UNIVERSITY OF ALBERTA

RESPONSES OF BRASSICA SPECIES TO FILTRATE FROM CULTURED *LEPTOSPHAERIA MACULANS* (DESM.) CES. et de NOT.

BY

SEAN ANGUS ROGERS ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Responses of Brassica species to filtrate from cultured *Leptosphaeria maculans* (Desm.) Ces. et de Not.** submitted by Sean Angus Rogers in partial fulfillment of the requirements for the degree of Master of Science in Plant Molecular Biology and Biotechnology.

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Abstract.

Blackleg fungus of canola, caused by *Leptosphaeria maculans* (anamorph: *Phoma lingam*), is a disease of concern among western Canadian producers. After foliar infection, blackleg fungus systemically spreads to the crown. Plant lodging results from stem girdling by the disease. Thus, severe yield loss in infested fields is possible. Most canola quality cultivars are susceptible to or have low tolerance to aggressive blackleg strains. However, many mustard Brassica species (e. g., *Brassica juncea* and *B. carinata*) are considered blackleg resistant.

The objective of this project was to assess a simplified model system for studying blackleg–Brassica interaction. Cotyledons of blackleg resistant and blackleg sensitive Brassicas were treated with filtrate from a still culture of the highly virulent isolate, Leroy. The examined species generally followed the published disease ratings based on pycnidiospore inoculation, although tissue damage was less extensive in the present study. Wounding at the time of treatment slightly hastened cotyledon response to culture filtrate. SDS-PAGE profiles (with and without urea present) of aqueous proteins from control and treated cotyledons were compared over time. A protein (M, 16.0–16.5 kDa) was putatively induced within 24 hours of treatment in the case of Domo (*B. juncea*, blackleg resistant), but not in the case of S67 (*B. carinata*, blackleg resistant). *In vivo* labelling of Domo cotyledons failed to conclusively establish evidence that the putative filtrate-induced protein was synthesized *de novo* after treatment. Northern analysis of Domo showed that lignification occurs in both control and filtrate-treated cotyledons at approximately equivalent levels, as determined by hybridization with cinnamoyl alcohol dehydrogenase (pTCAD4) from tobacco.

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- Dr. W. Schuch (ICI Seeds, Bracknell, UK) – pTCAD4.
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List of Abbreviations.

20:1	eicosenoic acid
22:1	erucic acid
A	androecium (floral formula)
ASIPC	area under the stem infection progress curve
bis	N,N'-methylenebi acrylamide
bp	base pairs
Bq	Bequerel
BSA	bovine serum albumin
C	corolla (floral formula)
CAD	cinnamoyl alcohol dehydrogenase
CAP	Common Agricultural Policy
CBB	Coommassie brilliant blue
cDNA	complementary DNA
CHS	chalcone synthase
Ci	curie
ddH_2O	distilled, deionized water
DEPC	dietyhyl pyrocarbonate
DTT	dithiothreitol
EC	Enzyme Commission
EEC	European Economic Community
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
FAO	Food and Agriculture Organization
G	gynoecium (floral formula)
GITC	guanidine isothiocyanate
GRAS	Generally Recognized As Safe
HPLC	high pressure liquid chromatography
HR	hypersensitive response
HRPG	hydroxyproline-rich glycoprotein
IAA	isoamyl alcohol
ITS	internal transcribed space
K	caylx (floral formula)
kb	kilobase

kDa	kiloDalton
MAb	monoclonal antibody
MOPS	3-(N-morpholino)propanesulphonic acid
M_r	relative molecular mass
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PDRAT	percentage disease rating
PEG	polyethylene glycol
PG	pathogenicity group
PMSF	phenylmethylsulphonyl fluoride
PR	pathogenesis-related
PVP	polyvinylpyrolidone
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNAase	ribonuclease A
Rubisco	ribulose bisphosphate carboxylase
SSC	standard saline citrate
SDS	sodium dodecyl sulphate
SPEB	soluble protein extraction buffer
ssDNA	single-stranded DNA
TE	tris-EDTA
TEMED	N,N,N'N'-tetramethylenediamine
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
VHO	very high output

1. CHAPTER 1. Literature Review.

1.1. Background.

1.1.1. History of oilseed Brassicas.

The mustard family Brassicaceae (Cruciferae), in particular the tribe Brassiceae, is of considerable economic importance. It contains cultivated crop plants including cabbage, Chinese cabbage, radish, turnip, mustard and oilseed rape (Gross, 1993). In addition, many troublesome weeds are categorized in the mustard family. Brassicas are among the earliest domesticated plants with some vegetable forms commonly used in Neolithic times. Direct references to Brassicas are found in ancient medical manuscripts of Indian, Chinese, Greek, and Roman origins, which describe the use of both oil and seed from mustard and rapeseed as remedies for stomach problems and skin diseases (Downey and Röbbelen,1989). Rapeseed oil was burned by the ancients as an illuminant (Thompson,1983). It was later used in soapmaking (Downey and Röbbelen, 1989; Gugel and Petrie, 1992) and in foods as edible oil (Canola Council of Canada , 1991b). The oldest reference to rapeseed is found in the Upanishads written in ancient Sanskrit circa 1500-2000 BC (Prakash and Hinata, 1980). Several herbal and medical European books from the Middle Ages mention Brassicas. In 1753, the Swedish botanist and taxonomist Linnaeus divided the Brassicas into two genera based on flower morphology (Prakash and Hinata, 1980). Several subsequent taxonomic classifications have been made since by others (reviewed by Prakash and Hinata, 1980).

The word mustard is derived from the Latin, *mustum ardens*, meaning hot must in reference to the European condiment made by mixing wine must with crushed black mustard (*Brassica nigra* (L.) Koch) seeds (Downey *et al.*, 1975). "Rape" is from the Latin *rapum, rapa* for turnip (Thompson, 1983; Taupman, 1966). Oilseed rape or rapeseed denotes plants, crops, or products of *B. rapa* L. (synonym *B. campestris* L.) or *B. napus* L., both existing in annual and biennial forms.

Oilseed rape, probably *B. rapa*, has been a field crop in Europe since the Middle Ages (Gugel and Petrie, 1992). Thompson (1983) speculated that "as oilseed rape, *B. napus* originated in Middle Europe possibly not more than 500-600 years ago from very few, partially self-fertile, chromosome-doubled hybrids, as naturally occurring genetic variation is relatively small." A Dutch

herbal book in 1578 gives the earliest reference to winter oilseed rape *B. napus* (Thompson, 1983). In the 16th and 17th centuries, winter oilseed rape *B. napus* was known in England as "cole seed". When the English fens were drained in the 17th century, oilseed rape was extensively grown for lamp oil (Thompson, 1983). During the 17th century, the English crushing industry not only met domestic oil demand but was able to export surplus oil (Gugel and Petrie, 1992). The demand for rapeseed oil was high as a result of the Industrial Revolution, driven by increasing use of steam engines. Rapeseed oil has traditionally been characterized by high erucic acid content (Thompson, 1983) endowing it with the ability to cling to water- and steam-washed metals better than other oils (Canola Council of Canada, 1991b). In the 1860's, German and French oilseed rape acreage was over 200 000 ha (Thompson, 1983). However, its demand in Europe sharply fell when transportation improvements led to increased competition from tropical oilseeds.

Oilseed Brassicas are one of the few edible oil crops available for growth in temperate zones or at higher elevations. Major areas of production are found in China, the Indian subcontinent, Canada, and Northern Europe (Downey and Röbbelen, 1989). Today, four Brassica species—*B. napus*, *B. rapa*, *B. juncea* (L.) Czern. (brown or Indian mustard), and *B. carinata* Braun (Abyssinian or Ethiopian mustard)—are among the world's most significant oil crops (Rimmer and van den Berg, 1992). Production and consumption of Brassica seed oil has grown faster between 1975 and 1985 than all oil crops other than palm oil (*Elaeis* spp.). For example, production in Britain of oilseed rape, mostly winter *B. napus*, rose from slightly over 5 000 ha in 1971 to 92 000 ha in 1980. This jump was a consequence of Britain's entry into the European Economic Community (EEC) in 1973. At that time, British producers became eligible for the prevailing world price set by the rapeseed marketing system of the EEC-controlled Common Agricultural Policy (CAP) (Thompson, 1983). Oilseed rape has been grown and actively bred for over 50 years in Sweden, France, Germany and Poland (Thompson, 1983). Ranking third most important oil crop after soybean and palm, it accounts for 13.2% of the world's edible oil (Downey and Röbbelen, 1989).

