BIOTRANSFORMATION OF THE PHYTOALEXINS BRASSININ, BRASSILEXIN AND CAMALEXIN BY
ALTERNARIA BRASSICICOLA

A Thesis Submitted to the
College of Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of
Master of Science
in the
Department of Chemistry
University of Saskatchewan
Saskatoon

By

Mohammad Showkatul Islam

© Copyright Mohammad Showkatul Islam, December, 2008. All rights reserved.
PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, the author has agreed that the libraries of this University may make it freely available for inspection. The author further agrees that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor who supervised the thesis work or, in her absence, by the Head of the Department of Chemistry, or the Dean of the College of Graduate Studies and Research. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without the author’s written permission. It is also understood that due recognition shall be given to the author and to the University of Saskatchewan in any scholarly use which may be made of any material in this thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

The Head
Department of Chemistry
University of Saskatchewan
Saskatoon, Saskatchewan (S7N 5C9)
ABSTRACT

Chemical investigation of the transformation of the crucifer phytoalexins brassinin, brassilexin and camalexin by the phytopathogenic fungus *Alternaria brassicicola* was carried out. The objectives of this study included: i) the isolation and characterization of the metabolites of biotransformation of brassinin (2), brassilexin (21) and camalexin (24) by *A. brassicicola*; ii) determination of the antifungal activity of these phytoalexins and their metabolites against *A. brassicicola*.

The phytoalexins were synthesized and characterized using HPLC retention time $t_R$, $^1$H NMR, $^{13}$C NMR, LC-MS and HRMS-ESI data. The metabolites of the biotransformation were also synthesized and characterized similarly. The metabolism of each phytoalexin and their metabolites was studied by analyzing broth extracts by HPLC. The percent inhibition of growth of *A. brassicicola* was determined by radial growth mycelial assays.

The biotransformation of brassinin (2) by *A. brassicicola* afforded $N_b$-acetyl-3-indolylmethylamine (46) via indole-3-methylamine (45) intermediate. Brassilexin (21) was metabolized to 3-(amino)methyleneindoline-2-thione (67) by the reduction of the isothiazole ring. Camalexin did not appear to be metabolized or the metabolism was very slow. The results of biotransformation and bioassay studies established that the metabolism of brassinin by *A. brassicicola* was a detoxification process. However, these studies using brassilexin (21) did not provide a rigorous conclusion. Camalexin showed strong inhibition of growth against *A. brassicicola* suggesting its importance in defense against this pathogen.
BIOGRAPHICAL SKETCH

April, 1969  Born in Brahmanbaria, Bangladesh

December, 1989 B. Sc. (Hons), Chemistry, University of Rajshahi, Bangladesh

December, 1990 M. Sc. (Inorganic Chemistry), University of Rajshahi, Bangladesh
(Held in 1994)

October, 1995 – March, 2004 Lecturer, Department of Chemistry, Chittagong University of Engineering & Technology, Bangladesh

December, 2003 M. Sc. (Environmental Engineering), Asian Institute of Technology, Thailand

April, 2004 – August, 2006 Lecturer, Curtin University of Technology, Malaysia

September, 2006 – August, 2008 Graduate Student, Department of Chemistry, University of Saskatchewan, Canada

September, 2008 – Present Research Technician, Department of Food and Bioproduct Sciences, University of Saskatchewan, Canada
Acknowledgements

I would like to express my sincere thanks and gratitude to my supervisor, Dr. M. Soledade C. Pedras, Thorvaldson Professor, Department of Chemistry, University of Saskatchewan for her support, valuable guidance, constructive comments and encouragement, without which the study would not have been possible.

It’s my great pleasure to take the opportunity of expressing my sincere gratefulness to the Advisory Committee Chair, Prof. Dale E. Ward for his valuable comments and wise suggestions during my thesis work. I am also grateful to other members of the committee as well as faculty in the Department. I would like to extend my sincere thanks to external examiner, Prof. Jonathan R. Dimmock for his kind review of my thesis as well as his valuable comments.

I wish to express my gratitude to the following past and present members of Pedras group: Dr. A. Adio, Dr. P. B. Chumala, Dr. M. Hossain, Dr. Z. Minic, Dr. O. Okeola, Dr. D. Okinyo, Dr. P. D. Thongbam, Dr. Y. Yu, Dr. Q. A. Zheng, Z. Du, S. Hossain, W. Jin, I. Khalaff, J. Liu, S. Shaikh, V. K. Sarma, R. B. Snitynsky and E. Yaya.

I would like to thank Dr. Keith Brown and Mr. Ken Thoms for their help and assistance. The financial support of the Department of Chemistry and the College of Graduate Studies and Research is gratefully acknowledged.

I am highly pleased to extend my warmest thanks to my family, relatives, friends and well-wishers for their mental and moral support.

Finally, I would like to express my sincere thanks to my wife Mrs. Hemani Akhter who went through a lot of difficulties and troubles during my graduate studies and research. Last but not least, loving thanks are due to my sweet sons for their understanding and patience throughout my study.
Dedication

To my parents,
Mr. Abdus Salam and Mrs. Latifa Begum

To my wife,
Mrs. Hemani Akhter

&

To my sons,
Mishkat and Muttaqi
# Table of Contents

CHAPTER 1: INTRODUCTION ................................................................. 1

1.1 General objectives ........................................................................ 1

1.2 Crucifer phytoalexins .................................................................. 2

1.2.1 Structures and biological activity ........................................... 3

1.2.2 Biotransformation of brassinin, brassilexin and camalexin by plant pathogenic fungi ................................................................. 9

1.2.2.1 Brassinin and related compounds ........................................ 10

1.2.2.2 Brassilexin and related compounds ..................................... 16

1.2.2.3 Camalexin and related compounds ..................................... 19

1.3 Alternaria brassicicola ................................................................. 24

1.3.1 Pathogen significance: Blackspot disease ............................ 24

1.3.2 Secondary metabolites ............................................................. 26

1.3.2.1 Metabolites of Alternaria brassicicola ............................... 27

1.3.2.2 Interaction with crucifers ................................................... 29

1.4 Summary and conclusion ............................................................ 31

CHAPTER 2: RESULTS AND DISCUSSION ....................................... 32

2.1 Syntheses of phytoalexins ............................................................. 32

2.1.1 Synthesis of brassinin ............................................................ 33

2.1.2 Synthesis of brassilexin ........................................................ 34

2.1.3 Synthesis of camalexin (24) .................................................. 34

2.2 Biotransformation of phytoalexins by Alternaria brassicicola ........ 35

2.2.1 Biotransformation of brassinin .............................................. 35
2.2.1.1 Biotransformation of indole-3-methylamine ........................................... 40
2.2.1.2 Biotransformation of $N_b$-acetyl-3-indolylmethylamine.......................... 42
2.2.2 Biotransformation of brassilexin.................................................................... 44
2.2.2.1 Biotransformation of 3-(amino)methyleneindoline-2-thione ................. 47
2.2.3 Biotransformation of camalexin..................................................................... 49
2.3 Antifungal activity............................................................................................... 51
2.4 Conclusion............................................................................................................ 52
CHAPTER 3: EXPERIMENTAL.................................................................................. 54
3.1 General methods ................................................................................................. 54
3.2 Syntheses of phytoalexins .................................................................................. 56
3.2.1 Synthesis of brassinin (2) .............................................................................. 56
3.2.1.1 Synthesis of $N_b$-acetyl-3-indolylmethylamine (46)................................. 58
3.2.2 Synthesis of brassilexin (21) ......................................................................... 59
3.2.2.1 Synthesis of 3-(amino)methyleneindoline-2-thione (66)......................... 61
3.2.3 Synthesis of camalexin (24) .......................................................................... 62
3.3 Biotransformations of phytoalexins by Alternaria brassicicola .................... 63
3.3.1 Preparation of minimal media .......................................................................... 63
3.3.2 Preparation of fungal cultures .......................................................................... 64
3.3.3 Time-course experiments .................................................................................. 64
3.4 Bioassays .............................................................................................................. 65
CHAPTER 3: REFERENCES .................................................................................... 66
List of Figures

Figure 1.1  Chemical structure of pisatin (1) ................................................................. 2

Figure 1.2  Crucifer phytoalexins: brassinin (2); brassitin (3); 1-methoxybrassinin (4); 4-methoxybrassinin (5); 1-methoxybrassitin (6); brassicanal A (7); brassicanal C (8); methyl 1-methoxyindole-3-carboxylate (9); caulilexin A (10); brassicanate A (11); cyclobrassinin (12); cyclobrassinin sulfoxide (13); sinalbin A (14); sinalbin B (15); indole-3-acetonitrile (16); caulilexin C (17); arvelexin (18); dioxibrassinin (19); brassicanal B (20); brassilexin (21); sinalexin (22); rutalexin (23); camalexin (24); 1-methylcamalexin (25); 6-methoxycamalexin (26); wasalexin A (27); wasalexin B (28); 1-methoxybrassenin A (29); 1-methoxybrassenin B (30); 1-methoxyspirobrassinin (31); spirobrassinin (32); 1-methoxyspirobrassinol (33); 1-methoxyspirobrassinol methyl ether (34); brussalexin A (35); rapalexin A (36); rapalexin B (37); dehydro-4-methoxycyclobrassinin (38); isalexin (39); erucalexin (40); caulilexin B (41) (Pedras et al., 2007a; 2007b, 2007c). ........................................... 5

Figure 1.3  Chemical structure of brassicicolin A (95) ......................................................... 27

Figure 1.4  Chemical structure of depudecin (96) .............................................................. 28

Figure 1.5  Chemical structures of fusicoccin (97) and cotylenol (98). ............................ 28

Figure 1.6  Chemical structures of brassiciscene A-F (99, 100, 101, 102, 103, 104) ... 29
List of Schemes

Scheme 1.1  Detoxification of brassinin (2) by *Leptosphaeria maculans*,
*Leptosphaeria biglobosa* and *Sclerotinia sclerotiorum* (Pedras and Ahiahonu, 2005;
Pedras et al., 2007d). ........................................................................................................... 11

Scheme 1.2  Detoxification of methyl tryptamine dithiocarbamate (49) by
*Leptosphaeria maculans* and *Leptosphaeria biglobosa* (Pedras and Ahiahonu, 2005;

Scheme 1.3  Detoxification of methyl tryptamine dithiocarbamate (49) by *Alternaria brassicae* and *Sclerotinia sclerotiorum* (Pedras and Okanga, 1998, 2000; Pedras et
al., 2004a). ................................................................................................................................ 13

Scheme 1.4  Detoxification of methoxybrassinin (4) and methyl 1-methoxytryptamine
dithiocarbamate (55) by *Sclerotinia sclerotiorum* (Pedras et al., 2004a). ..................... 14

Scheme 1.5  Detoxification of methyl 1-methyl tryptamine dithiocarbamate (57) by
*Sclerotinia sclerotiorum* (Pedras et al., 2004a; Pedras and Ahiahonu, 2005). ...... 15

Scheme 1.6  Detoxification of 2-naphthylmethyl dithiocarbamate (61) by *Sclerotinia
c sclerotiorum* (Pedras and Ahiahonu, 2005; Pedras et al., 2004a). ................................. 15

Scheme 1.7  Detoxification of methyl N(p-methylbenzyl) dithiocarbamate (63) by
*Leptosphaeria maculans* (Pedras et al., 1992). ................................................................. 16

Scheme 1.8  Detoxification of brassilexin (21) by *Leptosphaeria maculans* /
*Sclerotinia sclerotiorum* (Pedras, 2008). ............................................................................ 17

Scheme 1.9  Detoxification of methylbrassilexin (69) by *Leptosphaeria maculans* and
*Sclerotinia sclerotiorum* (Pedras, 2008; Pedras and Hossain, 2006). .......................... 18
Scheme 1.10  Detoxification of camalexin (24) by *Rhizoctonia solani*, *Leptosphaeria maculans* and *Alternaria brassicae* (Pedras and Liu, 2004; Pedras, 2004). ..........19

Scheme 1.11  Detoxification of camalexin (24) by *Sclerotinia sclerotiorum* (Pedras et al., 2004a; Pedras and Ahiahonu, 2005; 2002). .........................................................20

Scheme 1.12  Detoxification of 6-fluorocamalexin (77) by *Sclerotinia sclerotiorum* (Pedras and Ahiahonu, 2005; 2002, Pedras et al., 2004a).................................20

