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THE UNIVERSITY OF ALBERTA

Phytoalexin production in crucifers

by

Kenneth Lyle Conn

A THESIS

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Abstract

A disease assessment key for alternaria blackspot was prepared for leaves and siliques of rapeseed. The key for siliques was used to assess alternaria blackspot in central Alberta. Alternaria blackspot was severe in 1989, causing significant yield loss.

Various crucifers were screened to locate sources of resistance to *Alternaria brassicae*. An accession of *Brassica campestris* ssp. *rapifera* was more resistant than *B. napus*. An accession of *Eruca sativa* showed a hypersensitive response. *Camelina sativa* and *Capsella bursa-pastoris* were very resistant showing no symptoms on healthy leaves. Virulence of *A. brassicae* isolates was compared on *B. napus* cv. Westar. An Alberta isolate was the most virulent, followed by a French and then an Indian isolate. None of these isolates caused symptoms on *C. sativa*.

An accession of *B. campestris* ssp. *rapifera* produced the same phytoalexin as rapeseed but in larger quantities. *Camelina sativa* and *C. bursa-pastoris* produced larger amounts of different phytoalexins. Thus, the differences in susceptibility of these plants appeared to be due, at least in part, to qualitative and/or quantitative differences in phytoalexin production. The phytoalexins from *C. sativa* and *C. bursa-pastoris* were not associated with cell death. *Camelina sativa* and *C. bursa-pastoris* were sensitive to *A. brassicae* toxin. This demonstrated the significance of phytoalexins in their resistance to *A. brassicae*.

Camelina sativa was also more resistant to *Rhizoctonia solani* than was Westar. Percent emergence of *C. sativa* seedlings in *R. solani* infested soil was 22-33% greater than that for Westar. Large amounts of antimicrobial compounds were produced in *C. sativa* roots compared with very low amounts in Westar.

A rapid new purification procedure was developed for isolation and separation of the *C. sativa* phytoalexins. Aqueous extract was applied to an Extube and allowed to distribute over the specially modified diatomaceous earth, the organic metabolites eluted with $CHCl_3$, the $CHCl_3$ extracted with 5% HCl, the HCl made basic and passed through another Extube, and the $CHCl_3$ eluent subjected to vacuum liquid chromatography. Two new thiazoyl substituted indole alkaloids were isolated and named camalexin and methoxycamalexin. Their structure was generally similar to that of the fungicide thiabendazole. Camalexin reduced germ tube growth of *A. brassicae* conidia *in vitro*, autolysing apical cells. EC_{50} and MIC were approximately 6 and 80 μg/mL camalexin, respectively.

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General Introduction

A. Phytoalexins

1. Introduction

Usually any given pathogen can infect only a very limited number of plant species. Susceptibility of plants to pathogens is an exception rather than a rule. To achieve this, plants have developed many kinds of disease resistance, one of which is the production of phytoalexins (Gk. *phyton* meaning plant and Gk. *alexin* meaning warding-off substance). The concept of phytoalexins was first proposed in 1940 by Müller & Börger. Phytoalexins have been defined as "low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms" (Paxton, 1981). Phytoalexin production usually occurs only after penetration or attempted penetration into plant cells by an invading organism (Mansfield, 1982). It is often associated with the hypersensitive response (HR) (Dixon, 1986; Doke *et al.*, 1987; Hahlbrock & Scheel, 1987; Kuhn & Hargreaves, 1987; Rouxel, 1989). The HR is a defence reaction by some plants in which there is rapid localized necrosis of plant cells surrounding the infection site of the pathogen, restricting its development. These necrotic cells are thought to act as a reservoir for the accumulation of phytoalexins synthesized and exported from surrounding healthy cells (Keen, 1986). Their production is localized in and immediately around the site of infection. The speed and magnitude with which they are produced is often the important factor in disease resistance and not selective elicitation or toxicity of the phytoalexins (Kuć, 1987; Kuhn & Hargreaves, 1987). Phytoalexins have been shown to be produced by plants after attack by bacteria, fungi, nematodes, viruses, and insects (Bailey, 1982; Mansfield, 1982). There are many types of elicitors of the phytoalexin response. The biotic elicitors include glucans, proteins, glycoproteins, polysaccharides, and fatty acids; and the abiotic elicitors include UV radiation, low temperature, heavy metals, and detergents. A number of recent review articles deal with elicitors in detail (Callow, 1987; de Wit, 1986, 1987; Dixon, 1986; Dixon & Lamb, 1990; Ebel, 1986; Keen, 1986; Paxton, 1988; Ward, 1986). Elicitors function by directly eliciting phytoalexins or by causing damage to plant cells releasing endogenous molecules which elicit phytoalexins (de Wit, 1987; Kuć, 1987). Several hundred phytoalexins have been structurally characterized thus far from about 20 plant families

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(Bailey, 1987; Kuhn & Hargreaves, 1987) but a very large proportion of the plant kingdom has yet to be investigated. There is great diversity in chemical types of phytoalexins but generally there is uniformity within a plant family. New phytoalexins are continuously being discovered, like the recent discovery of a new sulfur-containing class of phytoalexins from cruciferous plants (discussed in a later section).

2. Role of Phytoalexins in Disease Resistance

There is a large body of evidence that demonstrates the role of phytoalexins in the resistance in many plant-pathogen systems. Rapid accumulation of phytoalexin following inoculation of plant tissue has been correlated with inhibition of fungal growth during the development of resistant reactions. An example is the broad bean-*Botrytis* system. Accumulation of phytoalexin within the epidermis of broad bean leaves occurred at the right time to explain the inhibition of growth of infection hyphae of *Botrytis* spp. (Mansfield, 1980).

Coinoculation of compatible and incompatible pathogen races has resulted in restricted development of both with production of high levels of phytoalexin. An example of this is the soybean-*Pseudomonas syringae* van Hall pv. *glycinea* system. Incompatible *P. syringae* pv. *glycinea* races elicited a HR on soybean leaves with the associated production of the phytoalexin glyceollin. Compatible *P. syringae* pv. *glycinea* races did not elicit this response and thus caused disease. When a mixture of incompatible and compatible *P. syringae* pv. *glycinea* races were inoculated onto soybean leaves, a HR and glyceollin accumulation occurred, restricting the bacterial population similar to that induced in leaves inoculated with only the incompatible race (Long *et al.*, 1985).

Compatible pathogens often chemically degrade or are more tolerant to the phytoalexins produced by their host plant than are other organisms. An example of this is the pea-*Nectria haematococca* Berk. & Br. system. The virulence of *N. haematococca* is due to its ability to detoxify the pea phytoalexin pisatin (VanEtten *et al.*, 1987). Another example is the chickpea-*Ascochyta rabiei* system. The virulence of *A. rabiei* is due to its ability to degrade the chickpea phytoalexin medicarpin (Kraft *et al.*, 1987).

Elicitors and suppressors from pathogens reproduce the same relative specificity for phytoalexin production as the living pathogen. An example of this is the soybean-*Phytophthora megasperma* Drechs. f.sp. *glycinea* (Hildeb.) Kuan & Erwin system. When tap roots of soybean seedlings were immersed into solutions of glucan elicitors from an incompatible race of *P. megasperma* f.sp. *glycinea*, this caused accumulation of the phytoalexin glyceolin along with an increase in resistance against a compatible race of *P. megasperma* f.sp. *glycinea* (Bonhoff & Grisebach, 1988). Another example is the potato-*Phytophthora infestans* (Mont.) deBary system, one of the most extensively studied

systems. Resistant potato cultivars responded with a HR associated with production of sesquiterpenoid phytoalexins while the response of susceptible cultivars was delayed allowing the fungus to spread throughout the tissue. Several races of *P. infestans* have been identified and are distinguishable by being virulent on certain cultivars of potato but not on others. Avirulent races elicited a HR while virulent races elicited a delayed response. When cell-free sonicates of the mycelium of any race were applied to a potato cultivar, including cultivars susceptible to all races of the fungus, symptoms typical of the HR were observed regardless of the race's virulence on that cultivar. Thus all races of the fungus contain a signal or elicitor of the HR, and all potato cultivars have the genetic potential to be resistant. It is not the elicitor of the HR that is responsible for race specificity. Inoculation of potato slices with living spores of a virulent race resulted in a delay in the reaction of host cell. Thus race specificity of *P. infestans* appears to be due to factors that suppress host resistance and have been termed "suppressors" (Kuć & Rush, 1985; Preisig & Kuć, 1987).

Phytoalexins have also been shown to be involved in another type of resistant response by plants, that is cross-protection and immunization (Dean & Kuć, 1985, 1987; Kuć, 1984, 1987; Kuć & Preisig, 1984; Kuć & Rush, 1985; Mansfield, 1982; Sequeira, 1984). There have been many reports of plants becoming resistant to microbial pathogens following previous exposure to other organisms. This protection can be direct physical or chemical antagonism between the protecting organism and the pathogen, or acquisition of resistance by the host. This acquired resistance involves many defense mechanisms, one of which can be the accumulation of phytoalexins. The acquired resistance can be either local or systemic. In local cross-protection, the plant responds with immediate local resistance. Systemic cross-protection or immunization is an induced systemic resistance which requires an initial stimulus that causes persistent biochemical changes in the plant (Dean & Kuć, 1987; Kuć, 1987). Then upon infection, the plant responds rapidly with any number of defense mechanisms, one of which can be the accumulation of phytoalexins. An example of this kind is the immunization of green beans by an incompatible race of *Colletotrichum lindemuthianum* (Sacc. & Magn.) Bri. & Cav. (Kuć, 1984; Kuć & Rush, 1985). In these studies, when the immunized green beans were challenged with a compatible race of *C. lindemuthianum*, its development was restricted by the rapid accumulation of isoflavonoid phytoalexins. Plant immunization has been achieved by limited inoculation with fungi, fungal wall components, bacteria, viruses, products from immunized plants, and by synthetic chemicals (Kuć, 1984, 1987).

Another type of cross-protection is provided by the symbiosis of plants and mycorrhizal fungi. Mycorrhizal fungi enhance the growth of plants and increase their resistance to root pathogens (Duchesne *et al.*, 1987). A lot of research is being carried out

on the use of mycorrhizal fungi for biological control of diseases. Mycorrhizal fungi do this by increasing the vigor of the plants and in at least some cases by eliciting the production of phytoalexins (Duchesne *et al.*, 1987). It is well documented that orchid mycorrhizae provide disease resistance in orchids by eliciting the production of phytoalexins (Arditti, 1979). There is also one report of vesicular-arbuscular mycorrhizal (VAM) fungi eliciting phytoalexins (Morandi *et al.*, 1984). Phytoalexins may be widely involved in mycorrhizal symbiosis but more research is needed to determine this.

3. Phytoalexins from Cruciferae

The first report of phytoalexins from the family Cruciferae was in 1986 from Japan. Takasugi *et al.* (1986) reported the elicitation of three phytoalexins (brassinin, methoxybrassinin, and cyclobrassinin; Fig. I-1) from Chinese cabbage heads inoculated with *Pseudomonas cichorii* (Swingle) Stapp or *Erwinia carotovora* (Jones) Holland. These were also the first phytoalexins reported that contained sulfur. There have now been eleven phytoalexins characterized (Table I-1, Fig. I-1) from nine cruciferous plants (Table I-2).

Phytoalexins from Cruciferae have been purified using thin-layer chromatography (TLC), column chromatography, and high-performance liquid chromatography (HPLC). The most common procedure has been repeated chromatography using silica gel TLC plus column chromatography using silicic acid and/or sephadex LH-20 (Monde *et al.*, 1990a, 1990b; Takasugi *et al.*, 1987, 1988). HPLC using C_{18} columns has been used for the final purification step in some cases (Dahiya & Rimmer, 1988b; Devys *et al.*, 1990; Kollmann *et al.*, 1989). However, HPLC has only limited value during identification of new phytoalexins as only a small amount of material can be handled by this technique. HPLC is most useful when used to study known phytoalexins in very small quantities (Dahiya & Rimmer, 1988b). Kollmann *et al.* (1989) reported the use of Extra-Sep reversed-phase cartridges for efficient clean-up of non-aqueous plant extracts prior to HPLC analysis of sulfur-containing phytoalexins.

The relationship between phytoalexin production in crucifers and resistance to diseases has thus far been investigated for only one system; the blackleg disease caused by *Leptosphaeria maculans* (Desm.) Ces. & De Not. Rouxel *et al.* (1989, 1990) showed that *Brassica napus* L. and *B. juncea* L. cultivars produced brassilexin in response to *L. maculans* or abiotic elicitors. They showed that *B. napus* cultivars, which are highly susceptible to *L. maculans*, accumulated lower levels of brassilexin than *B. juncea* cultivars, which show a hypersensitive response to *L. maculans*. This correlation between resistance to *L. maculans* and phytoalexin accumulation existed in the interspecific hybrid progeny of *B. napus* and *B. juncea* as well (Rouxel *et al.*, 1990). This correlation was also demonstrated by Dahiya & Rimmer (1989) while studying the accumulation of other phytoalexins. They showed that *B.*

napus cultivars accumulated lower levels of methoxybrassinin and cyclobrassinin when challenged with *L. maculans* or abiotic elicitors than *B. juncea* cultivars. Thus, it appears that phytoalexins may play a role in the differential susceptibility of *Brassica* spp. to *L. maculans*. Peterka & Schlösser (1990) also investigated this system. They were able to detect trace levels of brassilexin, cyclobrassinin, and methoxybrassinin in *B. napus* and *B. juncea* in response to *L. maculans* but did not find any correlation between resistance and phytoalexin production.

4. Phytoalexins as Control Agents for Pathogens

Phytoalexins as plant protectants

Use of phytoalexins as plant protectants has been discussed in several review articles (Brent, 1983; Callow, 1983; Kuhn & Hargreaves, 1987; Mansfield & Bailey, 1982; Salt & Kuć, 1985). There are only a few reports on the use of phytoalexins as plant protectants. Fawcett *et al.* (1969) used the broad bean phytoalexin wyerone as a fungicide. They showed that wyerone (100 μg/mL) sprayed onto the leaves of dwarf bean and broad bean provided significant protection against rust infection and chocolate spot disease, respectively. Ward *et al.* (1975) tested the pepper phytoalexin capsidiol as a fungicide. They showed that capsidiol (118 μg/mL) sprayed onto the leaves of tomato reduced the number of lesions caused by *P. infestans* by nearly 90% with protection lasting for at least eight days. Some attempts of using phytoalexins as fungicides have been unsuccessful. Rathmell & Smith (1980) used seven isoflavonoid phytoalexins as fungicides against six pathogens. They found that the phytoalexins failed to provide appreciable control of any of the pathogens at concentrations up to 100 μg/mL. This was in comparison with two commercial systemic fungicides benomyl and mancozeb which gave good control of the pathogens at 50 μg/mL.

These few studies indicate the possibility of using phytoalexins as plant protectants but there are a number of problems based on some of the properties of phytoalexins studied thus far. One drawback is that so far no phytoalexin has been shown to be systemic. Another is that the antibiotic activities of the phytoalexins studied thus far are lower than those of most synthetic fungicides (Callow, 1983). Also, some phytoalexins have been shown to be metabolized by microorganisms and plants (VanEtten *et al.*, 1982, 1989) and some phytoalexins have been shown to be phytotoxic (Smith, 1982). One exception is that capsicannol phytoalexins produced by *Capsicum annuum* L. fruits in response to glucan elicitors were shown to be associated with live cells with little or no necrosis occurring (Adikaram *et al.*, 1988). Another drawback is that large scale chemical synthesis of most phytoalexins thus far characterized is difficult (Brent, 1983; Callow, 1983). So far the phytoalexins orchinol from orchids (Stoessl *et al.*, 1974) and oryzalexins A, B, and C from

rice leaves (Mori & Waku, 1985) have been chemically synthesized. Thus, it appears that the majority of phytoalexins characterized so far are not appropriate for use as plant protectants. As the properties of more phytoalexins are investigated and new types discovered, like those from Cruciferae, phytoalexins may be found that have fewer of these negative properties. However, even if no phytoalexins can be found that are suitable as plant protectants, these compounds can provide clues for novel chemistries for new pesticides. Also, analogues of phytoalexins can be made that might overcome some of the negative properties described above.

Phytoalexin-elicitors as plant protectants

The recognition process in phytoalexin production is a likely target for use in disease control. This approach has been shown to be the mechanism by which some commercially available fungicides and herbicides act. Cartwright *et al.* (1980) showed that the ability of rice leaves to produce the phytoalexins, momilactones A and B, in response to infection with the blast fungus (*Pyricularia oryzae* Cav.) was enhanced by prior treatment of plants with a dichlorocyclopropane fungicide. It has been shown that part of the activity of the herbicide glyphosate is by making plants more susceptible to pathogens (Holliday & Keen, 1982; Johal & Rahe, 1984, 1988; Keen *et al.*, 1982). Glyphosate does this, in part, by suppressing the production of phytoalexins (Johal & Rahe, 1988, 1990).

Phytoalexin-elicitors have been successfully used to provide protection against pathogens. An example was given earlier in this chapter (soybean-*P. megasperma* f.sp. *glycinea* system). The use of some phytoalexin-elicitors as plant protectants has had detrimental effects on plants, for example, when fungus-derived glucan elicitors were applied frequently to the foliage of green bean and soybean plants (Kuć, 1987). They caused severe necrotization and stunting of plant growth. This is because some phytoalexins are phytotoxic (Smith, 1982). The phytoalexin-elicitors that cause detrimental effects on plants may still be useful, however, as herbicides.

Genetic manipulation of the phytoalexin response

Another way to use phytoalexins in the control of pathogens is through the manipulation of genes responsible for phytoalexin elicitation and biosynthesis. This becomes more plausible as our understanding of the biochemistry and genetics of host-pathogen interactions increases. These areas have been covered in depth in a number of recent review articles (Bailey, 1987; Callow, 1987; de Wit, 1986, 1987; Dixon, 1986; Dixon *et al.*, 1987; Dixon & Lamb, 1990; Hahlbrock & Scheel, 1987; Heath, 1987; Keen, 1986; Keen & Stackawicz, 1988; Lamb *et al.*, 1987). Several approaches to enhance the phytoalexin response are possible. One is to alter the genes in a plant so as to increase the speed and

quantity of phytoalexins produced in response to a pathogen, or to cause the plant to produce new phytoalexins. Another approach is to transfer genes from one plant to another to achieve these same results by either conventional breeding or by new biotechnological techniques. While there are many problems that would be encountered with these approaches, the potential gains are likely to be enormous.

Phytoalexins as pharmaceutical drugs

Since phytoalexins can protect plants against many types of pathogens it seems logical to wonder if they could do the same for animals. Gordon *et al.* (1980) undertook a study to determine whether some phytoalexins might serve as a new class of antifungal antibiotics useful in treatment of human mycoses. They showed that one or more of the phytoalexins maachiain, medicarpin, phaseollin, phaseollin isoflavan, pisatin, sativan, and vestitol inhibited the growth of 12 zoopathogenic fungi. The phytoalexin gossipol from cotton has been tested against trypanosomes which infest the blood of various vertebrates, including man, and cause sleeping sickness and Chagas' disease (Blanco, 1985; Turrens, 1986). Gossipol was found to reduce the growth of the trypanosome that causes Chagas' disease and to cause morphological alterations in the parasite (Blanco, 1985).

Phytoalexins have been tested in other areas of medicine as well. The phytoalexin gossipol has reversible antispermatogenic action and has been tested in China as a contraceptive in men (Blanco, 1985; Turrens, 1986). The phytoalexins maachiain from various Leguminosae and pinosylvin from pine were shown to be possible antineoplastic agents, inhibiting the growth of human lymphoblastoid cells (Skinnider & Stoessl, 1986). Crucifers are known to contain a number of compounds which have anticancer properties (Fenwick *et al.*, 1983). Some of these are indole compounds related to the phytoalexins identified from Cruciferae. The phytoalexin brassilexin has been shown to have anticancer activity (Devys & Barbier, 1991).

These few studies carried out so far have shown that there is a potential for the use of phytoalexins in medicine. The medical industry is always looking for new pharmaceutical drugs and phytoalexins may provide these or at least provide useful leads for new compounds. Of course, toxicity of phytoalexins to animals has to be considered, as there have been reports of some phytoalexins being toxic to animals (Smith, 1982).

B. Objectives of the Thesis

1. To prepare a disease assessment key for alternaria blackspot and conduct formal surveys in Alberta.
2. To screen various vegetable, oleiferous, and wild crucifers to locate sources of resistance to *Alternaria brassicae*.
3. To investigate if phytoalexins are involved in resistance of crucifers to *A. brassicae*.
4. To investigate if crucifers resistant to *A. brassicae* are also resistant to *Rhizoctonia solani* and if phytoalexins are involved.
5. To develop a method for purifying the phytoalexins for chemical identification.

C. Tables, Figures, and Legends

Table I-1. Physical data and molecular formulae of phytoalexins from Cruciferae.

Phytoalexin†	Molecular formulae	Molecular weight	Melting point (°C)	UV max, nm (methanol)	References
brassicanal A	$C_{10}H_9NOS$	191	210-213	210,255,276,311	Monde *et al.*, 1990a
brassicanal B	$C_{12}H_{11}NO_2S$	233	169-170	207,220,252,277,315	Monde *et al.*, 1990a
brassilexin	$C_9H_6N_2S$	174	164-167	218,245,264,285	Devys *et al.*, 1988
brassinin	$C_{11}H_{12}N_2S_2$	236	132-133	218,236,268,287	Takasugi *et al.*, 1986, 1988
compound 5	$C_{14}H_{14}N_2OS_2$	290	amorphous	202,227,259,282,294	Takasugi *et al.*, 1988
cyclobrassinin	$C_{11}H_{10}N_2S_2$	234	136-137	204,227,259,284,294	Takasugi *et al.*, 1986, 1988
cyclobrassinin sulphoxide	$C_{11}H_{10}N_2OS_2$	250	188-190	?	Devys *et al.*, 1990
methoxybrassinin	$C_{12}H_{14}N_2OS_2$	266	viscous oil	218,241,267,287,297	Takasugi *et al.*, 1986, 1988
4-methoxybrassinin	$C_{12}H_{14}N_2OS_2$	266	amorphous	219,265	Monde *et al.*, 1990b
methoxybrassitin	$C_{12}H_{14}N_2O_2S$	250	94-96	219,247,273,287,295	Takasugi *et al.*, 1988
spirobrassinin	$C_{11}H_{10}N_2OS_2$	250	158-159	215,250,300	Takasugi *et al.*, 1987

†The structural formulae for these phytoalexins is given in Figure I-1.

Table I-2. Crucifers that have been shown to produce phytoalexins.

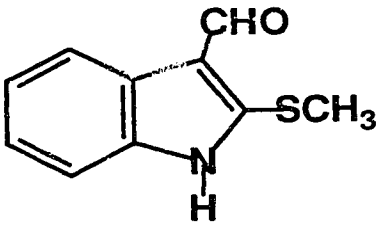
Plant	Elicitors	Phytoalexins	References
Arabidopsis thaliana	abiotic *Pseudomonas syringae* pv. *syringae*	not identified	Tsugi *et al.*, 1990
Brassica campestris ssp. *oleifera*	*Leptosphearia maculans*	cyclobrassinin methoxybrassinin	Dahiya & Rimmer, 1989
B. campestris ssp. *pekinensis*	*Erwinia carotovora* *P. cichorii* UV irradiation	brassicanal A brassicanal B brassinin compound 5 cyclobrassinin methoxybrassinin methoxybrassitin	Monde *et al.*, 1990a Takasugi *et al.*, 1986, 1988
B. carinata	abiotic	brassilexin	Rouxel *et al.*, 1990
B. juncea	abiotic *Alternaria brassicae* *L. maculans*	brassilexin cyclobrassinin cyclobrassinin sulphoxide methoxybrassinin	Dahiya & Rimmer, 1989 Devys *et al.*, 1988, 1990 Peterka & Schlösser, 1990 Rouxel *et al.*, 1990
B. napus	abiotic *L. maculans*	brassilexin cyclobrassinin methoxybrassinin	Dahiya & Rimmer, 1988a, 1989 Peterka & Schlösser, 1990 Rouxel *et al.*, 1990
B. nigra	abiotic	brassilexin	Rouxel *et al.*, 1990

(continued on next page)

Table I-2. Crucifers that have been shown to produce phytoalexins (continued).

Plant	Elicitors	Phytoalexins	References
B. oleracea var. *capitata*	*P. cichorii*	brassinin cyclobrassinin methoxybrassinin 4-methoxybrassinin methoxybrassitin spirobrassinin	Monde *et al.*, 1990b
rapeseed	*Albugo candida*	not identified	Dahiya & Woods, 1987
Rhaphanus sativus var. *hortensis*	*P. cichorii*	brassinin methoxybrassinin methoxybrassitin spirobrassinin	Takasugi *et al.*, 1987
R. sativus	*A. solani*	not identified	Dubey & Sarkar, 1987

Figure I-1. Structural formulae of phytoalexins from Cruciferae.
A. brassicanal A
B. brassicanal B
C. brassilexin
D. brassinin
E. compound 5
F. cyclobrassinin
(continued on next page)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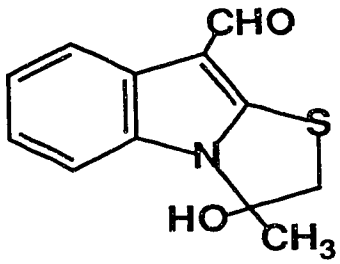
SCH3
N
H

A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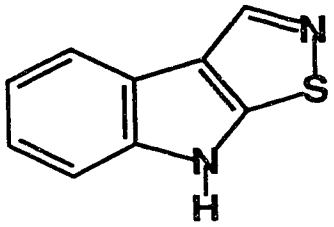
N
HO
CH3

B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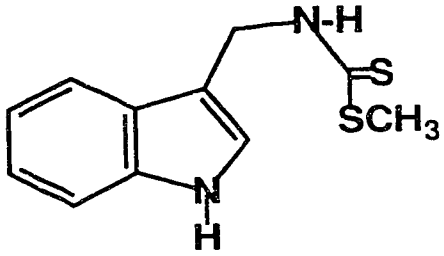
N
H

C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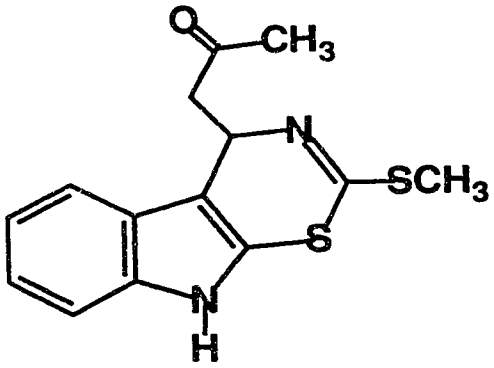
SCH3
N
H

D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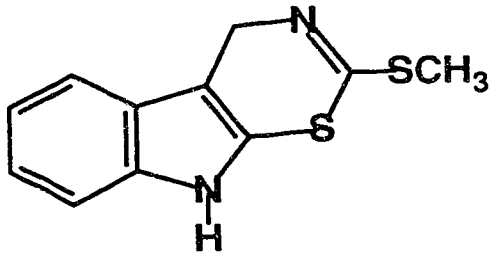
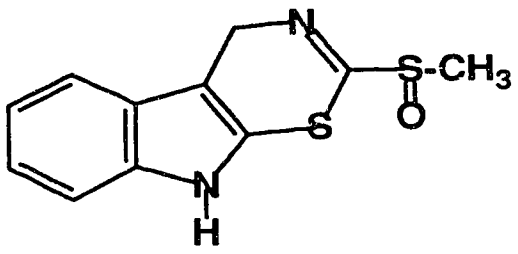
SCH3
S
N
H

E


N
SCH3
S
N
H

F

Figure I-1. Structural formulae of phytoalexins from Cruciferae (continued).
G. cyclobrassinin sulphoxide
H. methoxybrassinin
I. 4-methoxybrassinin
J. methoxybrassitin
K. spirobrassinin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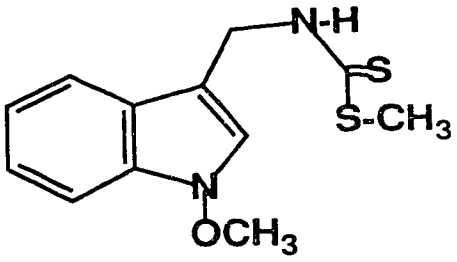
S
N
H

G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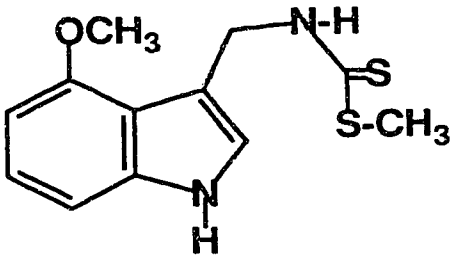
N
OCH3

H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N-H
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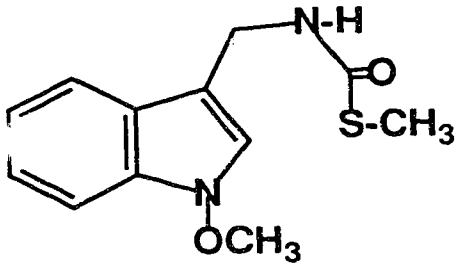
N
H

I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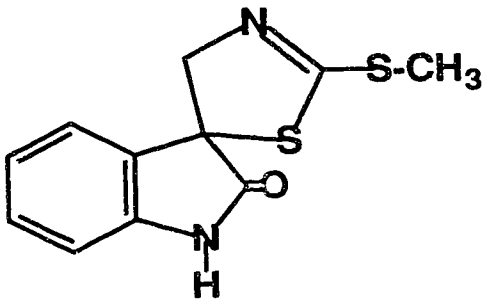
OCH3

J


N
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S
O
N
H

K

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Chapter II

Assessment of Alternaria Blackspot of Rapeseed

A. Introduction

Rapeseed is one of the more important oilseed crops in the world and is a major cash crop in western Canada. *Brassica campestris* L. and *B. napus* L. are the two oil producing species of rapeseed in the world. The two subspecies grown in Canada are *B. campestris* ssp. *oleifera* and *B. napus* ssp. *oleifera*. This is the only subspecies of *B. napus* discussed in this thesis and from here on is referred to as only *B. napus*. The name canola has been given to specific cultivars of rapeseed developed in Canada which have oil that is low in erucic acid (<5%) and meal that is low in glucosinolate (<3 mg/g of moisture-free and oil-free meal) (Vaisey-Genser & Eskin, 1982). Throughout this thesis both the terms rapeseed and canola will be used.

Alternaria brassicae (Berk.) Sacc., causal agent of the blackspot of rapeseed, is an economically important pathogen in western Canada and around the world (Kolte, 1985; Tewari, 1985). In several countries of Europe and southeast Asia this disease imposes a major constraint on optimum yields of this oilseed crop (Singh & Kolte, 1990). *Alternaria brassicae* produces a host specific toxin, destruxin B (Ayer & Peña-Rodriguez, 1987; Bains & Tewari, 1987), which is its main mode of pathogenesis (Bains & Tewari, 1987). The lesions on leaves consist of necrotic centers surrounded by chlorotic areas (Fig. II-1A). This leads to reduction in photosynthetic area, defoliation, and accelerated senescence. The pathogen also synthesizes abscisic acid (Dahiya *et al.*, 1988) which would aid in the accelerated senescence. Disease levels on leaves, through inoculum production, affect the disease severity on siliques. Lesions on siliques consist of necrotic spots with limited chlorotic areas in the early stages of lesion development (Fig. II-1B). Photosynthates from siliques are known to contribute significantly to the development of seeds in rapeseed (Allen *et al.*, 1971). Also, the blackspot lesions on siliques cause increased fruit shattering and often a direct infection of seeds through the silique wall (Fig. II-1C).

Disease assessment keys based on the host area affected have been prepared for many diseases (James, 1971, 1974). However, so far as we are aware, none is available for assessing alternaria blackspot of rapeseed and mustard. A 0-5 point rating scheme for

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A version of this chapter has been published:

Conn, K.L. and J.P. Tewari. 1991. Survey of alternaria blackspot and sclerotinia stem rot of canola in central Alberta in 1990. *Can. Plant Dis. Surv.* 71:96-97.

