

REVIEW PAPER

CAMALEXIN ACCUMULATION IN *Arabidopsis thaliana* LEAVES CHALLENGED BY *Botrytis cinerea*

Chong, K. P.¹, Mansfield, J. W.², Bennett, M. H.² and Rossiter, J. F.²

¹School of Science and Technology, Universiti Malaysia Sabah,
88999, Kota Kinabalu, Sabah, Malaysia

²Division of Biology, Imperial College, Wye, Ashford, Kent,
TN25 5AH, United Kingdom

ABSTRACT. Six weeks old lower leaves surfaces of *Arabidopsis thaliana* were inoculated with 20 µl inoculum of $2.5 \times 10^5 \text{ ml}^{-1}$ spores of *Botrytis cinerea* in 1/8 and 1/16 concentrations of Potato Dextrose Broth. Lesions produced were categorized to limited and spreading lesions for the study of phytoalexin accumulation. For limited lesions, accumulation of camalexin under the drop increased significantly from 5.13 µg/g tissue on day one to 17.4 µg/g tissues on day three but not on day four. The accumulation from 2 mm surrounding tissues also increased significantly for the first two days from 0.23 to 0.77 µg/g tissue. Most camalexin was detected in tissue containing lesions but low levels were detected from the surrounding ring of apparently symptomless tissue. In spreading lesions, the accumulation also increase significantly in tissues under the drop, 1 mm ring tissues surrounding drop and 1-2 mm ring tissues from drop at the first two days before drop after the tissue became completely colonized by *B. cinerea*.

KEYWORDS. *Arabidopsis thaliana*, *Botrytis cinerea*, Camalexin,

INTRODUCTION

Arabidopsis thaliana is a well-established model plant with a complete genomic sequence and a diverse secondary metabolite arsenal (Kliebenstein, 2004). This arsenal is comprised of anthocyanins, flavonoids, sinapoyl esters, glucosinolates, terpenoids, camalexin, and other tryptophan derivatives (Chapple *et al.*, 1994; Van Poecke *et al.*, 2001; Chen *et al.*, 2003). *Arabidopsis* has many advantages for genome analysis, including a short generation time, small size, large number of offspring, and a relatively small nuclear genome. These advantages promoted the growth of a scientific community that has investigated the biological processes of *Arabidopsis* and has characterized many genes (Meinke *et al.*, 1998). The characterization of these genes is not only relevant for plant biologists, but also affect agricultural science, evolutionary biology, bioinformatics, combinatorial chemistry, functional and comparative genomics, and molecular medicine (The *Arabidopsis* Genome Initiative, 2000). In recent years, *Arabidopsis* has been used in studying the impact of secondary metabolites in interactions with plant pathogens (Kliebenstein, 2004). Camalexin (3-thiazol-2 methyl-indole) and indolic secondary metabolite (Rhodes, 2003) is a type of phytoalexin that is involved in plant-pathogen interaction in *A.thaliana*. This phytoalexin is induced under pathogen attack or biotic elicitors that generate reactive oxygen species (Tsuji *et al.*, 1992; Reuber *et al.*, 1998; Roetschi *et al.*, 2001) or cell death (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. *B. cinerea*, the causal agent of gray mould is a facultative necrotrophic plant pathogen that is able to infect flowers, fruits, stems and leaves either through wounds or directly through intact cuticle (Doehlemann, *et al.*, 2005) Although both *Arabidopsis* and *B. cinerea* have been widely investigated (Bennet & Wallsgrove, 1994) but most studies have been concentrated on genetics rather than mechanism of resistance or effect of accumulation of secondary metabolites to plant resistance. Results from some experimental works showed that different types of lesion such as limited and spreading lesion on *Arabidopsis* leaves could be produced by *B. cinerea*, depending on the nutrient status within the inoculum droplet (Chong, 2005). In this study, we looked for the relation of camalexin accumulation in limited and spreading lesion to *Arabidopsis*'s resistance after challenged by *B. cinerea*.