Scheme 1.13  Detoxification of 6-methoxycamalexin (79) by *Sclerotinia sclerotiorum*:
           i) major metabolite, ii) minor metabolite (Pedras and Ahiahonu, 2005; 2002). ......21

Scheme 1.14  Detoxification of 6-fluoro-1-methylcamalexin (81) by *Sclerotinia sclerotiorum* (Pedras and Ahiahonu, 2005; 2002). ....................................................22

Scheme 1.15  Detoxification of 5-fluorocamalexin (84) by *Rhizoctonia solani* (Pedras and Liu, 2004; Pedras and Ahiahonu, 2005).................................................22

Scheme 1.16  Detoxification of 5-methylcamalexin (88) by *Rhizoctonia solani* (Pedras and Liu, 2004 Pedras and Ahiahonu, 2005). .................................................23


Scheme 2.1  Synthesis of brassinin (2). Reagents and conditions: (i) NH$_2$OH.HCl, Na$_2$CO$_3$, EtOH, 2 h, reflux, 60°C, 90%; (ii) NaBH$_4$, NiCl$_2$.6H$_2$O, MeOH, rt, 50%;
            (iii) Pyridine, Et$_3$N, CS$_2$, 0°C; (iv) MeI, 0°C, 55%;(v) Pyridine, Ac$_2$O, 1 h, 0°C,
            60%...........................................................................................................................33

Scheme 2.2  Synthesis of brassilexin (21). Reagents and conditions: (i) P$_4$S$_{10}$, NaHCO$_3$, THF, 81%; (ii) POCl$_3$, DMF; (iii) NH$_4$OH; (iv) I$_2$, Pyridine, 52%....34
Scheme 2. 3 Synthesis of camalexin (24). Reagents and conditions: (i) Mg, CH₃I, Et₂O; (ii) Benzene, 2-bromo-thiazole, 90 °C, 29%.................................................................34

Scheme 2. 4 Biotransformation of brassinin (2) via amine (45) in fungal cultures of *Alternaria brassicicola*. ...............................................................................................................37

Scheme 2. 5 Biotransformation of brassilexin (21) in cultures of *Alternaria brassicicola*. .........................................................................................................................44

Scheme 3.1 Synthesis of brassinin (2) 56
Scheme 3.2 Synthesis of N₅-acetyl-3-indolylmethylamine (46) 58
Scheme 3.3 Synthesis of brassilexin (21) 59
Scheme 3.4 Synthesis of 3-(amino)methyleneindoline-2-thione (66) 61
Scheme 3.5 Synthesis of camalexin (24) 62

List of Table:

Table 2.1 Percentage of growth inhibition of *Alternaria brassicicola* incubated with phytoalexins (2, 21 and 24) and metabolites (45, 46, 66) after 72 h of incubation under constant light. .................................................................................................................51
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac</td>
<td>Acetyl</td>
</tr>
<tr>
<td>Ac&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Acetic anhydride</td>
</tr>
<tr>
<td>A. brassicae</td>
<td><em>Alternaria brassicae</em></td>
</tr>
<tr>
<td>A. brassicicola</td>
<td><em>Alternaria brassicicola</em></td>
</tr>
<tr>
<td>A. thaliana</td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>B.</td>
<td><em>Brassica</em></td>
</tr>
<tr>
<td>br</td>
<td>Broad</td>
</tr>
<tr>
<td>13C NMR</td>
<td>Carbon-13 nuclear magnetic resonance</td>
</tr>
<tr>
<td>calc.</td>
<td>Calculated</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>EI</td>
<td>Electron impact</td>
</tr>
<tr>
<td>ESI</td>
<td>Electro-spray ionization</td>
</tr>
<tr>
<td>Et</td>
<td>Ethyl</td>
</tr>
<tr>
<td>Et&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Diethyl ether</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>FCC</td>
<td>Flash column chromatography</td>
</tr>
<tr>
<td>1H NMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HR</td>
<td>High resolution</td>
</tr>
<tr>
<td>HRMS</td>
<td>High Resolution Mass Spectrum</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>J</td>
<td>Coupling constant</td>
</tr>
<tr>
<td>L. maculans</td>
<td><em>Leptosphaeria maculans</em></td>
</tr>
<tr>
<td>m/z</td>
<td>Mass/charge ratio</td>
</tr>
<tr>
<td>Me</td>
<td>Methyl</td>
</tr>
<tr>
<td>MeI</td>
<td>Methyl iodide</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MHz</td>
<td>MegaHertz</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrum</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato dextrose agar</td>
</tr>
<tr>
<td>PDB</td>
<td>Potato dextrose broth</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PTLC</td>
<td>Preparative Thin Layer Chromatography</td>
</tr>
<tr>
<td>Py</td>
<td>Pyridine</td>
</tr>
<tr>
<td>R. solani</td>
<td><em>Rhizoctonia solani</em></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>rt</td>
<td>Room temperature</td>
</tr>
<tr>
<td>$t_R$</td>
<td>Retention time</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v</td>
<td>volume</td>
</tr>
<tr>
<td>w</td>
<td>weight</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

1.1 General objectives

*Alternaria brassicicola* (Schweinitz) Wiltshire is the causative agent of blackspot disease in a wide range of crucifer species in Canada and worldwide. The aim of my research project was to investigate the biological activities and biotransformations of crucifer phytoalexins by the blackspot fungus *A. brassicicola*. The chemical investigation of the metabolism of phytoalexins by *A. brassicicola* and evaluation of their antifungal activity will allow a better understanding of the role of phytoalexins in plant fungal diseases. My overall objectives included:

i) Biotransformations of the crucifer phytoalexins brassinin (2), brassilexin (21) and camalexin (24) by the phytopathogenic fungus *A. brassicicola*.

ii) Determination of the antifungal activity of these phytoalexins and their metabolites against *A. brassicicola*. 
1.2 Crucifer phytoalexins

Phytoalexins are secondary metabolites produced de novo by plants in response to various forms of biotic and abiotic stress including microbial attack (Pedras et al., 2007a). The term phytoalexin was introduced to describe induced antibiotic plant metabolites and is derived from the Greek words phyton = plant and alexein = protecting substance (Müller and Börger, 1940). The first phytoalexin, pisatin (1), was isolated from Pisum sativum (pea) infected with Sclerotinia fructicola (Cruickshank and Perrin, 1960).

![Chemical structure of pisatin (1).](image)

**Figure 1.1** Chemical structure of pisatin (1).

After discovery of the first phytoalexin, many other phytoalexins were identified belonging to different plant families. Phytoalexins from the same family of plants possess structural features indicating the same biosynthetic origin (Macías et al., 2007). Closely related plants produce phytoalexins of similar structures. For example, plants from Leguminosae produce predominantly isoflavanoid phytoalexins (Harborne, 1999; Ingham, 1982), plants from Solanaceae synthesize mostly terpenoid phytoalexins (Brooks and Watson, 1991; Jadhav et al., 1991), plants from Caryophyllaceae accumulate phytoalexins derived from anthranilamide (Niemann, 1993), and cruciferous plants accumulate sulfur containing indole phytoalexins (Pedras et al., 2003a; 2007a).
The biosynthetic precursor of crucifer phytoalexins, including camalexins (Glawischnig et al., 2004) is tryptophan (Pedras et al., 2003a, b).

Brassicaceae (Cruciferae), commonly known as crucifers, is the largest plant family in the plant kingdom. Crucifers are an economically important group of plants since they are used as vegetables, oil sources and condiments. Brassicaceae contains approximately 350 genera and about 3,700 species according to the Royal Botanic Gardens, Kew, England (Lawrence et al., 2008). Crucifer plants are present in almost every part of the world from tropics to the Arctic Circle. The greatest numbers of genera are abundant in temperate zones of the northern hemisphere.

1.2.1 Structures and biological activity

Phytoalexins from crucifers were first reported in 1986 by Takasugi and co-workers (Takasugi et al., 1986). Brassinin (2), 1-methoxybrassinin (4), and cyclobrassinin (12) were isolated from Chinese cabbage (B. campestris L. ssp. pekinensis) infected with *Pseudomonas cichorii* (Takasugi et al., 1986). After Takasugi’s report, close to 40 crucifer phytoalexins were isolated and reported (Figure 1.2) (Pedras et al., 2007a). The general structural feature of crucifer phytoalexins is the presence of indole or oxindole ring with substituents containing sulfur or nitrogen or both. The non-sulfur containing phytoalexins are 1-methoxyindole-3-carboxylate (9), isalexin (39), arvelexin (18), caulilexin B (41) and caulilexin C (17). Erucalexin (40) isolated from dog mustard (*Erucastrum gallicum*) is the first phytoalexin to have a
carbon substituent at C-2, whereas all other phytoalexins have carbon substituents at C-3. It is interesting that only cruciferous phytoalexins contain a dithiocarbamate or thiocarbamate group (2, 3, 4, 5, 6) are similar to widely used synthetic fungicides (Pedras et al, 2007a). Caulilexin A (10) is the first phytoalexin to possess a disulfide bridge (Pedras et al., 2006a). The first naturally occurring aromatic isothiocyanates, rapalexins A (36) and B (37), were isolated from B. rapa (canola) (Pedras et al, 2007a; Pedras et al., 2007b). Wasalexins A (27) and B (28) were isolated from wasabi (Wasabia japonica syn. Eutrema wasabi) (Pedras et al., 1999; Pedras et al, 2007a), arvelexin (18) was isolated from Thlaspi arvense (stinkweed) (Pedras et al., 2003a; Pedras et al, 2007a), isalexin (39), brassicanate A (11), and rutalexin (23) were isolated from B. napus, ssp. rapifera (rutabaga) (Pedras et al., 2004b; Pedras et al, 2007a), sinalbins A (14) and B (15) were first isolated from white mustard (Sinapis alba) (Pedras and Zaharia, 2000; Pedras et al, 2007a), brassicanals A (7), B (20) and C (8) were isolated from Chinese cabbage (Monde et al., 1990; Monde et al., 1991; Pedras et al, 2007a), camalexins (24, 25, 26) were isolated from Camelina sativa (Ayer et al., 1992; Pedras et al, 2007a), brassilexin (21) was isolated from Brassica juncea (Devys et al., 1988; Pedras et al, 2007a), sinalexin (22) was isolated from white mustard (Sinapis alba) infected with A. brassicaceae or elicited with CuCl2 (Pedras and Smith, 1997; Pedras et al, 2007a), brussalexin A (35) was isolated recently from brussels sprouts (B. oleracea var. gemmifera) (Figure 1.2) (Pedras et al., 2007c). Brussalexin A (35) is the first phytoalexin containing an allyl thiolcarbamate group.
Figure 1.2 *Crucifer phytoalexins*: brassinin (2); brassitin (3); 1-methoxybrassinin (4); 4-methoxybrassinin (5); 1-methoxybrassin (6); brassicanal A (7); brassicanal C (8); methyl 1-methoxyindole-3-carboxylate (9); caulilexin A (10); brassicanate A (11);
cyclobrassinin (12); cyclobrassinin sulfoxide (13); sinalbin A (14); sinalbin B (15); indole-3-acetonitrile (16); caulilexin C (17); arvelexin (18); dioxibrassinin (19); brassicanal B (20); brassilexin (21); sinalexin (22); rutalexin (23); camalexin (24); 1-methylcamalexin (25); 6-methoxycamalexin (26); wasalexin A (27); wasalexin B (28); 1-methoxybrassinenin A (29); 1-methoxybrassinenin B (30); 1-methoxyspirobrassinin (31); spiromassin (32); 1-methoxyspirobrassinol (33); 1-methoxyspirobrassinol methyl ether (34); brussalexin A (35); rapalexin A (36); rapalexin B (37); dehydro-4-methoxycyclobrassinin (38); isalexin (39); erucalexin (40); caulilexin B (41) (Pedras et al., 2007a; 2007b, 2007c).