Conn, K.L. and J.P. Tewari. 1990. Survey of alternaria blackspot and sclerotinia stem rot in central Alberta in 1989. *Can. Plant Dis. Surv.* 70:66-67.

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assessing alternaria blackspot has been developed by Kolte (1985) and is used widely in India. Part of this rating scheme, however, involves the visual assessment of the amount of host area affected. Based on the aforesaid considerations, disease assessment keys were prepared for leaves and siliques of rapeseed. The key for siliques was used to assess alternaria blackspot in central Alberta in 1989 and 1990.

B. Materials and Methods

1. Assessment key for alternaria blackspot

Leaves and siliques of rapeseed with alternaria blackspot were collected from the field and the symptoms studied (Fig. II-1A,B). Drawings of leaves and siliques with lesions were prepared. The necrotic centers of lesions were colored black and the surrounding chlorotic areas were indicated by dotted lines. Both necrotic and chlorotic areas were included in calculation of the diseased area. Percent area covered with lesions was calculated using a CalComp 9000 digitizer. The digitizer calculated the area of a simple closed polygon when the boundary of the polygon was digitized by tracing it with a cursor. Drawings of 1, 5, 10, 20, 30, and 50% areas covered by lesions were prepared.

2. Surveys of alternaria blackspot

Fifty-seven randomly selected fields of canola were surveyed in central Alberta during the middle of August, 1989. Fourty-eight of these fields were of *B. campestris* ssp. *oleifera* and nine were of *B. napus*. Sixty-three randomly selected fields of canola were surveyed in central Alberta during the third week of August, 1990. Fifty-nine of these fields were of *B. campestris* ssp. *oleifera* and four were of *B. napus*. The disease severity on siliques at two locations within each field, away from the edge or corners, was estimated visually using the assessment key (Fig. II-3), and the average recorded. Fields with less than 1% alternaria blackspot were categorized as having trace levels.

C. Results and Discussion

1. Assessment key for alternaria blackspot

Disease assessment keys for rapeseed leaves and siliques are given in Figures II-2 and II-3, respectively. Drawings showing more than 50% of the surface area covered with lesions

were not prepared because once the disease reached this severity the leaves or siliques were next to being dead. In some cases, siliques collected from the field had lesions mainly on the upper side. Proper adjustment was made if such was the case. The overall shape of leaves and siliques are similar in rapeseed and mustard; therefore these keys should permit blackspot assessment in both these crops. These keys should be usable for assessing some other diseases of rapeseed and mustard as well, such as white rust caused by *Albugo candida* (Pers. ex Chev.) Kuntze and white leaf spot caused by *Pseudocercosporella capsellae* (Ell. & Ev.) Deighton. These disease assessment keys will allow for consistant assessment by people all over the world. In places like India where alternaria blackspot is very severe, the keys for both leaves and siliques are needed (Singh & Kolte, 1990). Here in Alberta where the disease is not as severe, the key for siliques will likely be used more often. It was very easy to use this key in the surveys conducted in 1989 and 1990.

2. Survey of alternaria blackspot in 1989

Every field of canola surveyed had alternaria blackspot. Percent areas of siliques covered with lesions ranged from 1 to 50% (Fig. II-4) with a mean of 20%. The average for the nine fields of *B. napus* was 5% and for the 48 fields of *B. campestris* ssp. *oleifera* it was 22%. This supports the findings that *B. napus* is less susceptible to *A. brassicae* than *B. campestris* ssp. *oleifera* (Conn & Tewari, 1989; Skoropad & Tewari, 1977). Yield data were obtained from three fields in the Innisfail area that had been rated for alternaria blackspot and sclerotinia stem rot (the two most common diseases of rapeseed in central Alberta) (Table II-1). Yield data were also obtained from five fields in the Barrhead area in which disease had not been rated (Table II-1). Yields in the Barrhead fields were only about 52% of the farmers' expectations with an average of 19% dockage (Table II-1). Yields in the three Innisfail fields were only about 60% of the farmers' expectations with 11% dockage (Table II-1). In 1988, these same Innisfail farmers obtained 1970-2250 kg/ha, with 4% dockage. The crops in 1989 looked better than those in 1988 but yielded less. The levels of alternaria blackspot and sclerotinia stem rot in the Innisfail area were lower in 1988. This indicated that these diseases were likely the principal cause of yield loss in 1989. The relationship between level of disease and yield loss in the Innisfail fields shown in Table II-1 indicated that a greater portion of yield loss may have been due to alternaria blackspot rather than to sclerotinia stem rot, since an increase of sclerotinia stem rot from 10 to 30% did not appreciably affect yield. The timing of infection of *Sclerotinia sclerotiorum* (Lib.) de Bary in these fields was not known, however, and may have affected yield, but overall, sclerotinia stem rot did not appear to have caused significant yield losses in most of the fields in this survey. The severity of alternaria blackspot was evidenced by the fact that seeds of some siliques were colonized by *A. brassicae* (Fig. II-1C). The wet weather in

August may have allowed *A. brassicae* to grow inside siliques and led to higher dockage since many seeds were shrivelled. If *A. brassicae* caused even a 5% yield loss of rapeseed in Alberta in 1989, which would be a conservative estimate, it would be equal to a monetary value of approximately $25 million (Tewari, 1991).

3. Survey of alternaria blackspot in 1990

Every field of canola surveyed had alternaria blackspot. Percent areas of siliques covered with lesions ranged from trace levels to 20% (Fig. II-5). If the fields with trace levels are set to 0%, then the mean percent area of siliques covered with lesions was 2%. This decrease in disease severity of almost 10 fold from that of 1989 was probably due to weather. In 1989 the latter part of July and early part of August were wet, whereas in 1990 it was hot and dry during this period in most areas. This demonstrated how alternaria blackspot was influenced by weather. Yields in the Innisfail area in 1990 were about the same as in 1988. *Alternaria brassicae* did not cause any significant loss of yield in central Alberta in 1990.

Prior to these 1989 and 1990 surveys, there had not been any extensive surveys conducted in central Alberta, and in many years no formal surveys were carried out at all. A survey in central Alberta in 1987 of 25 rapeseed fields revealed the presence of some heavily infected fields (Tewari & Conn, 1988). Personal observations made in 1985, 1986, and 1988 of a few fields in central Alberta indicated low levels of alternaria blackspot in these years. Although alternaria blackspot is not severe every year in Alberta, a year like 1989 makes it an economically important disease of rapeseed in this province.

D. Tables, Figures, and Legends

Table II-1. Levels of alternaria blackspot and sclerotinia stem rot and yields of *Brassica campestris* ssp. *oleifera* fields in central Alberta in 1989.

Area	Alternaria blackspot (%)	Sclerotinia stem rot (%)†	Expected yield (kg/ha)	Actual yield (kg/ha)	Dockage (%)
Innisfail	30	30	2250	1240	11
Innisfail	30	10	2250	1400	11
Innisfail	30	10	2250	1400	11
Barrhead	*	*	1400	960	24
Barrhead	*	*	1690	1070	20
Barrhead	*	*	2250	1070	18
Barrhead	*	*	2250	960	24
Barrhead	*	*	1970	900	30

†For assessment of sclerotinia stem rot, the percentage of stems with symptoms was determined.
*Levels of diseases were not determined in these fields.
Thanks are due to Mr. J. Soldan, District Agriculturist, Alberta Agriculture, Barrhead, Alberta, for providing yield data from his area.

Figure II-1. Alternaria blackspot on *Brassica campestris* ssp. *oleifera* collected from fields in central Alberta.
A) Infected leaf. The lesions consist of necrotic areas surrounded by chlorotic areas (approx. x0.7).
B) Infected siliques. The lesions consist of necrotic spots generally with limited chlorotic areas in early stages of lesion development (approx. x2).
C) A silique with one half of the seeds colonized by *Alternaria brassicae* (approx. x3).

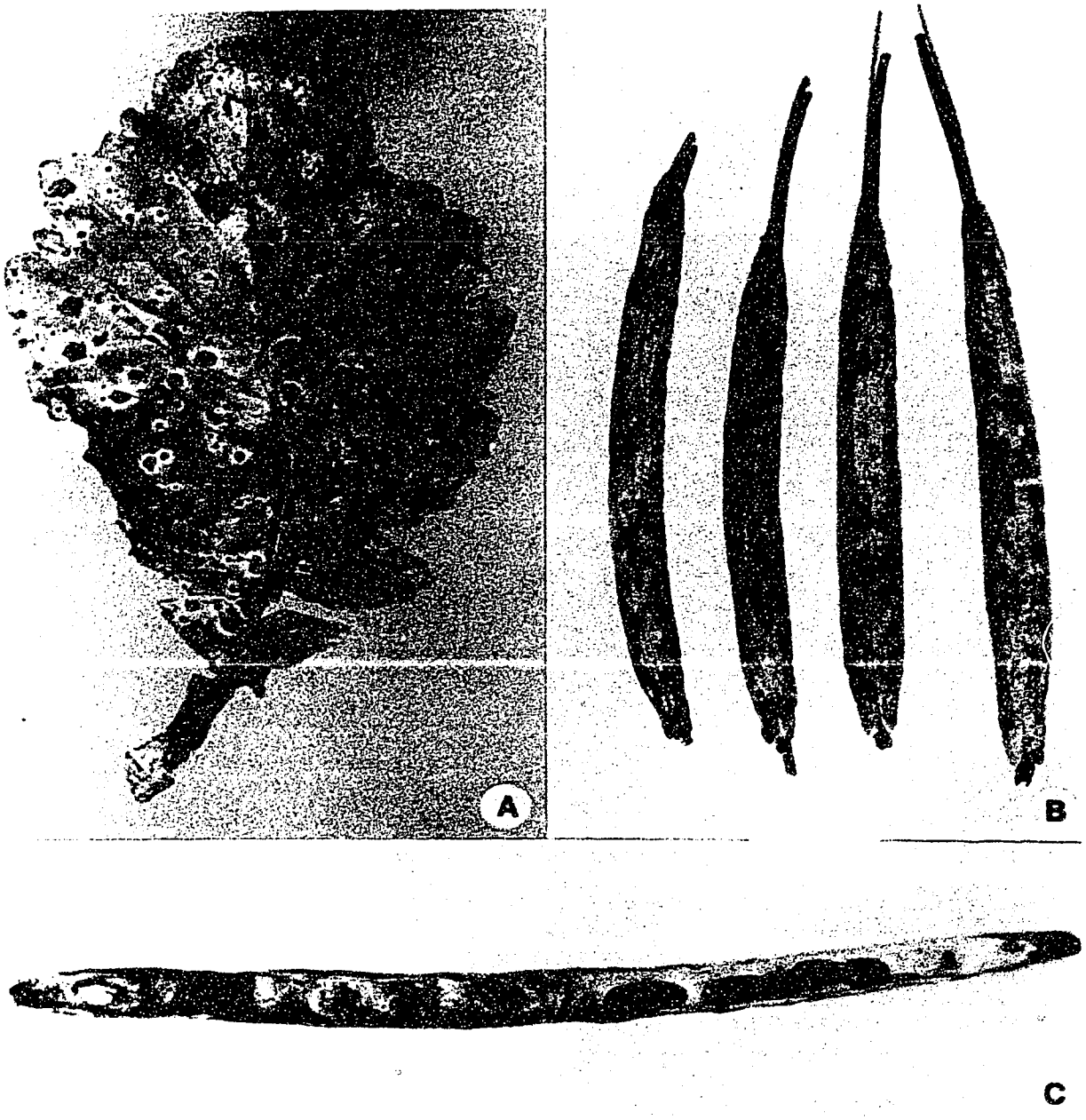

A
B
C

Figure II-2. Drawings of leaves showing 1, 5, 10, 20, 30, and 50% of the surface areas covered with blackspot lesions.
The dotted lines represent chlorotic areas surrounding the necrotic areas and are included as part of the diseased areas.

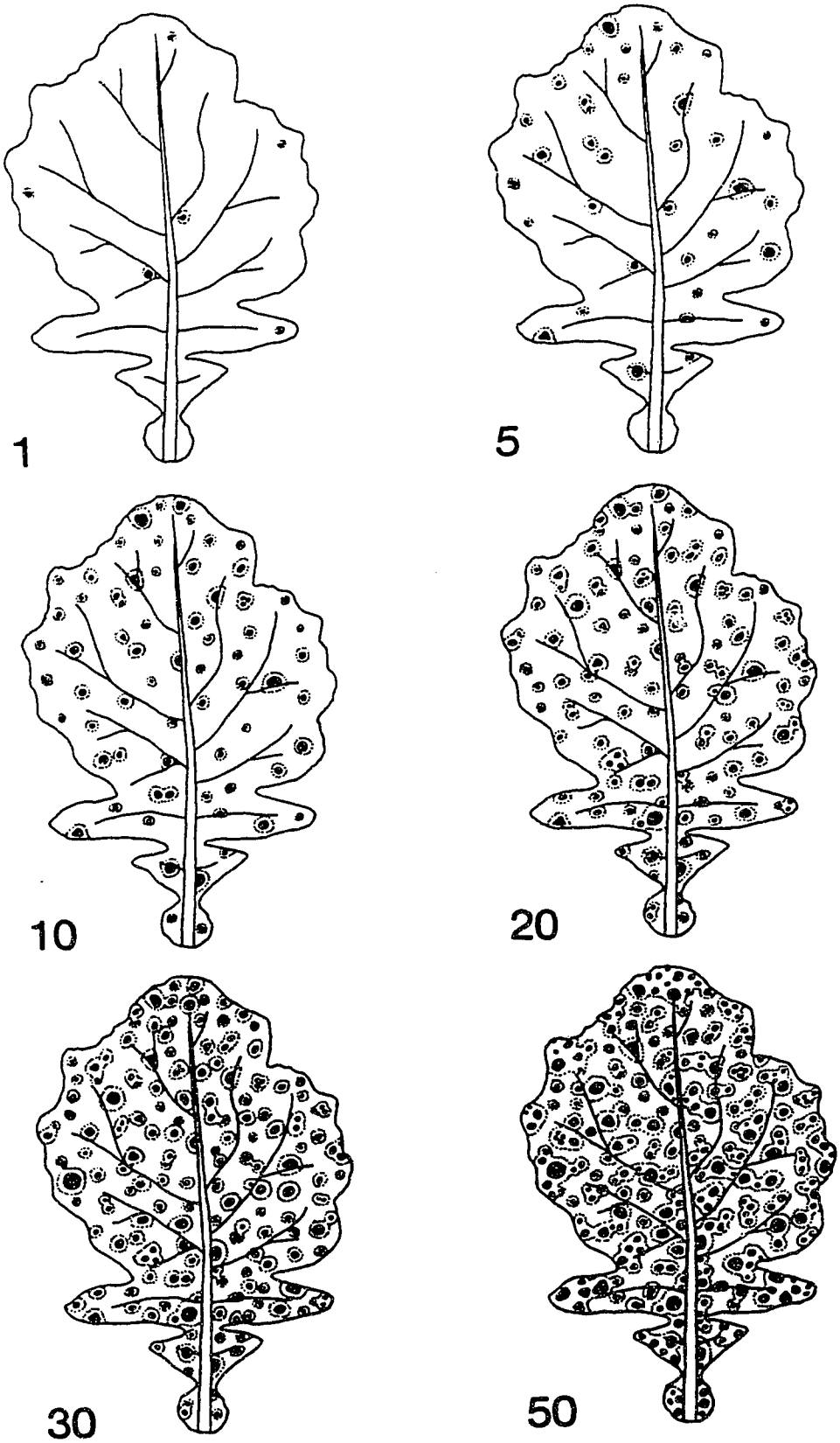
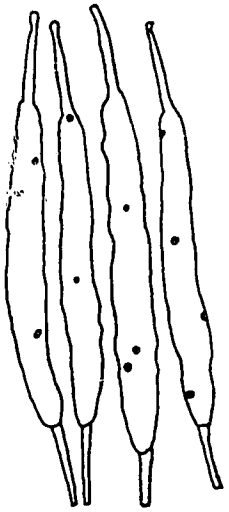
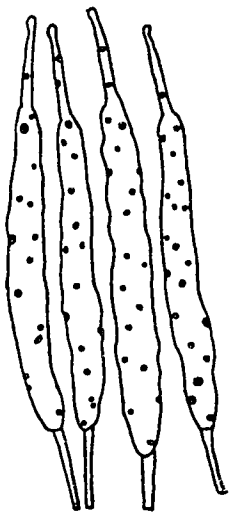

1
5
10
20
30
50

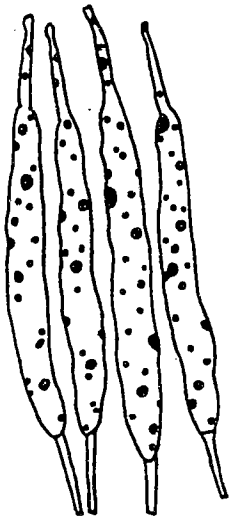
Figure II-3. Drawings of siliques showing 1, 5, 10, 20, 30, and 50% of the surface areas covered with blackspot lesions.
The dotted lines represent chlorotic areas surrounding the necrotic areas and are included as part of the diseased areas. The lesions on siliques generally did not have chlorotic areas in the early stages of lesion development.



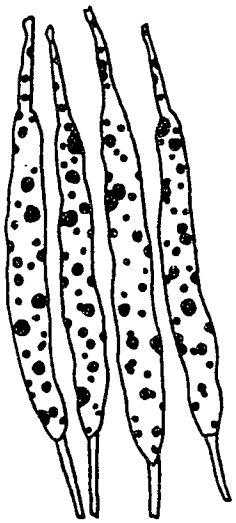
1



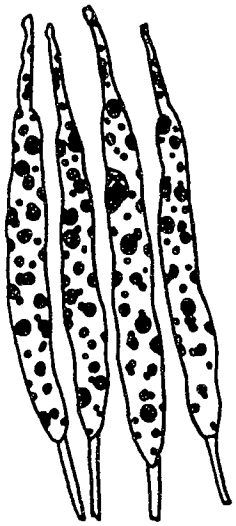
5



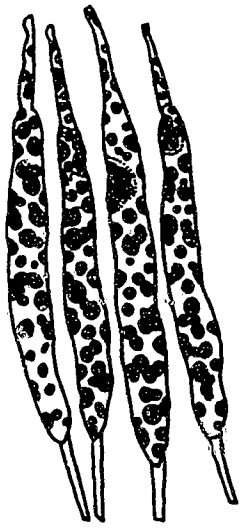
10



20



30



50

Figure II-4. Locations of fields of canola in central Alberta surveyed for alternaria blackspot in 1989.
The numbers represent percent areas of siliques covered with lesions.

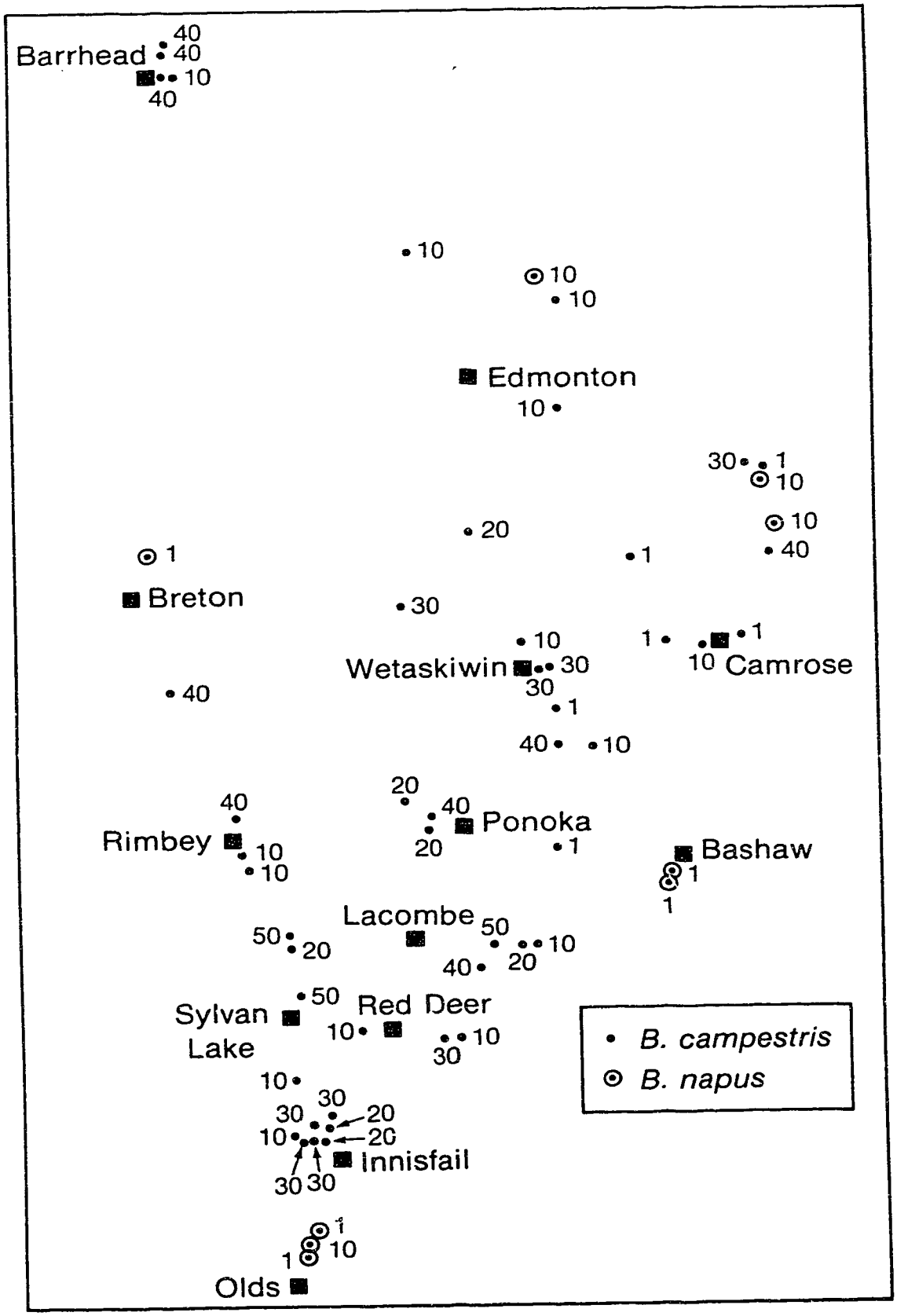
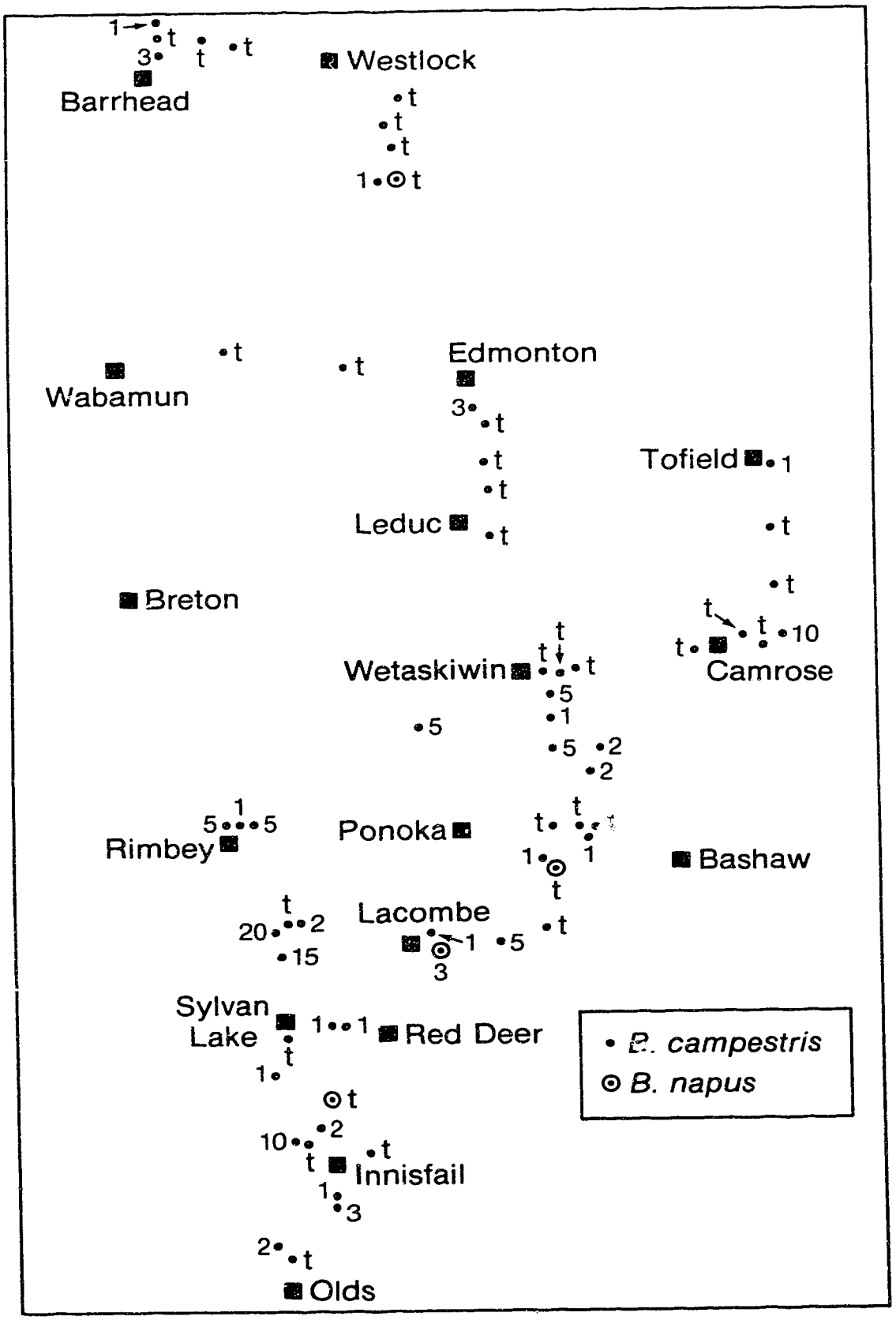

Barrhead
40
40
10
40
10
10
10
Edmonton
10
30
1
10
10
20
40
1
1
Breton
30
10
1
1
10
Camrose
Wetaskiwin
30
30
40
1
40
10
20
40
Ponoka
40
20
1
Rimbey
10
10
Bashaw
1
1
Lacombe
50
50
20
10
40
20
50
Red Deer
Sylvan
Lake
10
10
30
10
30
30
20
10
20
Innisfail
30
30
1
1
10
Olds
B. campestris
B. napus

Figure II-5. Locations of fields of canola in central Alberta surveyed for alternaria blackspot in 1990.
The numbers represent percent areas of siliques covered with lesions. Fields with less than 1% infection were categorized as having trace (t) levels.


Westlock
Barrhead
Edmonton
Wabamun
Tofield
Leduc
Breton
Camrose
Wetaskiwin
Ponoka
Rimbey
Bashaw
Lacombe
Sylvan Lake
Red Deer
Innisfail
Olds
B. campestris
B. napus

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Chapter III

Screening Crucifers for Resistance to *Alternaria brassicae*

A. Introduction

All cultivated *Brassica* spp. are susceptible to *Alternaria brassicae* (Singh & Kolte, 1990). Since there are no resistant lines of rapeseed or mustard, the only methods of reducing the level of alternaria blackspot is through cultural practices and the use of fungicides. Some of the cultural practices include sowing clean, pathogen-free seed, rotating *Brassica* spp. for a minimum of three years with non-crucifers, plowing under infected residue, and control of volunteer *Brassica* spp. (Nyvall, 1981). These cultural practices are generally followed by farmers in central Alberta and yet in 1989, when the weather conditions were ideal for this disease, there was a high incidence of alternaria blackspot (Fig. II-4, Chapter II). Thus, cultural practices alone are not enough to prevent severe incidences of alternaria blackspot.

A number of fungicides have been shown to give effective control of alternaria blackspot if applied at the silique infection phase of this disease, but it is not always practical or economical to do so (Singh & Kolte, 1990). Some farmers in less developed countries do not have ready access to chemicals or spray application equipment, or cannot afford them. As well, chemicals can be hazardous to the user and damaging to the environment. Another problem is that it is difficult to move through the canopy of a *Brassica* crop to apply a fungicide because of the height and dense branching of the crop. Spraying fungicides can be done with airplanes but this is expensive and not always available. A tramline approach using a tractor with a spray rig is another option of fungicide application in canola.

Thus, breeding for resistance to *A. brassicae* appears to be the best way of reducing the level of alternaria blackspot, as is true for diseases in general. However, to accomplish this, sources of resistance to *A. brassicae* have to be found. Wild plants and landraces are reservoirs of many traits and are good sources for resistance to diseases. This is because they have evolved together with pathogens and if they did not have some resistance to them, they would not have survived. Collection of landraces of *B. juncea* and screening for resistance to *A. brassicae* is being carried out in India under a program sponsored by the International Development and Research Centre, Ottawa (Dr. J.P. Tewari, personal communication). Prior to commencement of this project, there appeared to have been no

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A portion of this chapter has been published:
Tewari, J.P., Conn, K.L. and J.S. Dahiya. 1987. Resistance to *Alternaria brassicae* in crucifers. Proceedings of the Seventh International Rapeseed Congress, Poznań, Poland. 5:1085-1090.

known sources of high degrees of resistance to *A. brassicae* in *Brassica* spp. and no studies on resistance to *A. brassicae* in other genera of Cruciferae. Thus, various vegetable, oleiferous, and wild crucifers were screened to locate sources of resistance to *A. brassicae*.

Related to the susceptibility/resistance of crucifers is the question of virulence of different *A. brassicae* isolates. Alternaria blackspot is more severe in India and Europe than in western Canada. This raises the question as to whether this is due mainly to the more favorable environmental conditions that occur there, or whether the isolates of *A. brassicae* present there are more virulent than our Alberta isolates. There appears to have been very few studies comparing the virulence of *A. brassicae* isolates. Observations on several Alberta isolates indicated that they had about the same degree of virulence (K.L. Conn & J.P. Tewari, unpublished results). There are a couple of reports of variation in the virulence of *A. brassicae* isolates in Holland (Van Schreven, 1953) and in the United Kingdom (Mridha, 1983). Differences in cultural characteristics and host reactions of *A. brassicae* have been studied in detail in India establishing three distinct strains or races but their virulence was not investigated (Awasthi & Kolte, 1989; Kolte *et al.*, 1989). Thus, there appears to have been no comparison of isolates from different parts of the world, so the virulence of an isolate from India, an isolate from France and an Alberta isolate were compared. In addition to originating from different parts of the world, these three isolates differed from each other based on cultural and conidial characteristics (R.P. Awasthi & J.P. Tewari, unpublished results). Preliminary reports on portions of this chapter have been published (Conn *et al.*, 1987; Conn & Tewari, 1986).

B. Materials and Methods

1. Plant material

Seeds of various crucifers were obtained from the University of Alberta collection, from other researchers or from fields in central Alberta (see Table III-2). Plants were grown in a greenhouse at approximately 18/12°C (day/night). Leaves of some crucifers were also collected from fields in central Alberta (see Table III-2). Some weed seeds such as those of *Capsella bursa-pastoris* and *Thlaspi arvense* were soaked in gibberellic acid (1000 ppm) overnight in order to get them to germinate (Corns, 1960). Two non-host plants to *A. brassicae* (*Hordeum vulgare* L., barley; *Phaseolus vulgaris* L., bean) were also grown under greenhouse conditions.

2. Fungal material

Alternaria brassicae was isolated from an infected rapeseed leaf from central Alberta. Another *A. brassicae* isolate was isolated from a *B. juncea* leaf from India (provided by Dr. J.P. Tewari). Another *A. brassicae* isolate was obtained from Dr. H. Brun and Dr. M. Renard, France via Dr. J.P. Tewari. Cultures were grown in dark at room temperature for 8-10 days on V8 juice-rose bengal agar medium (Degenhardt *et al.*, 1974). Conidia were washed off the plates with distilled water, filtered through cheesecloth, centrifuged, washed twice, and resuspended in distilled water to concentrations of approximately 1 x 10^5/mL or 1 x 10^6/mL. Unless otherwise stated, experiments were conducted using the Alberta isolate of *A. brassicae*.

