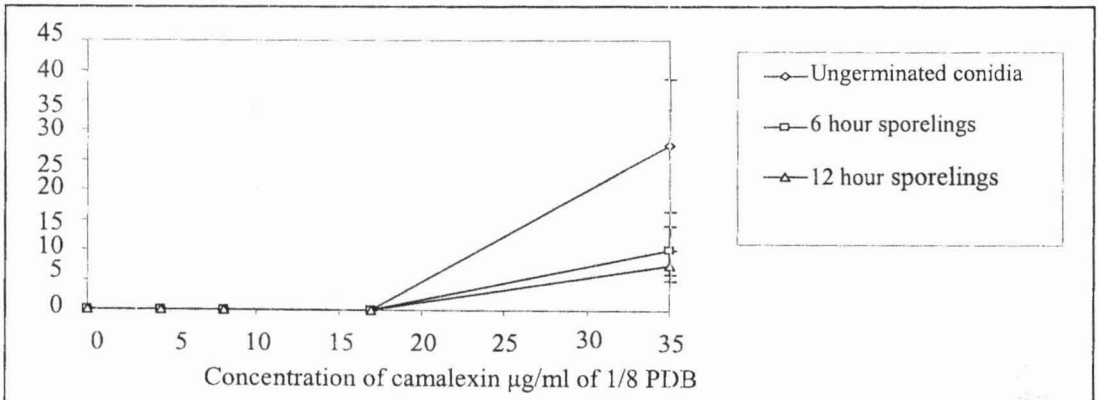

Fig 8: Toxicity of camalexin against different cells of *B. cinerea*. Death of spores and all cells in sporelings is recorded

The concentration of camalexin found in leave of *Arabidopsis* appeared to reach concentrations that were strongly inhibitory to *B. cinerea*. Involvement of phytoalexins in disease resistance has been widely studied for the past years. But whether or not phytoalexins are important in this resistance and to what degree is still an open question (Hammerschmidt 1999). There are some interactions in which the speed of accumulation of the inhibitors and their high level of toxicity argue strongly that they are the principal cause of restriction of microbial growth (Mansfield 2000).

In *Vicia faba* the roles of phytoalexins (wyerone, wyerone acid and wyerone expoxide) against *B. cinerea* have been well studied. Studies on the biochemical mechanisms underlying the success or failure of *Botrytis* to colonize the broad bean plant have been confined mainly to the role of phytoalexins in the restriction of fungi in limited lesions (Mansfield 1980).

In this *in vitro* experiment, we were allowed to make a direct comparison to different time when germ tubes of *B. cinerea* were challenged by camalexin, a prediction of the real camalexin induction time in the plant after pathogen attack. From the serial of experiments, it has been confirmed that an early induction of camalexin were more effective in restricting the fungal growth and increasing the percentage of mortality (younger germ tubes or conidia of *B. cinerea* found to be more susceptible). Higher accumulation of camalexin after *B. cinerea* infection is always associated with reduced lesion size in *Arabidopsis*, suggesting that camalexin may play a role in defence against this pathogen (Denby, Kumar and Kliebenstein 2004).

Wyerone acid was fungicidal to ungerminated conidia of *B. cinerea* but less active against conidia than sporelings with single celled germ tubes. Although wyerone expoxide has been shown to be slightly more inhibitory to germ tube growth than wyerone acid, their fungitoxicities were very similar. Both were more toxic than wyerone (Rossall, Mansfield and Huston 1980). By contrast, highest concentration tested of camalexin (35 µg/ml) only killed 27.5% of the conidia and conidia were found to be more sensitive than 6 and 12 hours old sporelings to camalexin. Differences in sensitivity may be caused by the presence of fewer receptor sites for phytoalexins in more tolerant cells (Rossall, Mansfield and Price 1977).

Highest concentration of camalexin tested (35 µg/ml), produced significantly higher percentage of mortality compared to other concentrations tested but still significantly produced longer germ tube than control. 50 µg ml^{-1} of camalexin was proved to completely inhibited growth of *B. cinerea* mycelium *in vitro* (Rossall, Mansfield and Huston 1980). In conclusion, sporelings and conidia of *B. cinerea* were more susceptible to higher concentration of camalexin. Camalexin (not like wyerone expoxide) found more inhibitory rather than fungitoxic in all concentrations tested, but whether increment of concentration to certain level will change camalexin to become very fungitoxic need further investigation.

Acknowledgements

The authors are very grateful for the scholarship given to Chong Khim Phin under Academic Staff Training Scheme of Universiti Malaysia Sabah.

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