Crucifer phytoalexins are bioactive plant products having inhibitory properties against different species of fungi. Crucifers under fungal attack, produce a complex blend of antimicrobial metabolites as induced chemical defenses. The rate of production and the amount of production of defense chemicals can affect plant resistance against pathogen invasion (Smith, 1996). Research studies using in situ localization and quantification revealed that phytoalexins can accumulate at the right time, concentration and location in plant tissues (Hammerschmidt and Dann, 1999). Antifungal activity of crucifer phytoalexins has been investigated using different bioassays (Pedras et al., 1998). Most crucifer phytoalexins are active against fungal pathogens at 0.1-0.5 mM. Brassinin (2) exhibited complete inhibition of spore germination of *Leptosphaeria maculans* and complete mortality of shrimp larvae (*Artemia salina*) at 0.5 mM (Pedras and Jha, 2006; Pedras et al., 2007a). Brassitin (3) was isolated from Japanese radish roots (*Raphani sativus var. hortensis*) elicited by *Pseudomonas cichorii* and was detected by inspection of its antifungal activity in a TLC bioassay (Monde et al., 1995). Brassitin (3) was sprayed onto leaves of *B. oleracea* for testing its influence on the oviposition behavior of cabbage fly, *Delia radicum* (L.) (Baur et al., 1998). Brassicanal
A (7) and brassicanal C (8) showed growth inhibition of ca. 70%, 50% and 30% in radial growth antifungal bioassays against *L. maculans*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*, respectively at a concentration of 0.5 mM (Pedras et al., 2006a; 2007a). Caulilexin A (10) displayed 50% growth inhibition against *L. maculans* whereas complete inhibition was observed against *S. sclerotiorum* and *R. solani* at the same concentration of 0.5 mM (Pedras et al., 2006a; 2007a). Caulilexin B (41) showed significantly less inhibition against *L. maculans*, *S. sclerotiorum* and *R. solani* at a concentration of 0.5 mM (Pedras et al., 2006a; 2007a). Caulilexin C (17) exhibited 75% and 30% inhibition against *L. maculans* and *S. sclerotiorum*, respectively, but complete inhibition against *R. solani* (Pedras et al., 2006a; 2007a). Brassicanate A (11) showed complete inhibition against *L. maculans*, *S. sclerotiorum* and *R. solani* at a concentration of 0.5 mM (Pedras et al., 2004a; 2007a). Cyclobrassinin (12) and spirobrassinin (32) showed strong inhibition against mycelial growth of *A. brassicae* and spore germination of *Cladosporium cucumerinum*, but these phytoalexins exhibited moderate antifungal activities against *L. maculans*, *R. solani*, *S. sclerotiorum*, *Botrytis cinerea*, *Fusarium nivale* and *Pythium ultimum* (Dahyia and Rimmer, 1988). Brassilexin (21) was found more inhibitory than brassinin and cyclobrassinin. Complete mycelial growth inhibition was observed against *L. biglobosa*, *L. maculans* and *S. sclerotiorum* at a concentration of 0.5 mM (Pedras et al., 2000; Pedras and Hossain, 2006; Pedras et al., 2007a). Camalexin (24) caused complete inhibition of various crucifer pathogens even at lower concentrations than brassilexin (Pedras et al., 2004a; Pedras and Khan, 2000; Pedras and Ahiahonu, 2002; Pedras et al., 2007a). Wasalexin A (27) and wasalexin B (28) exhibited antifungal activity against *L. maculans* causing ca. 50% spore germination inhibition (Pedras et al., 1999; Pedras et al., 2007a). Arvilexin (18) displayed 35%, 60% and 70%
inhibition in mycelial growth bioassays against *S. sclerotiorum*, *L. maculans* and *R. solani* respectively at a concentration of 0.5 mM (Pedras et al., 2006a; Pedras et al., 2007a). Sinalbin A (14) and sinalbin B (15) exhibited spore germination inhibition against *L. maculans* at 0.5 mM (Pedras and Zaharia, 2000; Pedras et al., 2007a). Rapalexin A (36) and B (37) were investigated for the inhibition of spore germination of *Albugo candida*; rapalexin A (36) was more inhibitory than rapalexin B (37) (Pedras et al., 2007b; Pedras et al., 2007a). Brussalexin A (35) showed higher inhibition against *S. sclerotiorum* than *A. brassicicola*, *L. maculans* and *R. solani* at 0.5 mM concentration (Pedras et al., 2007c). Erucalexin (40) exhibited complete inhibition against *R. solani* and only 40% inhibition against *S. sclerotiorum* in mycelial radial growth bioassays at 0.5 mM (Pedras et al., 2006b; Pedras et al., 2007a). Isalexin (39) displayed antifungal activity only against *L. maculans* in radial growth bioassay at 0.5 mM concentration while *S. sclerotiorum* and *R. solani* did not exhibit growth inhibition (Pedras et al., 2004a; Pedras et al., 2007a).

Crucifer phytoalexins appear to be cancer chemopreventive, antiproliferative and antitumor agents (Banerjee et al., 2008; Mezencev et al., 2003; Pilatova et al., 2005). Epidemiological studies suggest that cancer risk is reduced if crucifer vegetables are consumed in the diet (Pedras, 2008 and references within). The protective effect of the crucifer vegetables is due to the active metabolites produced by crucifer family such as isothiocyanates and indole-containing compounds (Glawischnig, 2007 and references within; Pedras, 2008). Metabolic detoxification of carcinogens undergoes three stages: i) activation of carcinogen by oxidation (Phase I); ii) conjugation to a more polar structure (Phase II); iii) ultimate excretion of the xenobiotics from the cell (Phase III).
Brassica diets have potential to increase the oxidative metabolism (Phase I) and to induce Phase II enzymes (Johnson et al., 1995; Mehta et al., 1995; Talalay et. el., 1995). Brassinin (2), cyclobassin (12) and spirobrassinin (32) inhibited mammary lesion formation in the mouse mammary gland organ culture model (Mehta et al., 1994). Cancer chemopreventive activity of brassinin (2) was also investigated by Mehta and coworkers in a dose-dependent experiment (Mehta et.al., 1995). Studies have been carried out to develop cancer chemopreventive drugs from derivatives of indole phytoalexins (Mezencev et al., 2008). Synthetic analogs of phytoalexins have also been investigated by Pedras’ group for designing “PALDOXIN” (PhytoALexin DetOXification INhibitor) toward the control of fungal diseases of crucifers (Pedras, 2008; Pedras et al., 2007a; Pedras et al., 2003b).

1.2.2 Biotransformation of brassinin, brassilexin and camalexin by plant pathogenic fungi

Detoxification is a process by which toxic metabolites are converted to less toxic or even nontoxic metabolites. Phytoalexins are toxic towards fungi, bacteria as well as plant cells (Darvill and Albersheim, 1984; Marinelli et al., 1995). Fungal cell membranes are most likely the site for toxic action of phytoalexins (Paxton, 1979; Smith, 1996), although the mode of action of phytoalexins has not been reported extensively. It is very likely that there are multiple modes of action for the disruption of cell membranes (Smith, 1996). Phytoalexins are very diverse in their structural variety and thus their toxicity covers a range of $10^{-6}$ to $10^{-4}$ M for inhibition. However, fungal
pathogens can void plant chemical defenses through metabolic detoxification and biotransformation (Pedras and Ahiahonu, 2005). As a result, plants become susceptible to pathogens. For fungal disease control, it is important to know how the fungus destroys or voids the defense chemicals through metabolic detoxification. A possible strategy for controlling fungal diseases is the inhibition of enzymes involved in the detoxification of phytoalexins (Pedras et al., 2004a). Biotransformation studies may open the opportunity to design inhibitors of these enzymatic reactions.

1.2.2.1 Brassinin and related compounds

Biotransformations of brassinin and related compounds have been studied extensively in Pedras’ group using phytopathogenic fungi L. maculans, L. biglobosa, S. sclerotiorum, R. solani and A. brassicae. Biotransformation studies of brassinin (2) and its homologues using L. maculans and L. biglobosa (Pedras and Ahiahonu, 2005; Pedras and Okanga, 2000; Pedras, 2008; Pedras and Taylor, 1993) showed that the metabolism takes place only in the side chain (Scheme 1.1). The biotransformation of brassinin (2) by L. maculans, virulent on canola, led to the formation of indole-3-carboxylic acid (43) via indole-3-carbaxaldehyde (42). Metabolism of brassinin (2) by L. biglobosa, avirulent on canola, took place via indole-3-methylamine (45) and Nβ-acetyl-3-indolylmethylamine (46) to indole-3-carboxylic acid (43) (Pedras and Taylor, 1991; Pedras and Taylor, 1993). However, biotransformation of brassinin (2) by the stem rot fungus S. sclerotiorum was completely different yielding an N-glucosylated product, 1-β-D-glucopyranosylbrassinin (44), without affecting the dithiocarbamate moiety (Pedras
and Ahiahonu, 2005; Pedras and Hossain, 2006; Pedras et al., 2004a). The presence of $(-\text{NH}-)$ moiety on the side chain of brassinin is the key for brassinin detoxification. In addition, research suggested that the removal of methylene bridge or the replacement of the $(\text{N})\text{H}$ with an $(\text{N})\text{CH}_3$ group could inhibit degradation of dithiocarbamates (Pedras, 2008). Brassinin (2) metabolism by phytopathogenic fungi $L.\text{ maculans}$, $L.\text{ biglobosa}$ and $S.\text{ sclerotiorum}$ is presented in Scheme 1.1.

**Scheme 1.1** Detoxification of brassinin (2) by *Leptosphaeria maculans*, *Leptosphaeria biglobosa* and *Sclerotinia sclerotiorum* (Pedras and Ahiahonu, 2005; Pedras et al., 2007d).
The selectivity of enzymes involved in this detoxification process was investigated using several analogs of brassinin (Pedras and Okanga, 1998, 2000; Pedras et al., 2004a). The metabolism of the brassinin analog, methyl tryptamine dithiocarbamate (49) by *L. maculans*, virulent on canola led to the formation of indole-3-acetic acid (48) as final product via indole-3-acetaldehyde (47) and another product 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (50), whereas *L. biglobosa*, avirulent on canola, metabolized methyl tryptamine dithiocarbamate (49) to indole-3-acetic acid (48) via tryptamine (51) and *N*$_b$-acetyltryptamine (52) (Pedras and Okanga, 1998, 2000), as shown in Scheme 1.2.

**Scheme 1.2** Detoxification of methyl tryptamine dithiocarbamate (49) by *Leptosphaeria maculans* and *Leptosphaeria biglobosa* (Pedras and Ahiahonu, 2005; Pedras and Okanga, 1998, 2000).
Similar to previous studies, metabolism of methyl tryptamine dithiocarbamate (49) by *A. brassicae* provided *N*$_h$-acetyltryptamine (52) as the major product and tryptamine (51) as minor product (Pedras and Okanga, 1998, 2000). Metabolism of methyl tryptamine dithiocarbamate (49) by *S. sclerotiorum* yielded *N*-glucosylated product of methyl tryptamine dithiocarbamate (53) (Pedras et al., 2004a) as shown in Scheme 1.3.

**Scheme 1.3** Detoxification of methyl tryptamine dithiocarbamate (49) by *Alternaria brassicae* and *Sclerotinia sclerotiorum* (Pedras and Okanga, 1998, 2000; Pedras et al., 2004a).
In most cases phytoalexins and phytoalexin analogs were metabolized by *S. sclerotiorum* to their respective *N*-glucosides (Pedras and Ahiahonu, 2005). However, metabolism of *N*-protected indole analogs, 1-methoxybrassinin (4) and methyl 1-methoxytryptamine dithiocarbamate (55) by *S. sclerotiorum* led to *O*-glucosylated metabolites (54, 56) (Pedras et al., 2004a) as shown in Scheme 1.4.

**Scheme 1.4** Detoxification of methoxybrassinin (4) and methyl 1-methoxytryptamine dithiocarbamate (55) by *Sclerotinia sclerotiorum* (Pedras et al., 2004a).

Metabolism of methyl 1-methyltryptamine dithiocarbamate (57) by *S. sclerotiorum* provided an oxidation product in addition to *O*-glucosylated product, as shown in Scheme 1.5.
Scheme 1.5 Detoxification of methyl 1-methyl tryptamine dithiocarbamate (57) by *Sclerotinia sclerotiorum* (Pedras et al., 2004a; Pedras and Ahiahonu, 2005).

Metabolism of S-methyl-2-naphthylmethyl dithiocarbamate (61) by *S. sclerotiorum* yielded methyl 5-(O-β-D-glucopyranosyl)-2-naphthylmethyl dithiocarbamate (62) as shown in Scheme 1.6. However, methyl 1-naphthylmethyl dithiocarbamate was transformed to several undetermined metabolites (Pedras et al., 2004a).

Scheme 1.6 Detoxification of methyl 2-naphthylmethyl dithiocarbamate (61) by *Sclerotinia sclerotiorum* (Pedras and Ahiahonu, 2005; Pedras et al., 2004a).
Previous biotransformation studies of $p$-substituted benzyl dithiocarbamate (63) in cultures of *L. maculans* showed that the dithiocarbamate moiety was transformed to the carboxylic acid group ($p$-toluic acid, 64) along with a minor metabolite $N(p$-carboxybenzyl) dithiocarbamate (65) (Pedras et al., 1992), as shown in the following scheme 1.7.