3. Screening crucifers for resistance to *Alternaria brassicae*

The cruciferous and non-host plants used had a variety of growth habits so it was not possible to always use leaves of the same physiological age. At least 5-10 leaves of each plant type were screened and each plant type was screened at least twice. All plants were not screened at the same time but each time a set of plants were screened, some crucifers of known susceptibility were included to make comparisons. Detached leaves were placed in humid chambers and a few droplets of an *A. brassicae* conidial suspension (1 x 10^6/mL) placed on one half of each leaf and drops of distilled water placed on the other half as a control. Leaves were incubated for 4-5 days at room temperature under continuous cool white fluorescent light (5.5 $\mu E/m^2/s$). The severity of symptoms was assessed visually and the plants sorted according to susceptibility/resistance. When neccessary, the extent of conidial germination was determined under a light microscope on leaf pieces stained with lactophenol cotton blue. Some plants were also screened against *A. brassicae* using injured leaves. Droplets of an *A. brassicae* conidial suspension and distilled water were placed on parts of leaves that had been gently scratched with the tip of a pasteur pipette.

4. Comparison of virulence of *Alternaria brassicae* isolates

Leaves of *B. napus* cv. Westar, *Camelina sativa*, *B. campestris* ssp. *rapifera* and *Armoracia rusticana* were spotted with the three isolates of *A. brassicae*. Measurements of lesion size were carried out for *B. napus* cv. Westar. Eighteen healthy leaves from 4-5 week old plants (growth stage four; Harper & Berkenkamp, 1975) were placed in humid chambers. The 36 leaf-halves were each spotted with two droplets (50 μL) of water or a conidial suspension (1 x 10^5/mL) of one of the three *A. brassicae* isolates. In this way 18 leaves were spotted with all possible combinations of the four treatments with three repeats for each treatment giving a total of 18 droplets per treatment. The diameter of the droplets was 5 mm. The diameters of the lesions (average of two perpendicular measurements per

lesion) were measured to include both the necrotic and chlorotic areas. This experiment was carried out three times.

C. Results and Discussion

1. Types of responses to *Alternaria brassicae*

The crucifers screened for resistance to *A. brassicae* were placed in three groups based on the type of response to this pathogen (Table III-1). The most common response was necrosis and chlorosis which covered a broad range of symptoms including limited and delayed necrosis and chlorosis (e.g. *Armoracia rusticana*; Fig. III-1D), to moderate necrosis and chlorosis (e.g. *B. napus* and *B. campestris* ssp. *oleifera*; Fig. III-1A,B), to rapid necrosis and extensive chlorosis (e.g. *B. campestris* var. yellow *sarson*; Fig. III-1E). Another response was a hypersensitive reaction by an accession of *Eruca sativa* (Fig. III-1F,G). Necrotic flecks (Fig. III-1G) appeared within the first day after inoculation and then remained of the same size until the leaves began to senesce after 4-6 days. This occurred only on healthy leaves. Some chlorosis occurred on older leaves or on plants that were not healthy. The most resistant response was one in which no symptoms occurred on healthy leaves and growth of *A. brassicae* was inhibited (e.g. *Camelina sativa*; Fig. III-1H,I,J). No symptoms appeared until leaves began to senesce after 4-6 days, and then only as localized necrotic flecks (Fig. III-1J). *Camelina sativa* leaves, stems, and siliques from plants obtained from central Alberta and from seeds obtained from Pakistan all showed this type of resistance. The resistant response of these crucifers was not due to a physical barrier because injuring the leaves prior to inoculation with *A. brassicae* did not reduce their resistance. This resistant response was different from that of two non-host plants (barley and bean) that were screened against *A. brassicae*. They showed an immune response in which no symptoms developed while growth of *A. brassicae* was not inhibited.

2. Screening crucifers for resistance to *Alternaria brassicae*

Table III-2 shows all the crucifers that were screened for resistance to *A. brassicae*. All *Brassica* spp. showed some degree of necrosis and chlorosis. The ranking of *Brassica* spp. (Table III-2) generally agreed with what is known, that *B. napus* is generally more resistant than *B. campestris* ssp. *oleifera* (Bansal *et al.*, 1990; Conn, 1986; Conn & Tewari, 1989; Degenhardt *et al.*, 1974; Petri, 1973; Skoropad & Tewari, 1977; Singh & Kolte, 1990; Tewari & Skoropad, 1976) which are more resistant than *B. juncea*, *B. nigra*, and *B. campestris* var. yellow *sarson* (Bains & Tewari, 1987; Bansal *et al.*, 1990; Singh & Kolte,

1990). Bansal *et al.* (1990) compared six *Brassica* spp. and found that generally *B. carinata* was the most resistant, followed by *B. oleracea*, *B. napus*, *B. campestris*, *B. juncea*, and *B. nigra*.

One *Brassica* sp. of particular interest was an accession of *B. campestris* ssp. *rapifera* that showed a limited and delayed necrosis and chlorosis (Table III-2). This was very different from the susceptibility of *B. campestris* in general. This accession of *B. campestris* ssp. *rapifera* appeared to be a good candidate for a source of resistance to *A. brassicae* that could be transferred to other Brassicas by conventional breeding methods. So this accession of *B. campestris* ssp. *rapifera* (Fig. III-2A,B) was passed on to Dr. G.R. Stringam (canola breeder, Dept. Plant Science, U of A) who crossed it with a *B. juncea* breeding line 90-131-4 (very susceptible to *A. brassicae*; Table III-2, Fig. III-2C,D). This *B. juncea* line is of interest because it's seed is low in erucic acid and glucosinolate, and it has resistance to *Leptosphaeria maculans* (Dr. Stringam, personal communication). Leaves of a F_1-B_1 cross (with an intermediate phenotype of the parents) provided by Dr. Stringam were screened against *A. brassicae* (Fig. III-2E,F). The amount of necrosis and chlorosis was intermediate between that found in the two parents. Thus, some resistance to *A. brassicae* was transferred to this F_1-B_1 cross. Hopefully, further work with these lines will yield a *B. juncea* with resistance to *A. brassicae* and *L. maculans* and seeds that are low in erucic acid and glucosinolate.

The most resistant crucifers were weeds such as *C. sativa*, *C. bursa-pastoris* and *Neslia paniculata* (Table III-2). Resistance to *A. brassicae* has been reported for *C. sativa* (Grontoft, 1986). The very high degree of resistance to *A. brassicae* in these weeds will not be transferable to *Brassica* spp. by conventional breeding methods but it may be transferable by biotechnological techniques. One way to obtain new plants is though the fusion of protoplasts from different plants (somatic hybridization). Crosses between species, genera, and tribes of Cruciferae have been carried out. Examples of interspecific crosses are between *B. juncea*, *B. nigra*, and *B. carinata* and *B. napus* (Sjödin & Glimelius, 1989). This was done in order to transfer *L. maculans* resistance to *B. napus*. In this case, selection pressure by *L. maculans* toxin was also used at the same time. An example of an intergeneric cross is between *Eruca sativa* and *B. napus* to transfer aphid and drought resistance to *B. napus* (Fahleson *et al.*, 1988; Glimelius *et al.*, 1990). Other examples of intergeneric crosses are between *Diplotaxis harra* (Forsk) Boiss and *B. napus* to transfer cytoplasmic male sterility to *B. napus* (Klimaszewska & Keller, 1988) and between *Raphanus sativus* and *B. napus* to transfer shattering resistance to *B. napus* (Glimelius *et al.*, 1990). Examples of intertribal crosses are between *Barbarea vulgaris* R.Br. and *Thlaspi perfoliatum* L. and *B. napus* to transfer cold tolerance and high nervonic acid content to *B. napus*, respectively (Glimelius *et al.*, 1990). The cruciferous weeds resistant to *A. brassicae*

described in the present study would involve intertribal crossing with rapeseed but the above examples show that such wide crossing is possible. Sometimes conventional methods of hybridization have limited use for wide crosses because of pre- and post-fertilization barriers. To overcome this, embryo rescue can be used. An example is the interspecific cross of *Sinapis alba* with *B. napus* to transfer *A. brassicae* resistance (Ripley & Arnison, 1990). Another example is the intergeneric cross of *Eruca sativa* with *B. campestris* to transfer white rust and drought resistance (Agnihotri *et al.*, 1990). Another technique that has been used is *in vitro* ovule culture. Ovule culture has been used to make interspecific and intergeneric crosses in Cruciferae (Hossain *et al.*, 1988; Zenkteler, 1990).

Another biotechnological technique is the genetic engineering of plants using a vector such as *Agrobacterium tumefaciens* (Sm. & Towns.) Conn. If genes for a particular trait from one plant can be identified and isolated, they can be incorporated into a vector which can then transfer them to another plant. This technique appears to be feasable for incorporating genes into rapeseed because it has been shown that *A. tumefaciens* and *A. rhizogenes* can transform *B. napus* (Boulter *et al.*, 1990). Boulter *et al.* (1990) demonstrated that these *Agrobacterium* ssp. had introduced genes into rapeseed as evidenced by the presence of hairy roots and some other markers. These plants were fertile and when they were crossed with wild type rapeseed, the new genes were transferred to the progeny. Thus, if the genes coding for resistance in *C. sativa* to *A. brassicae* could be identified and isolated, they could possibly be transfered to rapeseed.

3. Virulence of *Alternaria brassicae* isolates

The virulence of the *A. brassicae* isolates on *B. napus* cv. Westar is shown in Figure III-3 and III-4. The Alberta isolate was the most virulent, followed by the French, and then the Indian isolate. The water controls did not develop any symptoms. A similar trend in virulence was seen on *B. campestris* ssp. *rapifera* and *A. rusticana*. None of these isolates caused symptoms on *C. sativa* (Fig. III-4). The similar susceptibility/resistance responses of these crucifers to the different *A. brassicae* isolates indicated that the screening done here for resistance against the Alberta isolate holds true for other isolates as well. Only a few isolates have been compared, but the results suggest that the greater severity of alternaria blackspot in Europe and India may be due to more conducive environmental conditions than any higher virulence of *A. brassicae* isolates.

D. Tables, Figures, and Legends

Table III-1. Response of leaves of some crucifers to *Alternaria brassicae*.

Response of healthy leaves	Code #	e.g.	Figure
necrosis and chlorosis*	3	*Brassica napus* *B. campestris* ssp. *oleifera*	III-1A,B,C
localized necrotic flecks**	2	*Eruca sativa*	III-1F,G
no symptoms; fungal growth inhibited	1	*Camelina sativa*	III-1H,I,J

* The degrees of necrosis and chlorosis covered a broad range of symptoms from limited and delayed necrosis and chlorosis (e.g. *Armoracia rusticana*; Fig. III-1D), to moderate necrosis and chlorosis (e.g. *B. napus* and *B. campestris* ssp. *oleifera*; Fig. III-1A,B), to rapid necrosis and extensive chlorosis (e.g. *B. campestris* var. yellow *sarson*; Fig. III-1E).
** Hypersensitive reaction

Table III-2. Screening of crucifers for resistance to *Alternaria brassicae*.

Crucifer	Common name	Source	Response code (see Table III-1)
Camelina sativa (L.) Crantz	false flax	Pakistan[2]/Alberta[3]	1
Capsella bursa-pastoris (L.) Medic.	shepherd's-purse	Alberta[3]	1
Neslia paniculata (L.) Desv.	ball mustard	Alberta[3]	1
Eruca sativa (Miller) Thell.	garden-rocket	Pakistan[2]	2
Armoracia rusticana Gaertn.	horse-radish	Alberta[3]	3*
Brassica campestris L. ssp. *rapifera*	forage turnip	U of A collection	3
B. hirta Moench cv. Gisilba	white mustard	U of A collection	3
Sinapis alba L. cvs. Kirby, Tilney	mustard	U of A collection	3
Thlaspi arvense L.	stinkweed	Alberta[3]	3
B. hirta cv. Sabre	white mustard	U of A collection	3
B. napus L. ssp. *oleifera* cvs. Westar, Regent	canola[1]	U of A collection	3
B. napus ssp. *oleifera* cv. Jet Neuf	rapeseed	U of A collection	3
B. napus ssp. *oleifera* line Norin 9	rapeseed	France[4]	3
B. napus ssp. *oleifera* lines DH12209, DH12229, DH12014	rapeseed	Saskatoon[5]	3
Erysimum cheiranthoides L.	wormseed mustard	Alberta[3]	3
B. campestris ssp. *rapifera* cv. Tyfon	turnip	U of A collection	3
B. carinata Braun	abyssinian mustard	U of A collection	3
S. arvensis	wild mustard	Alberta[3]	3
B. campestris ssp. *oleifera* cvs. Candle, Tobin	canola[1]	U of A collection	3
B. juncea (L.) Cosson cv. Blaze	Indian mustard	U of A collection	3
B. juncea cv. Leth 22A	Indian mustard	U of A collection	3
B. carinata line R8734, cv. Peela raya	mustard	Pakistan[2]	3
B. campestris var. *toria*	rapeseed	U of A collection	3
B. juncea cv. Domo	Indian mustard	U of A collection	3
B. juncea breeding line 90-131-4	Indian mustard[1]	U of A[6]	3
B. campestris var. yellow *sarson*	rapeseed	U of A collection	3**

* Crucifers with a response code of 3 are listed from most resistant (*) to most susceptible (**).

(continued on next page)

Table III-2. Screening of crucifers for resistance to *Alternaria brassicae* (continued).

[1]Seeds low in erucic acid and glucosinolate.
[2]Seeds from Pakistan via U of A collection.
[3]Seeds and leaves collected from central Alberta.
[4]Thanks are due to Dr. M. Renard, I.N.R.A., Amelioration des Plantes, Le Rheu, France, for seeds. This line has some resistance to *Sclerotinia sclerotiorum* (Brun *et al.*, 1987).
[5]Thanks are due to Dr. G. Séguin-Schwartz, Agriculture Canada, Saskatoon, for seeds. These lines have resistance to *Leptosphearia maculans* (Dr. G. Séguin-Schwartz, personal communication to Dr. J.P. Tewari).
[6]Thanks are due to Dr. G.R. Stringam, Dept. Plant Science, U of A, Edmonton, for seeds. This breeding line has resistance to *L. maculans* (Dr. G.R. Stringam, personal communication).

Figure III-1. Response of leaves of some crucifers to *Alternaria brassicae*.
Leaves were spotted with water on the left leaf-halves and with an *A. brassicae* conidial suspension on the right leaf-halves. Photographs were taken after 5 days.
A,B,C) Canola showing necrosis and chlorosis.
A) *Brassica napus* cv. Westar (approx. x1).
B) *B. campestris* ssp. *oleifera* cv. Candle (approx. x1). Note that the lesions were a little larger than those on *B. napus*.
C) Close-up of the lesion on *B. campestris* ssp. *oleifera* cv. Candle. Note that the necrosis extended outside the inoculum droplet (approx. x9).
(continued on next page)

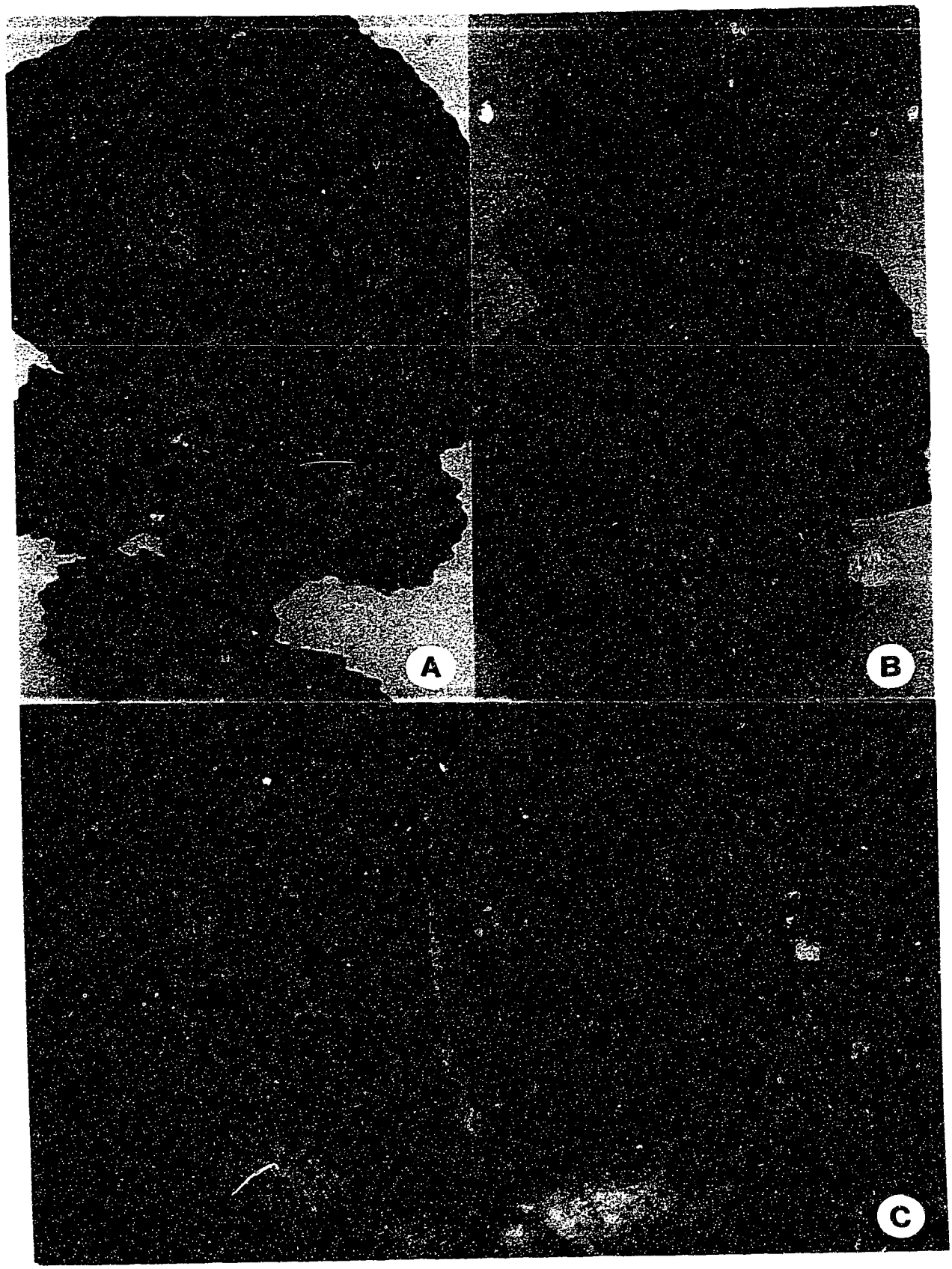

A
B
C

Figure III-1. Response of leaves of some crucifers to *Alternaria brassicae* (continued). **D)** *Armoracia rusticana* showing limited and delayed necrosis and chlorosis (approx. x1.2). **E)** *B. campestris* var. yellow *sarson* showing rapid necrosis and extensive chlorosis (approx. x1).
(continued on next page)

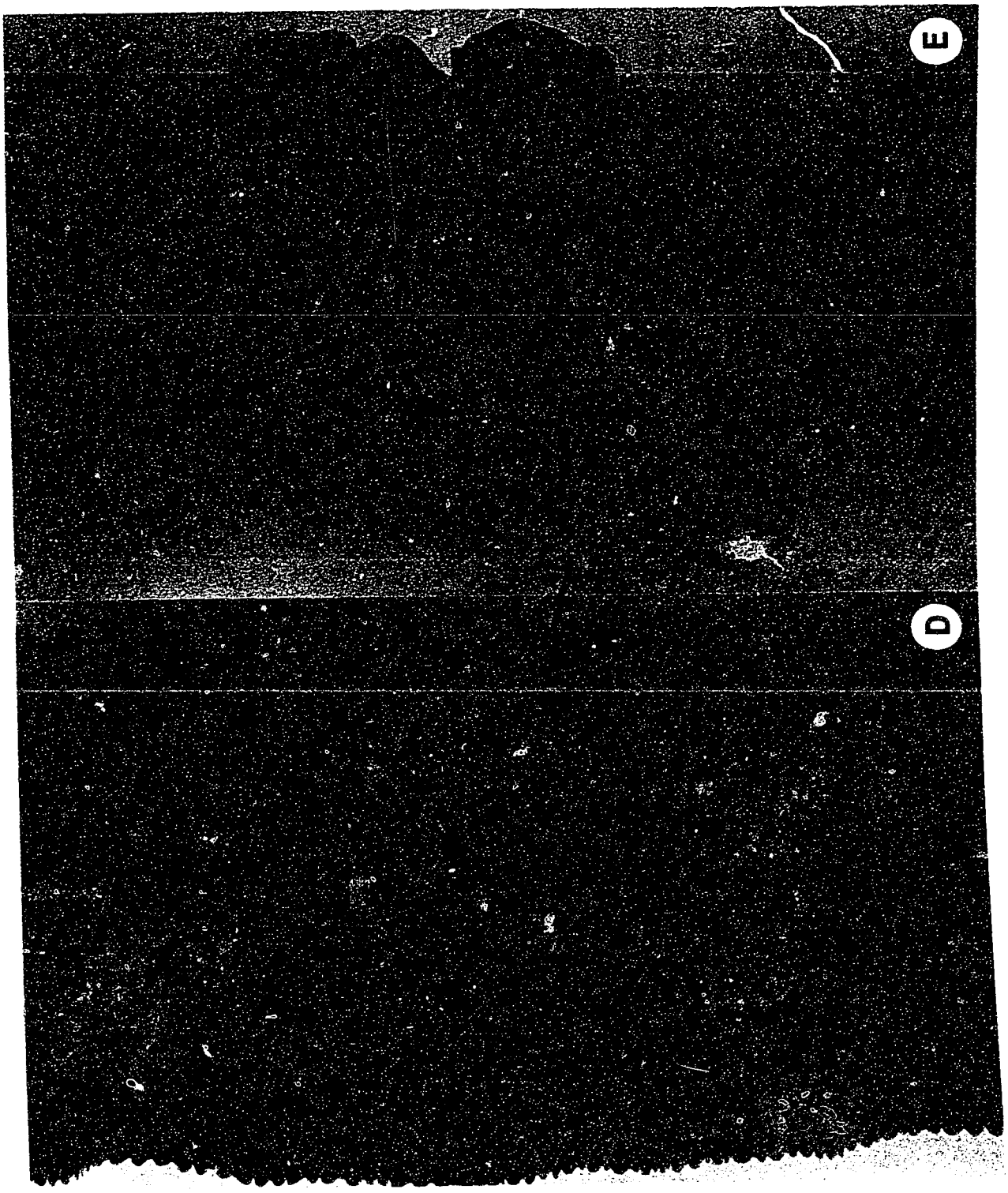

E
D

Figure III-1. Response of leaves of some crucifers to *Alternaria brassicae* (continued).
F) An accession of *Eruca sativa* showing a hypersensitive reaction (approx. x1).
G) *Eruca sativa* showing necrotic flecks within a droplet of *A. brassicae* conidial suspension (approx. x16). The necrotic flecks appeared within the first day after inoculation and remained the same size until the leaves began to senesce after 4-6 days.
(continued on next page)

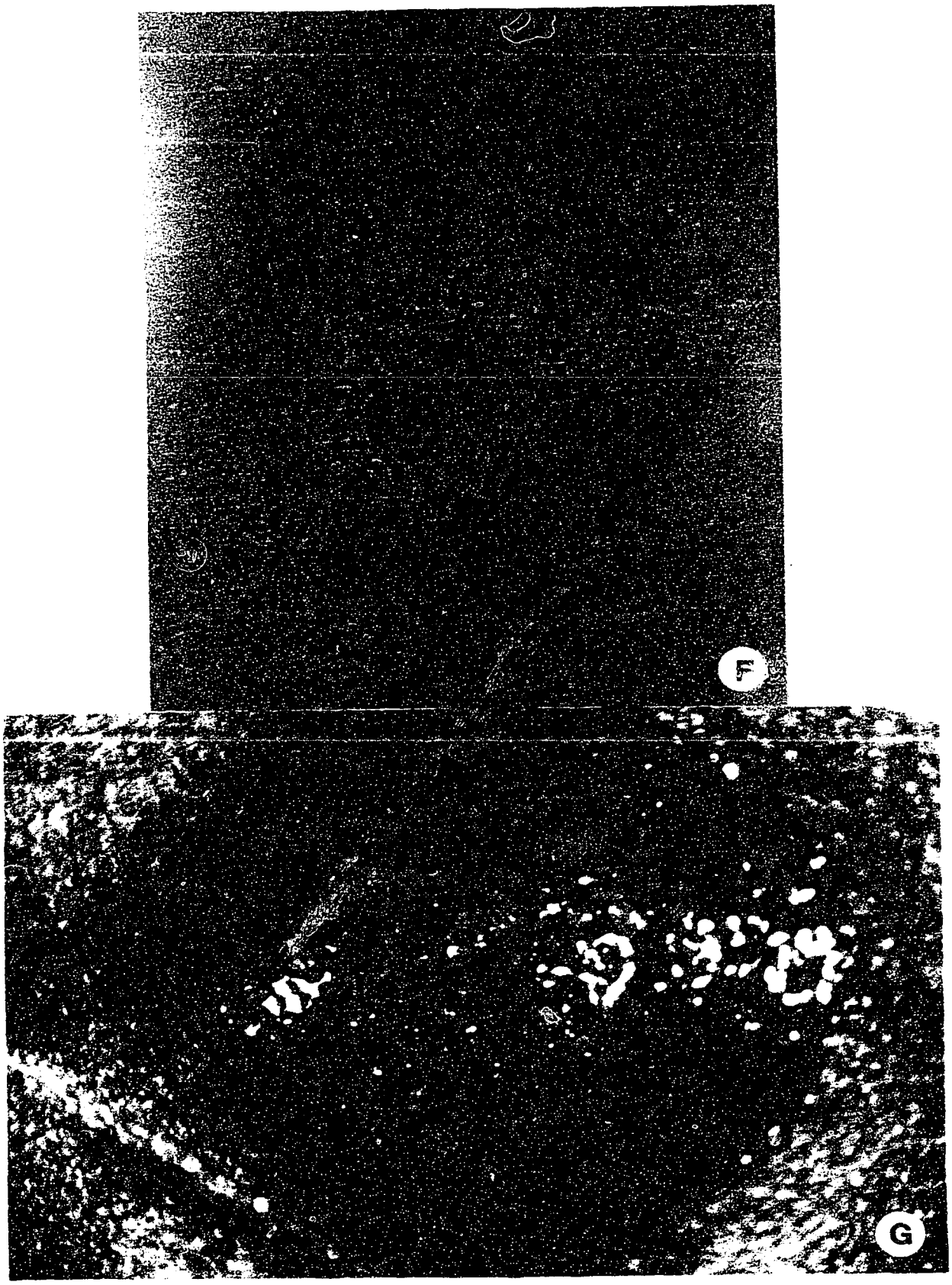

F
G

Figure III-1. Response of leaves of some crucifers to *Alternaria brassicae* (continued).
H) *Camelina sativa* showing no symptoms (approx. x2).
I,J) Droplet of *A. brassicae* conidial suspension removed from *C. sativa* leaf (I, arrowhead) revealing a few localized necrotic flecks that only appeared when leaves began to senesce after 4-6 days (J, arrowheads) (approx. x3, x25).

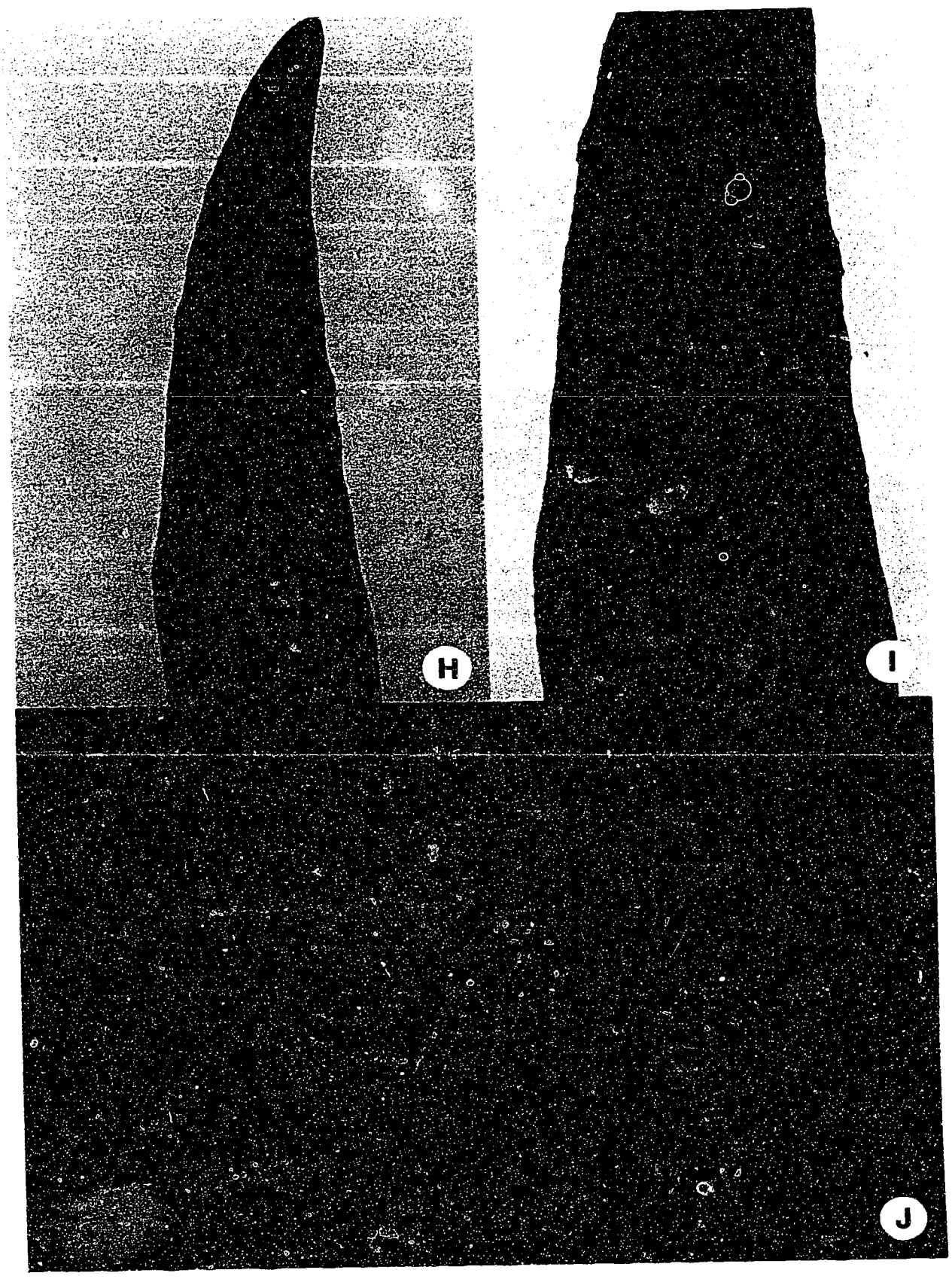

H
I
J

Figure III-2. Response of leaves of *Brassica campestris* ssp. *rapifera*, *B. juncea*, and a F_1-B_1 cross to *Alternaria brassicae*.
An accession of *B. campestris* ssp. *rapifera* with low susceptibility to *A. brassicae* (Table III-2) was provided to Dr. G.R. Stringam who crossed it with a *B. juncea* breeding line 90-131-4 (very susceptible to *A. brassicae*; Table III-2). Dr. Stringam then provided leaves of a F_1-B_1 cross with an intermediate phenotype of the parents. Leaves of both parents and the cross were then spotted with water or an *A. brassicae* conidial suspension. Photographs were taken after 3 days.
A,B) *Brassica campestris* ssp. *rapifera* leaves spotted with water (A) and an *A. brassicae* conidial suspension (B) (approx. x1). Note that *A. brassicae* caused very little necrosis and no chlorosis.
(continued on next page)


B
A

Figure III-2. Response of leaves of *Brassica campestris* ssp. *rapifera*, *B. juncea*, and a F_1-B_1 cross to *Alternaria brassicae* (continued).
C,D) *Brassica juncea* leaves spotted with water (C) and an *A. brassicae* conidial suspension (D) (approx. x1). Note that *A. brassicae* caused necrosis and extensive chlorosis.
(continued on next page)

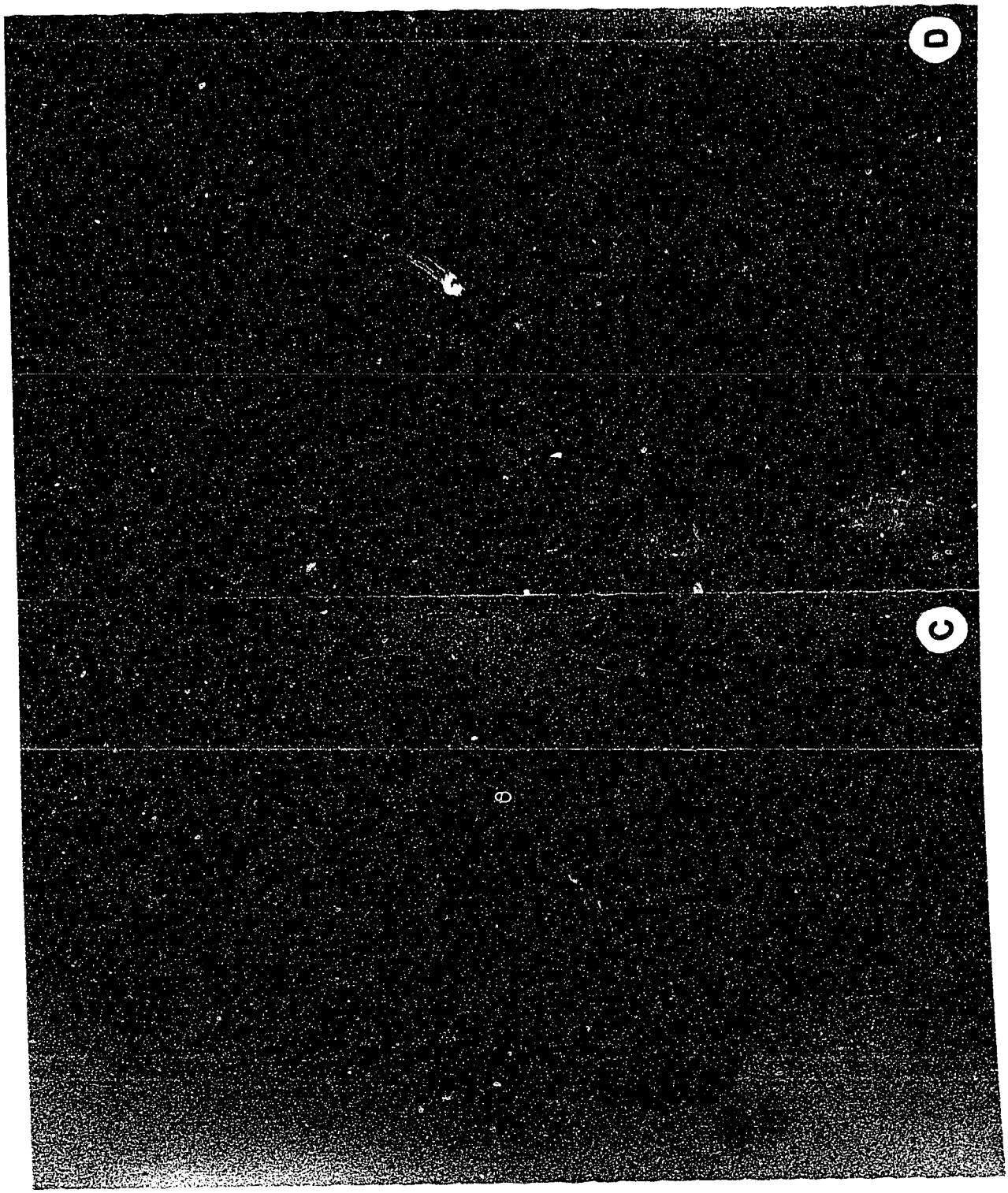

D
C
B

Figure III-2. Response of leaves of *Brassica campestris* ssp. *rapifera*, *B. juncea*, and a F_1-B_1 cross to *Alternaria brassicae* (continued).
E,F) Leaves of a F_1-B_1 cross between *B. campestris* ssp. *rapifera* and *B. juncea* spotted with water (E) and an *A. brassicae* conidial suspension (F) (approx. x1). Note that the amount of necrosis and chlorosis was intermediate between that found in the two parents.