MATERIALS AND METHODS

Plant

A. 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 photoperiod, a light intensity of 40 W/m² and a temperature of 20-21°C. After cotyledon development, seedlings were transplanted to individual pots (25 cm²). 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 1000ml 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 and 1/16 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 x 10⁵ 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 glasses (Suprasil) with a light path of 10mm were used with the spectrophotometer.

Leaf inoculation

Leaves of *Arabidopsis* were cut from six weeks old plants and inoculated with 2.5 x 10⁵ ml⁻¹ spores of *B. cinerea* in 1/8 and 1/16 concentrations of PDB. Each replication consists

of 3 leaves with 10 sites of inoculation. Each 20 µl inoculum was inoculated on the lower leaves surfaces. Sterile Double Distilled Water (SDDW) was inoculated as control. All inoculated leaves were incubated in sandwich boxes under high humidity. Lesions produced were scored and categorized using the key described by Rossall, (1978) in Figure 1 after inoculation.

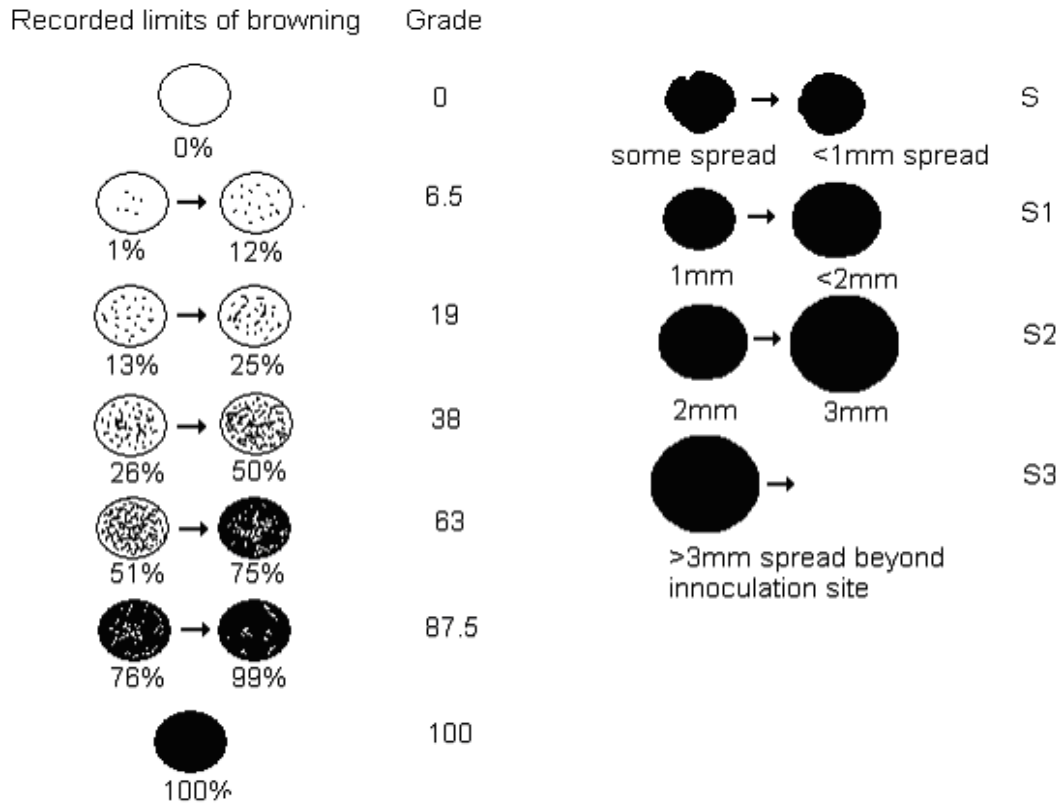


Figure 1. Key to infection grades of lesion caused by *B. cinerea* based on the percentage browning/blackening leaf surface beneath the inoculum droplet, and its spread beyond this area (Rossall, 1978)

Camalexin quantification by HPLC.