![Scheme 1.7](image)

**Scheme 1.7** Detoxification of methyl $N(p$-methylbenzyl) dithiocarbamate (63) by *Leptosphaeria maculans* (Pedras et al., 1992).

### 1.2.2.2 Brassilexin and related compounds

Metabolism of brassilexin (21) by *L. maculans* produced 3-formylindolyl-2-sulphonic acid (67) via 3-(amino)methyleneindoline-2-thione (66) (Pedras and Suchy, 2005; Pedras, 2008). Brassilexin (21) was metabolized to 1-$\beta$-D-glucopyranosylbrassilexin (68) by *S. sclerotiorum*. In addition, the phytoalexin brassicanal A (7) was formed via 3-(amino)methyleneindoline-2-thione (66) in the culture, as a minor metabolite after incubation with brassilexin (21) (Pedras and Hossain, 2006).
Scheme 1.8 Detoxification of brassilexin (21) by *Leptosphaeria maculans* and *Sclerotinia sclerotiorum* (Pedras, 2008).

The substrate specificity of the enzyme involved in the detoxification of naturally occurring brassilexin (21) was investigated by incubation of 1-methylbrassilexin (69) in *S. sclerotiorum*. Synthetic methylbrassilexin (69) was metabolized to 1-methyl-(oxy-\(\beta\)-D-glucopyranosyl)brassilexin (71) in the culture of *S. sclerotiorum* in about four days incubation period as shown in Scheme 1.9 (Pedras and Hossain, 2006). The rate of transformation in this case was slower than the transformation of naturally occurring brassilexin (21) and sinalexin (22). However, methylbrassilexin (69) was converted to enamine, 3-aminomethylene-1-methylindoline-2-thione (70) in cultures of *L. maculans* (Pedras and Suchy, 2005).
Scheme 1.9  Detoxification of methylbrassilexin (69) by *Leptosphaeria maculans* and *Sclerotinia sclerotiorum* (Pedras, 2008; Pedras and Hossain, 2006).
1.2.2.3 Camalexin and related compounds

Camalexin (24) was found stable in the fungal cultures of *L. maculans* and *A. brassicae* (Pedras and Khan, 1998; Pedras, 2004). Camalexin (24) was detoxified by *R. solani* via 5-hydroxycamalexin (72) to 5-hydroxyindole-3-carbonitrile (74) and 5-hydroxy-2-formamidophenyl-2'-thiazolylketone (73) (Scheme 1.10) (Pedras, 2004; Pedras and Ahiahonu, 2005; Pedras and Khan, 2000; Pedras and Liu, 2004).

Scheme 1.10 Detoxification of camalexin (24) by *Rhizoctonia solani*, *Leptosphaeria maculans* and *Alternaria brassicae* (Pedras and Liu, 2004; Pedras, 2004).

Camalexin was biotransformed by *S. sclerotiorum* via 6-hydroxycamalexin (75) instead of 5-hydroxycamalexin (72) to 6-oxy-(O-β-D-glucopyranosyl)camalexin (Scheme 1.11) (Pedras and Ahiahonu, 2005; 2002).
Scheme 1.11 Detoxification of camalexin (24) by *Sclerotinia sclerotiorum* (Pedras et al., 2004a; Pedras and Ahiahonu, 2005; 2002).

The selectivity and specificity of detoxifying enzymes of the stem rot fungus *S. sclerotiorum* were investigated by blocking the C-6 position of camalexin with -F and -OCH$_3$ substituents, as shown in Schemes 1.12 and 1.13. It was reported that 6-fluorocamalexin (77) was transformed to 6-fluoro-(1-β-D-glucopyranosyl)camalexin (78) (Pedras and Ahiahonu, 2005; 2002).

Scheme 1.12 Detoxification of 6-fluorocamalexin (77) by *Sclerotinia sclerotiorum* (Pedras and Ahiahonu, 2005; 2002, Pedras et al., 2004a).
6-Methoxycamalexin (79) was detoxified by S. sclerotiorum via two pathways: i) major pathway and ii) minor pathway. In the major pathway detoxification was accomplished via 6-hydroxycamalexin (75) to 6-oxy-(O-β-D-glucopyranosyl)camalexin (76) (Pedras and Ahiahonu, 2005; 2002). Methoxycamalexin (79) was partly converted to N-glucosylated product, 6-methoxy-(1-β-D-glucopyranosyl)camalexin (80) via a minor pathway (Pedras and Ahiahonu, 2005; 2002).

![Diagram of detoxification process]

**Scheme 1.13** Detoxification of 6-methoxycamalexin (79) by Sclerotinia sclerotiorum: i) major metabolite, ii) minor metabolite (Pedras and Ahiahonu, 2005; 2002).

6-Fluoro-1-methylcamalexin (81) was metabolized by S. sclerotiorum to metabolites 6-fluoro-1-methyl-(O-β-D-glucopyranosyl)camalexin (82) and 6-fluoro-1-methyl-indole-3-carbonitrile (83) (Pedras and Ahiahonu, 2005; 2002), at a slower rate than that observed for camalexin.
Scheme 1.14 Detoxification of 6-fluoro-1-methylcamalexin (81) by Sclerotinia sclerotiorum (Pedras and Ahiahonu, 2005; 2002).

The detoxifying enzyme specificity of R. solani was investigated by using the camalexin analogs 5-fluorocamalexin (84) and 5-methylcamalexin (88). As shown in Scheme 1.15, 5-fluorocamalexin was detoxified by R. solani producing the metabolites 2-(5-fluoro-3-indolyl)-oxazoline (85), 5-fluoroindole-3-carboxamide (86) and 5-fluoroindole-3-carbonitrile (87) (Pedras and Liu, 2004).

Scheme 1.15 Detoxification of 5-fluorocamalexin (84) by Rhizoctonia solani (Pedras and Liu, 2004; Pedras and Ahiahonu, 2005).

5-Methylcamalexin (88) was detoxified by R. solani producing the major metabolite, 5-(hydroxymethyl)camalexin (89) (Scheme 1.16). The minor metabolites identified were 2-(5-methyl-3-indolyl)-oxazoline (90) and 5-methylindole-3-carboxamide (91) (Pedras and Liu, 2004).
Scheme 1.16 Detoxification of 5-methylcamalexin (88) by *Rhizoctonia solani* (Pedras and Liu, 2004; Pedras and Ahiahonu, 2005).

1-Methylcamalexin (25), a phytoalexin from *Capsella bursa-pastoris* was metabolized by *R. solani* to 2-(1-methyl-3-indolyl)-oxazoline (92), 1-methylindole-3-carboxamide (93) and 1-methylindole-3-carbonitrile (94) (Pedras and Liu, 2004).


In my research project the biotransformations of the crucifer phytoalexins brassinin, brassilexin and camalexin were investigated using *A. brassicicola*. 
1.3 *Alternaria brassicicola*

*Alternaria brassicicola* is a necrotrophic fungus which causes blackspot disease in *Brassica* species (*Brassica napus* L. ssp. oleifera Metzg.). Scientific classification of *Alternaria brassicicola* (Schwein.) Wiltshire – Kingdom: Fungi; Phylum: Ascomycota; Class: Dothideomycetes; Subclass: Pleosporomycetidae; Order: Pleosporales; Family: Pleosporaceae; Genus: *Alternaria*; Species: *A. brassicicola*. This fungus exists almost everywhere – air, water and soil. Distribution: Cosmopolitan; Substrate: Leaves; Host: Brassicaceae; Disease Symptom: Dark brown to black circular leaf spots.

1.3.1 Pathogen significance: Blackspot disease

*A. brassicicola* is a destructive fungal pathogen that produces a complex mixture of toxic secondary metabolites. *A. brassicicola* causes severe yield losses in crops such as canola, mustard or rape with 20-50% yield reductions in infected fields (Rotem, 1994; Huang and Chung, 1993). Three *Alternaria* species namely i) *Alternaria brassicae*, ii) *Alternaria brassicicola* and iii) *Alternaria raphani* (*Alternaria japonica*) are responsible for blackspot disease (Verma and Saharan, 1994). *A. brassicicola* infects not only leaves but also other parts of the plant including seeds, pods and stems (Rotem, 1994). The necrotrophic nature of *A. brassicicola* is responsible for extensive damage to the plants. Disease propagation takes place from infected seeds to seedlings. The relationship between *Brassica* seeds internally infected by *A. brassicicola* and diseased seedlings was studied in England (Maude and Humpherson-Jones, 1980). This study showed that above 80% of commercial seeds were contaminated with *A. brassicicola* between 1976 and 1978. Insects also serve as transmission vectors for *A. brassicicola*
A. brassicicola spores kept in the leaf litter sporulate in the following season and initiate new infections (Humpherson-Jones and Maude, 1982). Dissemination of the disease during growing season is caused by rain and wind dislodged spores as well. Optimal conditions for sporulation and infection require the average period of 13 hours and a temperature range of 18 °C to 30 °C (Humpherson-Jones and Phelps, 1989, Rotem, 1994). Continuous moisture for 24 hours or longer practically guarantees infection (Rangel, 1945; Rotem, 1994). Relative humidity of 91.5% (at 20 °C) or higher produces large numbers of mature spores in 24 hours (Humpherson-Jones and Phelps, 1989). Thus, the presence of moisture in the form of free water or high relative humidity (at least 95%) is a requirement for germination (Degenhardt et al., 1982). Due to inadequate moisture, A. brassicicola rarely occurs in rapeseed on the cool prairies of Saskatchewan, Canada (Degenhardt et al., 1982, Rotem, 1994).

Blackspot disease can be controlled by a combination of chemical, biological and physical treatments. Chemical control includes use of fungicides in the infected fields. However, it poses great problem to the environment indicating the importance of other alternatives. Seed treatment with hot water is an effective method for controlling spores on seed coat, thus reducing the chance of seed-borne infection. However, hot water treatment depresses germination. Crop rotation with non-crucifer crops will help controlling the disease. Fields that are replanted soon after harvest have highest chances of a large amount of inoculum (Humpherson-Jones, 1989). Cultivars of Brassica species resistant to blackspot fungus can be used to combat disease epidemics (Howard et al., 1994; Verma and Saharan, 1994).
1.3.2 Secondary metabolites

The complete set of chemical reactions that occur in all living organisms for their growth, development and reproduction are known as primary metabolism. The essential chemical compounds such as sugars, amino acids, common fatty acids and nucleotides are synthesized by primary metabolism and are called primary metabolites. There exists another type of metabolism by which compounds of secondary utilities are produced. The production of such type of metabolites is initiated during particular stages of growth and development or during periods of stress, which include nutritional limitations and microbial attack (Mann, 1987). These compounds are known as secondary metabolites. Primary requirements of life for living organisms are maintained by primary metabolism whereas secondary metabolism produces compounds for secondary requirements such as ecological fitness, species selection, interspecies competition, mating response and defense against pathogens and predators.

The interaction between plant and pathogen is mediated partially by the production of secondary metabolites such as phytoalexins from plants and phytotoxins from fungi (Pedras et al., 2001). *A. brassicicola* produces a variety of secondary metabolites upon interaction with different species of crucifer (Otani et al., 1998). As well, crucifer plants utilize secondary metabolites for fighting against pathogens and other survival challenges.
1.3.2.1 Metabolites of *Alternaria brassicicola*

Phytopathogenic fungi produce a broad spectrum of secondary metabolites in culture and in the host. The isolation and identification of metabolites from culture filtrates of *A. brassicicola* yielded no phytotoxins. It was reported that *A. brassicicola* released proteins as well as cytokinins in vivo and in vitro (Suri and Mandahar, 1984; Otani et al., 1998). In 1969 Lidenfelser and Ciegler identified a secondary metabolite by antibiotic disc assay against *Bacillus subtilis* using the antibiotic complex obtained from *A. brassicicola* (Lidenfelser et al., 1969). The trivial name brassicicolin A was assigned to this antibiotic. Later in 1988 Gloer and co-workers isolated this compound from the culture of *A. brassicicola* and the structure of this compound was determined (Gloer et al., 1988). Brassicicolin A (95) was reported as naturally occurring novel isocyanide antibiotic from *A. brassicicola*, but its phytotoxic activity has not been evaluated.