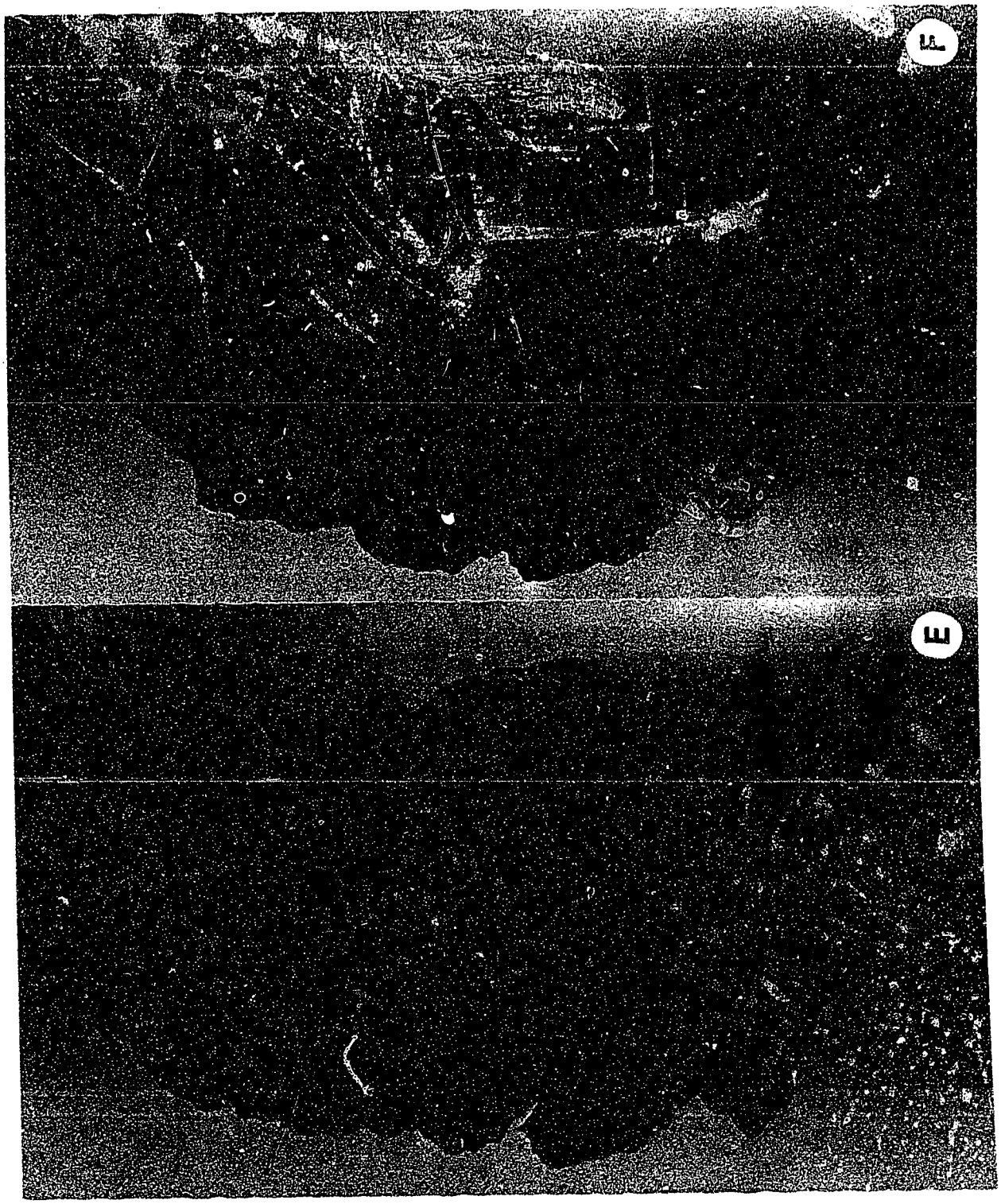

A
B

Figure III-3. Virulence of *Alternaria brassicae* isolates on *Brassica napus* cv. Westar leaves. Droplets of water or conidial suspensions of the Alberta, French, and Indian isolates of *A. brassicae* were spotted on leaves. The diameter of the droplets was 5 mm. Lesion diameters were measured to include both the necrotic and chlorotic areas. The water controls did not develop any symptoms. Note that the Alberta isolate was the most virulent, followed closely by the French, and then the Indian isolate. Vertical bars represent standard error of the mean of three experiments.

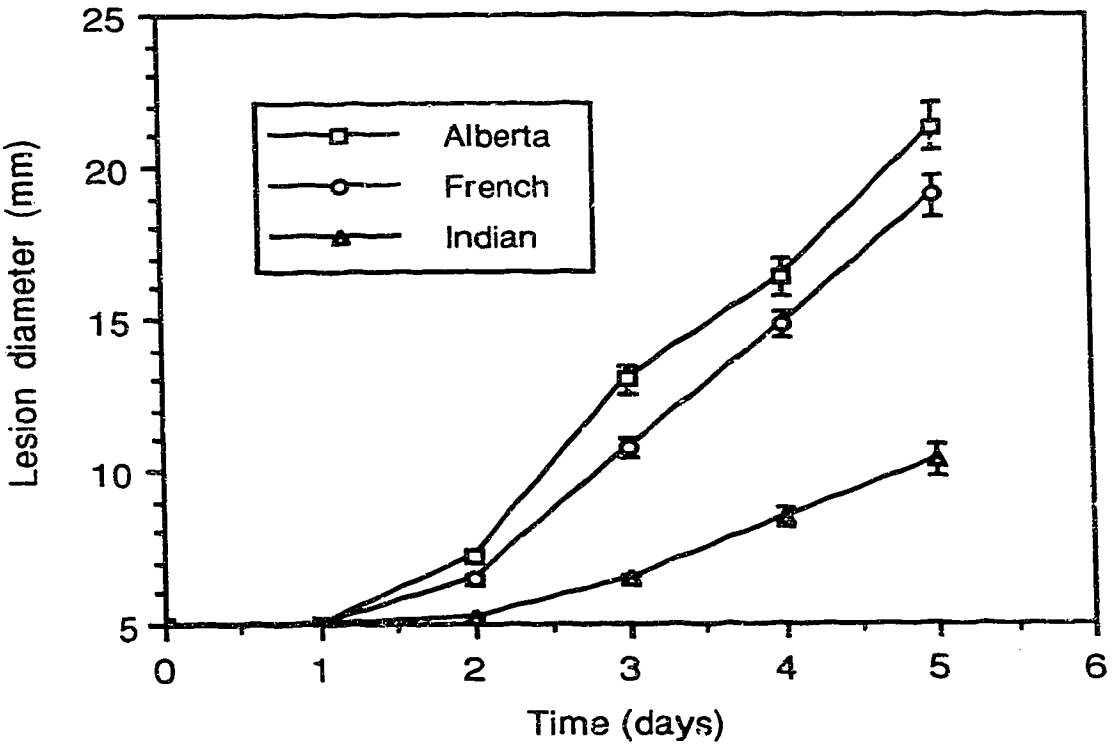
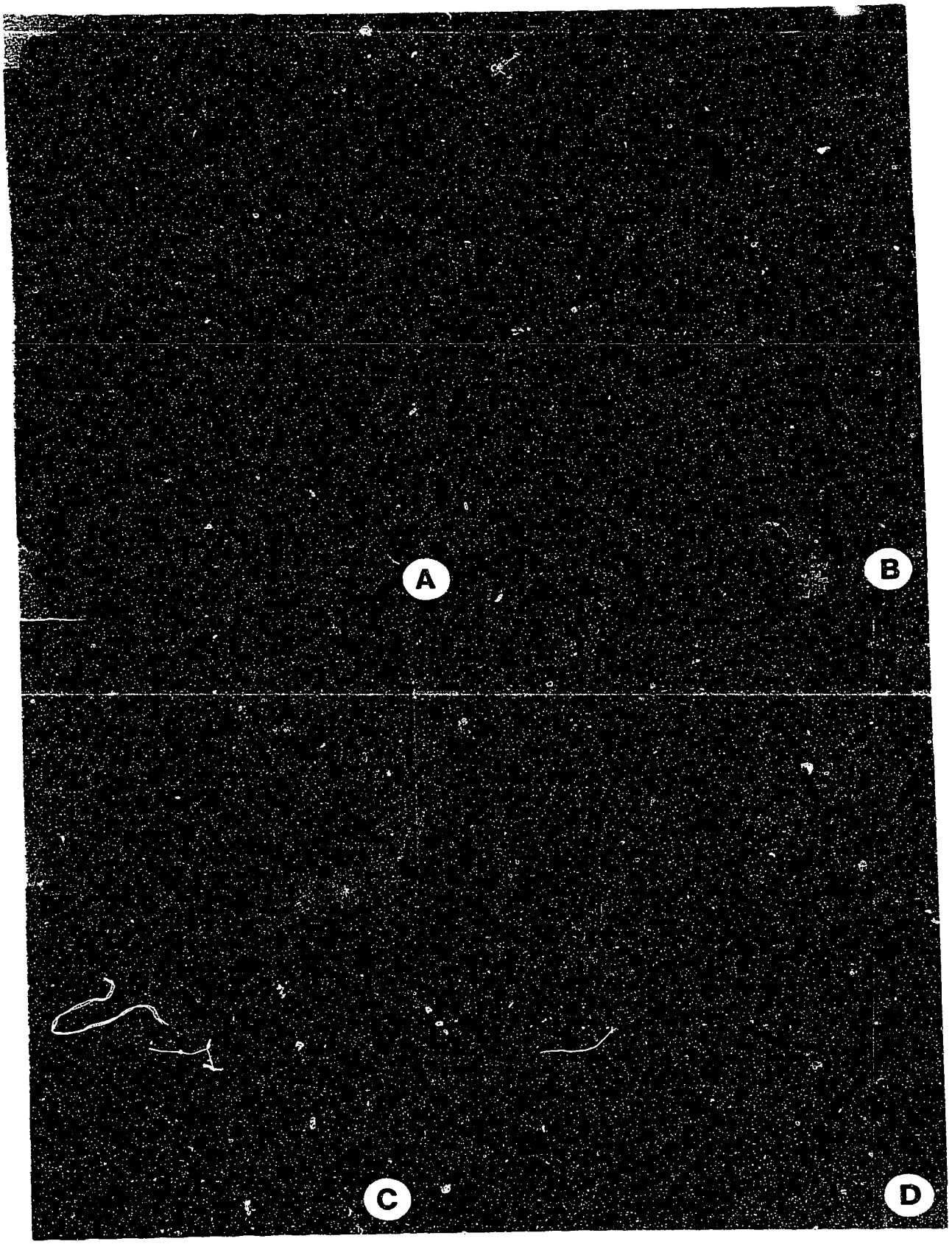

Alberta
French
Indian
Lesion diameter (mm)
Time (days)
25
20
15
10
5
0
1
2
3
4
5
6

Figure III-4. Virulence of *Alternaria brassicae* isolates on *Brassica napus* cv. Westar and *Camelina sativa* leaves.
A,B,C) *Brassica napus* cv. Westar leaves 4 days after spotting with a conidial suspension of *A. brassicae* isolates from Alberta (A,C; left leaf-halves), France (A,B; right leaf-halves), and India (B; left leaf-half, C; right leaf-half) (approx. x1). Note that the Alberta isolate was the most virulent, followed closely by the French, and then the Indian isolate.
D) *Camelina sativa* leaves 4 days after spotting with a conidial suspension of *A. brassicae* isolates from Alberta (first leaf), India (second leaf), and France (third leaf) (approx. x1.5). Note that none of the isolates caused symptoms on *C. sativa*.


A
B
C
D

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Chapter IV

Resistance to *Alternaria brassicae* and Phytoalexin-elicitation in Crucifers

A. Introduction

Little is known about mechanisms of resistance to *Alternaria brassicae* in Cruciferae. Prior to commencement of this project, there were only two mechanisms that had been observed. One of these mechanisms is differential susceptibility of crucifers to destruxin B, the host-specific toxin produced by *A. brassicae* (Bains & Tewari, 1987). Bains & Tewari (1987) observed that *Brassica nigra* and *B. campestris* were more sensitive to destruxin B than *B. napus* and *B. rapa*. Leaves of these plants had been gently scratched with the tip of a pasteur pipette before inoculation to remove any physical barrier. The reason for the differential susceptibility to destruxin B is not yet known.

The other mechanism of resistance observed in Cruciferae is based on differential amounts of epicuticular wax. The lower susceptibility of *B. napus* to *A. brassicae* as compared to *B. campestris* ssp. *oleifera* was shown to be due, in part, to greater amounts of epicuticular wax on the former (Conn, 1986; Singh & Kolte, 1990; Skoropad & Tewari, 1977; Tewari & Skoropad, 1976). The wax crystals formed a fluffy layer (Conn & Tewari, 1989a) which created a hydrophobic coating causing water droplets to roll off. Since inoculum of *A. brassicae* is largely water-borne, *B. napus* retained less inoculum than *B. campestris* (Conn, 1986; Tewari & Skoropad, 1976). The epicuticular wax also reduced germination of *A. brassicae* conidia on rapeseed leaves, likely by reducing the amount of leaf exudates that reached the conidia (Conn & Tewari, 1989b). This appeared to be due only to the amount and fluffy nature of the epicuticular wax since the chemistry of the epicuticular wax of *B. napus* and *B. campestris* ssp. *oleifera* was the same (Conn, 1986). As well, the epicuticular wax had no direct inhibitory effect on germination of *A. brassicae* conidia (Conn & Tewari, 1989b).

These mechanisms, however, did not appear to explain the high degree of resistance to *A. brassicae* observed in *Camelina sativa* and some other crucifers mentioned in Chapter III. The possibility of there being a physical barrier was ruled out as well since injuring these plants prior to inoculation did not make them more susceptible to *A. brassicae* (Chapter III). Thus, the possibility that phytoalexins were involved in the resistance of some crucifers to *A. brassicae* was investigated. Prior to commencement of this project,

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A portion of this chapter has been published:

Conn, K.L., Tewari, J.P. and J.S. Dahiya. 1988. Resistance to *Alternaria brassicae* and phytoalexin-elicitation in rapeseed and other crucifers. *Plant Sci.* 56:21-25.

Jejelowo, O.A., Conn, K.L. and J.P. Tewari. 1991. Relationship between conidial concentration, germling growth, and phytoalexin production by *Camelina sativa* leaves inoculated with *Alternaria brassicae*. *Mycol. Res.* 95:928-934.

there were no reports of phytoalexin production in Cruciferae. Preliminary reports on portions of this chapter have been published (Conn *et al.*, 1987, 1990; Tewari *et al.*, 1988).

B. Materials and Methods

1. Plant material

See Materials and Methods, Chapter III.

2. Fungal material

Alternaria brassicae conidia were obtained as previously described (Materials and Methods, Chapter III). A conidial concentration of approximately 1 x 10^6/mL was used for all experiments, except when the effect of conidial concentration on phytoalexin production was investigated and in the conidial germination experiment, when a number of concentrations were used. *Alternaria brassicae* was also grown in liquid V8 juice medium (Degenhardt, 1978) to obtain mycelium. Cultures were grown in 1 L flasks (50 mL medium) and in tubs (10 x 11 inches with 250 mL medium and covered with tinfoil) in dark at room temperature for 4-8 days. The mycelium was then removed, wrapped in cheesecloth and the liquid screezed out. The mycelium at this stage was used to determine whether any antimicrobial compounds came from the mycelium. When mycelium was used to elicit phytoalexins, some distilled water was added and it was placed in a blender for 3-4 seconds to make a mycelial suspension. Unless otherwise stated, experiments were conducted using the Alberta isolate of *A. brassicae*.

A *Cladosporium* sp. was isolated from infected tomato plants. Cultures were grown in dark at room temperature for 8-16 days on malt extract agar. Conidia were washed off the plates with double strength Czapek-Dox broth and filtered through cheesecloth.

3. Phytoalexin-elicitation

Detached plant material (leaves and in some cases stems and siliques as well) was placed in a humid chamber and the surface covered with droplets of a conidial suspension, or it was dipped into a mycelial suspension and then placed in a humid chamber. An equal amount of plant material was spotted with distilled water and placed in a humid chamber and used as a control. Unless otherwise stated, plant material was not injured before inoculation and was incubated at room temperature under continuous white fluorescent light (5.5 $\mu E/m^2/s$) for 2-10 days depending on the type of plant material. Plant material was extracted before the disease symptoms became too severe and before general senescence started.

The French and Indian isolates of *A. brassicae* were tested for phytoalexin-elicitation on *C. sativa* and compared with the Alberta isolate. Leaves were covered with either droplets of conidial suspensions or mycelial suspensions and incubated as described above. Leaves were extracted after 4 days. This experiment was carried out three times.

Several chemicals ($AgNO_3$, NaF, $NiCl_2$, NaCN, NaN_3, Li_2SO_4) were tested for phytoalexin-elicitation in *C. sativa* leaves. The chemical solutions (10^{-3} and 10^{-4} M for $AgNO_3$, 10^{-3} M for the rest) were prepared just prior to use. Droplets (20 μL) of these solutions were placed on leaves and the leaves incubated as described above, except for $AgNO_3$, which was incubated in both light and dark. Leaves were extracted after 4 days. This experiment was carried out two times. Un-injured leaves were used during the first run. In the second run, droplets of chemicals were placed on parts of leaves that had been gently scratched with the tip of a pasteur pipette.

4. Extraction of plant material

Plant material was extracted with 70% (v/v) aqueous CH_3OH for 3-5 minutes using the facilitated diffusion technique (Keen, 1978). Extracts were filtered, evaporated to dryness *in vacuo* at 40°C, and redissolved in CH_3OH or $CHCl_3$. These extracts were used for the TLC cladosporium bioassay most of the time, but the antifungal compounds in some extracts were partially purified before being used.

5. Partial purification of antifungal compounds

The vacuum liquid chromatography technique (Coll & Bowden, 1986; Pelletier *et al.*, 1986) was modified into a vacuum filtration technique. Thin-layer chromatography (TLC) grade silica gel 60 G (E. Merck) was packed into a sintered glass Buchner filter funnel to a height of 0.25-0.5 cm and covered with a filter paper. The dried extract was then redissolved in $CHCl_3$, loaded onto the silica gel under vacuum, and washed through with $CHCl_3$ (approximately 50 mL for a 3 cm diameter funnel). The eluent was concentrated and used for the TLC cladosporium bioassay. Passing a small amount of CH_3OH through the funnel cleaned the silica gel so that the same set-up could be used many times.

6. TLC cladosporium bioassay

Extracts were spotted on TLC plates (Whatman, K5 silica gel, 250 μm thickness) and developed in $CHCl_3$:CH_3OH (98:2 or 95:5, v/v). A dense conidial suspension of a *Cladosporium* sp. was sprayed on the plates and the plates incubated in a humid chamber in dark at room temperature. After 2-3 days, the plates were examined for zones of inhibition indicated by clear areas surrounded by fungal growth.

Samples of the phytoalexins, brassinin, cyclobrassinin and methoxybrassinin, from Chinese cabbage were received from Dr. M. Takasugi. TLC of these phytoalexins was carried out for comparison with the phytoalexins observed in this study.

7. Testing if antifungal compounds originated from *Alternaria brassicae*

Droplets (20 μL) of an *A. brassicae* conidial suspension were placed on glass slides in a humid chamber and allowed to germinate for a few hours. Droplets containing the germinated conidia and *A. brassicae* mycelium were extracted in the same way as plant material, and a TLC cladosporium bioassay done. This experiment was carried out two times.

8. Conidial germination experiments

Droplets (20 μL) containing approximately 1 x 10^2, 1 x 10^3, 1 x 10^4, 1 x 10^5, [illegible] 10^6 *A. brassicae* conidia were placed on glass slides and on *C. sativa* leaves and incubated as described above. At intervals, drops of lactophenol cotton blue were added to inoculum droplets and the conidial germination determined by counting at least 100 conidia in each of the three droplets for each treatment. The length of germ tubes in each of the three droplets were determined using 20 germlings. This experiment was carried out three times.

9. Biossay for antifungal activity of phytoalexins against *Alternaria brassicae*

Spots showing antifungal activity in the TLC cladosporium bioassay from *C. sativa* leaf extracts were scraped off TLC plates, extracted with CH_3OH and concentrated. These were spotted on small paper disks which were placed on V8 juice-rose bengal agar medium inoculated with *A. brassicae*. The plates were incubated for 2 weeks in dark at room temperature. Control disks were spotted with CH_3OH. This experiment was carried out two times.

10. Testing sensitivity of crucifers to destruxin B

Leaves of *B. napus* cv. Westar, *C. bursa-pastoris* and *C. sativa* were spotted with droplets (20 μL) of distilled water, *A. brassicae* conidial suspension and destruxin B (provided by Dr. J.P. Tewari) solutions (50 and 100 μg/mL of distilled water). The droplets were placed on parts of leaves that had been gently scratched with the tip of a pasteur pipette. Leaves were incubated as described above and scored for symptoms after 3 days. This experiment was carried out three times.

C. Results and Discussion

1. Phytoalexin-elicitation in *Camelina sativa*

The TLC cladosporium bioassay of extracts from *C. sativa* leaves spotted with an *A. brassicae* conidial suspension showed two antimicrobial spots that were not in the control (Fig. IV-1). This indicated the presence of two phytoalexins (Rf 0.33, 0.43; $CHCl_3$:CH_3OH 98:2) with the one at Rf 0.33 being produced in larger quantities (Fig. IV-1). It was determined later (Chapter VI) that the antifungal spot at Rf 0.33 was a mixture of two phytoalexins. These phytoalexins at Rf 0.33 were produced in appreciable amounts at all times and were the phytoalexins investigated further. All the phytoalexins produced by *C. sativa* fluoresced under UV light (254 nm) which facilitated monitoring their presence on TLC. The concentration of phytoalexin increased as the conidial concentration increased (Fig. IV-1). It was observed that leaves covered with a mycelial suspension of *A. brassicae* also produced these phytoalexins. The possibility that these antimicrobial compounds came from *A. brassicae* was investigated by extracting *A. brassicae* conidia and mycelium and performing a TLC cladosporium bioassay. The results showed that the antimicrobial compounds did not come from *A. brassicae*, and that they only appeared when leaves were spotted with *A. brassicae* (Fig. IV-2). Thus, these antimicrobial compounds were indeed phytoalexins. It was observed that the stems and siliques of *C. sativa* also produced these phytoalexins, and that they were produced when leaves were incubated in dark or light. However, plant material was always incubated in light because it remained healthy longer and thus produced more phytoalexins. The phytoalexins were detectable by the TLC cladosporium bioassay by 12 hours after inoculation of leaves, and continued to increase until leaves began to senesce, at which time the amounts stayed the same or declined. It was observed that phytoalexins could be extracted from the leaf area beneath droplets of an *A. brassicae* conidial suspension, as well as from within droplets of the conidial suspension, but not from the surrounding tissue. The phytoalexins in the droplets must have diffused from the leaf and could explain the reduced growth of *A. brassicae* on *C. sativa* that had been observed (Chapter III). The germination of *A. brassicae* conidia and growth of germ tubes on *C. sativa* was examined more closely. The effect of conidial concentration on germination and growth of germ tubes was examined on glass slides and on leaves. Germination over a range of concentrations of *A. brassicae* conidia are shown in Figure IV-3. By 6 hours, more than 90% of the conidia had germinated when there were 1×10^4 or fewer conidia per droplet on glass slides (Fig. IV-3A). When there were 1×10^5 conidia, the germination was significantly lower, and still lower when droplets contained 1×10^6 conidia (Fig. IV-3A). Thus, germination was inhibited as conidial concentration increased. A similar pattern was observed on leaf surfaces also (Fig. IV-3B). This crowding effect has

been observed for many fungi (Marko, 1981). On glass slides (Fig. IV-4A) and on leaf surfaces (Fig. IV-4B) the rate of growth of germ tubes also decreased as conidial concentration increased. However, for most treatments, percentage germination and germ tube length on leaf surfaces were appreciably lower than on glass slides (Figs. IV-3, IV-4), so crowding effect alone was not enough to explain the inhibition of *A. brassicae* conidia on leaf surfaces. To determine that these phytoalexins indeed had antifungal activity against *A. brassicae*, the phytoalexins (Rf 0.33) were extracted from TLC plates and a bioassay with paper disks done. *Alternaria brassicae* grew on the control disk (Fig. IV-5A) but not on the disk with the phytoalexin added (Fig. IV-5B). The latter disk had some conidia on it that did not germinate. Thus, it appeared that the phytoalexins produced by *C. sativa* were inhibitory to the growth of *A. brassicae*.

Phytoalexin-elicitation in *C. sativa* was also investigated against the French and Indian isolates of *A. brassicae*. As shown in Figures III-3 and III-4 (Chapter III), the Alberta isolate was the most virulent, followed closely by the French isolate, and the Indian isolate was the least virulent on *B. napus* cv. Westar. None of these isolates caused symptoms on *C. sativa* (Fig. III-4, Chapter III). Phytoalexin-elicitation could be seen only for the Alberta isolate when *C. sativa* leaves were spotted with conidial suspensions, but phytoalexin-elicitation could be seen for all three isolates when mycelial suspensions were used, although less so for the French and Indian isolates (Fig. IV-6). This was an unusual response because usually virulent isolates of a pathogen elicit less phytoalexins than weakly virulent or non-virulent isolates, as discussed in Chapter I. In this case, however, the more virulent Alberta isolate elicited greater amounts of phytoalexins. The reason for this is not known.

Abiotic elicitors have been shown to elicit phytoalexins in some plants, although usually at a lower level than biotic elicitors (Bailey, 1982). $AgNO_3$, for example, has been shown to elicit phytoalexins in several *Brassica* sp. (Dahiya & Rimmer, 1989; Rouxel *et al.*, 1989). Several chemicals that have been shown to elicit phytoalexins in plants (Sengupta & Sinha, 1987) were tested for phytoalexin-elicitation in *C. sativa* leaves. Chemicals are easier to obtain than *A. brassicae* conidia or mycelium, and thus could be used to elicit phytoalexins from large amounts of plant material, which is necessary for obtaining significant amounts of phytoalexins for chemical identification. None of the chemicals tested, however, elicited phytoalexins in *C. sativa* that could be detected by the TLC cladosporium bioassay. This is shown in Figure IV-7 for $AgNO_3$ where only *A. brassicae* elicited phytoalexins. $AgNO_3$ caused necrosis on *C. sativa* leaves causing stress to the leaves. The fact that no phytoalexin-elicitation could be induced by $AgNO_3$ was surprising since *A. brassicae* elicits large amounts of phytoalexins. Injuring the leaves did not elicit phytoalexins either. Injury causes phytoalexin production in some plants (Bailey, 1982).

When *C. sativa* leaves were spotted with the *Cladosporium* sp. used in the TLC bioassay, it elicited phytoalexins, although not quite as much as the Alberta isolate of *A. brassicae*. These results indicated that *C. sativa* responded to biotic elicitors, but did not appear to respond to abiotic elicitors.

An unusual feature of phytoalexin production by *C. sativa* was that there was no associated cell death. As shown in Figure III-1H,I,J (Chapter III), no symptoms appeared on *C. sativa* inoculated with *A. brassicae* until the leaves started to senesce after 4-6 days. While phytoalexin production has been shown to occur in live plant cells (Adikaram *et al.*, 1988; Mansfield *et al.*, 1974; Paxton *et al.*, 1974; Smith & Banks, 1986), it is almost always associated with necrosis and the hypersensitive response, as discussed in Chapter I. One other exception is that capsicannol phytoalexins produced by *Capsicum annuum* fruits in response to glucan elicitors were shown to be associated with live cells with little or no necrosis occurring (Adikaram *et al.*, 1988).

2. Phytoalexin-elicitation in other crucifers

The procedure used most of the time for detecting phytoalexins was extraction of plant material, concentration of extract to dryness, redissolving in CH_3OH and performing a TLC cladosporium bioassay. This method worked well most of the time. However, sometimes extracts contained a lot of non-polar compounds that interfered with the TLC cladosporium bioassay. They did this by causing the silica gel on the TLC plate to quickly become saturated with material before very much of the sample could be spotted. This could mask any small amount of phytoalexin in the sample. Redissolving the extract in $CHCl_3$ gave a sample that was a little cleaner. For an even cleaner sample a fast, easy, and inexpensive partial purification procedure was developed. The vacuum liquid chromatography technique (Coll & Bowden, 1986; Pelletier *et al.*, 1986) was modified into a vacuum filtration technique as described in the Materials and Methods of this chapter.