Typically, Brassica oilseeds yield over 40% oil on a dry weight basis and 38-44% high quality protein meal (Downey and Röbbelen, 1989). The meal is used in Asian countries as organic fertilizer, whereas in the West, it is used as feed for livestock and poultry.

The Canadian Brassica oilseed industry started during World War II in an effort to displace dependence of Allied merchant marine and naval forces upon war-restricted European sources of steam engine lubricants (Canola Council of Canada, 1991b). In Canada, two species of Brassica are currently grown for oil, *B. napus* (Argentine rape) and *B. rapa* (Polish rape). The parenthetical names are in deference to the unlicensed, mixed seed stocks deployed during the

war effort (Downey *et al.*, 1975). After the war, it was determined that oilseed rape was the best adapted edible oil crop for large-scale production in the Prairie Provinces and has since been the focus of major breeding programmes (Stefansson, 1983). In the 1960's, the first cultivars with low eicosenoic acid (20:1) and low erucic acid (22:1) were released (Canola Council of Canada, 1991b). Erucic acid causes cloudy oil and decreases spreadability of margarine. In addition, experiments with lab animals, especially rats, showed that 22:1 accumulated in heart muscles (lipidosis) leading to subsequent myocardial lesions. Although direct implications to human health remained uncertain, high erucic acid content was publicly perceived as undesirable since erucic acid is poorly digested by humans (Thompson, 1983). The use of the high protein, residual meal component as feedstock was initially hindered by the presence of glucosinolates (Canola Council of Canada, 1991b). Over 90 glucosinolates have been found in Brassicaceae (Downey and Rakow, 1987), although only four are predominant in *B. napus* (Thompson, 1983). Upon crushing, endogenous myrosinase converts glucosinolates to end products that are goitrogenic to monogastric animals, as they compete with iodine for uptake by the thyroid (Thompson, 1983). Although not goitrogenic to ruminants, these products taint the meal with a bitter taste rendering the meal unappetizing to livestock. In 1974, Stefansson released the first "double low" variety, Tower (Canola Council of Canada, 1991b), a landmark in the development of canola. The granting of Generally Recognized As Safe (GRAS) status to low-erucic acid (less than or equal to 2%) Brassica seed oil, by the US Food and Drug Administration in 1985, with subsequent permission to use the term canola, opened the large American market for the first time (Canola Council of Canada, 1991b).

Canola is a generic term for genetically improved Brassica oilseed. As such, it is defined by Canadian food acts, feed acts, and seed acts according to two consumable components, oil and residual meal (Canola Council of Canada, 1991b; Boulter, 1983). Under the Canada Agricultural Products Standards Act of Consumer and Corporate Affairs, oil from whole seed varieties of *B. napus* and *B. rapa* must contain less than or equal to 2% erucic acid (originally less than or equal to 5%) and meal less than or equal to 30 milligram equivalents of aliphatic glucosinolates per gram of oil-free, air-dried residual meal (Canola Council of Canada, 1991b; Rimmer and van den Berg, 1992; Boulter, 1983).

Although canola ranks first in production among the main oilseed crops in Canada, increase in production acreage of the oilseed sector is constrained via competition with other traditional agricultural crops for planting acreage (Agriculture Canada, 1991). In Ontario, winter varieties have found a niche, as spring canola quality varieties are less suitable for eastern Canadian growing conditions. However, in general the best suited land for canola production lies in the cooler, northern regions of Alberta, Manitoba or Saskatchewan where it vies for acreage

against wheat, barley, and an expanding variety of specialty crops (Agriculture Canada, 1991).

Nearly 50,000 Canadian farmers are canola producers (Agriculture Canada, 1991). Other industries associated with or stimulated by canola include the crushing industry with its ancillary processing sub-sector, handling and transportation sectors, producer-owned organizations (for example, Alberta Canola Producers Commission and Canola Council of Canada), and private enterprise. Private industry involvement in the development of new varieties has been stimulated by Plant Breeder's Rights legislation. In 1991, 10 private breeding companies existed in Canada (Canola Council of Canada, 1991a). Although breeding programmes are still carried out at the major Western Canadian universities, the majority of new varieties are developed by private companies (Canola Council of Canada, 1991b). Research in canola is further encouraged by various bodies. For example, the Canola Council of Canada granted 7 applicants $315 000 for various studies (Canola Council of Canada, 1991a).

There remains a significant but limited demand in the industrial oil market for very high (over 50%) erucic acid Brassica seed oil (Downey and Röbbelen, 1989). Most of this oil is fractionated with conversion of erucic acid to erucamide, a slippage agent in the plastics industry. There are minor applications for very high erucic acid oil as a lubricant in cold steel rolling and jet engines.

Brassica seed oil and meal are commodities of economic significance in the global marketplace. In terms of world production, Brassica seed oil has ranked third highest for fats and oil after soybean and palm since 1990/1991 and second as a source of protein meal after soybean since 1988/1989 (Agriculture Canada, 1992). Canada is a dominant player in the Brassica oilseed production market — fourth largest producer after China, the EEC, and India (Agriculture Canada, 1992), supplying 13.4% and 15.8% of total world production (24.4 and 27.2 million metric tonnes for 1990 and 1991, respectively) (FAO Yearbook, 1992).

Over 19 million hectares were sown to oilseed Brassica worldwide in 1991, with 16.5% (3.3 million hectares) in Canada (FAO Yearbook, 1992). Canola acreage in Canada increased to a record 4.1 million hectares in 1993 with predictions of 4.8 million hectares in 1994 (Edmonton Journal, 1994). Most of the arable land committed to canola production lies in western Canada, particularly in Alberta and Saskatchewan. Both provinces have grown over 1.0 million hectares since the mid-1980's (Agriculture Canada, 1991; Statistics Canada, 1993).

Canola yields vary according to seasonal climatic conditions for each season. In 1991, the five-year average yield was 23.5 bushels per acre (Canola Council of Canada, 1991b). The average provincial yield for Alberta in 1993 was a record 25.7 bushels per acre (Hansen, 1993)

In Canada, canola is sold on the open market as futures at the Winnipeg Commodity

Exchange. Although most canola producers have preferred to sell in the traditional method of prevailing price at time of delivery, more growers are expected to take advantage of guaranteed minimum prices offered by selling futures contracts, particularly when prices are high. Such was the situation in early 1994 when the price of canola exceeded well over $400.00 per metric tonne (Edmonton Journal, 1994). Prices have been considerably lower in the past with annual averages ranging about $300.00 ± $25.00 per tonne (Agriculture Canada, 1992). Direct economic benefit from canola production and processing has been estimated in excess of $1.5 billion annually (De Clerq *et al.*, 1989). Farm cash receipts of Alberta producers in 1991 exceeded $330 million (Alberta Treasury, 1992).

1.1.2. Economic impact of blackleg fungus on canola.

The blackleg fungus of Brassica, *Leptosphaeria maculans* (anamorph: *Phoma lingam*), has been documented for over 100 years as a source of yield reduction in cruciferous, especially horticultural crops (Gugel and Petrie, 1992). Blackleg is well known to be transmitted via seed and is, therefore, a world-wide problem for cole (*Brassica oleracea* L. var. *capitata*) seed producers in Canada, the United States, Europe, and Australia (Gabrielson, 1983). It has been a recurring problem in the US cabbage seed industry since reported by Pound in 1947 (Petrie, 1975). Severe damage to cabbage in the eastern American states due to blackleg occurred in 1966 (Petrie and Lewis, 1985). Blackleg has been a limiting factor in the use of turnip (*B. campestris* L. ssp. *rapifera* (Metzg.) Sinsk.) and swede (rutabaga) (*B. napus* L. ssp. *rapifera* (Metzg.) Sinsk) in New Zealand, Europe, and the US (Gabrielson, 1983; Petrie and Lewis, 1985).