Figure 1.3 Chemical structure of brassicicolin A (95)
Depudecin (96), an antitumor agent containing two epoxide groups was isolated from the culture broth of *A. brassicicola* (Matsumoto et al., 1992). The compound was also found to be a histone deacetylase inhibitor (Kwon et al, 2003).

![Depudecin](image)

**Figure 1.4** Chemical structure of depudecin (96)

Fusicoccin (97) was first isolated from the culture filtrate of the phytopathogenic fungus *Fussicoccum amygdali* Del. (Ballio et al., 1964). Similar structural feature also exists in cotylenol (98), which was isolated from the fungal culture of *Cladosporium spp*. (Sassa et al., 1972).

![Fusicoccin and Cotylenol](image)

**Figure 1.5** Chemical structures of fusicoccin (97) and cotylenol (98).
Six diterpenoid compounds were isolated from the culture filtrates of *A. brassicicola* possessing structural features similar to fusicoccin and cotylenol. These compounds were named as brassicene A-F (99, 100, 101, 102, 103, 104) by MacKinnon and coworkers (MacKinnon et al., 1999).

![Chemical structures of brassicene A-F](image)

**Figure 1.6** Chemical structures of brassicene A-F (99, 100, 101, 102, 103, 104).

### 1.3.2.2 Interaction with crucifers

The chemical interaction of *A. brassicicola* with crucifers results in the production of secondary metabolites by both organisms. The phytoalexin camalexin (24)
was detected in *Arabidopsis thaliana* as a defense response to pathogens (Tsuji et al., 1992; Glazebrook and Ausubel, 1994). The wild type *A. thaliana* (Columbia) inoculated with *A. brassicicola* produced a stronger camalexin (24) response than the phytoalexin-deficient *PAD3-1* mutants (Thomma et al., 1999). The enhanced susceptibility of *PAD3-1* mutants in comparison to the wild type species of *A. thaliana* against *A. brassicicola* was attributed to a deficiency of camalexin production (Glawischnig, 2007). *PAD3-1* mutants are defective in camalexin synthesis that causes increased susceptibility to infection (Thomma et al., 1999). The *PAD3* (*PhytoAlexin Deficient 3*) gene is the key determinant in the defense against *A. brassicicola* (Glawischnig, 2007; Thomma et al., 1999). Studies suggested that *PAD3-1* mutations not only changed the accumulation of camalexin (24) but also many defense related metabolites in response to the challenge against *A. brassicicola* (Narusaka et al., 2003).

Crucifers integrate multiple responses including production of glucosinolates, in response to the invasion of *A. brassicicola* (Halkier and Gershenzon, 2006; Glawischnig, 2007). The crucifers that are able to synthesize phytoalexins faster will be able to control the disease at the early stages. Thus, this group of plants will be resistant to the pathogen. If the fungus gets the advantage of spreading, the species will be disease sensitive. *A. brassicicola* produced phytotoxic fluids when incubated with different species of crucifer leaves (Otani et al., 1998). The spore germination fluid (SGF) as well as methanol extracts of culture filtrates of *A. brassicicola* tested for toxicity developed necrotic lesions on the leaves of infected crucifers (Otani et al., 1998; McDonald and Ingram, 1986; Oka et al., 2005). Culture filtrates of *A. brassicicola* were also found toxic to secondary embryoids of *Brassica napus ssp. Oleifera* (Metzg.), winter oilseed rape
(McDonald and Ingram, 1986). In their study, the methanol fraction of filtrates resulted in 90% death of the embryoids. Detached leaf tests using methanol extracts displayed chlorosis and necrosis, similar to that caused by the pathogen. Partially purified culture filtrates of *A. brassicicola* inhibited pollen germination of *B. napus* (Hodgkin and McDonald, 1986). Literature studies clearly indicated that extracts of *A. brassicicola* inhibited all stages of crucifer growth and development.

1.4 Summary and conclusion

Plants as sessile organisms are always exposed to a myriad of microbial and fungal invaders. Due to fungal invasion, the production of crucifer plants such as broccoli (*B. oleracea* var. *botrytis*), cabbage (*B. oleracea* var. *capitata*), cauliflower (*B. oleracea* var. *italica*), mustard (*B. juncea*) and canola is severely affected. *A. brassicicola* produces a complex blend of metabolites some of which are toxic to crucifer plants. In response, crucifer crops produce induced chemical defenses against *A. brassicicola*. Both organisms synthesize detoxifying enzymes to degrade each other’s chemical weapons. Isolation of detoxifying enzymes and detoxification metabolites will allow us to get a better understanding about pathogen invasion, host defense and virulence. Thus my research project was focused on the metabolism of crucifer phytoalexins by the phytopathogenic fungus *A. brassicicola*. In this introductory chapter I have summarized some of the relevant work published using *A. brassicicola* as well as biotransformation of crucifer phytoalexins by different fungi.
CHAPTER 2: RESULTS AND DISCUSSION

2.1 Syntheses of phytoalexins

For metabolism and biological activity studies, relatively large quantities of phytoalexins are necessary. Since it is difficult and time consuming to isolate larger quantities of these metabolites from plants, sufficient quantities required for the study of biotransformation and antifungal activity can be obtained through syntheses. There are several methods for the synthesis of each phytoalexin (Pedras et al., 2007a). For instance, three methods are known for brassinin synthesis. Phytoalexins used for this study were synthesized and characterized on the basis of spectroscopic analyses and comparison with authentic samples.
2.1.1 Synthesis of brassinin (2)

Brassinin (2) was synthesized starting from indole-3-carboxaldehyde (42) in a four step process (Pedras et al, 1992), as shown in Scheme 2.1.

Scheme 2.1 Synthesis of brassinin (2). Reagents and conditions: (i) NH₂OH.HCl, Na₂CO₃, EtOH, 2 h, reflux, 60°C, 90%; (ii) NaBH₄, NiCl₂.6H₂O, MeOH, rt, 50%; (iii) Pyridine, Et₃N, CS₂, 0°C; (iv) MeI, 0°C, 55%; (v) Pyridine, Ac₂O, 1 h, 0°C, 60%.
2.1.2 Synthesis of brassilexin (21)

Brassilexin was synthesized through Vilsmeier formylation of indoline-2-thione in a two-pot reaction starting from indoline-2-one (Pedras and Jha, 2005), as shown in Scheme 2.2.

\[
\begin{align*}
\text{N} & \quad \text{O} \\
\text{106} & \quad \xrightarrow{i} \quad \text{N} & \quad \text{H} \\
& \quad \text{S} & \quad \text{N} & \quad \text{H} \\
\text{21} & \quad \xrightarrow{\text{ii, iii}} \quad \text{N} & \quad \text{H} \\
& \quad \text{S} & \quad \text{N} & \quad \text{H} \\
& \quad \text{NH}_2 \\
\end{align*}
\]

Scheme 2.2 Synthesis of brassilexin (21). Reagents and conditions: (i) \( \text{P}_4\text{S}_{10}, \text{NaHCO}_3, \text{THF}, 81\% \); (ii) \( \text{POCl}_3, \text{DMF} \); (iii) \( \text{NH}_4\text{OH} \); (iv) \( \text{I}_2, \text{Pyridine}, 52\% \).

2.1.3 Synthesis of camalexin (24)

Synthesis of camalexin was performed following a published procedure (Ayer et al., 1992), as shown in Scheme 2.3.

\[
\begin{align*}
\text{N} & \quad \text{i, ii} \\
\text{108} & \quad \xrightarrow{} \quad \text{N} & \quad \text{H} \\
& \quad \text{S} & \quad \text{N} & \quad \text{H} \\
& \quad \text{H} & \quad \text{S} \\
\text{24} & \quad \\
\end{align*}
\]

Scheme 2.3 Synthesis of camalexin (24). Reagents and conditions: (i) \( \text{Mg, CH}_3\text{I, Et}_2\text{O} \); (ii) Benzene, 2-bromothiazole, 90 °C, 29\%.
2.2 Biotransformation of phytoalexins by *Alternaria brassicicola*

2.2.1 Biotransformation of brassinin

Brassinin (2) was synthesized as described in the experimental (Pedras et al., 1992) and its biotransformation in liquid cultures of *A. brassicicola* was studied over a period of time as detailed in the experimental section. Samples from liquid cultures of *A. brassicicola* incubated with brassinin (2, 0.1 mM) were collected at 0 h and at different time intervals and extracted with EtOAc (2 × 10 mL). The organic extracts were concentrated under reduced pressure and the residues were dissolved in CH$_3$CN (1mL) for HPLC analysis (Grad Screen method). Comparison of HPLC chromatograms of extracts of fungal cultures and control incubated with brassinin (2, 0.1 mM) indicated the emergence of a new metabolite and concomitant disappearance of brassinin (2) (Figure 2.1).
Figure 2.1  Chromatograms (Grad Screen method) of extracts of liquid cultures of Alternaria brassicicola in minimal media (fungal culture) and only minimal media (control) incubated with brassinin (2, 0.1 mM) for different time intervals (0 h, 24 h and 48 h).
It was observed that brassinin (2) was completely metabolized in fungal cultures to a major product ($t_R = 4.9$ min) within 48 hours. The structure of this metabolite was established by analyses of its spectroscopic data and synthesis as follows. Comparison of $^1$H NMR spectra with that of brassinin (2) as well as with synthetic $N_h$-acetyl-3-indolylmethylamine (46) ($t_R = 4.9$ min) indicated the presence of protons due to an indole moiety at $\delta$ 7.65 (d, $J$=8 Hz, 1H), 7.40 (d, $J$=8 Hz, 1H), 7.17 (m, 2H), two methylene protons at $\delta$ 4.62 (d, $J$=5 Hz, 2H) and a singlet at $\delta$ 2.00 (s, 3H). The molecular formula of this metabolite (C$_{11}$H$_{12}$N$_2$O) was determined by HRMS-ESI ($m/z$ found 188.0949 M$^+$, calc. 188.0947). Interestingly this metabolite was formed via indole-3-methylamine (45) intermediate (Scheme 2.4) similar to previous findings of brassinin biotransformation by L. maculans (Pedras et al., 2007d; Pedras and Taylor, 1993).

**Scheme 2.4** Biotransformation of brassinin (2) via amine (45) in fungal cultures of *Alternaria brassicicola*.  

---

37
The formation of indole-3-methylamine (45) intermediate was determined by HPLC analysis (AMINE2 method) of basic extracts of fungal cultures incubated with brassinin (2, 0.1 mM). The appearance of indole-3-methylamine (t_R = 6.4 min) in the chromatogram of basic extracts established that biotransformation of brassinin to N_h-acetyl-3-indolylmethylamine (46) occurred via indole-3-methylamine (45) intermediate. The retention time (t_R = 6.4 min) matches with standard amine. The presence of indole-3-methylamine (45) was also confirmed by comparison of 1H NMR data of the basic extracts with spectroscopic data of an authentic amine sample.

**Figure 2.2** Chromatograms of both basic and neutral extracts of cultures of *Alternaria brassicicola* incubated with brassinin (2, 0.1 mM) for a period of 8 h.
The brassinin (2) biotransformation and formation of \( N_b \)-acetyl-3-indolylmethylamine (46) curves as a function of time are depicted in Figure 2.3

![Graph showing curves for biotransformation of brassinin (2, 0.1 mM) and formation of \( N_b \)-acetyl-3-indolylmethylamine (46) in cultures of *Alternaria brassicicola*. Concentrations were calculated using calibration curves; each point is the average of experiment conducted in triplicate ± standard deviations.](image)

**Figure 2.3** Curves for biotransformation of brassinin (2, 0.1 mM) and formation of \( N_b \)-acetyl-3-indolylmethylamine (46) in cultures of *Alternaria brassicicola*. Concentrations were calculated using calibration curves; each point is the average of experiment conducted in triplicate ± standard deviations.

Biotransformation of brassinin by *A. brassicicola* occurred in the side chain similar to the phytopathogenic fungi *L. maculans* and *L. biglobosa* (Pedras and Okanga, 1998, 2000; Pedras and Ahiahonu, 2005). However, contrary to *L. maculans* and *L. biglobosa*, indole-3-carboxylic acid (43) was not detected as final detoxification product. It was shown from antifungal activity assay that the metabolite was a detoxification
product. Previous work showed that *S. sclerotiorum* yielded *N*-glucosylated metabolite rather than side chain modification of brassinin as shown in Scheme 1.1 (Pedras and Ahiahonu, 2005; Pedras et al., 2007d).