A reliable method was used for quantification of camalexin from leaf tissue using HPLC (Chong, 2005) to optimize the extraction. Three replicates were used and with ten sites for each replicate. Leaf tissues under the drop, 1 or 2 mm surrounding the drop inoculated with *B. cinerea* were excised from day 1 to day 4 and soaked overnight in 70% MeOH. Then, frozen at -20°C in an eppendorf tube for three hours and grinded with some sand in 500 µl of 70% MeOH in an eppendorf tube (1.5 ml) with a roughened plastic pestle until a fine suspension was obtained. The sample was then centrifuged at 13K rpm in an eppendorf centrifuge for 5 minutes, the supernatant transferred to a clean tube and pellet re-extracted with 500 µl of 70% MeOH another two times and separate supernatants were collected in different tubes. Camalexin was separated using HPLC and detected by fluorescence detector.

Quantification of camalexin accumulation

A standard calibration curve for camalexin quantification as described by Chong (2005) was used. The supernatants were injected into the HPLC column and run using isocratic solvent system 70:30 (water: acetonitrile) running for 15 min at 35°C with 0.5ml min⁻¹ flow rate and the fluorescence areas were recorded. The excitation (318 nm) and emission (385 nm) spectra of camalexin were used for the fluorescence detection. Areas under the camalexin peak were integrated with the concentration of camalexin in the standard curve described by Chong (2005) (Figure 2).

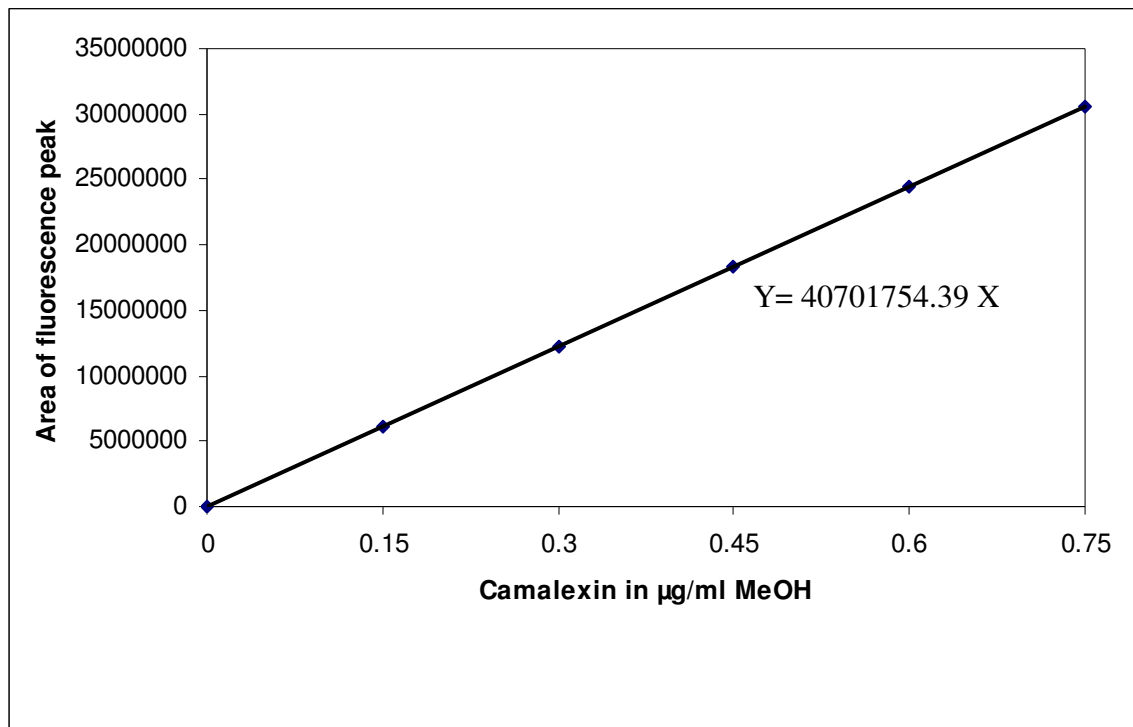


Figure 2: Integration output area under peak of fluorescence emission (Chong, 2005)