Oilseed Brassicas are susceptible to blackleg fungus which causes stem cankers near the crown of the plant, ultimately reducing yield through crop lodging. Severe epidemics in rapeseed were recorded in France in 1950 and again in 1966-1968 (Gugel and Petrie, 1992). In Germany, over 70% of winter rapeseed plants are affected some years (Gugel and Petrie, 1992). Severe incidence and yield losses to blackleg in English crops were reported in 1977 and 1978 (Newman, 1984). The most dramatic blackleg epidemic was in Australia where Canadian spring cultivars of rapeseed had been introduced as an alternative to wheat in 1968 (Gugel and Petrie, 1992). Within three years, the industry was eliminated because of blackleg (Roy and Reeves, 1975). Virulent blackleg has been reported in US canola in Kentucky and in 1991 in North Dakota (Lamey and Hershman, 1993). It is interesting to note that blackleg was not listed among the five

major fungal diseases of rape, as reviewed by Anderson and Olsson in 1961 (Thompson, 1983).

A weakly virulent strain of blackleg was found in Saskatchewan in 1961 (Gugel and Petrie, 1992). Losses to weakly virulent blackleg, even 1-2%, are rare (Thomas, 1984). However, a highly virulent strain was found in three widely separated central Saskatchewan fields in 1975 (Gugel and Petrie, 1992). Since that time, losses in Saskatchewan as a result of blackleg have totalled $300 to $500 million (Stringam *et al.*, 1992; Evans and Stevenson, 1993) with annual yield reductions of 10-50% (Kharbanda and Stevens, 1993). In the early 1980's, blackleg spread to Alberta, Manitoba and Ontario (Gugel and Petrie, 1992). Losses in Manitoba have been estimated at $50 million (Evans and Stevenson, 1993). From 1988-1992, overall yield losses in Manitoba were about 5% (Gugel and Petrie, 1992). In Alberta, losses are considerably lower as aggressive measures have been instigated to control the spread of blackleg Incidence remains high in Saskatchewan where 96% of the fields surveyed in 1991 were infested with virulent blackleg (Petrie, 1993a) Basal stem cankers were detected in 93% of the fields . Petrie (1993b) observed that since 1981 the spread of blackleg south to north, rated as increased prevalence and incidence, was much slower than east to west. Long-term sporulation potential (cued by climatic conditions) and shorter persistence of old canola stubble in northern areas were given as potential explanations.

Virulent blackleg was first identified in east central Alberta, near the Saskatchewan border in 1983 (Evans and Stevenson, 1993). It is now widespread in east central Alberta where up to one-half the canola fields in some municipalities are infected. Levels of incidence have generally remained at 10%, with an overall yield loss of less than 5%. As in Saskatchewan, the northerly spread of virulent blackleg in Alberta has been slower than that in the east-west direction. Although one-third of Alberta canola is grown in the Peace Region, the first incidence in that region was reported only in 1992 (Evans *et al.*, 1993). Several infected fields in the Peace Region were found in 1993, possibly related to more extensive surveying of that region (Evans, personal communication). Alberta losses to blackleg in the past few years have been minor, at about 1% of the total yield (Gugel and Petrie, 1992), valued under one million dollars (Evans and Stevenson, 1993).

Virulent blackleg was declared a pest by the Alberta Agricultural Pests Act of 1984, thereby empowering provincial organizations with the authority to take measures of prevention and control (Evans and Stevenson, 1993). Under these provisions, inspectors conduct standard surveys and take active measures pursuant to the Act. In 1988, Alberta Agriculture, the Municipal Agriculture Service Boards, and the canola industry initiated a concerted programme for blackleg control. Generally, all canola seed to be sown must be tested at approved facilities. Random field surveys are conducted and fields sown to canola should be under a four year roatation (Gugel

and Petrie, 1992).

1.2. Description of *Leptosphaeria maculans*.

1.2.1. Nomenclature.

Leptosphaeria maculans (Desmaziére) Cesari et de Notaris, order Pleosporales, is the sexual or teleomorphic stage of the bitunicate ascomycetous fungal pathogen causing blackleg or stem canker of Brassicas. The asexual or anamorphic stage is the deuteromycete, *Phoma lingam* (Tode ex Fries) Desmaziére, order Sphaeropsidales (Agrios, 1988; Sivaneson, 1984; Sjödin, 1989). *P. lingam* was first described as *Sphaeria lingam* by Tode in 1791 (Boerema, 1976), a saprophyte on dead red cabbage (Williams, 1992). In 1823, Fries 'sanctioned' this binomial in his starting-point book on mycological taxonomy, *Systema Mycologicum*. Because von Schweinitz had the first validly published reference after the starting-point date (1 January, 1821), some authors use "von Schw." in lieu of "Tode" (Boerema, 1976). After collecting the same fungus from living plants, Desmaziére reclassified it to the genus *Phoma* in 1849 (Williams, 1992). In 1863, Desmaziére also described *Leptosphaeria maculans* (Sjödin, 1989).

Various names have been applied to both phases of this pathogen, as a result of lack of communication among early taxonomists (Gabrielson, 1983). In addition, confusion was promoted by the highly variable conidial morphology. The conidia formed on living tissue are more typical of the species *Phoma* than are the black resistant conidial structures formed on woody host residue. Boerema and van Kesteren (1964) proposed the nomenclature of *Plenodomus lingam* (Tode ex Fr.) Hohn for the imperfect state (Petrie and Vanterpool 1968). However, most current authors use the older name, *Phoma lingam* (Tode ex Fr.) Desm. (Petrie, 1975).

In 1956, H. C. Smith of New Zealand first discovered the sexual stage of *P. lingam*, which he named *Leptosphaeria napi* (Fuckel) Sacc. (Gabrielson, 1983). After confirmation as being congruous with Desmaziére's 1863 description, this name was modified to the currently accepted *Leptosphaeria maculans* (Desm.) Ces. et de Not. (Smith and Sutton, 1964). Synonomy of the pathogen is further discussed elsewhere (Boerema, 1976; Sivaneson, 1984).

1.2.2. Life and Disease Cycles of *Leptosphaeria maculans*.

Blackleg is the most common English name of *Leptosphaeria maculans*. Because all parts of the host are attacked, other descriptive names have been applied, for example, canker and dry rot (Sivaneson, 1984; Evans and Thomas, 1993). In addition to stem-girdling crown cankers (collar necroses), the fungus causes damping-off of seedlings, leaf spots, infected seed stalks and siliques (thus invading seed), as well as root rot (Gabrielson, 1983).

Introduction of the disease into previously uninfected areas occurs when seed colonized by dormant mycelium is sown (Williams, 1992). Both conidia and ascospores can serve as sources of primary inoculum (Gabrielson, 1983). The anamorph stage consists of conidia on infested seed coats, infested crucifer residues or weed hosts; the teleomorph stage consists of pseudothecia on infected host residues. Additionally, the disease survives over winter either actively on crucifer weeds or via dormant mycelium on host residue. Viable conidia have been isolated from overwintered host stubble in Canada (Petrie and Vanterpool, 1968). Survival on woody field residues has been reported after 5 years (Gabrielson, 1983). However, persistence in soil is not long when host residue is absent. Hence, sanitation and reduction of alternate hosts (for example, volunteer canola or wild mustard) is part of routine disease control (Evans and Stevenson, 1993). Since pseudothecia and ascospores only form on parasitized woody material (that is, at the end of the growing season), only conidia act as the "classic vehicle" of secondary inoculum (Gabrielson, 1983).