### 2.2.1.1 Biotransformation of indole-3-methylamine

Time-course experiments were conducted to confirm the transformation of indole-3-methylamine (45) to *Nμ*-acetyl-3-indolylmethylamine (46) in cultures of *A. brassicicola*. Indole-3-methylamine (45) was synthesized as described in the experimental and incubated with cultures of *A. brassicicola* at 0.1 mM, as detailed in the experimental section. Samples from liquid cultures were collected at 0 h and at different time intervals and extracted with EtOAc (2 × 10 mL). The organic extracts were concentrated under reduced pressure and the residue was dissolved in CH$_3$CN (1 mL) for HPLC analysis (Grad Screen method). The transformation of indole-3-methylamine (45) to *Nμ*-acetyl-3-indolylmethylamine (46) was determined from the chromatograms of neutral extracts. The basic extracts were analyzed by HPLC (AMINE2 method) as detailed in the experimental section.
The curve for formation of the metabolic product, \( N_b \)-acetyl-3-indolylmethylamine (46) is shown in Figure 2.4.

\[ \text{\( N_b \)-acetyl-3-indolylmethylamine} \]

\[ \text{Concentration} [\text{mM}] \]

\[ \text{Time} [\text{days}] \]

**Figure 2.4** Curve for biotransformation of indole-3-methylamine (45, 0.1 mM) to \( N_b \)-acetyl-3-indolylmethylamine (46) in cultures of *Alternaria brassicicola*. Concentrations were calculated using calibration curves; each point is the average of concentrations from a single experiment conducted in triplicate ± standard deviations.

Conversion of indole-3-methylamine (45) by *A. brassicicola* to \( N_b \)-acetyl-3-indolylmethylamine (46) confirmed that bioconversion of brassinin (2) takes place via indole-3-methylamine (45), as shown in Scheme 2.4. The formation of the detoxification product by *L. biglobosa* also occurred via indole-3-methylamine (45) as shown in Scheme 1.1 (Pedras and Ahiahonu, 2005; Pedras et al., 2007d).
2.2.1.2  Biotransformation of \(N_b\)-acetyl-3-indolylmethylamine

\(N_b\)-acetyl-3-indolylmethylamine (46) was synthesized as described in the experimental and its biotransformation in liquid cultures of \(A. brassicicola\) was studied over a period of several days. Solutions of \(N_b\)-acetyl-3-indolylmethylamine (46) in acetonitrile (200 \(\mu\)L) were added to fungal cultures and were incubated. Control cultures were incubated in parallel. Samples from liquid cultures of \(A. brassicicola\) and control cultures incubated with \(N_b\)-acetyl-3-indolylmethylamine (46, 0.1 mM) were collected at 0 h and at different time intervals and extracted with EtOAc (2 \(\times\) 10 mL). The organic extracts were concentrated under reduced pressure and the residues were dissolved in CH\(_3\)CN (1 mL) for HPLC analysis (Grad Screen method). The basic extracts were analyzed by HPLC (AMINE2 method) as detailed in the experimental section. \(N_b\)-acetyl-3-indolylmethylamine (46) seems to be stable in the cultures over a period of 10 days. Further metabolism of \(N_b\)-acetyl-3-indolylmethylamine (46) by \(A. brassicicola\) was not observed in the HPLC chromatograms of both neutral and basic broth extracts (Grad Screen method and AMINE2 method, respectively). In addition, the antifungal activity experiments supported that \(N_b\)-acetyl-3-indolylmethylamine (46) was a detoxification product of brassinin (2).
The graph for the time-course experiment of (46) is shown in Figure 2.5

Figure 2.5  Curve for biotransformation of \(N_b\)-acetyl-3-indolylmethylamine (46, 0.1 mM) in cultures of \textit{Alternaria brassicicola}. Concentrations were calculated using calibration curves; each point is the average of concentrations from a single experiment conducted in triplicate ± standard deviations.

Indole-3-carboxylic acid (43) was the final metabolite in the detoxification of brassinin (2) by \textit{L. maculans} and \textit{L. biglobosa} as shown in Scheme 1.1. However, \(N_b\)-acetyl-3-indolylmethyamine (46) was the final product for the detoxification of brassinin (2) by \textit{A. brassicicola}. It is likely that \textit{A. brassicicola} transforms 46 to metabolites not extractable with organic solvents due to their higher solubility in water.
2.2.2 Biotransformation of brassilexin

Brassilexin (21) was synthesized as described in the experimental and its biotransformation in liquid cultures of *A. brassicicola* was studied over a period of time. Solutions of brassilexin in acetonitrile were added to fungal cultures and were incubated. Control cultures were incubated in parallel. Samples from liquid cultures of *A. brassicicola* and controls incubated with brassilexin (21, 0.1 mM) were collected at 0 h and at different time intervals and extracted with EtOAc (2 × 10 mL). The extracts were concentrated under reduced pressure and the residues were dissolved in CH$_3$CN (1 mL) for HPLC analysis (Grad Screen method). Brassilexin (21) was stable in control medium over 5 days. Metabolism of brassilexin (21, 0.1 mM) by *A. brassicicola* was completed within 24 h. Comparison of HPLC chromatograms of extracts of fungal cultures and control cultures indicated the emergence of a major peak (t$_R$ = 7.2 min) with disappearance of brassilexin. The new peak (t$_R$ = 7.2 min) was due to a known compound, 3-(amino)methyleneindoline-2-thione (66) formed by reduction of the isothiazole ring of brassilexin (21) (Pedras and Suchy, 2005) in the fungal culture. The metabolism of brassilexin (21) by *A. brassicicola* to 3-(amino)methyleneindoline-2-thione (66) is shown in Scheme 2.5.

![Scheme 2.5 Biotransformation of brassilexin (21) in cultures of *Alternaria brassicicola*](image-url)
3-(Amino)methyleneindoline-2-thione (66) was synthesized to corroborate its identity, as described in the experimental. Biotransformation of brassilexin (21, 0.1 mM) over a period of time in the cultures of *A. brassicicola* is shown in figure 2.6.

![Graph showing biotransformation of brassilexin](image)

**Figure 2.6** Curve for biotransformation of brassilexin (21, 0.1 mM) in cultures of *Alternaria brassicicola*. Concentrations were calculated using calibration curves; each point is the average of concentrations from a single experiment conducted in triplicate ± standard deviations.

The metabolite (66) is less stable and readily oxidized to form brassilexin (21). Synthetic 3-(amino)methyleneindoline-2-thione (66) was also unstable in silica column and in solvent solutions. Chromatographic separation did not afforded pure compound and thus a calibration curve for the determination of concentration of 3-
(amino)methyleneindoline-2-thione (66) was not prepared. The curve for the formation of 3-(amino)methyleneindoline-2-thione (66) is shown in Figure 2.7 using mAU instead of concentrations.

![Figure 2.7](image)

**Figure 2.7** Curve for the formation of 3-(amino)methyleneindoline-2-thione (66) in the biotransformation of brassilexin (21, 0.1 mM) by *Alternaria brassicicola*. A calibration curve for concentration determination was not prepared; each point is the average of areas in mAU (milliabsorbance units) from a single experiment conducted in triplicate ± standard deviations.

Detoxification of brassilexin (21) by *A. brassicicola* yielded 3-(amino)methyleneindoline-2-thione (66) similar to *L. maculans* and *S. sclerotiorum* as shown in Scheme 1.8 (Pedras, 2008).
2.2.2.1 Biotransformation of 3-(amino)methyleneindoline-2-thione

3-(Amino)methyleneindoline-2-thione (66) was synthesized as described in the experimental and its biotransformation in liquid cultures of *A. brassicicola* was studied over a period of time. Solutions of 3-(amino)methyleneindoline-2-thione (66) in acetonitrile were added to fungal cultures and were incubated. Control cultures were carried out in parallel. Samples from liquid cultures of *A. brassicicola* incubated with 3-(amino)methyleneindoline-2-thione (66, 0.1 mM) and control cultures were collected at 0 h and at different time intervals and extracted with EtOAc (2 × 10 mL). The extracts were concentrated under reduced pressure and the residues were dissolved in CH₃CN for HPLC analysis (Grad Screen method). 3-(Amino)methyleneindoline-2-thione (66) was almost completely metabolized in the cultures of *A. brassicicola* within ca. 24 h.

Synthetic enamine (66) used for feeding experiment was not pure and it contained brassilexin (21) as was determined by ¹H NMR. The spectroscopic data presented in the experimental section was assigned due to the presence of units from multiple entities in the tautomeric mixture of 3-(amino)methyleneindoline-2-thione, 3-(amino)methylene-2-thiol and 3-(methylimino)indole-2-thiol (Pedras and Okanga, 1998). The initial concentration for feeding was determined on the basis of the ratio of the area of protons from ¹H NMR. The concentration of this compound (66) was initially increased in the culture due to the faster reduction of the isothiazole ring of brassilexin (21) than transformation of 66, and then decreased.
The result of time-course experiments of 3-(amino)methyleneindoline-2-thione (66) metabolized in fungal cultures is shown in Figure 2.8.

![Graph showing the biotransformation of 3-(amino)methyleneindoline-2-thione](image)

**Figure 2.8** Curve for the biotransformation of 3-(amino)methyleneindoline-2-thione (66, 0.1 mM) in cultures of *Alternaria brassicicola* (in minimal media). The calibration curves for concentration determinations were not prepared due to instability of 3-(amino)methyleneindoline-2-thione (66) in solvent systems as well as in silica; each point is the average of concentrations from a single experiment conducted in triplicate ± standard deviations.

3-(Amino)methyleneindoline-2-thione (66) was not a stable product and the compound exists as mixture of tautomers (Pedras and Okanga, 1998). Thus my attempt to establish 3-(amino)methyleneindoline-2-thione (66) as detoxification product of brassilexin (21) was unsuccessful.
2.2.3 Biotransformation of camalexin

The phytoalexin camalexin (24) is a strong inhibitor of growth against *A. brassicicola*. Camalexin (24) was synthesized as described in the experimental and its metabolism in liquid cultures of *A. brassicicola* was studied over a period of several days. Solutions of camalexin in acetonitrile were added to fungal cultures and were incubated. Control cultures were carried out in parallel. Samples from liquid cultures of *A. brassicicola* and controls incubated with camalexin (24, 0.1 mM) were collected at 0 h and at different time intervals and extracted with EtOAc. The extracts were concentrated under reduced pressure and the residues were dissolved in CH$_3$CN for HPLC analysis (Grad Screen method). I was not able to detect or identify any metabolites from the extracts of fungal cultures incubated with camalexin (24, 0.1mM). The process of metabolism of camalexin (24) may be very slow or the quantity of metabolites formation may be negligible or very polar (more water soluble). Longer incubation period may lead to the identification of metabolites for camalexin (24) metabolism. However, camalexin (24) was not metabolized by *L. maculans, A. brassicae* and pathogenic bacteria (Pedras and Khan, 1998). The decrease in concentration of camalexin (24) with increasing time of incubation may also be due to the absorption of camalexin (24) by cell membranes of mycelia. After 10 days, all mycelia from the culture flasks were filtered, air-dried and extracted with methanol to recover camalexin potentially trapped in mycelia cells. The methanol extracts were concentrated under reduced pressure and the residue was dissolved in methanol for HPLC analysis (Grad Screen method). The recovery of camalexin was 24% from mycelial cells with respect to the initial amount; altogether ca. 66% camalexin (24) was recovered.
The curve for time-course experiments of camalexin (24) is shown in figure 2.9.

**Figure 2.9**  Curve for biotransformation of camalexin (24, 0.1 mM) in cultures of *Alternaria brassicicola*. Concentrations were calculated using calibration curves; each point is the average of concentrations from a single experiment conducted in triplicate ± standard deviations.

Camalexin (24) biotransformation is different from that reported for *R. solani* (Scheme 1.10) (Pedras, 2004). However, this fact was not completely established perhaps due to the slow metabolism of camalexin (24) by *A. brassicicola*. Camalexin (24) was also metabolized by *S. sclerotiorum* but not metabolized by *L. maculans* or *A. brassicae* (Pedras et al., 2004a; Pedras and Ahiahonu, 2005; 2002; Pedras and Liu, 2004).
2.3 Antifungal activity

Antifungal activity of phytoalexins can be determined by conducting different types of bioassay such as fungal radial growth assay using PDA or minimal media on wells of tissue culture plates or Petri dishes (Pedras and Jha, 2006), fungal spore germination on TLC plates (Pedras and Sorenson, 1998) and filter paper disc assay (Lazarevic et al., 2001). In my study, mycelial radial growth bioassay on PDA media was chosen for the determination of percent inhibition of growth. At all times fungal radial growth assay results were reproducible and each bioassay was carried out in triplicate, using at least three independent experiments. The final concentrations of assay compounds were $5.0 \times 10^{-4}$ M, $2.0 \times 10^{-4}$ M and $1.0 \times 10^{-4}$ M in PDA media. The results of the antifungal activity using mycelial radial growth are summarized in the following Table 2.1.