Phytoalexin-elicitation by *A. brassicae* on a number of crucifers was examined. Some of these are shown in Figure IV-8 along with *C. sativa*. The TLC cladosporium bioassay showed two antimicrobial spots from *C. bursa-pastoris* indicating the presence of two phytoalexins (Rf 0.43, 0.67; Fig. IV-8D) with one (Rf 0.43) being in common with *C. sativa*. The TLC cladosporium bioassay showed one weak antimicrobial spot from *B. napus* cv. Altex indicating the presence of one phytoalexin (Rf 0.67; Fig. IV-8F). Three other canola cultivars tested (*B. napus* cv. Westar, *B. campestris* spp. *oleifera* cvs. Candle, Tobin) also produced a weak antimicrobial spot at Rf 0.67. The accession of *B. campestris* ssp. *rapifera* that showed a higher level of resistance to *A. brassicae* than canola (Table III-2, Chapter III) was compared with canola for phytoalexin production. The TLC cladosporium bioassay showed that it produced the same phytoalexin (Rf 0.67; Fig. IV-8H) as *B. napus*

cv. Altex, but in larger amounts. Thus, it appeared that the differences in susceptibility of these plants to *A. brassicae* may be due, at least in part, to qualitative and/or quantitative differences in phytoalexin production. Phytoalexin-elicitation by *A. brassicae* was also observed in some other crucifers such as *Armoracia rusticana*, *Erysimum cheiranthoides*, *Sinapis arvensis* and *Neslia paniculata*. The only plant from Table III-2 (Chapter III) that showed a high level of resistance to *A. brassicae*, but showed no apparent phytoalexin production, was *Eruca sativa*. Some other factor of the hypersensitive response shown by *E. sativa*, other than phytoalexin production, must be responsible for its resistance.

Shortly after the commencement of this project, the first report of phytoalexin production in crucifers came from Japan identifying three phytoalexins from cabbage (Takasugi *et al.*, 1986). The Rf-values of phytoalexins observed in this study (Fig. IV-8) were compared with the phytoalexins from Chinese cabbage; brassinin (Rf 0.60), cyclobrassinin (Rf 0.67) and methoxybrassinin (Rf 0.71) ($CHCl_3$:CH_3OH 98:2). The phytoalexin observed for canola and *B. campestris* ssp. *rapifera* had the same Rf-value (0.67) as cyclobrassinin, indicating that they might be the same. The phytoalexins from *C. sativa* and *C. bursa-pastoris* at Rf 0.33 and 0.44, however, were different from brassinin, cyclobrassinin, and methoxybrassinin. It was also observed that only the phytoalexins from *C. sativa* and *C. bursa-pastoris* at Rf 0.33 and 0.44 fluoresced under UV light.

3. Sensitivity of resistant crucifers to destruxin B

Alternaria brassicae produces the toxin, destruxin B (Ayer & Peña-Rodriguez, 1987; Bains & Tewari, 1987), which is host-specific and the primary agent of its pathogenicity (Bains & Tewari, 1987). The action of this toxin has been studied on several crucifers of varying susceptibility to *A. brassicae* as well as on several non-crucifers (Bains & Tewari, 1987). It had not, however, been tested on crucifers with a very high degree of resistance to *A. brassicae*, like *C. sativa* and *C. bursa-pastoris*. Thus, the effect of destruxin B on these crucifers was investigated and compared to that of *B. napus* cv. Westar. Both *A. brassicae* and destruxin B caused necrosis and chlorosis on leaves of *B. napus* cv. Westar (Fig. IV-9A,B), whereas, only destruxin B caused necrosis and chlorosis on leaves of *C. bursa-pastoris* (Fig. IV-9C,D) and *C. sativa* (Fig. IV-9E,F,G). There was a small amount of necrosis in some of the *C. bursa-pastoris* water controls (Fig. IV-9C) which was likely due to scratching of the leaf. The leaves had been scratched prior to inoculation to ensure that destruxin B would diffuse into the leaves rapidly. The rest of the water controls did not develop any symptoms. These results showed that *C. sativa* and *C. bursa-pastoris* were resistant to *A. brassicae* but were sensitive to destruxin B. If *A. brassicae* had the opportunity to grow on these plants and produce destruxin B, these plants would be susceptible to this pathogen. These results reinforce the significance of phytoalexins in the

resistance of *C. sativa* and *C. bursa-pastoris* to *A. brassicae*.

These results on *C. sativa* and *C. bursa-pastoris* also raise questions about the definition of host-specific toxins like destruxin B. By definition, plants inoculated with host-specific toxins should show the same degree of symptoms as they would if inoculated with pathogens that produce the toxins. This is the case with destruxin B and *A. brassicae* on several crucifers and non-crucifers (Bains & Tewari, 1987), but not on *C. sativa* and *C. bursa-pastoris*.

D. Figures and Legends

Figure IV-1. TLC cladosporium bioassay from *Camelina sativa* leaves spotted with different concentrations of *Alternaria brassicae* conidia.
Shown are extracts from leaves 4 days after spotting with water (A), 1 x 10^2 (B), 1 x 10^3 (C), 1 x 10^4 (D), 1 x 10^5 (E), or 1 x 10^6 (F) *A. brassicae* conidia (approx. no./20 μL droplet). The bioassay showed two antifungal spots indicating that *C. sativa* produced two phytoalexins (Rf 0.33, 0.43; arrowheads). The control (A) showed no antimicrobial spots. Note that the amount of phytoalexins increased as the conidial concentration increased. The minor phytoalexin (Rf 0.43) can be seen only at the highest conidial concentration and was not resolved at lower conidial concentrations perhaps due to elicitation in smaller quantities. The final position of the solvent ($CHCl_3$:CH_3OH 98:2) front is indicated by an asterisk.

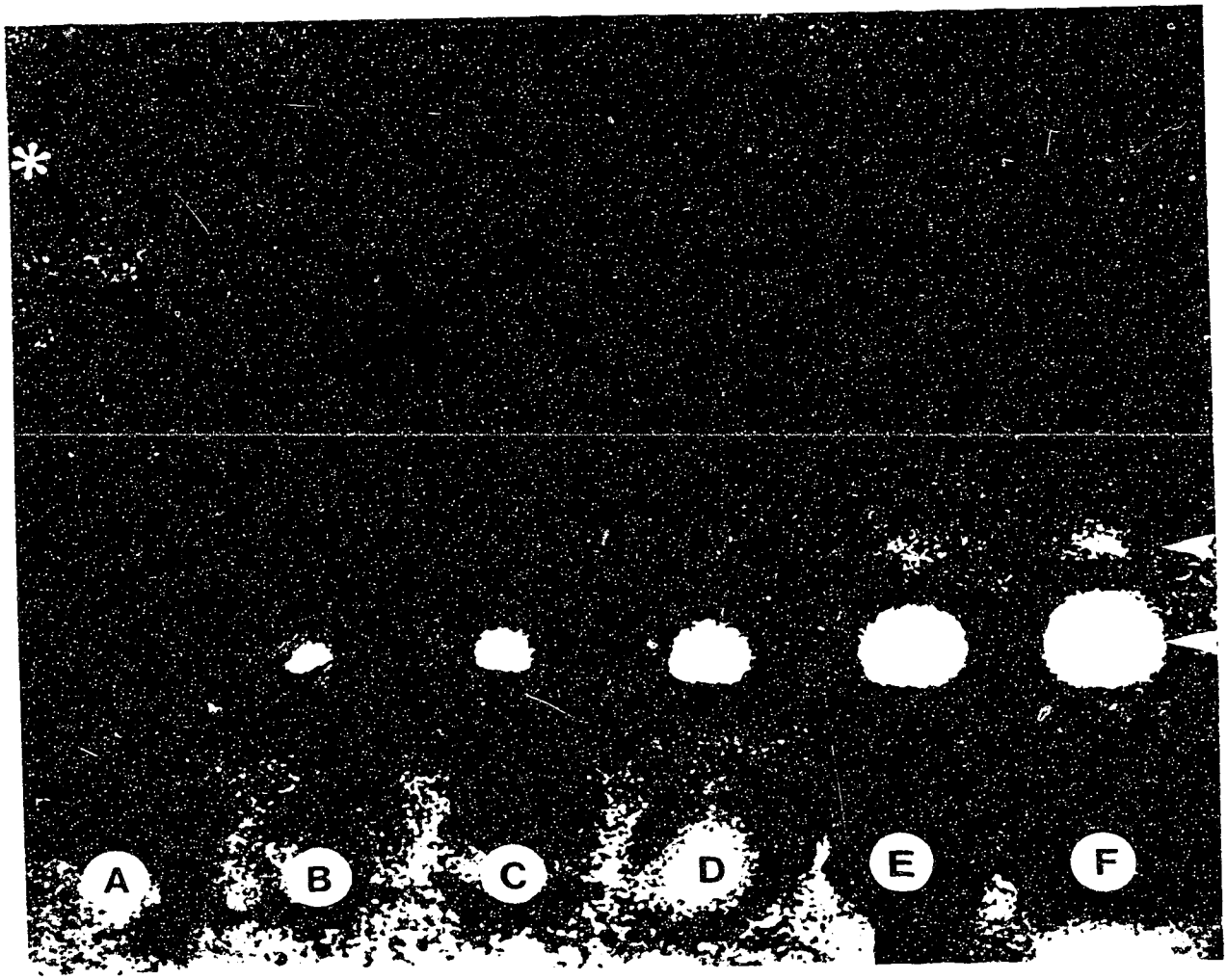

*
A
B
C
D
E
F

Figure IV-2. TLC cladosporium bioassay from *Camelina sativa* leaves spotted with *Alternaria brassicae* conidia; and from *A. brassicae* conidia and mycelium. Shown are extracts from leaves 3 days after spotting with an *A. brassicae* conidial suspension (A); and extracts from germinated *A. brassicae* conidia (B) and mycelium (C). This bioassay showed that the antimicrobial compounds were not derived from *A. brassicae*, and that they were indeed phytoalexins produced by *C. sativa* (Rf 0.33; A). The final position of the solvent ($CHCl_3$:CH_3OH 98:2) front is indicated by an asterisk.

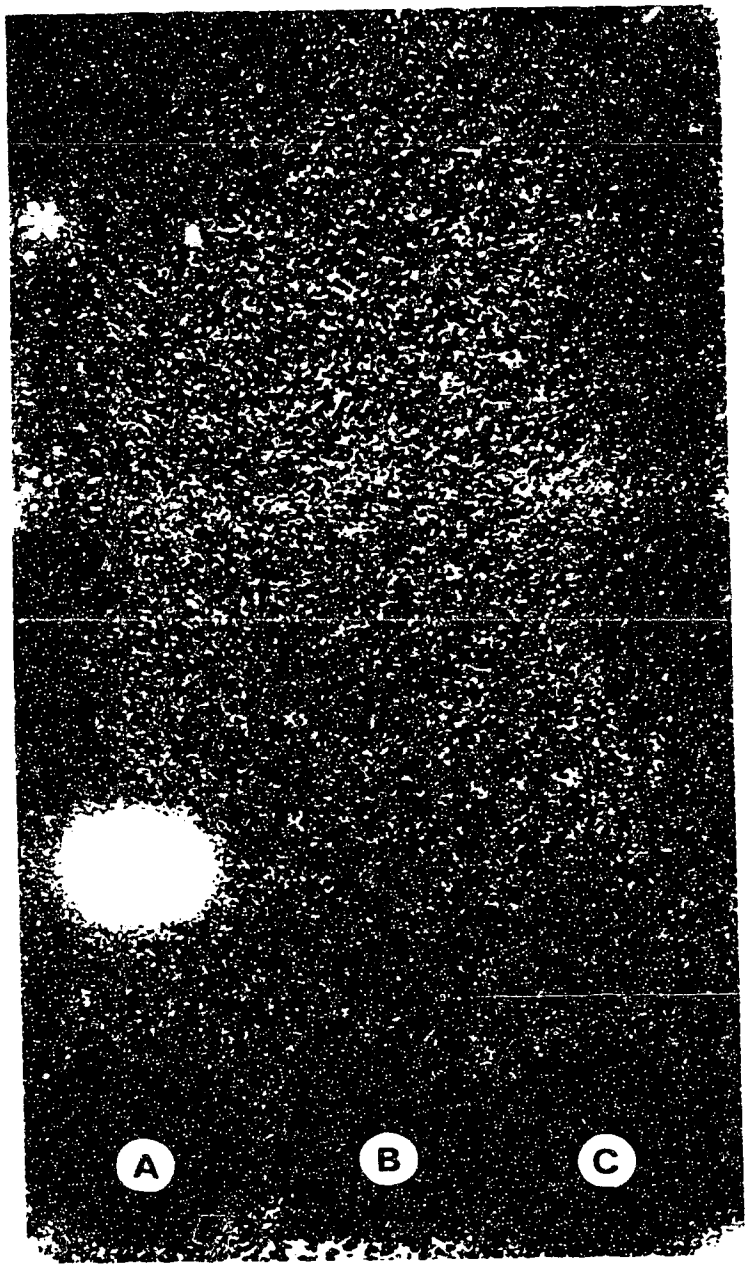
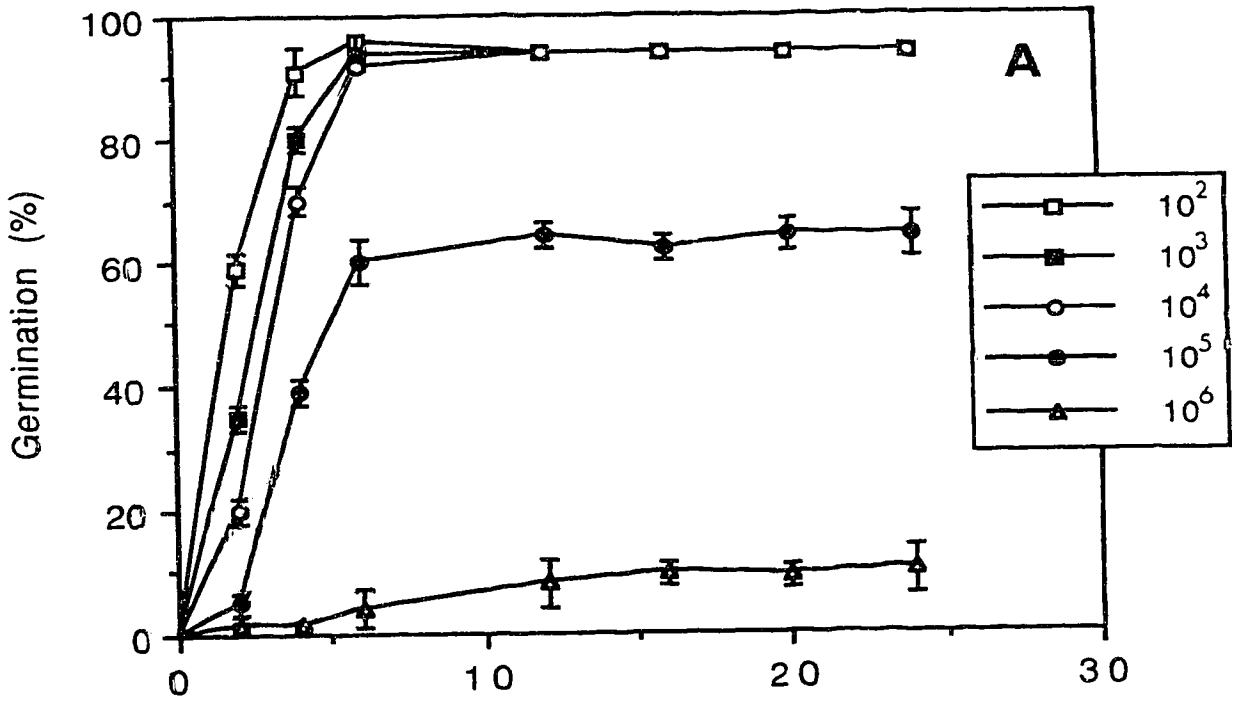

A
B
C

Figure IV-3. Germination of *Alternaria brassicae* conidia on glass slides (A) and on *Camelina sativa* leaves (B).
Droplets contained 1 x 10^2, 1 x 10^3, 1 x 10^4, 1 x 10^5, or 1 x 10^6 conidia. Note that germination of conidia was inhibited as the conidial concentration increased, and that germination was inhibited to a greater extent on leaves as compared to glass slides. Vertical bars represent standard error of the mean of three experiments.


A
Germination (%)
100
80
60
40
20
0
0
10
20
30
10^2
10^3
10^4
10^5
10^6

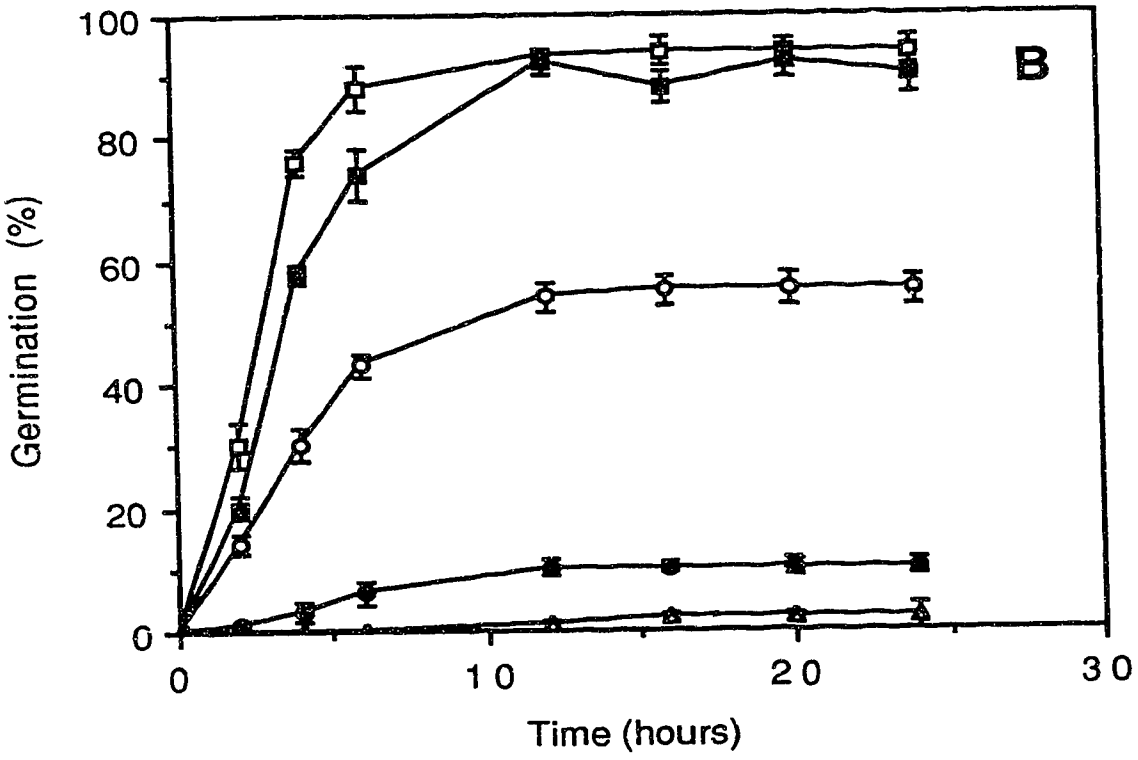

B
Germination (%)
100
80
60
40
20
0
0
10
20
30
Time (hours)

Figure IV-4. Growth of germ [illegible] *ernaria brassicae* conidia on glass slides (A) and on *Camelina sativa* leaves (B).
Droplets contained 1 x 10^2, 1 x 10^3, 1 x 10^4, 1 x 10^5, or 1 [illegible] e that length of germ tubes was reduced as the conidial concentration increased. [illegible] that germ tube length was reduced to a greater extent on leaves as compared to glass slides. Vertical bars represent standard error of the mean of three experiments.

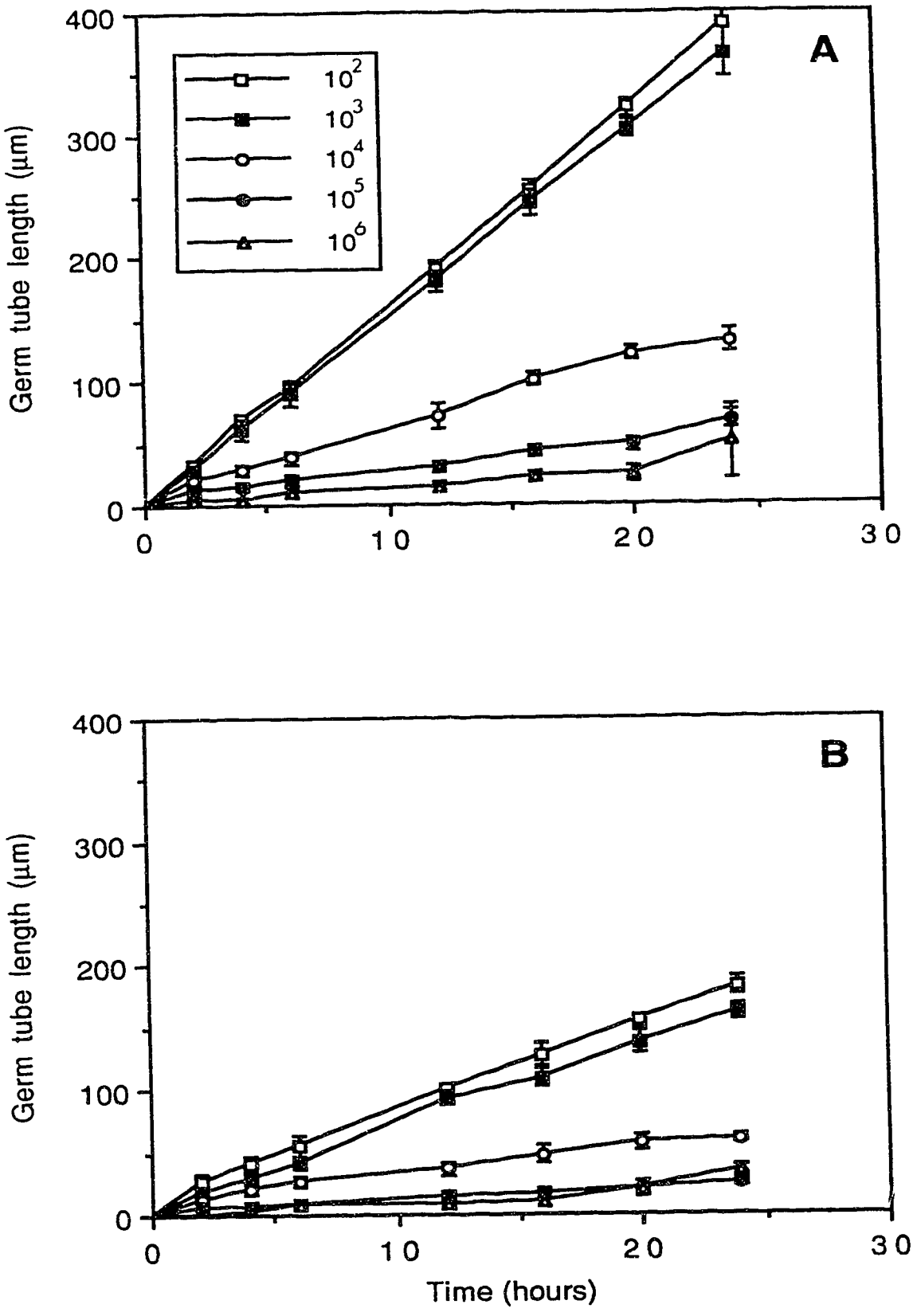

A
B
Germ tube length (µm)
Time (hours)
10^2
10^3
10^4
10^5
10^6
0
100
200
300
400
0
10
20
30

Figure IV-5. Assay for antifungal activity against *Alternaria brassicae* by phytoalexins from *Camelina sativa* leaves.
Partially purified phytoalexins (Rf 0.33) extracted from TLC plates were spotted on a paper disk. The disk, along with a control disk spotted with CH_3OH, were placed on a culture of *A. brassicae* for 2 weeks. *Alternaria brassicae* grew on the control disk (A) but not on the disk with the phytoalexins (B). The latter disk had some conidia on it that did not germinate.

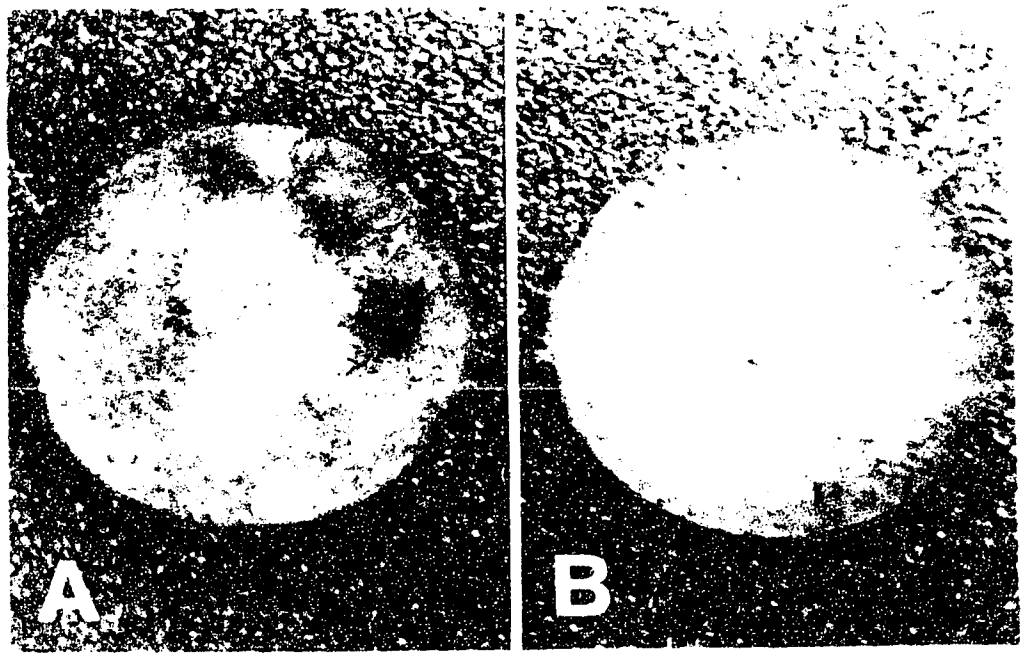
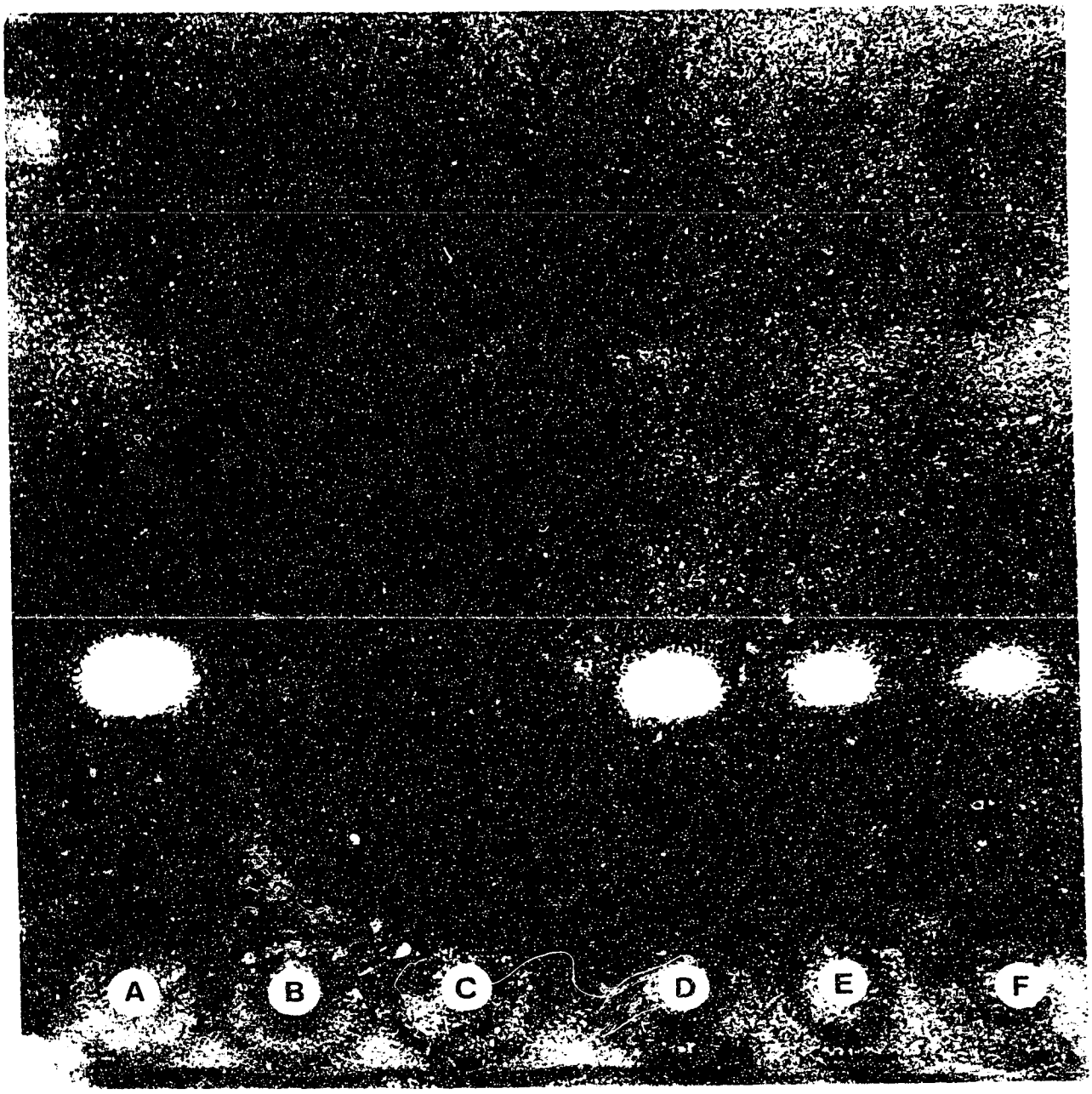

A
B

Figure IV-6. TLC cladosporium bioassay from *Camelina sativa* leaves spotted with *Alternaria brassicae* isolates.
Shown are extracts from leaves 4 days after spotting with conidial suspensions (A,B,C) or mycelial suspensions (D,E,F) of *A. brassicae* isolates from Alberta (A,D), India (B,E), and France (C,F). Note that phytoalexin-elicitation (Rf 0.33) could be seen only for the Alberta isolate when conidial suspensions were used, but that phytoalexin-elicitation could be seen for all three isolates when mycelial suspensions were used. The final position of the solvent ($CHCl_3$:CH_3OH 98:2) front is indicated by an asterisk.


A
B
C
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F

Figure IV-7. TLC cladosporium bioassay from *Camelina sativa* leaves spotted with *Alternaria brassicae* or $AgNO_3$.
Shown are extracts from leaves 3 days after spotting with water (A), *A. brassicae* conidial suspension (B), or $AgNO_3$ (10^{-3} M) (C). Note that only *A. brassicae* elicited phytoalexins in *C. sativa* (Rf 0.40; B). The final position of the solvent ($CHCl_3$:CH_3OH 95:5) front is indicated by an asterisk.