Under suitable conditions (temperature, radiation, and relative humidity), conidia and pseudothecia produce rainsplash dispersed conidia and ascospores, respectively. Ascospores can be dispersed by wind for several kilometers (Thomas, 1984). Germ tubes from conidia and ascospores enter the host through stomata and wounds. As many *Phoma* spp. are soil-inhabitating fungi, it was speculated erroneously that *L. maculans* had a disease cycle similar to soil inhabiting wilt-producing fungi (Rimmer and van den Berg, 1992). Hammond *et al.* (1985) were the first to demonstrate a direct systemic link between leaf infection and stem cankers. They deduced the following sequence: colonization of leaves from germinating conidia; latent infection of leaves; formation of leaf lesions; systemic biotrophic spread in leaf lamina leading to latent infections of the stem; and, ultimately formation of stem cankers. This sequence was confirmed by subsequent studies (Hammond and Lewis, 1986). Systemic growth of the fungus through the lamina and petiole into the main stem is the major pathway of infection (Hammond *et al.*, 1985). There is, therefore, a correlation between the incidence of leaf lesions and stem infections. This relationship may be exploited as an indicator of disease severity and potential yield loss of a

standing crop. However, direct stem invasion under Saskatchewan field conditions has also been reported (Xi *et al.*, 1991).

Based on ultrastructural studies, Abadie and Boudart (1982) suggested intercellular hyphal growth. They further proposed that the most important feature of subsequent infection was cell wall hydrolysis by fungal polysaccharases allowing mycelial penetration. This event was coincidental with the first visible symptoms. After colonization of the intercellular spaces in the spongy mesophyll of the lamina, hyphae spread down the petiole either via xylem vessels or between cells of the xylem parenchyma and cortex (Hammond *et al.*, 1985). The fungal infection changes from unimpeded biotrophic growth to necrotrophic growth upon reaching stem tissue (Hammond and Lewis, 1987). Death of invaded stem cortical cells produces the stem canker. During canker formation, the periderm and cortical tissues dissociate (Brunin, 1972). Brunin (1972) also observed "intense and anarchic lignification" with ensuing formation of necrotic islands. This lignification was not similar to that of healthy, normal tissue. Lesions or cankers are sunken with a black or purple border at the stem base or the point of leaf attachment (Gugel and Petrie, 1992). Since nutrient and moisture flow are restricted, premature seed ripening occurs (Gugel and Petrie, 1992; Brun and Jacques, 1991). Seeds under pod lesions may be shrunken or shrivelled and are pale gray (Thomas, 1984). Seed viability is affected; the earlier the pod infection, the less likely the seed is to be viable. Girdling of the stem by the canker may result in lodging (Gugel and Petrie, 1992).

Morphological descriptions of the pseudothecial loculoascomycete *L. maculans* and its anamorph, *P. lingam*, have been published (Punithalingam and Holliday, 1972; Gabrielson, 1983; Williams. 1985; Williams, 1992). The mycelia are septate, either pigmented or hyaline. Conidia are single-celled, aseptate, biguttulate, hyaline and shortly cylindrical (1-2 x 3-5 µm) borne in either pseudoparenchymatous (thin-walled) or pseudoschlerenchymatous (thick-walled) pycnidia (200-600 µm diameter). Thus, two pycnidial forms exist. Type I pycnidia are formed in lesions of living tissue, in culture or in recently killed tissue (Williams, 1992). Typically, they possess a papilla (neck) and tend to be uniformly oval. Pycnidia formed on woody plant residues are usually the sclerotiod Type II form (Williams, 1992). Type II pycnidia are black, immersed to erumpent, may not have a papilla, irregularly shaped, and have walls consisting of several layers. Under favorable environmental cues, pinhead-sized pycnidia ooze copious quantities of conidia in a pink or purple mucilaginous matrix (Gugel and Petrie, 1992). Pycnidia are strongly saprophtytic and, therefore, are easily cultured on many different culture media.

Ascospores are more effective as inoculum than conidia (Rimmer and van den Berg, 1992). Ascospores are biseriate, five-septate, guttulate, and cylindrical to ellipsoidal (35-70 x 5-8 µm) with rounded ends. At maturity, they are yellow-tan in colour. Late in the growing season or

in the following year, pseudothecia form on basal stems of the host (Gladders and Musa, 1980). Details of the mechanisms of environmental or substrate conditions triggering the induction of ascospore formation are unknown. Pseudothecia are black, cylindrical (300-500 µm in diameter), immersed to erumpent, osteolate, pseudoschlerenchymatous, and globose, bearing clavate to cylindrical, eight-spored bitunicate asci (80-125 x 15-22 µm). Filiform, septate, hyaline paraphyses may form in association with the ascocarps. Ascospores are released at the time of greatest crop susceptibilty. For example, they are discharged in the fall (September to November) in Europe (Schram and Hoffman, 1991) and in the summer (May to August) in Australia and western Canada (Hall, 1992). Ascopspore release by virulent strains has been observed in Canada as early as May (McGee and Petrie, 1979). Dispersal of airborne ascospores is under environmental influence of temperature (8-12°C) and moisture (high dew or high humidity). Range of ascospore dispersal is from 5 to 8 kilometres (Thomas, 1984), thus ascospore production is necessary for severe outbreak (Gabrielson, 1983).

Mating control of *L. maculans* is known to be bipolar heterothallic (Venn, 1979; Boudart, 1981)—that is, sexual compatibility is controlled by a single diallelic gene (designated by either A/a or +/-). *L. maculans* isolates have been characterized as being weakly virulent (non-aggressive) or highly virulent (aggressive). Strain variability was first detected in New Zealand by Cunningham in 1927 (Badawy *et al.*, 1991). *In vitro* matings of compatible virulent isolates generally produced viable ascospores, even when mated isolates were of widely separated geographic origin (Petrie and Lewis, 1985). Attempts at interstrain mating have not been successful (Gabrielson, 1983). No evidence of interstrain crossing in nature has been reported (Williams, 1992). A protocol for reliable and consistent *in vitro* production of pseudothecia and ascospores has recently been published (Mengistu *et al.*, 1993). This should facilitate genetic characterization of single ascospore isolates.

Partial parasexuality was demonstrated by Petrie (1969) by using complementary auxotrophic mutants on minimal media. No haploidization was observed nor could it be induced chemically. The stable diploids revealed somatic segregation of morphological markers.

In addition to the two major Brassica virulence groups, further host specialization has been reported. The other strains are commonly isolated from cruciferous weeds such as *Thlaspi arvense* L. (stinkweed), *Sisymbrium loeslii*, *Descurania richardsonii*, and *Lepidium* species (Petrie, 1969). Generally, the weed isolates resemble the weakly virulent Brassica strain in morphology and pathogenicity to rapeseed but are more virulent on their weed host. Some crucifer weed isolates have occasionally been found in rape (Petrie and Vanterpool, 1966; 1968). Cruciferous weeds, therefore, potentially provide inoculum for Brassicas(Gugel and Petrie, 1992). Other weed

hosts have been noted to include members from the families Compositae (Asteraceae), Onagraceae, and Gentianaceae (Petrie and Vanterpool, 1968). However, virulence is varied and complex as indicated by several conflicting reports in the literature regarding cross-infectivity of many isolates (Gabrielson, 1983).

Variation in pathogenicity of *L. maculans* has been reported in Canada (McGee and Petrie, 1978), Australia, England (Newman, 1984), and the U.S.A. (Mengistu *et al.*, 1991a,b). Although McGee and Petrie (1978) found no significant differences in pathogenicity between virulent isolates from Australia and North America, Cargeeg and Thurling (1980b) refuted these results. They found a continuous response suggestive of polygenic control in both host and pathogen. They also proposed that one or more virulence genes having relatively large effect may explain the several observed instances of clear specific interaction.

Differential cultivar-pathotype interactions have been used to develop systems of classification of *L. maculans* isolates. Lack of acceptance by researchers of a standard set of differential cultivars has hindered comparisons among studies. However, the four pathogenicity groups (PGs) of Koch *et al.* (1991) based on the cotyledon reactions of *B. napus* cultivars Westar, Quinta, and Glacier has progressively gained wide usage. On the basis of this scheme, all single ascospore isolates from western Canada consistently scored in the PG2 category (infecting Westar but neither Glacier nor Quinta) (Mengistu *et al.*, 1991a,b; Rimmer and van den Berg, 1992; Stringam *et al.*, 1992; Lange, 1993). PG2 isolates have also been reported in northeast North Dakota (Lamey and Hershman, 1993). When west Australian isolates were mated *in vitro* in half diallel crosses, some PG2 isolates showed high fertility when paired with PG3 and PG4 isolates (Mengistu *et al.*, 1993). Since virulent isolates from distant points of origin have been shown to be highly compatible, many Canadian researchers are concerned that aggressivity has the potential to increase in Canadian isolates, particularly if foreign aggressive isolates are inadvertently introduced (Petrie and Lewis, 1985).