Data Analysis

Variance 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

Demonstration of the accumulation of camalexin at the right time and place to explain restriction of fungal growth is required to demonstrate a role of phytoalexin in defending *Arabidopsis* from *B. cinerea* invasion. The relationship between camalexin accumulation and lesion formation was reported in two subsections; limited lesions and spreading lesions.

Limited lesions

Using 1/16 PDB for spore suspension led to the production of limited lesions (Figure 3) at all sites. Most were at category scoring 5-74% (Rossall, 1978) after 4 days. The accumulation of camalexin in mixed limited lesions (scoring 5-74%) is shown in Figure 4.

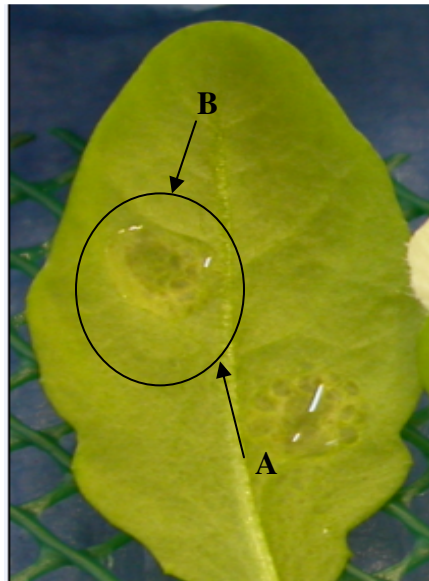
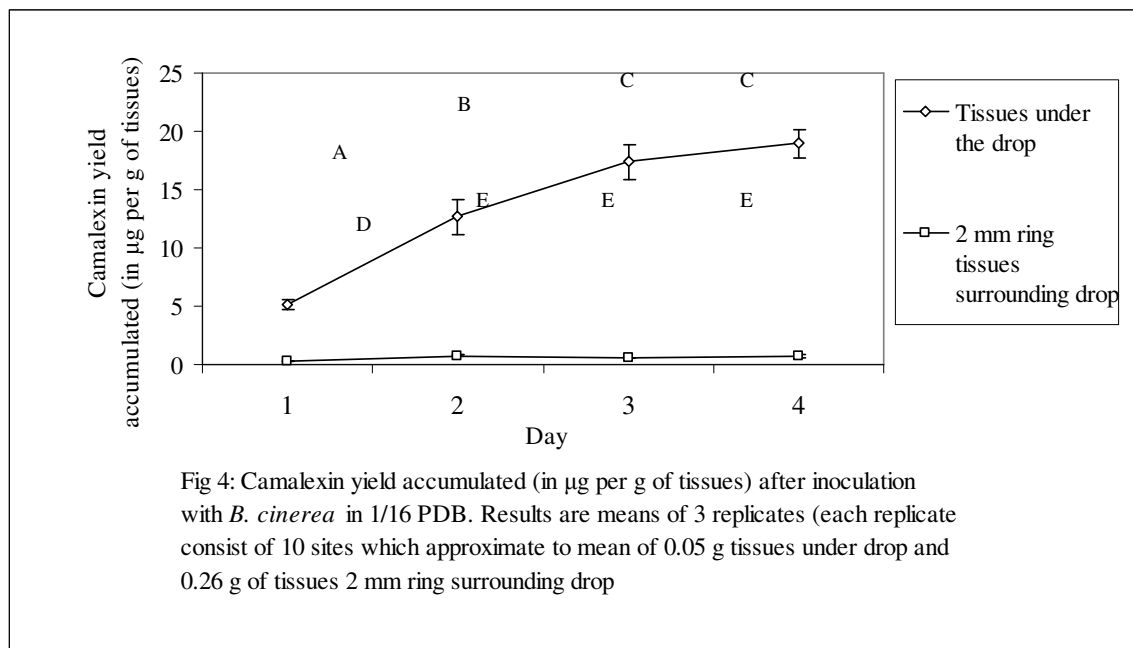