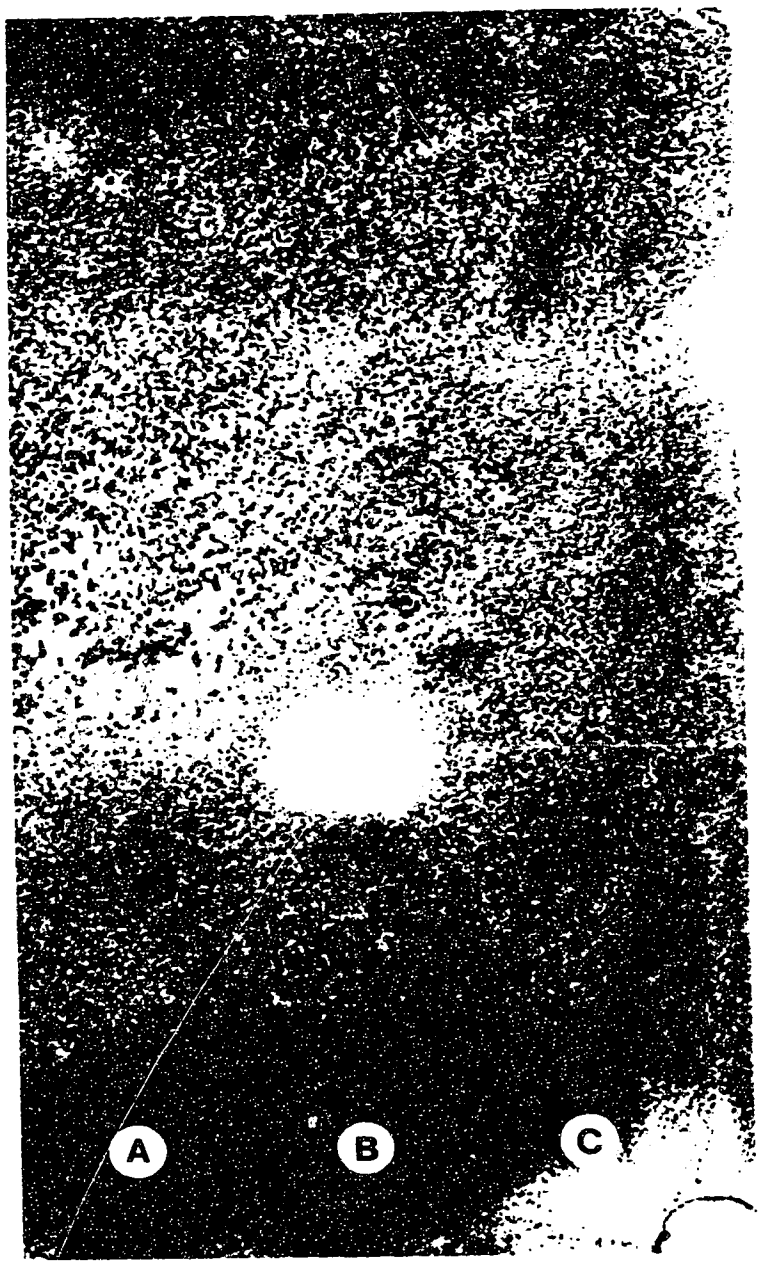
A
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Figure IV-8. TLC cladosporium bioassay from *Camelina sativa*, *Capsella bursa-pastoris*, *Brassica napus* cv. Altex, and *B. campestris* ssp. *rapifera* leaves spotted with *Alternaria brassicae*.
Shown are extracts from leaves of *C. sativa* (A,B), *C. bursa-pastoris* (C,D), *B. napus* cv. Altex (E,F), and *B. campestris* ssp. *rapifera* (G,H) 4 days after spotting with water (A,C,E,G) or an *A. brassicae* conidial suspension (B,D,F,H). The bioassay indicated that *C. sativa* produced two phytoalexins (Rf 0.33, 0.43; B) (arrowheads) as did *C. bursa-pastoris* (Rf 0.43, 0.67; D). It indicated that *B. napus* cv. Altex and *B. campestris* ssp. *rapifera* produced one phytoalexin (Rf 0.67; F and H, respectively). All the controls (A,C,E,G) showed no antimicrobial spots. The final position of the solvent ($CHCl_3$:CH_3OH 98:2) front is indicated by an asterisk.

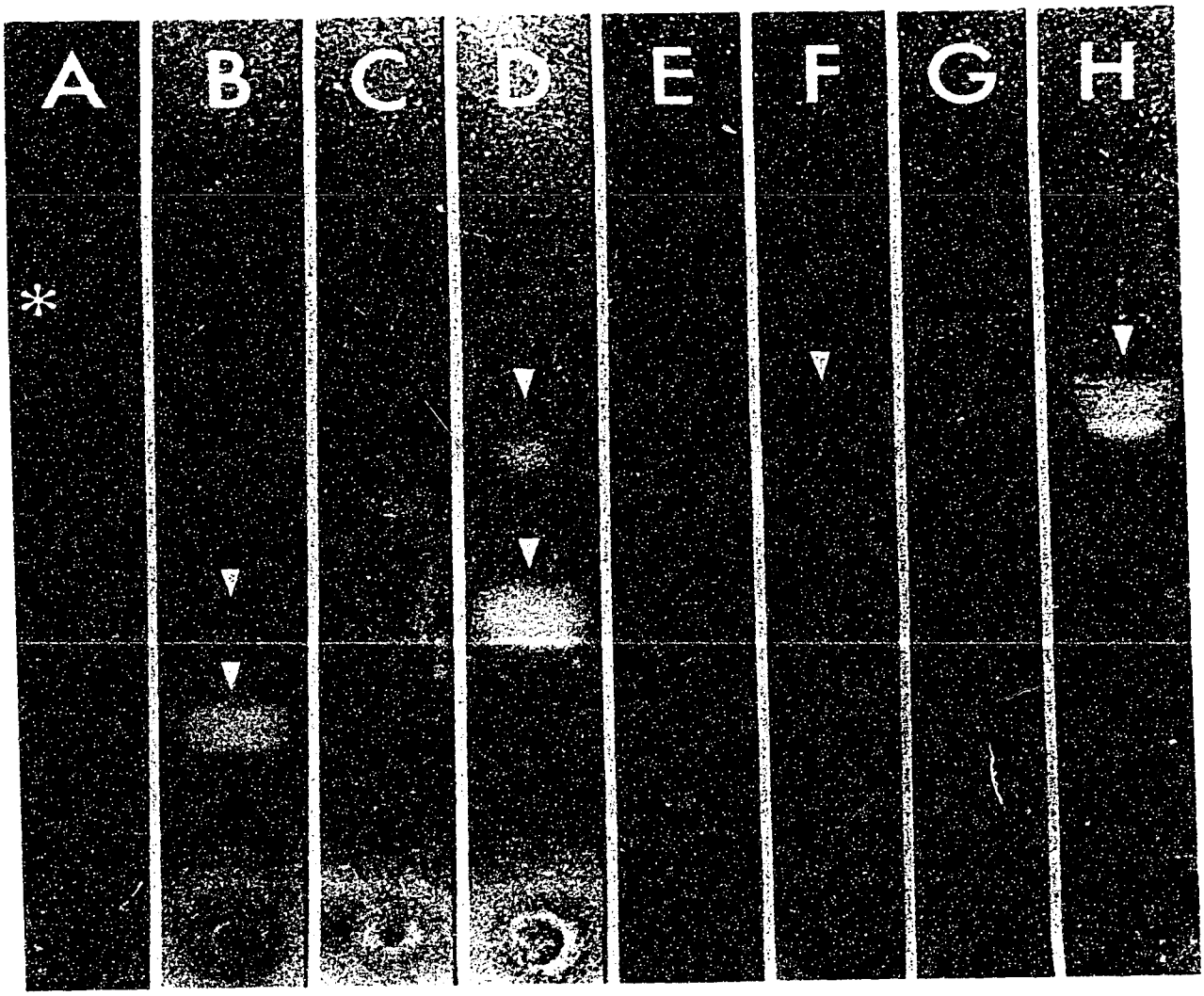
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Figure IV-9. Sensitivity of *Brassica napus* cv. Westar, *Capsella bursa-pastoris*, and *Camelina sativa* leaves to *Alternaria brassicae* and destruxin B.
A,B) Left and right leaf-halves of *B. napus* cv. Westar 3 days after spotting with an *A. brassicae* conidial suspension and water, respectively (A); and with an *A. brassicae* conidial suspension and destruxin B solution (50 μg/mL), respectively (B) (approx. x0.75). Note that both *A. brassicae* and destruxin B caused necrosis and chlorosis.
C,D) Left and right leaf-halves of *C. bursa-pastoris* 3 days after spotting with an *A. brassicae* conidial suspension and water, respectively (C); and with an *A. brassicae* conidial suspension and destruxin B solution (50 μg/mL), respectively (D) (approx. x1). The necrosis in the water control was likely due to scratching of the leaf. Note that *A. brassicae* did not cause any more symptoms than in the control, whereas, destruxin B caused necrosis and chlorosis.
(continued on next page)

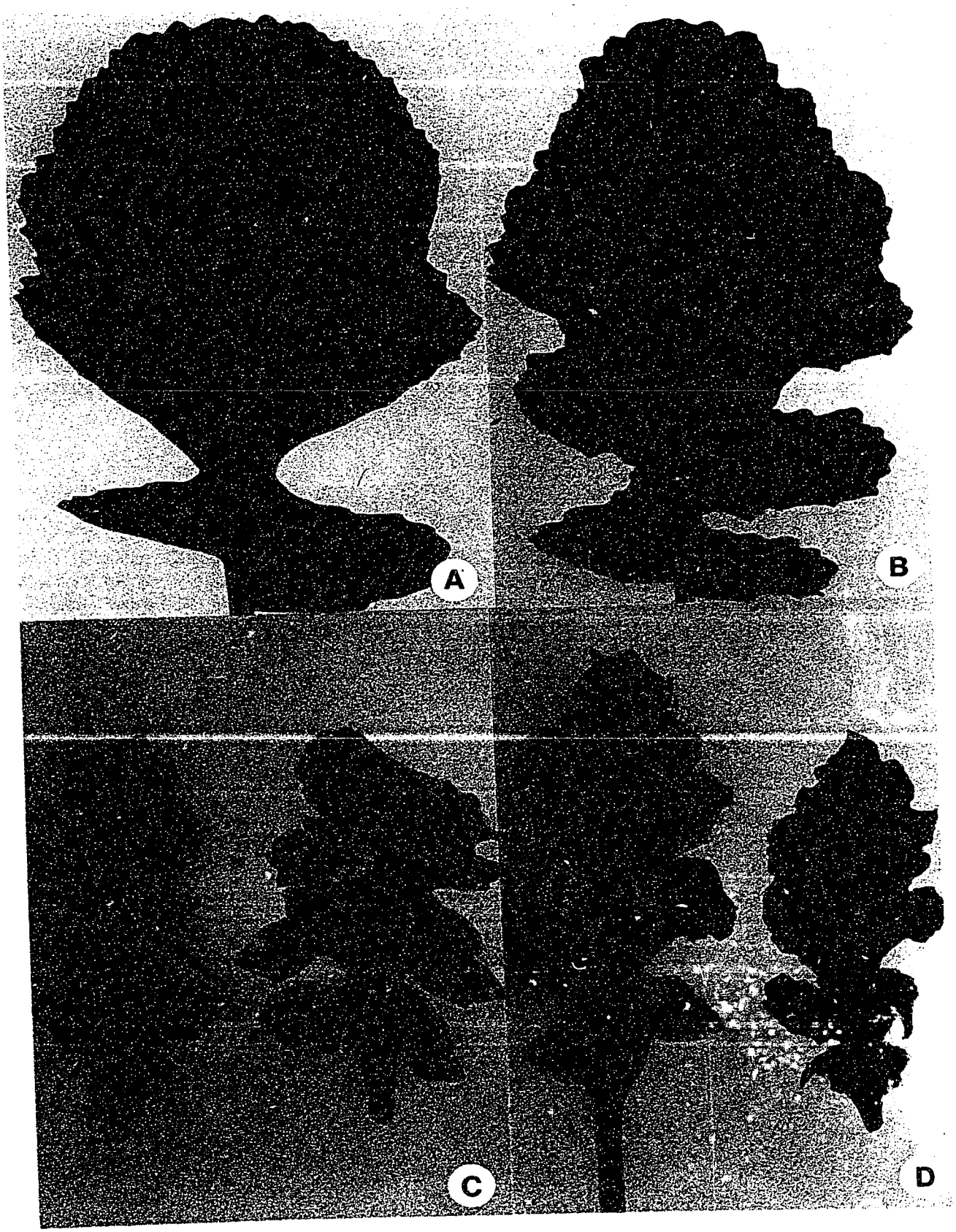
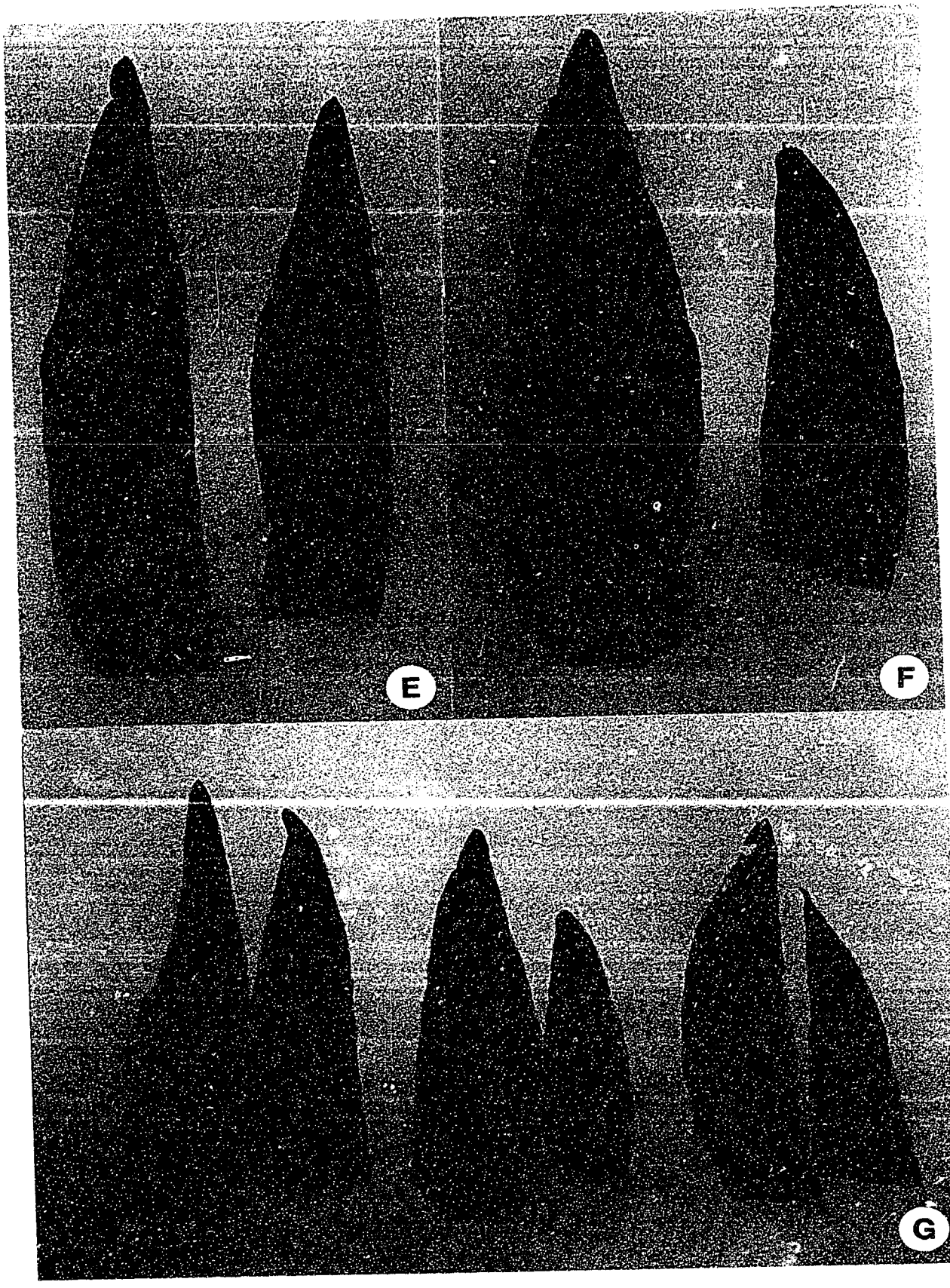

A
B
C
D

Figure IV-9. Sensitivity of *Brassica napus* cv. Westar, *Capsella bursa-pastoris*, and *Camelina sativa* leaves to *Alternaria brassicae* and destruxin B (continued).
E,F) Left and right leaf-halves of *C. sativa* 3 days after spotting with an *A. brassicae* conidial suspension and water, respectively (E); and with an *A. brassicae* conidial suspension and destruxin B solution (50 μg/mL), respectively (F) (approx. x1.5).
G) Left and right leaf-halves of *C. sativa* 5 days after spotting with an *A. brassicae* conidial suspension and water, respectively (first set of leaves); with an *A. brassicae* conidial suspension and destruxin B solution (50 μg/mL), respectively (second set of leaves); and with an *A. brassicae* conidial suspension and destruxin B solution (100 μg/mL), respectively (third set of leaves) (approx. x1). Note that *A. brassicae* did not cause any symptoms, whereas, destruxin B caused necrosis and chlorosis. The third set of leaves showed some chlorosis on the left side due to destruxin B causing the leaves to senesce.


E
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G

E. References

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Chapter V

Resistance to *Rhizoctonia solani* and Phytoalexin-elicitation in *Camelina sativa*

A. Introduction

Rhizoctonia solani Kühn is a soil-borne fungus with a very widespread distribution. It can survive in soil as microsclerotia or mycelium and saprophytically on plant residues for many years. It is a destructive pathogen on a great number of plants, causing seed decay, damping-off, stem cankers, root rots, fruit decay and foliage diseases (Nyvall, 1981). Isolates of *R. solani* have been separated into anastomosis groupings (AGs) based on the concept that *R. solani* is not a single species but is instead a collection of genetically isolated noninterbreeding populations (Anderson, 1982; Ogoshi, 1987). Ten AGs have been reported so far and generally isolates of each AG parasitize a specific group of plants (Anderson, 1982; Ogoshi, 1987). *Rhizoctonia solani* AG 2 parasitizes cruciferous plants and results in seed rot and damping-off, root lesions, and crown rot (Anderson, 1982). This AG has been further divided into AG 2-1 and AG 2-2. Both infect canola but AG 2-1 has been more frequently isolated from canola in western Canada and has been reported to be more virulent on canola than other AGs (Gugel *et al.*, 1987; Hwang *et al.*, 1986; Tewari *et al.*, 1987; Yitbarek, 1987; Yitbarek *et al.*, 1987). Virulence of *R. solani* is also influenced by soil temperature. AG 2 isolates are most virulent on canola seedlings when night temperatures are around 7°C (Kaminski & Verma, 1985; Yitbarek, 1987; Yitbarek *et al.*, 1987). High soil moisture also contributes to the virulence of AG 2 but is not as influential as soil temperature (Teo *et al.*, 1988; Yitbarek, 1987).

Rhizoctonia solani is the principal causal agent of pre- and post-emergence damping-off and root rot of canola in western Canada, though *Fusarium* spp. and *Pythium* spp. are associated as well (Berkenkamp & Vaartnou, 1972; Calman, 1990; Gugel *et al.*, 1987; Hwang *et al.*, 1986; Petri, 1985; Teo *et al.*, 1988; Tewari *et al.*, 1987; Yang, 1989; Yitbarek *et al.*, 1987). Pre-emergence damping-off refers to death of seed and/or seedlings before emergence, and post-emergence damping-off refers to seedling death after emergence. Symptoms on seedlings include constrictions and discoloration (Fig. V-3). In Alberta, damping-off and root rot are most severe in the Peace River region causing estimated yield losses of 20-30% (Ellis, 1983; Harrison, 1988; Sippell *et al.*, 1985; Yitbarek, 1987). All presently registered canola cultivars are susceptible to *R. solani* (Harrison, 1988; Gugel *et al.*, 1987). Many cultivars and lines of rapeseed have been screened for resistance to *R. solani* (Acharya *et al.*, 1984; Harrison, 1987; Yang, 1989). Resistance to *R. solani* has been investigated in other crucifer crops as well such as radish and cabbage (Humaydan *et al.*, 1976; Williams & Walker, 1966; Williams *et al.*, 1968; Yang, 1989). A great variation in susceptibility has been observed but high degrees of resistance against *R. solani* have not

been found. Since *C. sativa* had been shown to be very resistant to *A. brassicae*, the question arose as to whether it would also have resistance to *R. solani*, a soil-borne pathogen. Thus, the susceptibility of *C. sativa* was compared to *B. napus* cv. Westar.

Several mechanisms of resistance to *R. solani* have been demonstrated. These include the amount of epicuticular wax on rice sheaths (Massaquoi & Rush, 1987), cuticle thickness (Stockwell & Hanchey, 1983), lignification of lesion borders (Stockwell & Hanchey, 1987), calcium content (Stockwell & Hanchey, 1982), and production of phytoalexins in bean hypocotyls (Smith *et al.*, 1975). Little work has been done on mechanims of resistance to *R. solani* in crucifers. Yang (1990) found that *Sinapis alba* was more resistant than *B. napus* and that cuticle thickness may play a role. Yang (1990) also studied calcium content of several crucifers but found no association with resistance. Thus, the possibility that phytoalexins might be involved in susceptibility/resistance of *B. napus* cv. Westar and *C. sativa* roots to *R. solani* was investigated. Preliminary reports on portions of this chapter have been published (Conn *et al.*, 1987, 1988; Tewari *et al.*, 1988).

B. Materials and Methods

1. Plant material

Seeds of *B. napus* cv. Westar and *C. sativa* were surface sterilized by dipping them into CH_3CH_2OH for 5 seconds and then into 1% NaOCl for 1 minute. The seeds were then subjected to three serial sterile distilled water washes and air-dried. Siliques of *C. sativa* from plants grown in the greenhouse were also surface sterilized as described above and the seeds aseptically removed.

2. Fungal material

A virulent isolate (AG 2-1) and a weakly virulent isolate (AG 4) of *R. solani* isolated from fields in Alberta were obtained from Dr. J.P. Tewari. Cultures were grown in dark at room temperature for 5 days on potato-dextrose agar (PDA). The isolates were also grown in potato-dextrose broth for 5 days. A dried and ground inoculum of the virulent isolate of *R. solani* grown on rye seeds was also used (Calman, 1990).

3. Growth of seedlings on cultures of *Rhizoctonia solani*

Surface sterilized seeds of *B. napus* cv. Westar and *C. sativa* were placed on cultures of *R. solani* or on PDA for a control. The plates were incubated in darkness at room

temperature for 3-4 days. Seeds from surface sterilized siliques of *C. sativa* were also placed on PDA.

4. Emergence of seedlings in *Rhizoctonia solani* infested soil

Peat pots (2 x 2 inches) were filled 3/4 full with soil (loam:sand:peat-moss; 2:2:3). Surface sterilized seeds of *B. napus* cv. Westar and *C. sativa* were placed in the pots (25 seeds/pot). The seeds were covered with 3/4 inch of uninoculated soil or with soil inoculated with dried *R. solani* inoculum (19 or 38 cfu/g soil). Twelve replicates were used for each treatment. The pots were placed in a growth cabinet in a completely randomized design for 10 days with 16 hours light and day/night temperatures of 18/7°C, respectively. The percent emergence was then determined along with percent plants emerged without symptoms. This experiment was carried out two times. The percent emergence in each experiment was recalculated to adjust the controls to 100% before analyses. Comparisons between *B. napus* cv. Westar and *C. sativa* treatments were made with the Mann-Whitney (Wilcoxon) test.

5. Extraction of roots

Seedlings were grown on cultures of *R. solani* as described above. The stems and cotyledons were removed and the roots extracted with CH_3OH for 3-5 minutes as described in Chapter IV. In one experiment, the stems and cotyledons were also extracted. TLC cladosporium bioassays were carried out as described in Chapter IV.

6. Testing if antifungal compounds originated from *Rhizoctonia solani*

Mycelium of the *R. solani* isolates was extracted in the same way as the roots and TLC cladosporium bioassays done. This experiment was carried out two times.

7. Bioassay for antifungal activity of phytoalexins against *Rhizoctonia solani*

Spots showing antifungal activity in the TLC cladosporium bioassay from *C. sativa* roots were scraped off TLC plates, extracted with CH_3OH and concentrated. The extracts were redissolved in water and 200 μL placed in sterile metal cylinders that had been placed on PDA plates inoculated with *R. solani*. The metal cylinders were pushed slightly into the agar. The extract usually diffused out of the metal cylinder into the agar within 1-2 days. The plates were incubated as described above. This experiment was carried out two times.

C. Results and Discussion

On cultures of the weakly virulent isolate of *R. solani*, no symptoms occurred on *C. sativa* seedlings while a small amount of discoloration occurred on *B. napus* cv. Westar roots (Fig. V-1). On cultures of the virulent isolate of *R. solani*, *C. sativa* seedlings had some discoloration of the roots while *B. napus* cv. Westar seeds were totally destroyed (Fig. V-1). *Camelina sativa* was also more resistant to the virulent isolate of *R. solani* in infested soil (Table V-1; Fig. V-2). The percent emergence of *C. sativa* seedlings was 22-33% greater than for *B. napus* cv. Westar seedlings (Table V-1). Practically all *B. napus* cv. Westar seedlings that emerged had symptoms, whereas only 81-92% of *C. sativa* seedlings had symptoms (Table V-1). Figure V-3 shows symptoms on seedlings caused by the virulent isolate of *R. solani*. Thus, *C. sativa* was not completely resistant to *R. solani*, like it was to *A. brassicae*, but it was significantly more resistant than *B. napus* cv. Westar.

The TLC cladosporium bioassay from *C. sativa* roots revealed three antimicrobial spots (Rf 0.33, 0.43, 0.75) (Fig. V-4). These spots occurred in the control as well as from seedlings challenged with *R. solani*. Thus, they did not appear to fit the definition of a phytoalexin. The possibility that surface sterilization of the seeds with NaOCl caused the production of these compounds was investigated. NaOCl has been shown to cause alterations to barley seeds such as causing separation of the pericarp from the underlying tissues and damaging the aleurone tissue (Goudey *et al.*, 1987; Tittle *et al.*, 1988). This kind of alteration would cause stress on seeds and stress caused by abiotic factors has been shown to cause the production of phytoalexins (Bailey, 1982). *Camelina sativa* seeds from surface sterilized siliques were germinated on PDA and extracted. The same three spots appeared on the TLC cladosporium bioassay plates ruling out the possibility that NaOCl elicited the antimicrobial compounds. It is possible that some other unknown stress on the seedlings caused the production of these compounds. The spot at Rf 0.33 fluoresced under UV light and appeared to be the same as the antimicrobial spot at Rf 0.33 from *C. sativa* leaves. It was determined later (Chapter VI) that indeed this spot was a mixture of the same two phytoalexins as from *C. sativa* leaves. It can be seen in Figure V-4 that this spot at Rf 0.33 was larger for seedlings challenged with the weakly virulent isolate of *R. solani* than for the control indicating that it could possibly be called a phytoalexin. The size of the other two antimicrobial spots (Rf 0.43, 0.75) remained the same for both the weakly virulent isolate of *R. solani* and the control indicating that they may indeed be preformed compounds. The sizes of these three antimicrobial spots were less for the virulent than the avirulent isolate of *R. solani* (Fig. V-4). This was likely due to the virulent isolate causing some disease on *C. sativa* leaving a smaller amount of healthy root tissue to produce the antimicrobial compounds.

No consistant antimicrobial spots showed up on the TLC cladosporium bioassay plates from *B. napus* cv. Westar seedlings challenged with the weakly virulent isolate of *R. solani*. Occasionally, a faint spot was observed near the origin of the TLC plate. There was never enough of the seedlings of *B. napus* cv. Westar left after challenging with the virulent isolate of *R. solani* to extract. No seedlings from the infested soil experiment were extracted to determine what compounds were present.

The possibility that these antimicrobial compounds came from *R. solani* was investigated by extracting *R. solani* mycelium and performing a TLC cladosporium bioassay. The results showed that the antimicrobial compounds did not come from *R. solani*.

The stems and cotyledons of *C. sativa* seedlings grown on *R. solani* were also extracted and tested for the presence of these antimicrobial compounds. No antimicrobial compounds were seen in these extracts. This was expected since the stems and cotyledons were not in contact with *R. solani*.

To determine if the antimicrobial compounds had antifungal activity against *R. solani*, they were extracted from TLC plates and a bioassay done. The compounds inhibited the growth of both isolates of *R. solani* to about the same extent (Fig. V-5). This suggested that the virulent isolate of *R. solani* was able to cause some disease on *C. sativa* probably because it attacked the seedlings very quickly, and not because it was resistant to these compounds. Thus, it appeared that the greater resistance to *R. solani* in *C. sativa* seedlings as compared to *B. napus* cv. Westar, may be due to greater amounts of antimicrobial compounds present in *C. sativa*, either preformed and/or phytoalexins.

D. Tables, Figures, and Legends

Table V-1. Growth of *Brassica napus* cv. Westar and *Camelina sativa* in *Rhizoctonia solani* infested soil.

R. solani (cfu/g soil)		Emergence (%)† Expt. 1	Emergence (%)† Expt. 2	Symptomless (%)‡ Expt. 1	Symptomless (%)‡ Expt. 2
38	*B. napus* cv. Westar	40	36	0	1
	C. sativa	73*	59*	19*	10*
19	*B. napus* cv. Westar	$	61	$	2
	C. sativa	$	83*	$	8*

†Data were recalculated to adjust controls to 100% before analyses.
‡The percent seedlings without symptoms was determined on seedlings that had emerged.
$Treatments with 19 cfu/g soil were not conducted in the first experiment.
*Treatments significantly different at P = 0.05 based on the Mann-Whitney (Wilcoxon) test.

Figure V-1. Germination of *Brassica napus* cv. Westar and *Camelina sativa* seeds on cultures of *Rhizoctonia solani*.
Seeds were placed on PDA as a control (A) and on cultures of the weakly virulent (B) and virulent (C) isolates of *R. solani*. Photographs were taken after 4 days.
Brassica napus cv. Westar (upper photograph)
Camelina sativa (lower photograph)
Note that *B. napus* cv. Westar seeds were totaly destroyed by the virulent *R. solani* isolate while *C. sativa* seedlings showed only limited browning of the roots.

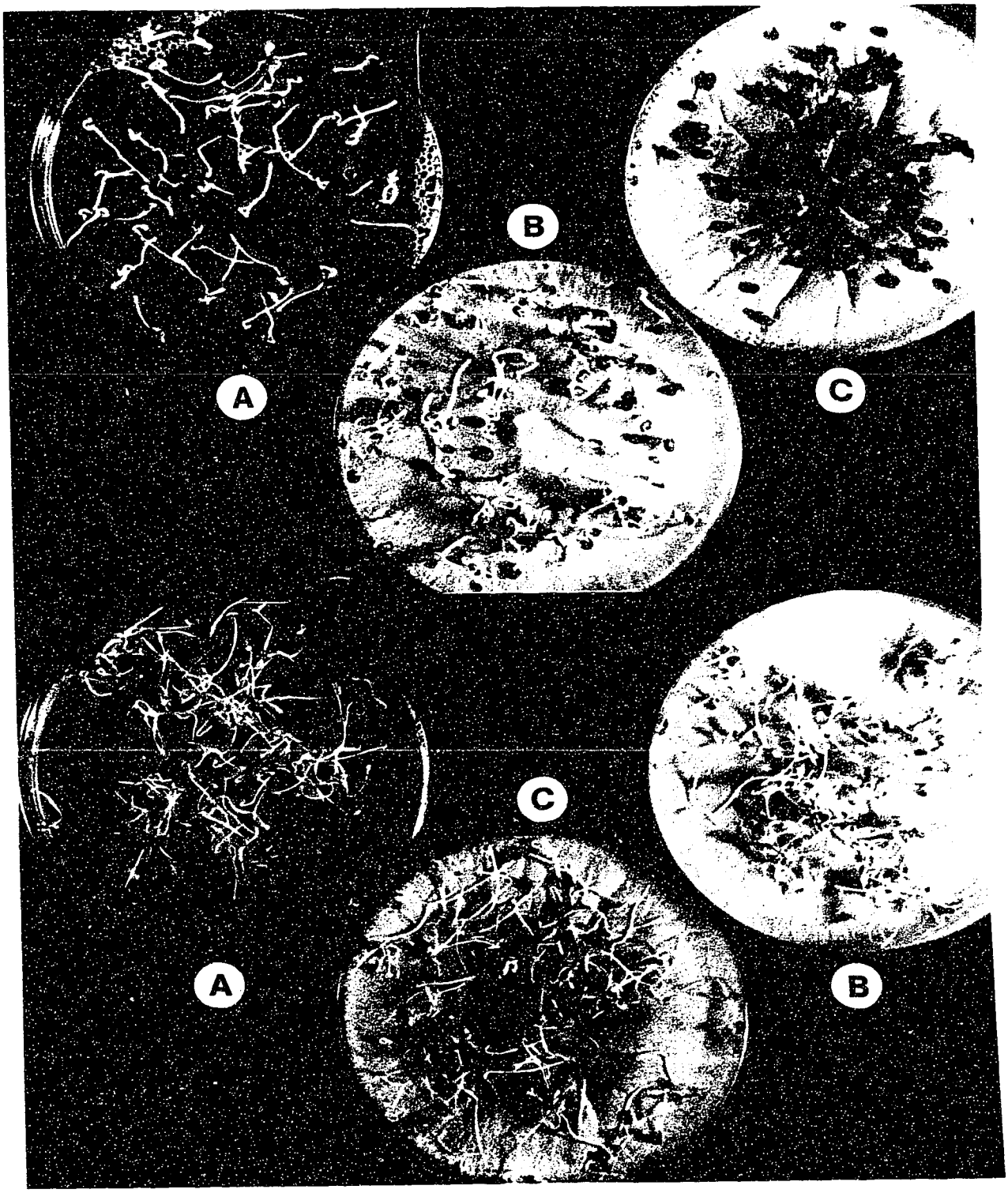

A
B
C
A
C
B

Figure V-2. Damping-off of *Brassica napus* cv. Westar and *Camelina sativa* seedlings in *Rhizoctonia solani* infested soil.
Shown are pots with *B. napus* cv. Westar (A,B) and *C. sativa* (C,D) seedlings in control soil (A,C) and *R. solani* (virulent isolate) infested soil (B,D) (approx. x1). Photographs were taken after 10 days. Note that fewer *B. napus* cv. Westar seedlings emerged than *C. sativa* seedlings (see Table V-1).