Several differential characteristics exist between aggressive and non-aggressive strains. In still liquid culture, all non-aggressive isolates produce a yellow-brown pigment whereas no pigment is formed by aggressive isolates (McGee and Petrie, 1978; Koch *et al.*, 1989). Slow or inhibited growth on various laboratory media, as well as lack of pigment production, are reported for aggressive isolates (Delwiche, 1980). Under controlled growth conditions, differences in conidia germ tube length can be used to distinguish nonaggressive isolates (long germ tube length) from aggressive ones (shorter, unbranched germ tubes) (Petrie, 1988). The characteristics of colony growth rate, conidia germination, and pigment production on modified Czapek's medium are routinely used in standard assays of seedlots for blackleg disease infestation (Kharbanda and Stevens, 1993). In addition, only aggressive strains produce phytotoxins of the sirodesmin family.

Therefore, phomenoic acid isolated from *in vitro* cultured mycelia, which is closely linked to sirodesmin production, is used as a characteristic of aggressive isolates (Balesdent *et al.*, 1992). Further, nonaggressive isolates have an additional fast-migrating band for malate dehydrogenase (EC 1.1.1.37) compared to aggressive isolates (Hill *et al.*, 1984). Also, aggressive isolates rapidly metabolize the Brassica phytoalexin, brassinin, whereas nonaggressive isolates display a much slower rate of brassinin metabolism (Pedras and Taylor, 1993a).

Differences between the two strains have been found in the level of production and isoelectrophoretic banding patterns of certain cell wall-degrading enzymes (Easton and Rossall, 1985; Annis and Goodwin, 1991; Balesdent *et al.*, 1992; Hassan *et al.*, 1991). Generally, high activities of cellulase, α- and β-glucanase, and polygalacturonase were only detected in culture filtrates of aggressive isolates whereas no distinct differences in activities of pectate lyase, protease or lipase were found between aggressive and nonaggressive isolates (Hassan *et al.*, 1991). Further evidence that pectate lyase is not likely to play a significant role includes the fact that it was found at high levels in culture filtrates from both strain types, yet was not extracted from canker material (Easton and Rossall, 1985). On the other hand, high levels of fungal polygalacturonase were found in infected tissue of very susceptible plants, indicating its potential role in canker formation (Easton and Rossall, 1985). Isoelectric focusing of polygalacturonase showed more bands for nonaggressive isolates than for aggressive ones (Annis and Goodwin, 1991). Other degradative enzymes (carboxymethyl cellulase, α-arabinosidase, and β-galactosidase) accumulated to high levels after compatibility between host and pathogen was established (Easton and Rossall, 1985). In SDS-PAGE analysis of LiCl-extractable surface proteins from the mycelia of different *P. lingam* strains, all aggressive isolates shared an obvious 17 kiloDalton (kDa) band and a less prominent 26 kDa band whereas nonaggressive isolates had a 16 kDa band in common (Hassan *et al.*, 1991). Another potential diagnostic distinction lies in the fact that esterase from aggressive isolates have different banding patterns than esterase extracted from nonaggressive isolates (Balesdent *et al.*, 1992). In addition to proteins expressed in normally grown fungus cells, quantitative and qualitative differences in heat shock protein (hsp) profiles of aggressive and nonaggressive isolates, as well as DNA polymorphisms of hsp 70-related genes, have been demonstrated (Chakeraborty *et al.*, 1992). Although calcium is known to play a role in the regulation of several resistance symptoms (Hammond and Lewis, 1986), it was observed to be sequestered by the pathogen in both compatible and incompatible reactions of the *L. maculans*-rapeseed interactions (Tewari and Conn, 1989). Thus, calcium accumulation is induced by both strains.

Monoclonal antibodies (MAbs), prepared from mycelial slurries of aggressive and nonaggressive *L. maculans*, can be used to distinguish the two strains (Stace-Smith *et al.*, 1993).

Although a large number of hybridomas must be laboriously screened to find one expressing adequate specificity, MAbs may be used to distinguish pathotypes in seed mixtures and other plant tissues. This would obviate the necessity to isolate pure cultures from seed being tested for blackleg infestation. Presently, standard seed assay procedures for blackleg are based on the presence of the fungus in cultures derived from the examined seedlot (Kharbanda and Stevens, 1993).

DNA analysis of the two strains also reveal differences. Electrophoretic karyotyping of *L. maculans* on transverse alternating field electrophoresis indicated that aggressive strains had 6-8 distinct bands (genome size 8.6 x 10^6 bp), whereas nonaggressive strains had 12-14 distinct bands (genome 1.6 x 10^7 bp) (Taylor *et al.*, 1991). Similarly, strain differences of karyotype and chromosome gene allocations were found via contour-clamped homogenous electric fields (CHEF) electrophoresis (Morales *et al.*, 1993a). DNA fingerprinting studies using restriction fragment length polymorphism (RFLP) have been performed by several groups (Johnson and Lewis, 1990; Hassan *et al.*, 1991; Koch *et al.*, 1991; Meyer *et al.*, 1992). All researchers report that the two pathotypes are distinguishable from each other, although neither pathotype is completely homologous within its own group. Hassan *et al.* (1991) found a high occurrence of linear plasmid-like DNA molecules in aggressive isolates. Imperfect fungi are known to carry supernumerary dispensable chromosomes (B chromosomes) (Miao *et al.*, 1991).

Application of the polymerase chain reaction (PCR) to DNA analysis further displays differences between the pathotypes. Xue *et al.* (1992) developed PCR primer pairs specific to aggressive (isolate Leroy) and nonaggressive (isolate Unity) isolates. They proposed that an amplified 220 base pair fragment, generated from Leroy-infected canola tissue, could be used to identify the presence of aggressive isolates in plant tissue prior to visible symptoms. Goodwin and Annis (1991) showed via random amplified polymorphic DNA (RAPD) that aggressive and nonaggressive pathotypes isolated from western Canada were distinguishable from each other, and were also distinct to a third nonaggressive pathotype isolated from Ontario.

Sequences of 5.8S rDNA and internal transcribed spacer (ITS) sequences of isolates of *L. maculans* have been compared (Morales *et al.*, 1993b). The 5.8S rDNA sequences of all isolates are identical. Phylogenetic analysis of ITS sequences show that highly virulent isolates are statistically different from weakly virulent isolates.

Because so many differential characteristics have been found between aggressive and nonaggressive pathotypes, many workers have expressed the opinion that the two pathotypes represent distinct species or semispecies, especially in regard to the evidence of major genetic distance between them.

1.2.3. Disease Screening.

Many methods have been used to inoculate and evaluate resistance to *L. maculans* (reviewed by Rimmer and van den Berg, 1992). To be beneficial in a large breeding programme, screening methods must be accurate, fast, and reliably identify resistance that can be confirmed in the field. Unfortunately, definitive methodology for the *L. maculans*-Brassica system has not been developed (Williams, 1992). Since evaluation in infested nurseries is limited to one generation per year with selection efficiency ultimately dependant upon weather-responsive disease development, many programmes use large-scale screening of plant material under controlled growth conditions (Rimmer and van den Berg, 1992), to abbreviate time-consuming selection (Gugel *et al.*, 1990).

The cotyledon assay (De March *et al.*, 1986; Delwiche, 1980; Williams, 1985) has achieved widespread use as test material for *L. maculans*-Brassica interaction. Although this method permits assessment of a large number of plants or isolates, there are conflicting opinions regarding resistance or virulence based solely on results of the cotyledon assay (Rimmer and van den Berg, 1992). Cotyledon assays may assess only a portion of isolate pathogenic potential as only limited plant material is screened (Williams, 1992). Also, potential resistance mechanisms associated with later plant developmental stages, for example, lignification, may not be encompassed by the genetic expression at the cotyledonary stage.