**Table 2.1** Percentage of growth inhibition of *Alternaria brassicicola* incubated with phytoalexins (2, 21 and 24) and metabolites (45, 46, 66) after 72 h of incubation under constant light.

<table>
<thead>
<tr>
<th>Name of assay compound</th>
<th>Concentrations (M)</th>
<th>% Inhibition$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brassinin (2)</td>
<td>$5.0 \times 10^{-4}$</td>
<td>39±2</td>
</tr>
<tr>
<td></td>
<td>$2.0 \times 10^{-4}$</td>
<td>31±2</td>
</tr>
<tr>
<td></td>
<td>$1.0 \times 10^{-4}$</td>
<td>22±1</td>
</tr>
<tr>
<td>Indole-3-methylamine (45)</td>
<td>$5.0 \times 10^{-4}$</td>
<td>16±1</td>
</tr>
<tr>
<td></td>
<td>$2.0 \times 10^{-4}$</td>
<td>5±1</td>
</tr>
<tr>
<td></td>
<td>$1.0 \times 10^{-4}$</td>
<td>N.I.</td>
</tr>
<tr>
<td>$N_6$-acetyl-3-indolylmethylamine (46)</td>
<td>$5.0 \times 10^{-4}$</td>
<td>N.I.</td>
</tr>
<tr>
<td></td>
<td>$2.0 \times 10^{-4}$</td>
<td>N.I.</td>
</tr>
<tr>
<td></td>
<td>$1.0 \times 10^{-4}$</td>
<td>N.I.</td>
</tr>
<tr>
<td>Name of assay compound</td>
<td>Concentrations (M)</td>
<td>% Inhibitiona</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Brassilexin (21)</td>
<td>5.0 × 10⁻⁴</td>
<td>85±2</td>
</tr>
<tr>
<td></td>
<td>2.0 × 10⁻⁴</td>
<td>59±2</td>
</tr>
<tr>
<td></td>
<td>1.0 × 10⁻⁴</td>
<td>50±3</td>
</tr>
<tr>
<td>3-(Amino)methyleneindoline-2-thione b (66)</td>
<td>5.0 × 10⁻⁴</td>
<td>92±3</td>
</tr>
<tr>
<td></td>
<td>2.0 × 10⁻⁴</td>
<td>59±1</td>
</tr>
<tr>
<td></td>
<td>1.0 × 10⁻⁴</td>
<td>47±1</td>
</tr>
<tr>
<td>Camalexin (24)</td>
<td>5.0 × 10⁻⁴</td>
<td>C.I.</td>
</tr>
<tr>
<td></td>
<td>2.0 × 10⁻⁴</td>
<td>85±1</td>
</tr>
<tr>
<td></td>
<td>1.0 × 10⁻⁴</td>
<td>53±1</td>
</tr>
</tbody>
</table>

a [The percentage of inhibition was calculated using the formula: % inhibition = 100 – \{(growth on amended/growth in control) × 100\} ± standard deviation]; results are the means of at least three independent experiments; C.I., Complete Inhibition; N.I., No Inhibition.

b Synthetic enamine was not obtained in pure form since the compound was not stable in silica as well as in solvent solutions. The purity of the compound may be the cause for high inhibition in bioassay results as expected with respect to brassilexin.

### 2.4 Conclusion

The antifungal activity and biotransformation of crucifer phytoalexins against *A. brassicicola* were investigated. The phytoalexins used for feeding and bioassay experiments were synthesized as detailed in the experimental. The purity of products was confirmed by spectroscopic analyses. The metabolism of brassinin (2) gave a known metabolite (46) which was isolated, characterized as well as synthesized for structural determination. Finally, the antifungal bioassay and feeding experiments confirmed that the transformation was a detoxification process. In addition, it is clear from this study that the putative intermediate in the transformation of brassinin (2) is an amine (45) similar to the metabolism occurring in other fungi (Pedras and Okanga, 2000).
The metabolism of brassilexin (21) gave a metabolite which was a known compound isolated earlier (Pedras and Suchy, 2005). This metabolite (66) was synthesized and used for both bioassay and feeding experiments to confirm the metabolism and detoxification process. The metabolism of brassilexin (21) was not in agreement with the expected trend of phytoalexin detoxification. The discrepancies in the result of bioactivity and metabolism can be attributed to the instability and unavailability of the detoxification product in the pure form for biotransformation.

Camalexin metabolism was very slow and any metabolite from biotransformation of camalexin in the culture of A. brassicicola was not detected within 10 days of incubation period. Antifungal activity showed that camalexin (24) was a strong growth inhibitor against A. brassicicola whereas brassinin (2) displayed lower inhibition than brassilexin and camalexin. The percent inhibition of growth against A. brassicicola caused by brassilexin (21, 0.5 mM) was 85, suggesting that brassilexin is a reasonable inhibitor. Kagan and co-workers reported that TLC bioassay results of camalexin showed poor inhibitory effect against A. brassicicola (Kagan et al., 2002). However, the mycelial radial growth bioassay in my study suggested that camalexin (24) is a strong inhibitor of A. brassicicola. Brassinin (2) metabolism was completed in the cultures of A. brassicicola over a period of 48 h. Brassilexin (21) was metabolized in cultures of A. brassicicola within ca. 24 h. I was not able to detect any metabolite from camalexin metabolism within 10 days incubation period which left the possibility of future work of the metabolism study for longer incubation periods.
CHAPTER 3: EXPERIMENTAL

3.1 General methods

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON, Canada. All solvents were HPLC grade and used as such, except phosphorous oxychloride (POCl₃), benzene and ether which were redistilled. Solvents used in syntheses were dried over the following drying agents prior to distillation and use: benzene over CaH₂ and diethyl ether over sodium/benzophenone.

Analytical Thin Layer Chromatography (TLC) was carried out on precoated silica gel TLC aluminum sheets (Merck, Kieselgel 60 F₂₅₄, 5 × 2.5 cm × 0.2 mm). Compounds were visualized by exposure to UV (254 nm) after elution with a suitable solvent system. Finally plates were charred by dipping in a 5% aqueous (w/v) phosphomolybdic acid solution containing 1% (w/v) ceric sulphate and 4% (v/v) H₂SO₄, followed by heating on a hot plate. Preparative Thin Layer Chromatography (PTLC) was performed on silica gel glass plates (Merck, Kieselgel 60 F₂₅₄, 20 × 20cm × 0.25 mm). Flash Column Chromatography (FCC) was carried out on silica gel (Merck, grade 60, and mesh size 230-400, 60 Å).

HPLC analyses were carried out with Agilent 1100 series high performance liquid chromatographic systems equipped with a quaternary pump, automatic injector and diode array detector (wavelength range 190-600 nm), degasser and Eclipse XSB C-18 columns (5 µm particle size, 150 x 4.6 mm diameter), equipped with an in-line filter.
The mobile phase consisted of a linear gradient of water and acetonitrile (75:25 to 25:75 in 35 min to 0:100 in 5 min, Grad Screen method) and a flow rate of 1 mL/min. Samples for HPLC analysis were dissolved in CH₃CN (ca. 0.2 mg/mL to 0.5 mg/mL) and filtered through a cotton plug.

HPLC analysis for amine detection (AMINE2 method) was carried out with an Agilent 1200 series high performance liquid chromatographic system equipped with quaternary pump, automatic injector and diode array detector (wavelength range 190-600 nm), degasser and an Eclipse XDB-CN column (5 µm particle size, 150 × 4.6 mm diameter), equipped with an in-line filter. The mobile phase consisted of a linear gradient of 0.5% NH₄OH in isopropanol and hexane (85:15 to 100:0 in 15 min and 0:100 to 85:15 in 5 min, AMINE2 method) and a flow rate of 1 mL/min. Samples for HPLC analysis were prepared by dissolving in CH₃CN (ca. 0.2 mg/mL to 0.5 mg/mL) and passing through a cotton plug.

HPLC-MS-ESI analysis was carried out with an Agilent 1100 series HPLC system equipped with an autosampler, binary pump, degasser, and a diode array detector connected directly to a mass detector (Agilent G2440A MSD-Trap-XCT ion trap mass spectrometer) with an ESI source. Chromatographic separation was carried out at room temperature using an Eclipse XSB C-18 column (5 µm particle size, 150 x 4.6 mm diameter). The mobile phase consisted of a linear gradient of 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B) [75:25 to 25:75 (A:B) in 35 min and 0:100 (A:B) in 5 min] and a flow rate of 1mL/min. Samples were prepared by dissolving in acetonitrile (ca 0.2 mg/mL to 0.5 mg/mL) and passing through a cotton plug.
NMR spectra were recorded on Bruker Avance 500 MHz spectrometers. For $^1$H NMR (500 MHz), the chemical shifts (δ) are reported in parts per million (ppm) relative to TMS. The δ values were referenced to CHCl$_3$ in CDCl$_3$ at 7.27 ppm and CHD$_2$CN in CD$_3$CN at 1.94 ppm. Multiplicities are denoted by the following symbols: s = singlet, d = doublet, dd = doublet of doublets, m = multiplets and br = broad. Spin coupling constants ($J$ values) are reported to the nearest 0.5 Hz. For $^{13}$C NMR (125.8 MHz), δ values are referenced to CDCl$_3$ (77.23 ppm) and CD$_3$CN (118.69 ppm).

Mass Spectra [high resolution (HR), electron impact (EI)] were obtained on a VG 70 SE mass spectrometer at electron beam energy of 70 eV using a solids probe.

### 3.2 Syntheses of phytoalexins

#### 3.2.1 Synthesis of brassinin (2)

![Scheme 3.1 Synthesis of brassinin (2)](image_url)
An aqueous solution of NH₂OH.HCl (952 mg, 13.7 mmol) and Na₂CO₃ (803 mg, 7.6 mmol) in water (14 mL) was added to a solution of indole-3-carboxaldehyde (42) (1.0 g, 6.9 mmol) in EtOH (30 mL). After stirring for 3 hours at 60 °C, EtOH was removed under reduced pressure and ice cold water was added to the residue. The resulting precipitate was filtered off and air dried to afford indole-3-carboxaldehyde oxime (105) (995 mg) in 90% yield (Pedras et al., 1992).

Sodium borohydride (140 mg, 3.7 mmol) was added in small portions to a solution of indole-3-carboxaldehyde oxime (105) (200 mg, 1.25 mmol) and hexahydrated nickel chloride (298 mg, 1.25 mmol) in methanol (25 mL) with vigorous stirring. The reaction mixture was filtered off (under gravity filtration) after 15 minutes of stirring and the volume was reduced to approximately 1/3 under reduced pressure. The resulting mixture was diluted with water and basified with aqueous NH₄OH (28%, pH 9). The basified solution was extracted using EtOAc (3 x 50 mL), the organic extracts were combined, dried over Na₂SO₄ and evaporated under reduced pressure to yield the crude amine. The crude product was purified by FCC eluting with CH₂Cl₂-MeOH-NH₄OH (80:20:1) to afford indole-3-methylamine (45) in 50% yield (Kutschy et al., 1998).

**Indole-3-methylamine (45):**

HPLC tᵣ = 6.0 min (AMINE2 Method).

¹H NMR (500 MHz, CD₃CN): δ 9.19 (br s, 1H), 7.61 (d, J=8 Hz, 1H), 7.38 (d, J=8 Hz, 1H), 7.13 (m, 2H), 7.04 (dd, J=7.5, 7.5 Hz, 1H), 3.95 (s, 2H), 2.05 (s, 2H).
Carbon disulfide (70 µL, 1.5 mmol) was added to a solution of indole-3-methylamine (45) (220 mg, 1.5 mmol) and triethylamine (217 µL, 3 mmol) in pyridine (2.3 mL) at 0°C. After stirring for 20 minutes, CH₃I (93 µL, 1.5 mmol) was added and the resulting reaction mixture was stirred for an additional 1 hour. The reaction mixture was acidified with 1.5 M H₂SO₄ and extracted with Et₂O (2 x 25 mL). The organic extracts were combined, dried over Na₂SO₄ and concentrated under reduced pressure using toluene. The residue was purified by FCC on silica gel eluting with EtOAc-hexane (40:60) to afford brassinin (2) which was finally crystallized from CH₂Cl₂, white crystals in 55% yield (Pedras et al., 1992).