Figure V-3. Damping-off symptoms on *Brassica napus* cv. Westar and *Camelina sativa* seedlings caused by *Rhizoctonia solani*.
Shown are *B. napus* cv. Westar (A,B,C,D) and *C. sativa* (E,F,G,H) healthy seedlings (A,E) and seedlings showing constricted and discolored hypocotyls and roots (B,C,D,F,G,H) due to the virulent isolate of *R. solani* (approx. x2).

Figure V-4. TLC cladosporium bioassay from *Camelina sativa* roots challenged with *Rhizoctonia solani*.
Shown are extracts from roots 4 days after placing seeds on PDA (A) and on cultures of the weakly virulent (B) and virulent (C) isolates of *R. solani* as shown in Fig. V-1. The bioassay showed three antimicrobial spots (Rf 0.33, 0.43, 0.75) for all three treatments. The final position of the solvent ($CHCl_3$:CH_3OH 98:2) front is indicated by an asterisk.

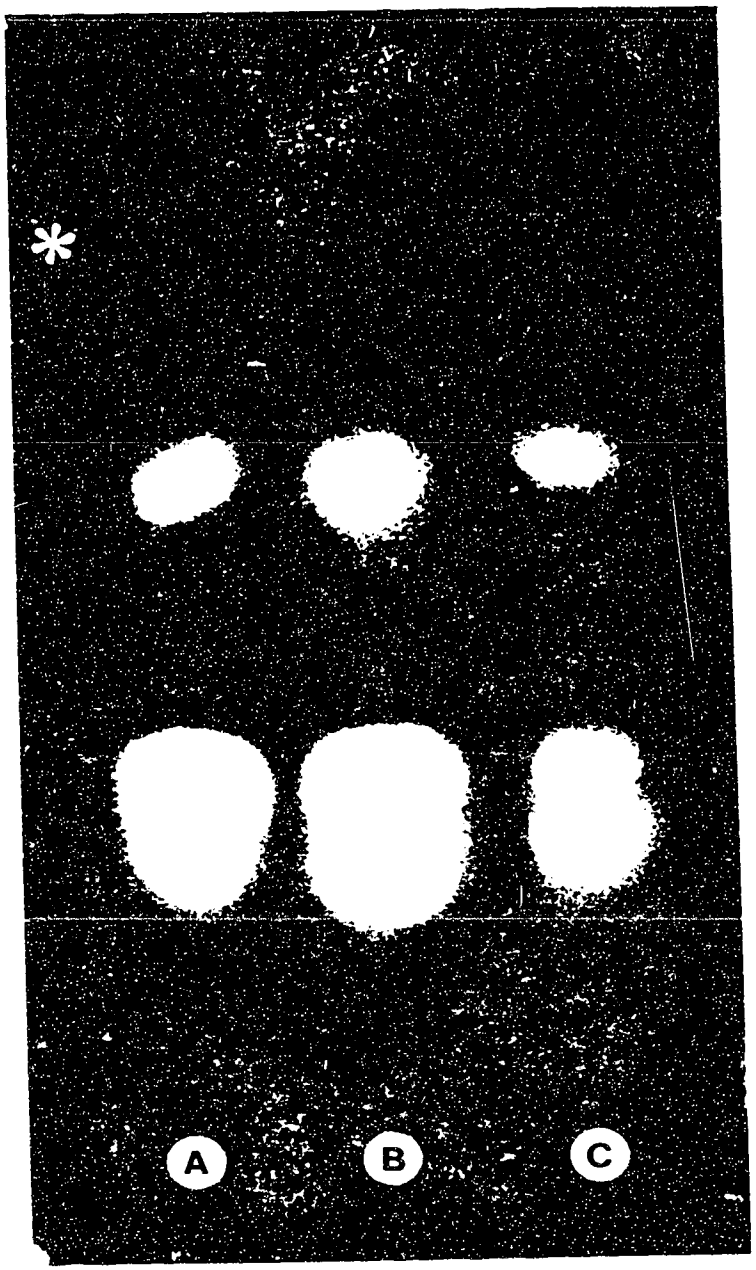
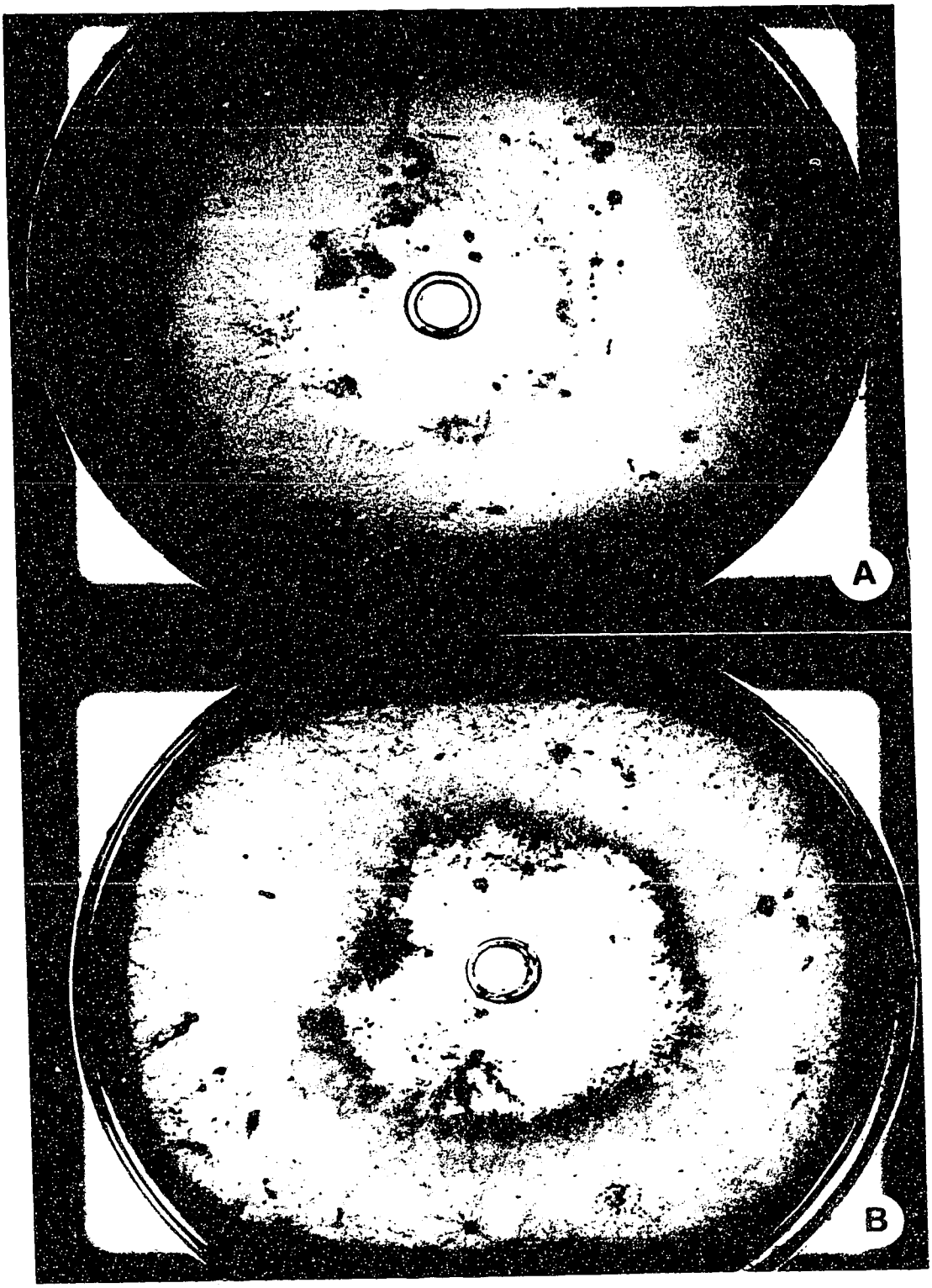

*
A
B
C

Figure V-5. Assay for antifungal activity against *Rhizoctonia solani* by antimicrobial compounds from *Camelina sativa* roots.
Partially purified antimicrobial compounds (Rf 0.33, 0.43, 0.75) extracted from TLC plates were placed in metal cylinders on cultures of the weakly virulent (A) and virulent (B) isolates of *R. solani*. Photographs were taken after 5 days. Note that growth of both isolates was inhibited to about the same extent.


A
B

E. References

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Chapter VI

Isolation and Separation of Phytoalexins from *Camelina sativa* and *Capsella bursa-pastoris*

A. Introduction

Shortly after phytoalexin production in rapeseed, *Camelina sativa*, and *Capsella bursa-pastoris* was observed in the present investigation, the first report of phytoalexin production in crucifers came from Japan identifying three phytoalexins from cabbage (Takasugi *et al.*, 1986). These phytoalexins were sulfur-containing indole alkaloids; brassinin, methoxybrassinin, and cyclobrassinin (Fig. I-1, Chapter I). The alkaloids constitute a very broad group of heterocyclic bases which are widely distributed in the plant kingdom. Based on Rf values, the phytoalexins from *C. sativa* and *C. bursa-pastoris* leaves (Rf 0.33, 0.43) were shown to be different from the phytoalexins from cabbage (Chapter IV). Since the phytoalexins from cabbage were the only phytoalexins identified from Cruciferae at that time, it was considered likely that the phytoalexins from *C. sativa* and *C. bursa-pastoris* were as yet unidentified. Also, since phytoalexins within a plant family are often chemically similar, it was considered likely that the phytoalexins from *C. sativa* and *C. bursa-pastoris* were similar in structure to those identified from cabbage. Taking this into consideration, a procedure for isolation and purification of the phytoalexins from *C. sativa* and *C. bursa-pastoris* was developed.

A portion of this chapter has been published:
Browne, L.M., Conn, K.L., Ayer, W.A. and J.P. Tewari. 1991. The camalexins: new phytoalexins produced in the leaves of *Camelina sativa* (Cruciferae). *Tetrahedron* 47:3909-3914.

B. Materials and Methods

1. Fungal material

Alternaria brassicae (Alberta isolate) conidia (1 x 10^6/mL) and mycelium were obtained as previously described (Materials and Methods, Chapter IV). *Rhizoctonia solani* (weakly virulent isolate) was grown on PDA as previously described (Materials and Methods, Chapter V).

2. Inoculation and extraction of plant material

Camelina sativa and *C. bursa-pastoris* plants were grown under greenhouse conditions. Excised leaves, stems, and siliques were placed in humid chambers and their surfaces were covered with *A. brassicae* conidial droplets, or were dipped into a mycelial suspension and then placed in humid chambers. The plant material was incubated for 2-5 days at room temperature under continuous cool white fluorescent light (5.5 $\mu E/m^2/s$) and extracted as previously described (Materials and Methods, Chapter IV).

Non-sterilized *C. sativa* seeds were placed on 4 day old cultures of *R. solani* and incubated in darkness at room temperature for 3-4 days. The stems and cotyledons were removed and the roots extracted with CH_3OH for 3-5 minutes as described in Chapter V.

3. Isolation and separation of the phytoalexins from *Camelina sativa* leaves

A flow diagram for this procedure is shown in Figure VI-1. Aqueous CH_3OH extract (12 L) from inoculated plant material was concentrated *in vacuo* to 250 mL. This was then applied to an Extube (300 mL capacity) and allowed to distribute over the specially modified diatomaceous earth (Analytichem). After 5 minutes the organic metabolites were eluted with $CHCl_3$ (700 mL). The $CHCl_3$ eluant was concentrated to 10 mL and extracted with 5% HCl (3 x 20 mL) for 10 minutes. The aqueous acidic extract was cooled and basified to pH 10 with NaOH pellets, then applied to an Extube (100 mL capacity). After 5 minutes the organic bases were eluted with $CHCl_3$ (400 mL). This extract was concentrated to 3 mL, combined with two other extracts obtained in the same manner (total 9 mL), then subjected to vacuum liquid chromatography (VLC) (Coll & Bowden, 1986; Pelletier *et al.*, 1986) over TLC grade silica gel 60 G (E. Merck; 6 cm ID x 4 cm). The phytoalexins were carefully eluted with $CHCl_3$ (100 mL fractions) while monitoring the chromatography with UV light in the dark. A small amount of the minor phytoalexin (Rf 0.43) was obtained but not in sufficient quantities for identification. Fractions 7-9, containing the major phytoalexin (Rf 0.33) contaminated with a small amount of other material, were combined and concentrated to 3 mL, then again subjected to VLC over silica gel 60 HR (extra pure,

E. Merck #7744, 60 Å; 4 cm ID x 3 cm) eluting with $CHCl_3$ (40 mL fractions). Fractions containing the major phytoalexin showed a single spot on TLC when sprayed with phosphomolybdic acid and heated (Bobbitt, 1963). However, during chromatography it was observed that color development with phosphomolybdic acid (without heating), UV maxima, and HPLC analysis (C-18 reverse phase (Alltech), 250 x 4.6 mm; eluant: $CH_3OH:H_2O$ 1:1; chart speed 1 cm/min; Varian 5000 HPLC) were different between early and late fractions, indicating that the major component was a mixture of two components. Careful VLC fractionation with collection of small fractions led to the separation and isolation of these two fluorescent phytoalexins. These phytoalexins were provided to Dr. L.M. Browne and Dr. W.A. Ayer, Dept. of Chemistry, U of A, who carried out spectral analyses and structural elucidation.

4. Isolation and separation of the phytoalexins from *Capsella bursa-pastoris* leaves

The procedure for isolation and separation of the phytoalexins (Rf 0.43, 0.67) from *C. bursa-pastoris* leaves was the same as that from *C. sativa* leaves up to the step when VLC was used. At this stage, the extract was applied to a silica gel column (40-140 mesh, J.T. Baker Chemical Co., 4 x 35 cm) using $CHCl_3:CH_3OH$ (95:5) at a flow rate of 1.5 mL/minute. Progress of the elution was monitored with UV light in the dark. A small amount of a minor phytoalexin (Rf 0.67) was isolated but this was not sufficient to allow further purification and identification. The major phytoalexin (Rf 0.43) was collected, concentrated to dryness, and redissolved in water:CH_3OH (75:25). This was applied to a 10% C-18 silica gel column (35-70 μm, Toronto Research Chemicals Inc., 3 x 30 cm) using water:CH_3OH (75:25) at a flow rate of 1.5 mL/minute. The phytoalexin was again monitored with UV light in the dark. TLC cladosporium bioassays of the fractions revealed that the material had been separated into a non-fluorescent antimicrobial component and a fluorescent non-antimicrobial component. A small amount of this non-fluorescent phytoalexin was isolated but not enough to allow identification because of difficulties in obtaining sufficient *C. bursa-pastoris* plants.

5. Isolation and separation of the antimicrobial compounds from *Camelina sativa* roots

The procedure for isolation and separation of the antimicrobial compound at Rf 0.33 from *C. sativa* roots was the same as for the phytoalexins from *C. sativa* leaves. This antimicrobial compound was found to be a mixture of the same two phytoalexins as those found in the leaves of *C. sativa* at Rf 0.33. Extraction with 5% HCL removed the phytoalexins at Rf 0.33 but the other two antimicrobial compounds (Rf 0.43, 0.75) remained behind in the $CHCl_3$. The extracts containing these antimicrobial compounds (Rf 0.43, 0.75) were provided to Mrs. GuiJuen Ji, Dr. L.M. Browne and Dr. W.A. Ayer, Dept. of

Chemistry, U of A, for further purification and identification.

6. Conidial germination experiments

To determine the antifungal effects of camalexin on *A. brassicae*, conidia (final concentration approximately 1 x 10^5/mL) were suspended in sterile distilled water (sdw), sdw:CH_3OH (98:2, v/v), or in sdw:CH_3OH:camalexin. Droplets (20 μL) of conidial suspensions were placed on glass slides in a humid chamber. At intervals, drops of lactophenol cotton blue were added to conidial droplets and the conidial germination determined by counting at least 100 conidia in each of the three droplets for each treatment. To determine the effects of camalexin on germ tube growth, conidia were first allowed to germinate for 4 hours in sdw. CH_3OH and camalexin were then added (final concentration of CH_3OH = 2%) and the germinating conidial suspensions incubated for a further 20 hours. Effective concentration (50%) or EC_{50} was the concentration of camalexin that reduced germ tube growth by 50% compared to germ tubes in the control treatment and the minimum inhibitory concentration (MIC) was the concentration of camalexin that completely inhibited germ tube growth. These experiments were carried out three times.

C. Results and Discussion

1. Isolation and separation of the phytoalexins from *Camelina sativa* leaves

Many thousand *C. sativa* leaves were extracted in order to obtain enough purified phytoalexin for structural elucidation. One of the first problems encountered was to find an efficient method of transferring the phytoalexins from large volumes of aqueous extract (e.g. 100 L) into organic solvent. A small volume of aqueous extract (e.g. 1 L) could easily be taken to dryness and redissolved in CH_3OH or $CHCl_3$, but large volumes were not easily handled because of the high concentration of solutes. When concentrated to dryness, it was difficult to wash the phytoalexins out of the residue. The extract could be worked up in small portions but this was a slow process. To overcome this, Extubes (containing a specially modified diatomaceous earth) were used to transfer the phytoalexins into $CHCl_3$ which could then be easily concentrated. In addition, this step partially purified the extract, removing a large amount of unwanted material. The phytoalexins gave a positive test when sprayed with Dragendorff's reagent (Bobbitt, 1963) indicating the presence of basic nitrogen. Thus, partitioning between $CHCl_3$ and 5% HCl was used for the next step to help isolate these bases. The step was fast and simple removing a lot of additional material. The acidic extract was then made basic and passed through another Extube to transfer the phytoalexins

quickly back into $CHCl_3$. Initially, the next step in the purification procedure involved chromatography using a silica gel column followed by a 10% C-18 reverse phase silica gel column. This yielded a small amount of pure phytoalexin but it required several days to run one small sample. It then took several more days to clean the columns and to get them ready for another sample. This column separation was replaced with vacuum liquid chromatography (VLC) (Coll & Bowden, 1986; Pelletier *et al.*, 1986) which was found to work exceptionally well. This technique is essentially a preparative layer chromatographic separation run on a column, the elution of which is activated by vacuum. This could handle a very large sample (e.g. several grams of material) in 1-2 hours and gave separation of TLC quality. This technique could accomplish in one day what it took 3-4 weeks to achieve with silica gel columns. The VLC technique usually involves starting off with a non-polar solvent and then increasing the polarity in successive fractions (Coll & Bowden, 1986). In this investigation, however, it was found that the use of $CHCl_3$ for all fractions resulted in separation of the phytoalexins making the procedure even simpler. When fractions containing the major phytoalexin (Rf 0.33) from VLC were spotted on TLC plates and sprayed with phosphomolybdic acid and heated, a single spot was observed. However, it was noticed that color development with phosphomolybdic acid was different between early and late fractions when no heat was applied. Early fractions gave a purple spot and late fractions gave a brown spot. Also, the UV maximum for the early fractions was at 318 nm while it was at 295 and 325 nm for the late fractions. With HPLC, the early and late fractions had slightly different retention times of 9.9 and 10.1 minutes, respectively. These observations established that the phytoalexin at Rf 0.33 was actually a mixture of two phytoalexins. The phytoalexin from the early fractions comprised approximately 85% of the mixture. A few mg of this major component were crystalized from CH_3OH giving clear rectangular crystals. The major component (30 mg) and the minor component (5 mg) were provided to Dr. L.M. Browne and Dr. W.A. Ayer for spectral analyses and structural elucidation. The spectral analyses of these phytoalexins is reported in the Appendix. These phytoalexins were found to be new thiazoyl substituted indole alkaloids differing from each other by only a methoxyl group. The major component ($C_{11}H_8N_2S$; m.w. 200) was named camalexin (Fig. VI-2A) and the minor component ($C_{12}H_{10}N_2OS$; m.w. 230) was named methoxycamalexin (Fig. VI-2B). They were named after their source plant (*Camelina*) and the fact that they were phytoalexins. Camalexin and methoxycamalexin were somewhat similar to the other phytoalexins identified from Cruciferae (Table I-1, Fig. I-1, Chapter I) in that they were sulfur-containing indole alkaloids. These phytoalexins appeared to be the first reported naturally occurring antifungal compounds containing a 2-substituted thiazole. Thiabendazole (Fig. VI-2C), a 4-substituted thiazole (Woodcock, 1977), is a synthetic systemic fungicide first developed as an anthelmintic agent and antiinfective agent in animals

(Brown *et al.*, 1961). It has been used extensively to control a variety of plant diseases since the early 1970's. Another compound (Fig. VI-2D) which is even more similar to camalexin had been synthesized in a search for compounds with a biological profile similar to that of thiabendazole (Sarodnick & Kempter, 1979). It is interesting that nature had already produced antimicrobial compounds (camalexin and methoxycamalexin) similar to the synthetic antimicrobial compounds produced by man.

It is also interesting to note that these phytoalexins are easy to synthesize, especially camalexin (Dr. W.A. Ayer, personal communication). A possible biosynthesis of camalexin by *C. sativa* is through the condensation of indole-3-carboxaldehyde with cysteine followed by cyclization and decarboxylation. Indole-3-carboxaldehyde has been isolated from *B. oleracea* and has been proposed to be a precursor of brassilexin (Devys & Barbier, 1991). Both camalexin and methoxycamalexin have been synthesized (Dr. YuTing Ma and Dr. W.A. Ayer, unpublished results). This is unusual for phytoalexins because large scale chemical synthesis of most phytoalexins thus far characterized is difficult (Brent, 1983; Callow, 1983). Thus far the phytoalexins orchinol from orchids (Stoessl *et al.*, 1974) and oryzalexins A, B, and C from rice leaves (Mori & Waku, 1985) have been chemically synthesized.

2. Isolation and separation of the phytoalexins from *Capsella bursa-pastoris* leaves

A small amount of the major phytoalexin (Rf 0.43) from *C. bursa-pastoris* was purified using the silica gel and C-18 silica gel columns. TLC cladosporium bioassay of the fractions revealed that the material had been separated into a non-fluorescent antimicrobial component and a fluorescent non-antimicrobial component. Thus, unlike camalexin and methoxycamalexin, this phytoalexin did not fluoresce. There was not enough of this phytoalexin isolated to carry out identification because of difficulties in obtaining sufficient *C. bursa-pastoris* plants. This was due to difficulties encountered in growing *C. bursa-pastoris* in the greenhouse.

3. Isolation and separation of the antimicrobial compounds from *Camelina sativa* roots

The antimicrobial compound at Rf 0.33 from *C. sativa* roots was isolated and purified using the same procedure as for the phytoalexins from *C. sativa* leaves. This antimicrobial compound was found to be a mixture of camalexin and methoxycamalexin. Extraction with 5% HCL removed these phytoalexins at Rf 0.33 but the other two antimicrobial compounds (Rf 0.43, 0.75) remained behind in the $CHCl_3$. The extracts containing these antimicrobial compounds (Rf 0.43, 0.75) were provided to Mrs. GuiJuen Ji, Dr. L.M. Browne and Dr. W.A. Ayer, Dept. of Chemistry, U of A, for further purification and identification. They identified the antimicrobial compound at Rf 0.75 as being methyl

1-methylindole-3-carboxylate (Mrs. GuiJuen Ji, Dr. L.M. Browne and Dr. W.A. Ayer, unpublished results) (Fig. VI-2E), a simple derivative of indole-3-carboxylic acid. They have separated the antimicrobial compound at Rf 0.43 into several compounds, some being decomposition products. These non-aromatic compounds have so far not been identified (Mrs. GuiJuen Ji, Dr. L.M. Browne and Dr. W.A. Ayer, unpublished results). It was initially thought that the antimicrobial compound at Rf 0.43 from *C. sativa* roots was probably the same as the phytoalexin at Rf 0.43 from *C. sativa* and *C. bursa-pastoris* leaves but this was not the case.

4. Effect of camalexin on *Alternaria brassicae* conidia

TLC cladosporium bioassay of camalexin and methoxycamalexin revealed that they had approximately the same level of antimicrobial activity. The resolution in TLC cladosporium bioassay was approximately 0.25 μg camalexin per spot (Fig. VI-3). This was similar to the resolution of approximately of 0.125 μg per spot for brassilexin in the TLC cladosporium bioassay (Rouxel *et al.*, 1989). The *in vitro* effects of camalexin on germination of *A. brassicae* conidia were examined on glass slides (Fig. VI-4). Over 90% of conidia germinated in sdw after 6 hours incubation. The presence of 2% CH_3OH in droplets caused no significant reduction in percentage germination. Camalexin reduced the rate of germination of conidia. With 2 μg/mL camalexin, germination reached 90% after 16 hours and with 4 to 20 μg/mL camalexin, germination reached 90% by 24 hours. Germination was almost completely inhibited by 40 and was completely inhibited by 80 μg/mL camalexin. At 80 μg/mL, conidia failed to germinate even after further incubation of up to 84 hours. When camalexin was added to germinated *A. brassicae* conidia, it inhibited germ tube growth. The EC_{50} was approximately 6 μg/mL (3×10^{-5} M) and the MIC was approximately 80 μg/mL (4×10^{-4} M) (Fig. VI-5). This level of antimicrobial activity fell within the range of most phytoalexins (10^{-5} to 10^{-4} M) (Smith, 1982). In addition, the apical cells of many of the germ tubes autolysed in the presence of camalexin as evidenced by the lack of uptake of lactophenol cotton blue stain (Fig. VI-6). This indicated that camalexin may be fungicidal. The majority of phytoalexins are considered to be fungistatic (Smith, 1982) but some have been shown to be fungicidal. The broad bean phytoalexins wyerone, wyerone acid, and wyerone epoxide were shown to be lethal to the germlings of *Botrytis cinerea* and *B. fabae*, causing death of apical cells (Rossall *et al.*, 1980). Another example includes the phytoalexins orchinol and dehydroorchinol that were shown to kill the apical cells of *Monilinia fructicola* by causing rupture of hyphal tips (Ward *et al.*, 1975). Membranes appear to be a common target for phytoalexins. A number of phytoalexins have been shown to rapidly alter the structural integrity of various cell membranes and cause dysfunction of a variety of membrane-associated processes in fungal, bacterial, plant, and

animal cells (Yoshikawa *et al.*, 1987). Thiabendazole, thought to be an antimitotic agent (Sijpesteijn, 1977), also effects germ tubes of fungi. It has been shown to reduce germ tube elongation of *Penicillium atrovenetum* G. Smith (Gottleib & Kumar, 1970) and to cause malformation of the germ tubes of *Venturia inaequalis* (Cke.) Wint. apud Thuem. (Koch, 1971).

D. Figures and Legends

Figure VI-1. Flow diagram for the isolation and separation of phytoalexins from *Camelina sativa* leaves.

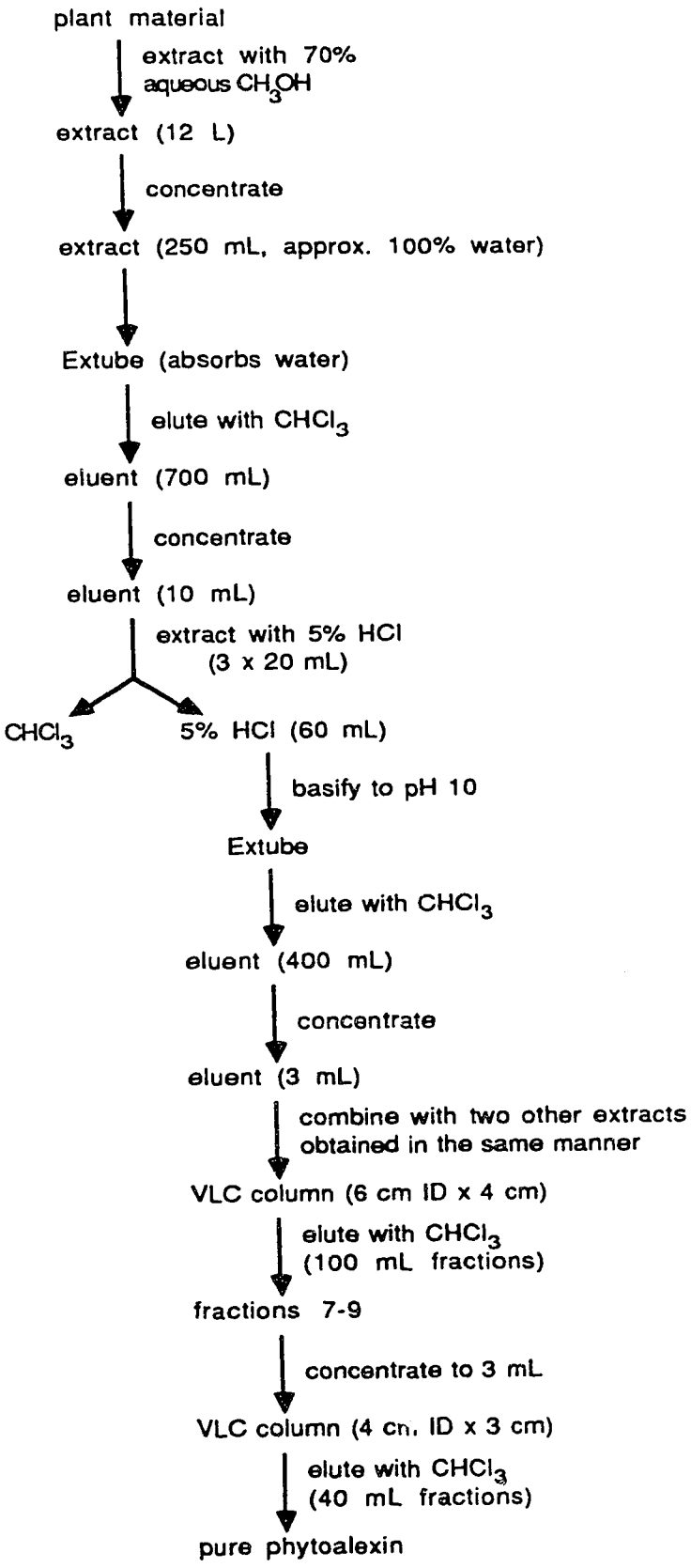
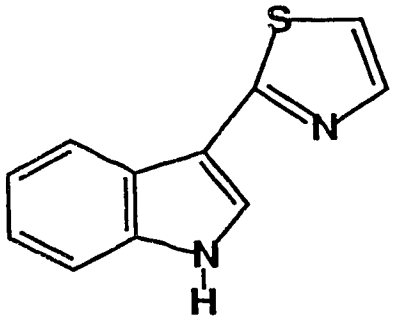
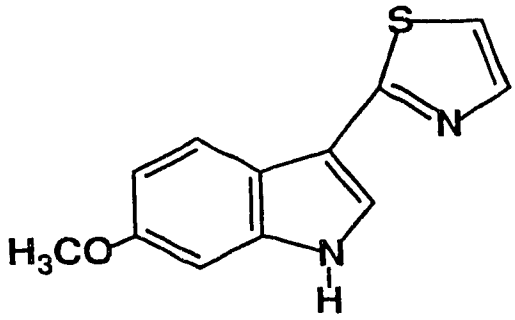

plant material
extract with 70% aqueous CH_3OH
extract (12 L)
concentrate
extract (250 mL, approx. 100% water)
Extube (absorbs water)
elute with $CHCl_3$
eluent (700 mL)
concentrate
eluent (10 mL)
extract with 5% HCl (3 x 20 mL)
$CHCl_3$
5% HCl (60 mL)
basify to pH 10
Extube
elute with $CHCl_3$
eluent (400 mL)
concentrate
eluent (3 mL)
combine with two other extracts obtained in the same manner
VLC column (6 cm ID x 4 cm)
elute with $CHCl_3$ (100 mL fractions)
fractions 7-9
concentrate to 3 mL
VLC column (4 cm ID x 3 cm)
elute with $CHCl_3$ (40 mL fractions)
pure phytoalexin

Figure VI-2. Structural formulae of camalexin, methoxycamalexin, thiabendazole, an analogue of thiabendazole, and methyl 1-methylindole-3-carboxylate.