Via the cotyledon assay, Williams and Delwiche (1979) determined simple yet highly heritable genetic control of cotyledon response. This indicated the involvement of different variety-specific resistance factors. Subsequently, Delwiche (1980) reported genetic control by two dominant resistance genes in the cotyledons of varieties tested. Polygenic control of seedling response has been found in other studies (Cargeeg and Thurling, 1980b). Sjödin and Glimelius (1988) proposed that at the less differentiated cotyledonary stage, there would be a higher degree of sensitivity to the disease, as well as fewer morphological differences between accessions, than at later growth stages. Correlations of cotyledon lesions with adult reaction has been divergently reported. Tewari and Conn (1989) found the cotyledon assay inconsistently predicted the reactions at later growth stages. However, Stringam *et al.* (1992) found that resistance assessed at the cotyledon stage persisted to the adult stage. In a comparative study on the effectiveness of inoculation and rating methods, McNabb *et al.* (1993) found the highest precision was with cotyledon inoculation and cotyledon rating. Similar results have been reported (Sjödin and Glimelius, 1988). However, with senescence of cotyledons, ratings tend to coalesce (Zhu *et al.*,

1993), suggesting that timing of assessment after inoculation, as well as testing of additional plant material, would be required for thorough evaluation of all resistance mechanisms (McNabb *et al.*, 1993).

Lines selected as resistant under greenhouse conditions are not always resistant in infested nurseries (Gabrielson, 1983). Reasons for the poor observed correlation have been proposed to include late assessment of early maturing lines (permitting extensive fungal development on senescent plants) (Rimmer and van den Berg, 1992), low levels of field inoculum or infection resulting from climatic response of the pathogen (Cargeeg and Thurling, 1980a), and adult resistance factors not expressed at the time of inoculation (McNabb *et al.*, 1993). However, interpretation of reported results is not consistent among reviewers. For example, Gugel *et al.* (1990) cited studies of Cargeeg and Thurling (1980a,b), Newman and Bailey (1987), and Wittern and Krüger (1985) as indicating a poor correlation. These same studies have been cited elsewhere as examples that resistance assessment in the greenhouse correlates well with observed field ratings (Rimmer and van den Berg, 1992). Other studies corroborate good to strong positive correlation of resistance assessment when using cotyledon evaluation in the greenhouse compared to field evaluation of infected plants (Sawatsky, 1989; Stringam *et al.*, 1992). Hence, "greenhouse screening for blackleg resistance/tolerance is a reliable predictor of field resistance" (Stringam *et al.*, 1992; Bansal *et al.*, 1994).

Various fungal sources have served as inoculum. Cargeeg and Thurling (1980a,b) used mycelium as inoculum stating that cultures often produced inadequate quantities of conidia to be sufficiently effective. Since that study, reliable protocols for conidia culture have been developed which readily produce adequate inoculum (Kolte, 1985). Some workers have used ascospores as inoculum (Cargeeg and Thurling, 1979, 1980a,b; Wittern and Krüger, 1985). However, since *L. maculans* is heterothallic, such inoculum is heteogenous and yields variable results. Inoculation of intact cotyledons has resulted in a great many escapes (reviewed by Rimmer and van den Berg, 1992), hence the inoculation procedure routinely uses wounding prior to application of inoculum (Williams, 1985; De March *et al.*, 1986), which "sharpens the evaluation" (Sjödin and Glimelius, 1988).

Elicitation of host defense responses may be mimicked by nonintrusive abiotic treatments thus obviating use of the parasite. Abiotic elicitors used on Brassica material include silver nitrate, cupric chloride (Rouxel *et al.*, 1990a,b) and mercury chloride (Dahiya and Rimmer, 1989).

Comparison between results from different workers suffers from the lack of an internationally accepted set of test cultivars (Badawy *et al.*, 1991). One set of *B. napus* cultivars often used is Quinta, Glacier, and Westar (Koch *et al.*, 1991; Mengistu *et al.*, 1991). According this scheme, all Manitoba and Saskatchewan ascospore isolates from canola were PG2 (virulent on

Westar, avirulent on Glacier and Quinta) (Mengistu *et al.*, 1991a,b). One PG4 isolate from Canada was scored in this study but it was not isolated from canola. Resistance to PG4 isolates (extremely virulent) was detected only in Brassica species carrying the b-genome.

Pathogenicity on cotyledons is also part of the standard diagnostic procedure in testing seedlots (Kharbanda and Stevens, 1993). In pathogenicity tests, the highly virulent single ascospore isolate, Leroy, is often used as a positive control for the fungus (Stringam *et al.*, 1992) and *B. napus* cv. Westar for the positive susceptible host (Gugel *et al.*, 1990).

Scoring schemes are usually based on the amount of host tissue affected and the presence of conidia. In cotyledon assays, lesion areas are often converted to a scale rating (De March *et al.*, 1986) which may then be converted to percent disease rating (PDRAT) (Gugel *et al.*, 1990). Typically, Brassica species carrying the b-genome produce low PDRAT scores, i.e., are evaluated resistant. As a close relationship exists between incidence of leaf infection in seedlings and incidence of stem lesions at harvest (Gladders and Musa, 1980), field rating systems contain elements for stem infection and for crown canker development. The area under the stem infection progress curve (ASIPC) has been reported to be a reliable measure of the interaction between pathogen, plant, and environment (Xi, 1991; Xi *et al.*, 1991). However, upper stem infection may be a poor indicator of crown symptom severity for individual plants and thus may only be useful as a general indicator of plot infection level (Newman and Bailey, 1987). A high correlation between internal and external crown symptoms has been reported (Newman and Bailey, 1987). Therefore, field evaluations should include assessment of internal symptoms. Plants may not display external stem symptoms (Gugel *et al.*, 1990) despite extensive internal rotting (Xi *et al.*, 1992). Also, several lines of symptomless *B. juncea* are capable of having roots from which *L. maculans* can be isolated (Keri, 1991).

1.2.4. Epidemiology and Control.

Natural epidemics of *L. maculans* follow five sequential phases: (1) latent infection of leaf lamina, (2) leaf lesion expression, (3) symptomless (biotrophic) growth down the petiole, (4) latent infection of the stem, and (5) stem canker development (Hammond and Lewis, 1986). The systemic process by which the fungus enters the stem via the leaf lamina and petiole has been elucidated (Hammond *et al.*, 1985). Colonization can precede appearance of necrotic symptoms in leaves and stems by several weeks. The infection sequence can operate over a very wide

range of temperatures and a long period, from the beginning of rapid stem extension and, to at least the third sequence phase, until leaf nine (Hammond and Lewis, 1986). This is approximately the time of ascospore discharge (Gladders and Musa, 1980).

The biotrophic phase is characterized by 'latent infection' (Hammond and Lewis, 1986; Gugel *et al.*, 1990). This phase may be shortened by warmer temperatures, higher relative humidity, longer photoperiod and injury to the host at the time of infection, but is not affected by age of the host nor inoculum density (Vanniasingham and Gilligan, 1989). As detectable 'latent infection' is influenced by climate and host resistance, field resistance should be assessed more than once in the season (Xi *et al.*, 1991). In addition, *L. maculans* has been isolated from symptomless field plants, suggesting that 'latent infection' may be an intermediary stage.

Factors influencing disease incidence and severity have been reviewed (Hall, 1992). Changes in timing and duration of the early phases of disease infection are ultimately manifested in the extent of incidence and severity of stem cankers. Rapeseed is most susceptible before the six-leaf stage (McGee and Petrie, 1979). Incidence of petiole and stem infection is higher in young plants, with stems most susceptible at cotyledon, one- and two-leaf stage (Hall, 1992). Thus, later infection results in less severe stem lesion, possibly by a shorter duration of the stem lesion phase or some developmentally related stem resistance (Hammond and Lewis, 1986).