Figure 3: Limited lesions on *Arabidopsis* leaves after infected with *B. cinerea* spores in 1/16 PDB. A: Tissues under the drop. B: 2 mm ring tissues surrounding drop



For limited lesions, accumulation of camalexin under the drop increased significantly from 5.13 $\mu\text{g/g}$ tissue on day one to 17.4 $\mu\text{g/g}$ tissue on day three but not on day four.

The accumulation from 2 mm surrounding tissues also increased significantly for the first two days from 0.23 to 0.77 $\mu\text{g/g}$ tissue. Most camalexin was detected in tissue containing lesions but low levels were recovered from the surrounding ring of apparently symptomless tissue.

Spreading lesions

In order to study changes in camalexin concentration during the formation of spreading lesions (Figure 5), nutrient levels in droplets were increased to 1/8 PDB. The accumulation of camalexin in spreading lesions is shown in Figure 6.

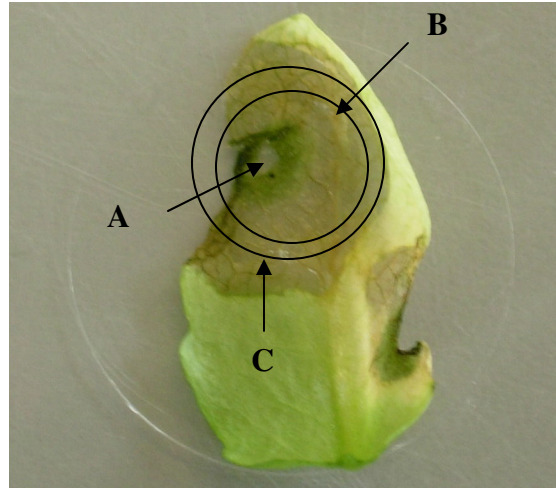
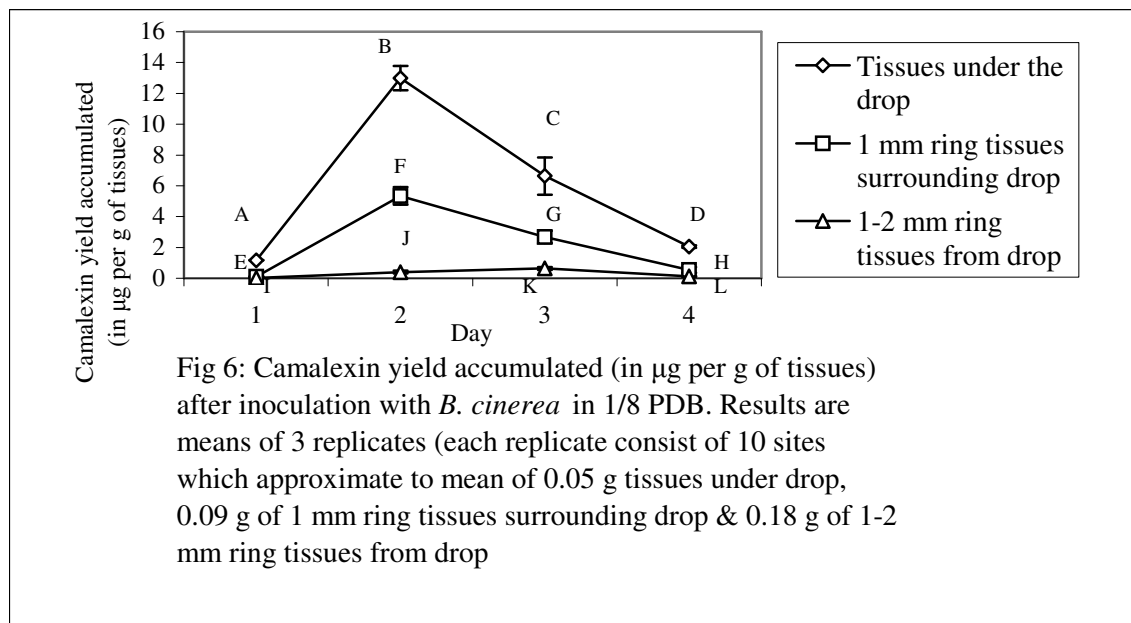