**Brassinin (2):**

HPLC $t_R = 20.5$ min (Grad Screen method).

$^1$H NMR (500 MHz, CD₃CN): $\delta$ 9.25 (br s, 1H), 8.25 (br s, 1H), 7.63 (d, $J$=8 Hz, 1H), 7.42 (d, $J$=8 Hz, 1H), 7.31 (d, $J$=2 Hz, 1H), 7.16 (dd, $J$=8, 8 Hz, 1H), 7.09 (dd, $J$=8, 8 Hz, 1H), 5.04 (d, $J$=5 Hz, 2H), 2.55 (s, 3H).

$^{13}$C NMR (125.8 MHz, CD₃CN): $\delta$ 199.1 (s), 137.7 (s), 128.1 (s), 126.1 (s), 123.2 (d), 120.7 (d), 119.8 (d), 120.7 (d), 112.9 (d), 111.8 (s), 43.5 (s), 18.5 (s).

### 3.2.1.1 Synthesis of N₆-acetyl-3-indolylmethylamine (46)

![Scheme 3.2](image)

**Scheme 3.2** Synthesis of N₆-acetyl-3-indolylmethylamine (46)
To a solution of indole-3-methylamine (45) (29 mg, 0.2 mmol) in pyridine (500 µL, 6.1 mmol) at 0°C, Ac₂O (85 µL, 0.89 mmol) was added. The reaction mixture was stirred for an additional 1 hour at room temperature. Pyridine was removed by evaporating the solvent under reduced pressure using toluene. The crude product was purified by crystallization from hexane and CH₂Cl₂, white crystals (60%) of \(N\beta\)-acetyl-3-indolylmethylamine (46).

\(N\beta\)-acetyl-3-indolylmethylamine (46):

HPLC \(t_R = 4.9\) min (Grad Screen method).

\(^1\)H NMR (500 MHz, CD₃CCl₃): \(\delta 8.12\) (br s, 1H), 7.65 (d, \(J=8\) Hz, 1H), 7.40 (d, \(J=8\) Hz, 1H), 7.25 (m, 1H), 7.17 (m, 2H), 5.61 (br s, 1H), 4.62 (d, \(J=5\), 2H), 1.99 (s, 3H).

\(^{13}\)C NMR (125.8 MHz, CD₃CN) \(\delta_C 170.7\) (s), 137.9 (s), 128.0 (s), 124.8 (s), 123.0 (s), 120.4 (s), 120.0 (s), 114.2 (s), 112.7 (s), 35.5 (s), 23.3 (s).

3.2.2 Synthesis of brassilexin (21)

\includegraphics{scheme33.png}

Scheme 3.3  Synthesis of brassilexin (21)
A mixture of 2-oxindole (500 mg, 3.75 mmol) and P₂S₅ (1.0 g, 2.25 mmol) in THF (25 mL) was stirred for 15 minutes at room temperature. Sodium bicarbonate (631 mg, 7.5 mmol) was then added to the reaction mixture with stirring. The resulting solution was allowed to stir for 4 hours at room temperature and filtered (by gravity). The excess THF was removed by evaporation under reduced pressure. Ice-cold water was added to the residue with vigorous stirring to yield a light yellow product. The precipitate was filtered and air dried to get indoline-2-thione (107) (458 mg, 81%) (Kamila and Biehl, 2004).

Indoline-2-thione (107) (200 mg, 1.3 mmol) was added to a mixture of freshly distilled POCl₃ (250 µL, 2.6 mmol) in DMF (1.5 mL) at 45˚C with stirring. After 20 minutes, the reaction mixture was placed on an ice bath to cool down to 0˚C. Aqueous NH₄OH (28%, 15 mL) was added dropwise with constant stirring and the mixture was allowed to warm up to room temperature. The resulting solution was extracted with Et₂O (3 x 30 mL) and the organic extracts were combined, dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was dissolved in pyridine (4 mL) and I₂ (160 mg, 0.63 mmol) was added to the reaction mixture. After one hour of stirring, the reaction mixture was acidified with 1.5 M H₂SO₄ and extracted with Et₂O (2 x 25 mL). The organic extracts were combined, dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was purified by FCC on silica gel eluting with EtOAc-hexane (20:80) to afford brassilexin (21) in 52% yield (Pedras and Jha, 2005).
Brassilexin (21):

HPLC $t_R = 13.4$ min (Grad Screen method).

$^1$H NMR (500 MHz, CD$_3$CN): $\delta$ 9.85 (br s, 1H), 8.69 (s, 1H), 7.90 (d, $J=8$ Hz, 1H), 7.55 (d, $J=8$ Hz, 1H), 7.32 (dd, $J=8$, 8 Hz, 1H), 7.22 (dd, $J=8$, 8 Hz, 1H).

$^{13}$C NMR (125.8 MHz, CD$_3$CN): $\delta$ 160.5 (s), 148. 9(s), 145.6 (s), 128.6 (s), 125.0 (s), 121.8 (s), 121.3 (s), 121.1 (s), 113.4 (s).

3.2.2.1 Synthesis of 3-(amino)methyleneindoline-2-thione (66)

\[
\begin{align*}
\text{Indoline-2-thione (107)} & \rightarrow \text{3-(amino)methyleneindoline-2-thione (66)} \\
\end{align*}
\]

Indoline-2-thione (107) (50 mg, 0.33 mmol) was added to a mixture of freshly distilled POCl$_3$ (64 $\mu$L, 0.7 mmol) in DMF (350 $\mu$L, 5.0 mmols) at 45°C with stirring. After 45 minutes, the reaction mixture was placed on an ice bath to cool down to 0°C. Aqueous NH$_4$OH (28%, 10 mL) was added dropwise with constant stirring (pH 11) and the temperature was brought to room temperature. The resulting solution was extracted with Et$_2$O (2 x 20 mL) and the organic extracts were combined, dried over Na$_2$SO$_4$ and concentrated under reduced pressure. The crude product was purified by FCC on silica gel eluting with MeOH-CH$_2$Cl$_2$ (2:98) to afford 3-(amino)methyleneindoline-2-thione (66) (52%).
3-(Amino)methyleneindoline-2-thione (66):

HPLC $t_R = 7.2$ min (Grad Screen method).

$^1$H NMR (500 MHz, CD$_3$CN): $\delta$ 11.01 (br s, 1H), 9.8 (br s, 1H), 8.25 (dd, $J=15$, 8 Hz, 1H), 7.46 (d, $J=8$ Hz, 1H), 7.31 (m, 1H), 7.07 (m, 3H).

3.2.3 Synthesis of camalexin (24)

Methyl iodide (295 µL, 4.75 mmol) was added slowly by injection at room temperature under argon atmosphere to magnesium turnings (77 mg, 3.2 mmol) in dry ether (15 mL). After all of the magnesium had reacted (disappeared), the ether was distilled off and dry benzene (7 mL) was added. A solution of indole (200 mg, 1.7 mmol) in benzene (1 mL) was added to the solution of methyl magnesium iodide in benzene and stirred for 15 minutes after which 2-bromothiazole was added. After refluxing at 90 ºC for 24 h, the reaction mixture was cooled to room temperature and extracted with EtOAc ($3 \times 20$ mL). The EtOAc extracts were combined, dried over Na$_2$SO$_4$ and concentrated under reduced pressure and the residue was separated by FCC on silica gel eluting with EtOAc-hexane (20:80) to afford camalexin (24) in 29% yield (Ayer et al., 1992).
**Camalexin (24):**

HPLC $t_R = 15.3$ min (Grad Screen method).

$^1$H NMR (500 MHz, CD$_3$CN): $\delta$ 9.78 (br s, 1H), 8.28 (d, $J=6.5$, 1H), 7.9 (s, 1H), 7.8 (s, 1H), 7.55 (d, $J=7$ Hz, 1H), 7.37 (d, $J=2$ Hz, 1H), 7.28 (dd, $J=7$, 7 Hz, 2H).

$^{13}$C NMR (125.8 MHz, CD$_3$CN): $\delta$ 164.6 (s), 143.9 (s), 138.0 (s), 127.0 (s), 125.9 (s), 124.1 (s), 122.4 (d), 121.7 (s), 117.3 (s), 113.3 (s), 112.6 (d).

**3.3 Biotransformations of phytoalexins by *Alternaria brassicicola***

**3.3.1 Preparation of minimal media**

Solution 1 (100 mL) containing 31.2 g/L KNO$_3$, 7.5 g/L K$_2$HPO$_4$, 7.5 g/L KH$_2$PO$_4$, 1.0 g/L NaCl and 2.8 g/L asparagine was added to a solution of glucose (15.0 g) in 700 mL of distilled water. Solution 3 (1 mL) containing 0.39 g/L ZnSO$_4$.7H$_2$O, 0.08 g/L CuSO$_4$.5H$_2$O, 0.41 g/L MnSO$_4$.4H$_2$O, 0.018 g/L MoO$_3$ (85%), 0.54 g/L ferric citrate and 0.38 g/L Na$_2$B$_4$O$_7$.10H$_2$O was added to it. The mixture was diluted up to 900 mL using distilled water and autoclaved. Solution 2 (100 mL), containing 1.0 g/L CaCl$_2$.7H$_2$O and 5.0 g/L MgSO$_4$.7H$_2$O was prepared separately and autoclaved. After autoclaving, the two solutions were allowed to cool to room temperature before mixing them together. A sterile solution 4 (1 mL) containing 100 g/L of thiamine was finally added to prepare minimal media (Pedras et al., 1997).
3.3.2 Preparation of fungal cultures

*Alternaria brassicicola* (ATCC 96866) spores were grown on potato dextrose agar (PDA) media in sterile polystyrene Petri dishes (100 × 15 mm). Petri dishes were then incubated under constant light at 22°C ±1°C. After eight days, spores were collected and stored at -20°C. Erlenmeyer flasks (250 mL) containing 100 mL of minimal media were inoculated each time with eight-day old spores (10^6 spores/100 mL) and incubated on a shaker (@ 120 rpm) under constant light at a temperature 22°C for 24 hours.

3.3.3 Time-course experiments

Time-course experiments were carried out to determine minimum concentrations and optimum periods for the formation of metabolites in fungal cultures. Initial experiments suggested that the minimum concentrations for phytoalexin biotransformation at different incubation periods were 1.0 × 10^{-4} M. For conducting time-course experiment, phytoalexins dissolved in acetonitrile (200 µL) were added to the fungal cultures (final concentration, 1.0× 10^{-4} M) as well as to the flasks containing only minimal media (control media); only acetonitrile (200 µL) was added to one of the fungal culture flasks (control fungus). Samples were collected (10 mL) from the culture flasks at 0 h and at different time intervals and extracted with EtOH (2×10 mL). Organic extracts were concentrated under reduced pressure and the residue was dissolved in 1mL CH$_3$CN for HPLC analysis. The aqueous layer was basified with NH$_4$OH (28%, pH 9), extracted with 5% methanol in CH$_2$Cl$_2$, concentrated under reduced pressure and the
residue was dissolved in 1mL of CH$_3$CN for HPLC analysis employing AMINE2 method.

3.4 Bioassays

Six-well tissue culture plates were employed for bioassay studies and sterilized by UV light exposure (1 h) before introducing the assay medium. Solutions of phytoalexins and other compounds (final concentrations of 5.0 × 10$^{-4}$ M, 2.0 × 10$^{-4}$ M and 1.0 × 10$^{-4}$ M) in CH$_3$CN were added to warm PDA medium in a Falcon® tube. After mixing (vortex), PDA mixture was poured into wells. In control wells only solvent (1% CH$_3$CN) with PDA medium (vortex mixing) were introduced. Eight-day old mycelial plugs of $A$. brassicicola were obtained by growing them on PDA at 22° ± 1°C under constant light in Petri dishes. In each well, 4-mm PDA plugs of mycelium were placed on the center of the wells, upside down, and incubated at 22° ± 1°C under constant light for 72 h. Mycelial radial growth was monitored over the period and the percent inhibition of growth was calculated. Bioassay experiments were carried out in triplicate at least three independent experiments for each bioassay.
CHAPTER 3: REFERENCES