Moisture conditions have a major effect on blackleg development. Low incidence of disease has been observed in dry seasons. Warmer weather favours disease development. *L. maculans* grows well from 12 to 18°C, typical for the average temperatures of western Canada and France during the early growth stages of canola (McGee and Petrie, 1979). The presence of anthers and wounding or injury by pests or herbicides increase incidence and severity of stem infection (Hall, 1992).

The inoculum source is a factor affecting disease severity. Conidia germinate more slowly than ascospores (Wittern and Krüger, 1985). Conidia production increases with longer duration of leaf wetness, warm temperatures (20-25°C), and increased light intensity (Vanniasingham and Gilligan, 1989). However, Petrie (1978) considered conidia to be secondary inoculum, via rain splashing. Conidia have not been trapped as airborne spores (McGee, 1977), although this particular study was apparently designed to collect ascospores, not conidia (Hall, 1992). Conidia may serve as primary inoculum over short distances (Barbetti, 1976), although ascospores are the main form of primary inoculum. Ascospore release is typically within a few hours of rain although high dew or high relative humidity may also trigger discharge. Pseudothecia readily form on woody residue of infected plants 1-10 months after harvest and will persist as long as plant residue is present (Hall, 1992). Hence, ascospore discharge is earlier the second year after crop growth (McGee and Petrie 1979). McGee and Petrie (1979) stated that, based on rare incidence

of early discharge in Saskatchewan, the virulent strain of *L. maculans* occurred only infrequently. It is noteworthy that in the intervening fifteen years since that report, the presence of virulent blackleg has dramatically risen, occurring in 96% of surveyed Saskatchewan fields in 1991 (Petrie, 1993a).

Control of the disease falls into two categories, exclusion from uninfected areas and management on infested land. Producers can prevent introducing blackleg by sowing seed that has been tested in an approved laboratory and which has been treated with a recommended fungicide (Evans and Thomas, 1993; Evans and Stevenson, 1993). However, no extant seed treatments prevent seedling infection 15 days after sowing (Kharbanda, 1992). A crop rotation of three years with absolute suppression of volunteer canola, wild mustard, and other cruciferous weeds is the single most effective cultural practice (Gugel and Petrie, 1992). Additionally on infested land, tillage of canola residue as deeply as possible followed by three seasons of shallow tillage or direct seeding is recommended. Canola should not be planted within one kilometre of infested land for three years. Burning of stubble has been used in Australia and England but is not recommended because of air pollution, loss of organic matter in soil, and high potential of losing control of the fire (Gugel and Petrie, 1992). Foliar applications of fungicides have had varied success (Gugel and Petrie, 1992). Xi *et al.* (1991) found that flutriafol was taken up and was present in the plant throughout the growing season yet had little effect on infection early in the season. The development of blackleg resistant canola cultivars is viewed as the ultimate control.

1.2.5. Culture Filtrate and Phytotoxins.

Both culture filtrate and purified phytotoxins have been used in strategies to identify Brassica germplasm resistant to pathogens. For example, lines derived from secondary embryoids of winter rapeseed lines carrying stable resistance to toxins from partially purified culture filtrates of *Alternaria brassicicola* were selected *in vitro* (Newsholme *et al.*, 1989). However, no correlation was found between insensitivity to selection medium and resistance of regenerated plants. *In vitro* application of culture filtrate and phytotoxins from *L. maculans*, as a selective agent for Brassica resistance to blackleg, has been tried by several groups. Results have been contradictory.

Culture filtrate from the aggressive isolate Leroy causes bifacial necrotic lesions on *B. napus* cotyledons (Sacristán, 1982; De March *et al.*, 1986) but is not phytotoxic on roots or hypocotyls (De March *et al.*, 1986). Culture filtrates from aggressive strains are more phytotoxic

than those from nonaggressive strains (Sacristán, 1985; De March *et al.*, 1986; Pedras *et al.*, 1987; Newsholme *et al.*, 1989). However, culture filtrates are generally regarded as nonselective and not host-specific (Badawy and Hoppe, 1989a). Sacristán (1982, 1985) found that, for the purpose of *in vitro* selection, use of toxic culture filtrate was superior to infection and co-cultivation with *P. lingam* as it gave a greater inhibitory effect on seedlings, as well as cell and embryogenic cultures of rape. Furthermore, Sacristán (1985) reported that toxic culture filtrate was efficacious in selection of blackleg tolerant or resistant callus and embryogenic rape cultures which had been mutagenized. The proportion of resistant plants was higher in the selected regenerants, however segregation of resistance was still reported at R_3. Apparently heritable resistance was never achieved by Sacristán (Hill and Williams, 1988). An objection raised against the conclusions reached by that study was the fact that a very low number of regenerants were obtained (Newsholme *et al.*, 1989). In addition, components of culture filtrates may not be implicated in the initial interaction between Brassicas and blackleg fungus. The role of toxic constituents of culture filtrate may come into play after compatibility in the host-pathogen interaction has been established (De March *et al.*, 1986), or may be the result of some synergistic effect of culture filtrate components (but not sirodesmin PL) (Newsholme *et al.*, 1989). Secondary embryoids of *B. napus* ssp. *oleifera* selected on media containing *P. lingam* culture filtrate quickly lost putative resistance when placed on non-selective media (Newsholme *et al.*, 1989). More recently, culture filtrate was used in selective media of microspore-derived haploid embryos which were scored for survival after six to eight weeks (Jedryczka *et al.*, 1991). Doubled regenerant plants screened for resistance to culture filtrate under greenhouse conditions showed either delay of symptom development or hypersensitive reaction, that is, localized islets of necrotic lesions contained at the site of infection.

Phytotoxins are secondary metabolites produced by many microbial pathogens, including *L. maculans*, which disrupt normal, healthy growth of plants. The first report of a metabolite in the filtrate of cultured *L. maculans*, inhibitory to *B. rapa* growth, was by Bousquet *et al.* (1977). Sirodesmin PL, the major metabolite, as well as its deacetyl derivative, were identified in the culture filtrate (Férézou *et al.*, 1977). Structural elucidation revealed sirodesmin PL and deacetylsirodesmin PL were congruous with the family of epipolythiopiperazine-2,5-diones previously isolated from the degradative wood saprophyte, *Sirodesmium diversum* Cooke (Broadbent *et al.*, 1974; Curtis *et al.*, 1977). Sirodesmin PL, identical to a minor metabolite (sirodesmin G) of *S. diversum*, is an epimer of the major *S. diversum* metabolites, sirodesmin A, B, and C (Bu' Lock and Clough, 1992). The biosynthesis of sirodesmins have been elucidated (Férézou *et al.*, 1977; Curtis *et al.*,1977; Bu' Lock and Clough, 1992). Biosynthesis is via rearrangement of an L-tyrosine-L-serine dicyclopeptide derivative with all 9 carbon atoms of

L-tyrosine incorporated into the final structure (Bu' Lock and Clough,1992).

Sirodesmins have been detected in infected host tissue (Boudart, 1978; Badawy and Hoppe, 1989b). Sirodesmin PL is the major component extracted from culture filtrate (50-70% w/w). It is heat sensitive (Sjödin, 1989), and is active at $2x10^{-4}$ M based on cotyledon assay (Pedras *et al.*, 1989). Other minor phytotoxins have been identified: sirodesmins B and C (Badawy and Hoppe, 1989b); sirodesmin H (Pedras *et al.*, 1988); sirodesmins J and K, and 3-(methylthio)phomamide (Pedras *et al.*, 1990); and phomalirazine (Pedras *et al.*, 1989). The sirodesmin phytotoxins have a common skeletal structure, differing in extent of acetylation, deacetylation and/or number of sulphur atoms in the sulphide bridge. Phomalirazine, isolated from the most polar active fraction, constitutes less than 1% (w/w) of the total extract, is active at 10^{-5} M, and is probably an intermediate in sirodesmin biosynthesis. Under certain cultural conditions, nonaggressive (weakly virulent) isolates produce phomaligols and phomaligadiones that are structurally unrelated to sirodesmins (Pedras *et al.*, 1993). Phytotoxins of the blackleg fungus have been reviewed elsewhere (Pedras and Séguin-Swartz, 1992).