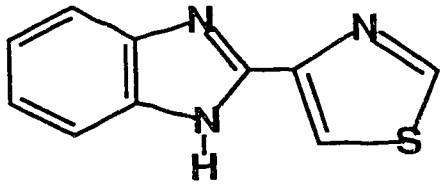
A. camalexin
B. methoxycamalexin
C. thiabendazole; a well known systemic fungicide.
D. This compound has been synthesized in a search for compounds with a biological profile similar to that of thiabendazole.
E. methyl 1-methylindole-3-carboxylate



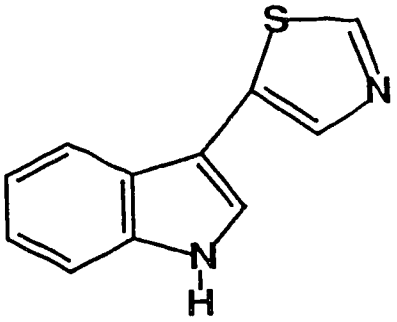
A



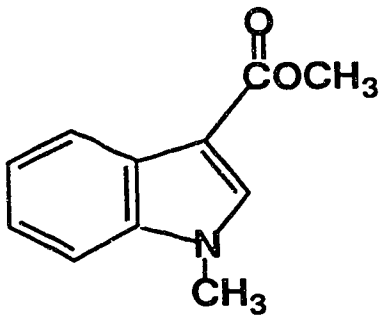
B



C



D



E

Figure VI-3. TLC cladosporium bioassay of known amounts of camalexin. Droplets (10 μL) containing 1 (A), 0.5 (B), 0.25 (C), and 0.12 μg (D) of camalexin were deposited on a TLC plate. The lower limit of the bioassay was approximately 0.25 μg camalexin per spot. The final position of the solvent ($CHCl_3$:CH_3OH 98:2) front is indicated by an asterisk.

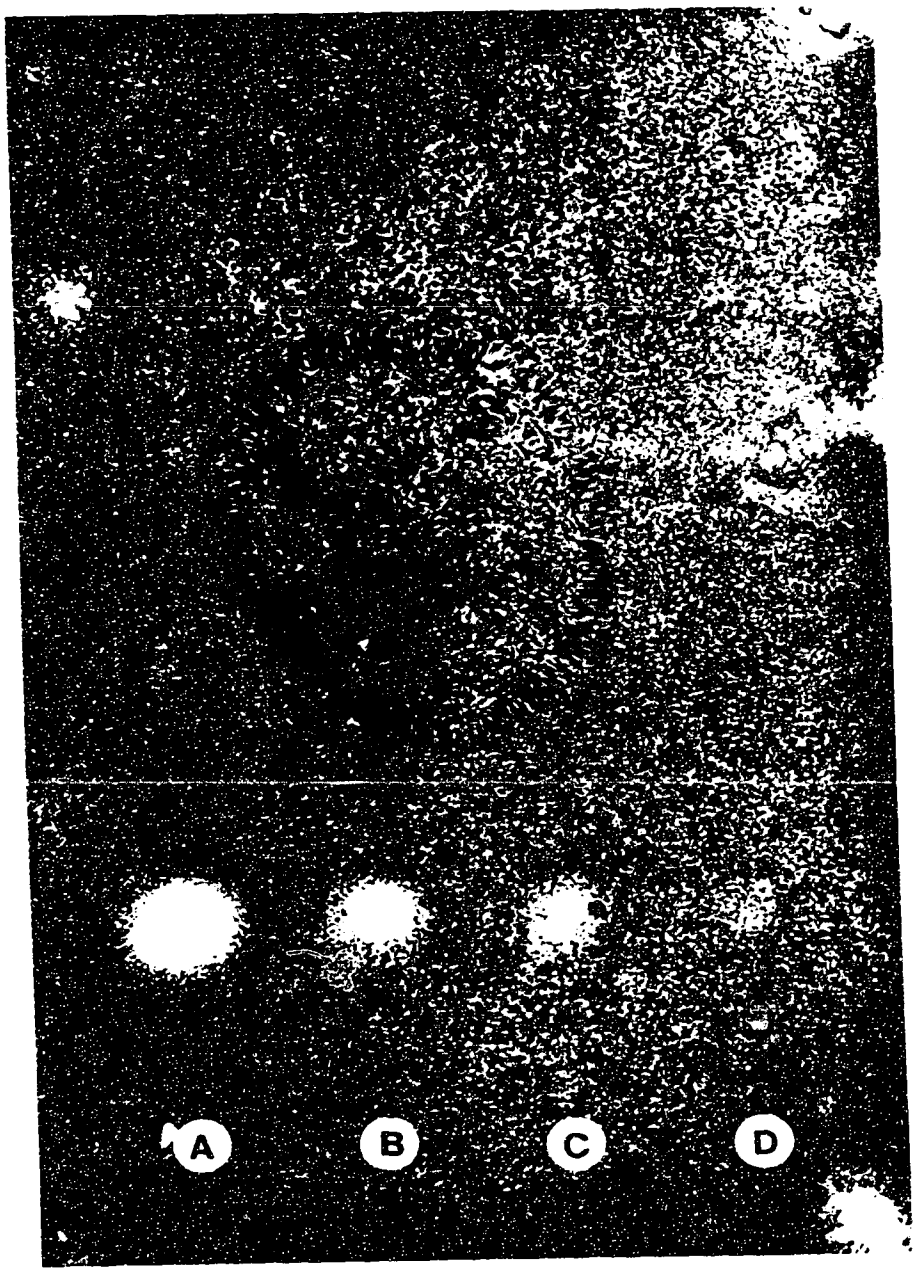
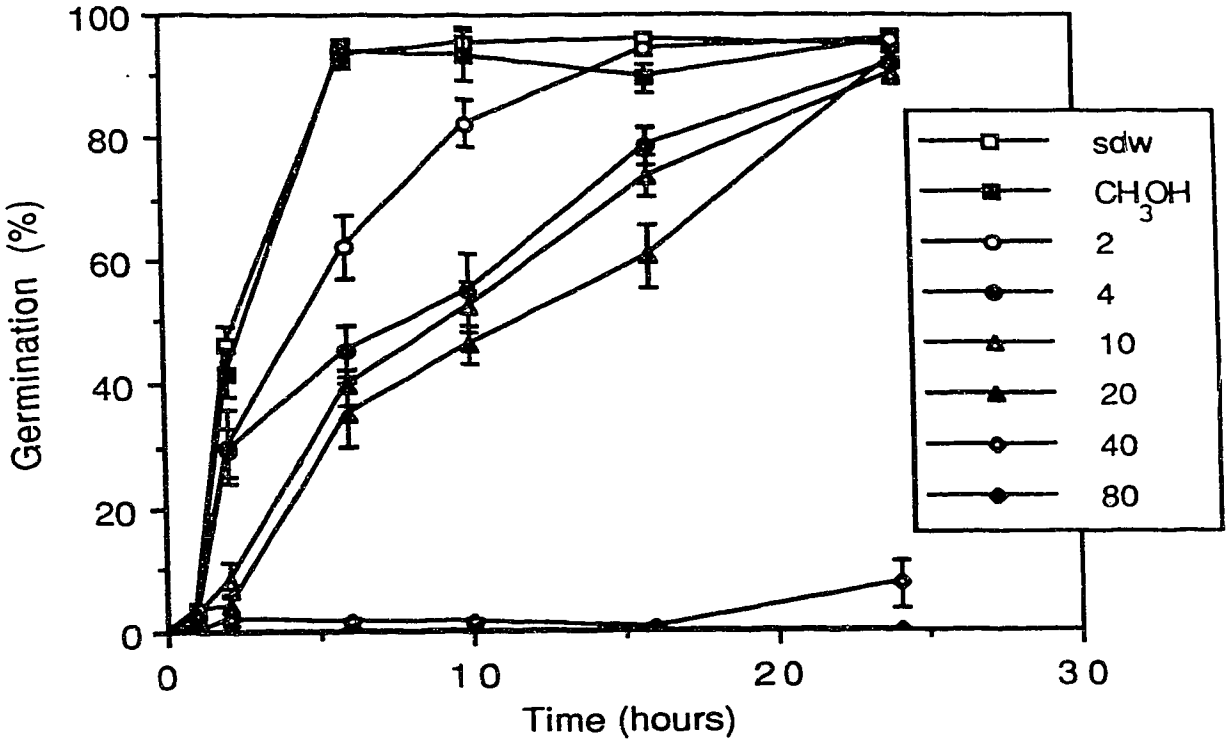

A
B
C
D

Figure VI-4. Effect of camalexin on germination of *Alternaria brassicae* conidia. Droplets deposited onto glass slides contained conidia in sterile distilled water (sdw), sdw:CH_3OH (98:2), sdw:CH_3OH + 2, 4, 10, 20, 40, or 80 μg/mL of camalexin. Vertical bars represent standard error of the mean of three experiments.

Figure VI-5. Effect of camalexin on germ tube growth of *Alternaria brassicae* conidia. Note that the EC_{50} and MIC of camalexin were approximately 6 and 80 μg/mL, respectively.


100
80
60
40
20
0
Germination (%)
0
10
20
30
Time (hours)
sdw
CH3OH
2
4
10
20
40
80

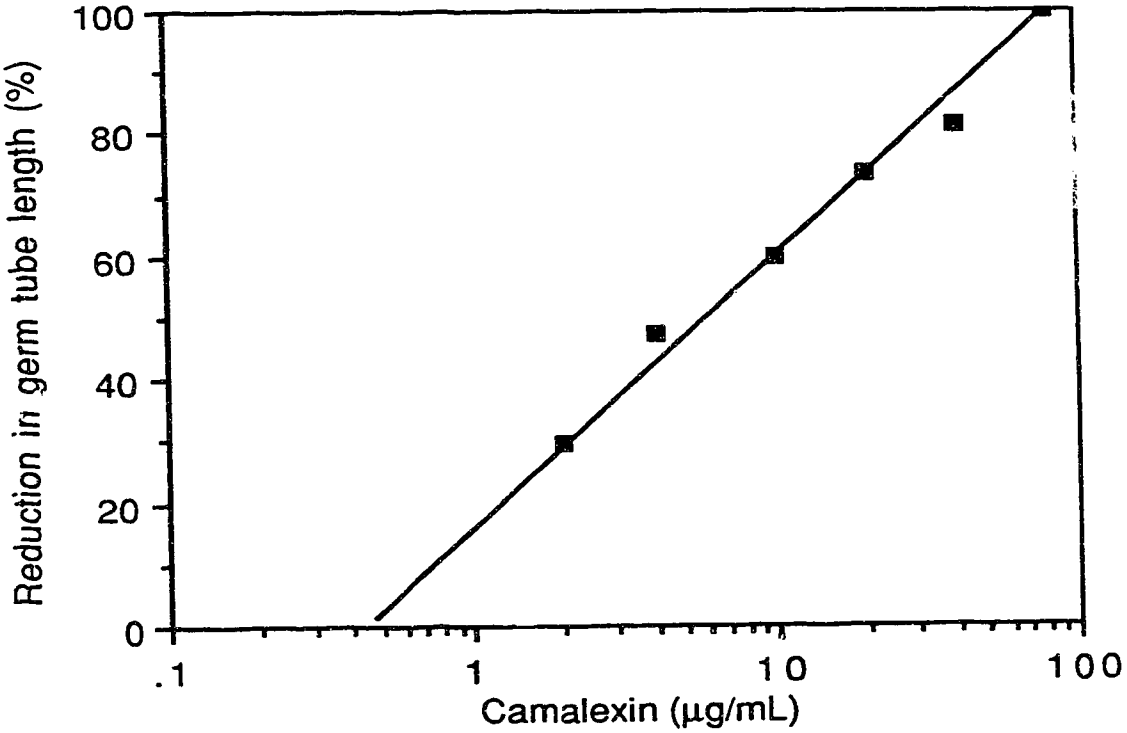

100
80
60
40
20
0
Reduction in germ tube length (%)
.1
1
10
100
Camalexin (µg/mL)

Figure VI-6. Effect of camalexin on *Alternaria brassicae* conidia. Droplets deposited onto glass slides contained conidia in sterile distilled water (A) and in solutions of 50 (B) and 100 μg/mL (C) of camalexin. Photographs were taken after 7 (B) and 20 hours (A,C). Note that camalexin caused the apical cells of germ tubes to autolyse as evidenced by the lack of uptake of lactophenol cotton blue stain (B,C). (A,B,C; approx. x100, x160 and x160, respectively).

A

B

C

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Chapter VII
General Discussion and Conclusions

A. General Discussion

A disease assessment key for alternaria blackspot was prepared for leaves and siliques. The overall shape of leaves and siliques are similar in rapeseed and mustard, therefore these keys should permit blackspot assessment in both these crops. Also, these keys should be usuable for assessing some other diseases of rapeseed and mustard as well, such as white rust and white leaf spot. These keys will allow for consistant disease assessment by different people. A 0-5 point rating scheme for assessing alternaria blackspot is widely used in India but part of it involves the visual assessment of the amount of host area affected (Kolte, 1985). The key for siliques was used to assess alternaria blackspot in central Alberta and proved easy to use. Every field of canola surveyed had alternaria blackspot. This disease was severe in 1989, causing significant yield loss. The severity of alternaria blackspot was evidenced by the fact that seeds of some siliques were colonized by *Alternaria brassicae*. If *A. brassicae* caused even a 5% yield loss of rapeseed in Alberta in 1989, which would be a conservative estimate, it would be equal to a monetary value of approximately $25 million (Tewari, 1991a). Alternaria blackspot is not severe every year in Alberta but a year like 1989 makes it an economically important disease of rapeseed in this province. Prior to the surveys conducted here, there had generally not been any extensive surveys conducted in central Alberta, and in many years, no formal surveys were carried out at all.

In several countries of Europe and southeast Asia this disease is severe every year and imposes a major constraint on optimum yields of rapeseed and mustard (Singh & Kolte, 1990). All cultivated *Brassica* spp. are susceptible to *A. brassicae* (Singh & Kolte, 1990). Breeding for resistance to *A. brassicae* is the best way of reducing the level of alternaria blackspot but to accomplish this, sources of resistance to *A. brassicae* have to be found. Prior to commencement of this project, there appeared to have been no known sources of high degrees of resistance to *A. brassicae* in *Brassica* spp. and no studies on resistance to *A. brassicae* in other genera of Cruciferae. Thus, various vegetable, oleiferous, and wild crucifers were screened to locate sources of resistance to *A. brassicae*. The crucifers were placed in three groups based on the type of response to this pathogen. The most common response was a broad range of necrosis and chlorosis. Another response was a hypersensitive reaction by an accession of *Eruca sativa*. Necrotic flecks appeared within the first day after inoculation and then remained of the same size until the leaves began to senesce. The most resistant response was one in which no symptoms occurred on healthy leaves and growth of *A. brassicae* was inhibited (e.g. in *Camelina sativa* and *Capsella bursa-pastoris*). No

symptoms appeared until leaves began to senesce, and then only as necrotic flecks.

Of particular interest was an accession of *B. campestris* ssp. *rapifera* that showed a limited and delayed necrosis and chlorosis. This was very different from the symptoms in *B. campestris* in general. This accession of *B. campestris* ssp. *rapifera* appeared to be a good candidate for a source of resistance to *A. brassicae* that could be transferred to other *Brassica* spp. by conventional breeding methods, and indeed Dr. G.R. Stringam was able to cross it with *B. juncea*. Leaves of a F_1-B_1 cross were screened against *A. brassicae*. The amount of necrosis and chlorosis was intermediate between that found in the two parents. Thus, some resistance to *A. brassicae* was transferred to this F_1-B_1 cross.

The very high level of resistance against *A. brassicae* found in *C. sativa* and *C. bursa-pastoris* will not be transferable to *Brassica* spp. by conventional breeding methods but it may be transferable by biotechnological techniques such as somatic hybridization. These weeds would involve intertribal crossing with rapeseed which would not be easy but intertribal crosses between *Barbarea vulgaris* and *Thlaspi perfoliatum* and *B. napus* have been reported (Glimelius *et al.*, 1990). Other techniques that could be used are embryo rescue (Agnihotri *et al.*, 1990) and *in vitro* ovule culture (Zenkteler, 1990). It may also be feasable to use genetic engineering to transfer this resistance to rapeseed by using a vector such as *Agrobacterium tumefaciens* (Boulter *et al.*, 1990).

Little is known about mechanisms of resistance to *A. brassicae* in Cruciferae. Prior to commencement of this project, there were only two mechanisms that had been observed. One of these mechanisms is differential susceptibility of crucifers to destruxin B, the host-specific toxin produced by *A. brassicae* (Bains & Tewari, 1987). The other mechanism is based on differential amounts of epicuticular wax (Conn, 1986; Conn & Tewari, 1989; Tewari & Skoropad, 1976). These mechanisms, however, did not appear to explain the high degree of resistance to *A. brassicae* observed in *C. sativa* and *C. bursa-pastoris*. Thus, the possibility that phytoalexins were involved in the resistance of some crucifers to *A. brassicae* was investigated. An accession of *B. campestris* ssp. *rapifera* which was more resistant to *A. brassicae* than rapeseed, produced the same phytoalexin as rapeseed but in a larger quantity. The very resistant *C. sativa* and *C. bursa-pastoris* produced larger amounts of different phytoalexins. Thus, the differences in susceptibility of these plants appeared to be due, at least in part, to qualitative and/or quantitative differences in phytoalexin production. Phytoalexin production is now the third mechanism of resistance to *A. brassicae* that has been observed in Cruciferae. Some other mechanisms of resistance to *A. brassicae* in crucifers, such as subcuticular growth and calcium sequestration are also now known and the resistance is thought to be layered and multicomponent (Tewari, 1991b). Prior to commencement of this project, there were no reports of phytoalexin production in Cruciferae. The significance of phytoalexins in the resistance of *C. sativa* and *C.*

bursa-pastoris to *A. brassicae* was reinforced by the fact that they were found to be sensitive to destruxin B. Thus, if *A. brassicae* had the opportunity to grow on these plants and produce destruxin B, these plants would be susceptible to this pathogen.

Virulence of *A. brassicae* isolates originating from different parts of the world were compared on *B. napus* cv. Westar. An Alberta isolate was the most virulent, followed by a French, and then an Indian isolate. None of these isolates caused symptoms on *C. sativa*. If these isolates were representative of their areas of origin, it would indicate that the greater severity of alternaria blackspot in Europe and India may be due to more conducive environmental conditions than any higher virulence of *A. brassicae* isolates. These isolates were also compared for phytoalexin-elicitation in *C. sativa*. The Alberta isolate elicited the most, followed by the French, and then the Indian isolates. This was an unusual response because usually virulent isolates of a pathogen elicit less phytoalexins than weakly virulent or non-virulent isolates. In this case, however, the more virulent Alberta isolate elicited greater amounts of phytoalexin. The reason for this is not known at the present time. This appeared to be the first comparison of the virulence of *A. brassicae* isolates from different parts of the world.

Rhizoctonia solani is the principal causal agent of pre- and post-emergence damping-off and root rot of canola in western Canada. In Alberta, it is most severe in the Peace River region causing estimated yield losses of 20-30% (Ellis, 1983; Harrison, 1988; Sippell *et al.*, 1985; Yitbarek, 1987). All presently registered canola cultivars are susceptible to *R. solani* (Harrison, 1988; Gugel *et al.*, 1987). A large variation in susceptibility to *R. solani* has been observed in *Brassica* spp. but high degrees of resistance against *R. solani* have not been found (Acharya *et al.*, 1984; Harrison, 1987: Yang, 1989). Since *C. sativa* had been shown to be very resistant to *A. brassicae*, the question arose as to whether it would also have resistance to *R. solani*, a soil-borne pathogen. *Camelina sativa* was more resistant to a virulent isolate of *R. solani* than *B. napus* cv. Westar. Percent emergence of *C. sativa* seedlings in *R. solani* infested soil was 22-33% greater than for *B. napus* cv. Westar. Thus, *C. sativa* was not completely resistant to *R. solani*, like it was to *A. brassicae*, but it was significantly less susceptible than *B. napus* cv. Westar. Large amounts of antimicrobial compounds were produced in *C. sativa* roots compared with very low amounts in *B. napus* cv. Westar. These compounds appeared to be a mixture of phytoalexins and preformed compounds. Thus, the differential susceptibility of *C. sativa* and *B. napus* cv. Westar to *R. solani* appeared to be due, at least in part, to qualitative and/or quantitative differences in the presence of antimicrobial compounds.

The cruciferous weeds such as *C. sativa* and *C. bursa-pastoris* appear to have a general resistance against a number of pathogens. In this study they were shown to be very resistant to *A. brassicae* and *C. sativa* was shown to have some resistance to *R. solani*.

Camelina sativa has also been shown to be very resistant to *Leptosphaeria maculans*, the causal agent of blackleg of rapeseed (K.L. Conn & J.P. Tewari, unpublished results). *Capsella bursa-pastoris* has also been shown to be resistant to *L. maculans* (Gugel *et al.*, 1990). It is interesting that *C. sativa* and *C. bursa-pastoris* are very resistant to both *A. brassicae* and *L. maculans*. No *Brassica* spp. are resistant to both. For example, *B. juncea* is generally more resistant to *L. maculans* than *B. napus* but *B. napus* is generally more resistant to *A. brassicae* than is *B. juncea*. This general resistance of *C. sativa* and *C. bursa-pastoris* may be due to phytoalexin production as being the major mechanism of resistance in these plants to *A. brassicae*. Since these plants have resistance to several pathogens, it would make transferring their resistance to rapeseed and mustard more worthwhile.

A rapid new purification procedure was developed for isolation and separation of *C. sativa* phytoalexins. It involved applying aqueous extract to an Extube and allowing it to distribute over the specially modified diatomaceous earth, the organic metabolites eluted with $CHCl_3$, the $CHCl_3$ extracted with 5% HCl, the HCl made basic and passed through another Extube, and the $CHCl_3$ eluent subjected to vacuum liquid chromatography. This procedure will likely work for the extraction of phytoalexins from many crucifers. This procedure led to the isolation of two new thiazoyl substituted indole alkaloids which were named camalexin and methoxycamalexin. These phytoalexins appeared to be the first reported naturally occurring antifungal compounds which contained a 2-substituted thiazole. The fungicide thiabendazole is a 4-substituted thiazole (Woodcock, 1977). Another compound which is even more similar to these phytoalexins had been synthesized in search of compounds with a biological profile similar to that of thiabendazole (Sarodnick & Kempter, 1979). It is interesting that nature had already produced antimicrobial compounds like these phytoalexins that were similar to synthetic antimicrobial compounds produced by man.

It is also interesting to note that these phytoalexins are easy to synthesize, especially camalexin (Dr. W.A. Ayer, personal communication). Both camalexin and methoxycamalexin have been synthesized (Dr. YuTing Ma & Dr. W.A. Ayer, unpublished results). This is unusual for phytoalexins because large scale chemical synthesis of most phytoalexins thus far characterized is difficult (Brent, 1983; Callow, 1983).

Another unusual aspect of these phytoalexins is that their production in *C. sativa* was not associated with cell death, not even a hypersensitive reaction. While phytoalexin production has been shown to occur in live plant cells (Adikaram *et al.*, 1988; Smith & Banks, 1986), it is almost always associated with necrosis and the hypersensitive response. One other exception is that capicannol phytoalexin produced by *Capsicum annuum* fruits was shown to be associated with live cells with little or no necrosis occurring (Adikaram *et al.*, 1988). Since these phytoalexins from *C. sativa* were not associated with plant cell death and

their chemical synthesis is easy, they have a greater potential to be used as chemical control agents for pathogens than the previously known phytoalexins. So far the phytoalexins wyerone and capsidiol have been used with some success as fungicides (Fawcett *et al.*, 1969; Ward *et al.*, 1975). The systemicity of camalexin and methoxycamalexin in plants, if any, is not known at this time. Their usefulness would be limited if they are not systemic. The use of these phytoalexins as pharmaceutical drugs should also be explored because the phytoalexin brassilexin has been shown to have anticancerous activity (Devys & Barbier, 1991).

B. Conclusions

1. A disease assessment key for alternaria blackspot was prepared. It proved easy to use and should allow for consistant assessment of this disease by different people.
2. Sources of resistance to *Alternaria brassicae* have been found. Dr. G.R. Stringam was able to cross an accession of *Brassica campestris* ssp. *rapifera*, with a higher level of resistance than rapeseed and mustard, with *B. juncea* to transfer this resistance to *B. juncea*. Some crucifers with a very high level of resistance to *A. brassicae* were found and this resistance may potentially be transferable to rapeseed with biotechnological and other techniques.
3. Differences in the virulence of *A. brassicae* isolates were found. An Alberta isolate was the most virulent, followed by a French, and then an Indian isolate.
4. Differences in the susceptibility/resistance of some crucifer leaves appeared to be due, at least in part, to qualitative and/or quantitative differences in phytoalexin production.
5. *Camelina sativa*, which was very resistant to *A. brassicae*, also had some resistance to *Rhizoctonia solani*. This difference appeared to be due, at least in part, to qualitative and/or quantitative differences in the presence of antimicrobial compounds in the roots.
6. A rapid new purification procedure was developed for isolation and separation of the phytoalexins from *C. sativa*. This procedure will likely work for the extraction of phytoalexins from many crucifers.
7. Two new phytoalexins from *C. sativa* were isolated and named camalexin and methoxycamalexin. The structural chemistry on these phytoalexins was done by Dr. L.M. Browne and Dr. W.A. Ayer. Their general structure was similar to that of the fungicide, thiabendazole.
8. Since these phytoalexins were not associated with plant cell death and their chemical synthesis is easy, they have a greater potential to be used as chemical control agents for pathogens than the previously known phytoalexins.

C. Suggestions for Future Work on this Topic

1. Compare the virulence of more *A. brassicae* isolates from around the world.
2. Investigate the mechanisms of differential phytoalexin-elicitation by *A. brassicae* isolates.
3. Identify the phytoalexin-elicitor compounds in *A. brassicae*.
4. Screen more crucifers for phytoalexin production. Utilize the procedure developed in this program for the isolation and purification of other phytoalexins.
5. Test *C. sativa* and *C. bursa-pastoris* against other rapeseed pathogens to see just how broad their resistance is.
6. Try to transfer the resistance in *C. sativa* to rapeseed.
7. Investigate the mode of action of camalexin and methoxycamalexin.
8. Determine whether camalexin and methoxycamalexin are systemic in plants and determine how broad their antimicrobial activity is.
9. Investigate the potential of camalexin and methoxycamalexin as chemical control agents.

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Appendix

Spectral analyses of camalexin and methoxycamalexin.

General. Mass spectra were recorded on an AEI model MS-50 mass spectrometer. The formulas of all peaks reported were determined by high resolution measurements. IR spectra were recorded on a Nicolet 7199F.T. interferometer. ^{1}H and ^{13}C NMR spectra were determined on Brüker WH-300, WM-360, or WH-400 spectrometers with either Aspect 2000 or 3000 computer systems. Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected. Skellysolve B refers to Skelly Oil Co. light petroleum, b.p. 62-70°C. All chromatography solvents were distilled prior to use.

Camalexin: Isolated as colorless crystals (methanol), m.p. 134-137°C from benzene; UV λmax (CH_3OH) 214 (e=22,200), 274 (e=7,900), 318 (e=13,800) nm; FTIR (KBr) 3432, 3154, 3084, 2921, 1621, 1562,1484, 1456, 1347, 1306, 1295, 1244, 1209, 1146, 1134, 1128, 1098, 1054, 919, 759, 738, 706 cm^{-1}; HRMS (EI, probe, 150°C) m/z 200.0403 meas, 200.0437 calc for $C_{11}H_8N_2S$ (100), 172 ($C_{10}H_6NS$, 3), 155 ($C_{10}H_7N_2$, 3), 142 ($C_9H_6N_2$, 26), 128 (C_9H_6N, 2), 115 (C_8H_5N, 12); (CI NH_3) m/z 203 (13), 202 (36), 201 (100, M^++1), 200 (59, M^+); ^{1}H NMR (360 MHz, $CDCl_3$) δ9.00 (br s, D_2O exchangeable, N-H), 8.16 (m, H-4, {7.31, 7.19, 7.22}), 7.77 (d, J=3Hz, H-4', {7.17}), 7.74 (d, J=2.5 Hz, H-2, {NH}), 7.31 (m, H-7, {8.16, 7.22}), 7.22 (m, H-6), 7.19 (m, H-5), 7.17 (d, J=3Hz, H-5'); (Benzene-d_6) 8.68 (d, H=8Hz, H-5), 7.71 (d, J=3Hz, H-4', {7.17}), 7.32 (d, J=2.5 Hz, H-2), 7.26 (dt, J=1,7Hz, H-5), 7.18 (dt, J=1,7Hz, H-6), 6.93 (d, J=8Hz, H-7), 6.61 (d, J=3Hz, H-5'); ^{13}C NMR (75.5 MHz, $CDCl_3$, APT) δ163.4 (s, C-2'), 142.2 (d, C-2), 136.5(s, C-7a), 124.8 (s, C-3a), 124.6 (d, C-4'), 123.2 (d, C-5), 121.5 (d, C-6), 120.5 (d, C-4), 116.0 (d, C-5'), 112.2 (s, C-3), 111.7 (d, C-7).

Methoxycamalexin: Isolated as an oil, crystallizes from methanol-Skellysolve B , m.p.157-159°C; UV λmax (CH_3OH) 218 (e=21,700), 296 (e=11,500), 324 (e=11,250) nm; FTIR (CHCl3 cast) 3270, 3120, 2954, 2925, 2854, 1629, 1540, 1456, 1318, 1300, 12489, 1200, 1167, 1111, 1094, 1084, 1050 cm^{-1}; HRMS (EI, probe, 150°C) 230.0515 meas, 230.0511 calc for $C_{12}H_{10}N_2OS$ (100), 215 ($C_{11}H_7N_2OS$, 73), 200 ($C_{11}H_8N_2S$, 15), 187 ($C_{10}H_7N_2S$, 22), 157 ($C_9H_5N_2O$, 7), 129 ($C_8H_5N_2$, 12), 102 (C_7H_4N, 5); (CI-NH_3) 231.64 (M^++H, 13), 230.64 (M+, 100), 200 (M^+-30, 54), 195 (14), 123 (48); ^{1}H NMR (360 MHz, $CDCl_3$) δ8.32 (br s, D_2O exchangeable, N-H {7.77}),8.13 (d, J=8Hz, H-4 {6.95}), 7.82 (d, J=3Hz, H-4'{7.23}), 7.77 (d, J=2.5Hz, H-5' {8.32}), 7.23 (d, J=3Hz, H-5' {7.82}), 6.95 (m, H-5 {8.13}), 6.89 (m, H-7 {8.13}), 3.88 (s, OCH_3); ^{13}C NMR ($CDCl_3$), 163.2 (s, C-2'), 157.2 (s, C-6), 142.6 (d, C-2), 137.2 (s, C-7a), 123.2 (d, C-4'), 121.4 (d, C-4), 119.1 (s, C-3a), 115.8 (d, C-5'), 112.6 (s, C-3), 111.3 (d, C-5), 94.9 (d, C-7), 55.7 (q, OCH_3).