Figure 5: Spreading lesions on *Arabidopsis* leaves after infected with *B. cinerea* spores in 1/8 PDB. A: Tissues under the drop. B: 1 mm ring tissues surrounding drop. C: 1-2 mm ring tissues from drop



Accumulation of camalexin under the drop and tissues one mm surrounding drop showed a significant increase from day one to day two, but started to decrease significantly after day two to four. Camalexin accumulation in tissue 1-2 mm from drop

increased significantly from 0.03µg/g of tissues on day one to 0.64 µg/g of tissues on day three, and decreased significantly to 0.13 µg/g of tissues after day three. In conclusion, higher amounts of camalexin accumulated under the sites, which were initially attacked, compared to the surrounding tissues. The concentration of camalexin increased as tissues were infected reaching a maximum after two days when about 75% of the challenged sites were necrotic. Concentration decreased as tissue became completely colonized by *B. cinerea*.

DISCUSSION

Results show that accumulations of camalexin in both limited and spreading lesions of *Arabidopsis* were more in tissues under the inoculate droplets compared to surrounding tissues. The accumulation was highly localized. In resistant genotype of cocoa, a remarkable localization of sulphur in scattered xylem parenchyma cells were found during the colonization by *Verticillium dahlia* (Durrands and Cooper, 1998). Bailey and Deverall, (1971) reported, the bean phytoalexins were found to be remarkably localized to cells which had undergone the HR. High concentrations of lacinilene C and 2,7-dihydroxycadalene were found in cotton cotyledons inoculated with an avirulent strain of *Xanthomonas campestris* pv. *malvacearum* (Pierce and Essenberg, 1987). The overall conclusion was that more than 90% of the most active phytoalexins recovered from whole cotyledons are concentrated in dead, fluorescent cells at infection sites (Essenberg *et al.*, 1992; Pierce *et al.*, 1996).

It remains to be determined if the accumulations of camalexin was mainly in the dead cells after invasion by *B. cinerea* but synthesis probably took place in the surrounding symptomless cells. Accumulations of camalexin in several non-spreading types of lesion formed by *B. cinerea* in a low concentration of nutrient were significantly higher than in spreading lesions. The outcome of the interaction between *B. cinerea* and tissues of *Arabidopsis* inoculated under the laboratory conditions may depend on three major factors: i) The numbers of cells killed by the fungus before the accumulation of camalexin. ii) The sensitivity of *B. cinerea* hyphae to the camalexin. iii) The ability of the fungus to detoxify camalexin. With a stimulatory effect from a higher concentration of nutrient (1/8 PDB), *B. cinerea* perhaps have a greater ability to kill *Arabidopsis* cells (reducing sites producing camalexin) and metabolize camalexin. We suggest high concentration of nutrient may also decrease the sensitivity of *B. cinerea* to camalexin. This is correlated with the increase of camalexin in spreading lesions reaching a maximum after two days when about 75% of the challenged sites were necrotic and concentration decreased as tissue became completely colonized by the fungus. With lower stimulatory effect (in 1/16 PDB), less cells were killed instantly after the attack, more sites were available for the production of camalexin and *B. cinerea* has a lower ability to metabolize camalexin leading to phytoalexin accumulation to restrict invasion and finally produce limited lesions.

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