Only aggressive strains of *L. maculans* excrete phytotoxic metabolites during *in vitro* culture (Koch *et al.*, 1989; Pedras and Séguin-Swartz, 1992). Aggressive isolates from Canada, England, and France produced identical phytotoxins, mainly sirodesmin PL and deacetylsirodesmin PL (Pedras *et al.*, 1990). Four main metabolites—sirodesmin PL, sirodesmin C, and their deacetyl derivatives—are responsible for the phytotoxic activity of the culture filtrate from three aggressive strains with differential interactions (based on *B. napus* cvs. Quinta and Jet Neuf) (Badawy and Hoppe, 1989a). Extractions from culture filtrate of the weakly virulent isolate, Unity, were not biologically active, contained no TLC- nor HPLC-detectable phytotoxins, and had fatty acids, mainly oleic acid, as major components (Pedras *et al.*, 1990). Likewise, culture filtrates from *Thlaspi* strains did not reveal any phytotoxic metabolites upon extraction (Pedras and Séguin-Swartz, 1992).

A novel chemical signal (a pentadipsipeptide, phomamide) produced only by germinated spores of 20 to 60 hour-old cultures of virulent isolates has been reported (Pedras and Taylor, 1993b). Phomalide appears to be host-selective, causing symptomatic-like leaf lesions on canola (susceptible) but not on mustard (*B. juncea*). Fungal biosynthesis of sirodesmins inhibits production of phomalide. Thus, plant cell damage promoted by phomalide of germinating spores may facilitate rapid fungal invasion and colonization. Additionally, preliminary results of co-cultivation of fungal isolates with microspore-dervied haploid embryos, separated by a fine membrane, indicate a degree of selection, presumably by a diffusable product of fungal origin (Stringam, personal communication). The selection appears to correspond to resistance or

sensitivity of the microspore donor line.

Phomenoic acid and phomenolactone, both of which are moderately antimicrobial and antifungal, have been isolated from mycelia of *L. maculans* after prolonged incubation (Topgi *et al.*, 1987). However, mycelial extracts of virulent isolates usually display no biological activity (Pedras and Séguin-Swartz, 1992). Phomenoic acid is isolated only from strains of *L. maculans* that produce sirodesmin PL (Balesdent *et al.*, 1992).

Confusing results regarding bioactivity of purified phytotoxins have been reported by various workers, but generally, tissue from cruciferous sources are more sensitive than that from non-hosts (Koch *et al.*, 1989; Sjödin *et al.*, 1988; Badawy and Hoppe, 1989a). Sirodesmins are known to inhibit spore germination and mycelial growth of other fungi (Poiret *et al.*, 1985), a fact which may account for its non-host activity. The minimum concentration of sirodesmin PL required to produce lesions on cotyledons and leaves is greater than that of host-specific toxins (Pedras and Séguin-Swartz, 1992). Boudart (1978) reported a hypovirulent mutant that produces high levels of sirodesmin PL, and thus concluded there was no correlation between aggressivity and ability to produce sirodesmin PL. A lack of quantitative difference in response to toxin between two cultivars of *B. napus* (Brutor and Jet Neuf) and *B. juncea* cv. Aurea suggests that resistance to the fungus may not be correlated to resistance to purified sirodesmin PL or its derivatives (Rouxel *et al.*, 1988).

Extreme sensitivity of mesophyll protoplasts from different cruciferous species to sirodesmin PL has been detected at concentrations greater than 1 mM (Pedras and Séguin-Swartz, 1992). The same researchers reported a very narrow window of selection when microspore-derived haploid embryos were assayed for sensitivity to sirodesmin PL and deacetylsirodesmin PL; no survival at concentrations greater than 10 µM, 80-90% survival at 0.01 µM. Using phomalirazine and sirodesmin PL, the viability of *B. napus* cv. Westar (blackleg susceptible) and *B. juncea* cv. Cutlass (blackleg resistant) was greatly reduced in both species at concentrations between 5-10 µM for either compound (Pedras and Séguin-Swartz, 1992). Sjödin (1989) also found the selective concentration range of sirodesmin PL to be narrow, 1-5 µM for cell aggregates and 1-3 µM on intact plants. Sjödin and Glimelius (1989) reported that sirodesmin PL was selective on leaves and cell aggregates at concentrations under 10 µM. However, Pedras and Séguin-Swartz (1992) concluded that results of assays at concentrations less than 20 µM were not statistically significant. Badawy and Hoppe (1989a) concurred with Sjödin *et al.* (1988) that sirodesmin PL was more phytotoxic than deacetylsirodesmin PL whereas no differences in phytotoxicity between these two compounds was found by Pedras *et al.* (1987).

The toxic effects of sirodesmin PL are related to chlorosis and collapse of

parenchymatous tissue (Rouxel *et al.*, 1988). It was earlier proposed that sirodesmin PL contributed to rapid tissue degradation via osmotic disequilibrium (Boudart, 1978). Cell-wall degrading enzymes are known to be involved with necrosis (Boudart, 1978; Easton and Rossal, 1985) but this stage is later than host-pathogen compatibility recognition (De March *et al.*, 1986).

Rouxel *et al.* (1988) found strong and rapid inhibition of ^{14}C-uridine incorporation into RNA of embryogenic *B. napus* tissue occurred when 5.2 µM sirodesmin PL was included in culture medium. Membrane function, cell respiration, as well as DNA and protein synthesis remained unaltered in this study. As active plant defense responses can be regulated by *de novo* RNA and protein synthesis, Rouxel *et al.* (1988) concluded that sirodesmin PL was a potential inhibitor of RNA synthesis, and that such delay or suppression of host defense responses may account for the symptomless biotrophic phase. Antagonism of sirodesmin PL was displayed by IIB series metals (in particular zinc) with partial resistance to blackleg symptoms *in planta* observed following zinc treatment (Rouxel *et al.*, 1990a).

Despite the abundance of conflicting results anent the direct involvement of phytotoxins or culture filtrate constituents in plant disease, the fact remains that a close correlation between aggressiveness and phytotoxin production is irrefutable. This suggests that the phytotoxins produced by *L. maculans* are involved in "disease development" (Koch *et al.*, 1989). Furthermore, non-host selective toxins may facilitate pathogen growth as a result of tissue damage or through the suppression or delay of host defense responses (Badawy and Hoppe, 1989a).

1.3. Brassica Description.

1.3.1. Botany of Brassicas.

"These Brassicas are the most baffling plants."

L.H. Bailey (1922)

The Brassicaceae (or Cruciferae) is a large dicotyledonous family commonly called the mustard family. It is consists of about 3000 species in approximately 375 genera (Blackmore and Tootill, 1984). Its members are predominantly found in northern temperate latitudes. Most are

herbs with alternate leaves. The Brassicas have been well reviewed (Downey *et al.*, 1980; Prakash and Hinata, 1980; Downey and Rakow, 1987; Downey and Röbbelen, 1989) with a well summarized table of Brassica species and related genera in Downey *et al.* (1980).

Four Brassica species are grown world-wide as a source of edible vegetable oil: biennial or winter annual *Brassica napus* in Europe and China, summer annual *B. napus* or *B. rapa* in western Canada, *B. juncea* (rai) in India—some Indian regions also are sown to *B. rapa* (yellow and brown sarsons and toria) , and *B. carinata* in east Africa (Rimmer and van den Berg, 1992). Although the spring form of *B. rapa* tolerates spring frosts and has a relatively short growing season (80-95 days), it is lower yielding in western Canada than is spring *B. napus* (Downey and Rakow, 1987). Hence, slightly more *B. napus* is cultivated in western Canada (De Clerq *et al.*, 1989).

Wild forms of *B. napus* are not documented, but the general consensus is that the species originated in Europe (Downey and Röbbelen, 1989). *B. rapa* has the widest distribution among Brassica oilseeds, having a primary center of origin in Himalaya and secondary centers of Mediterranian Europe and Asia. Asian types differ from their European counterparts in plant morphology and profiles of fatty acids and glucosinolates. However, conspecificity of Indian and European forms is substantiated by protein and chemotaxonomic evidence (Downey and Röbbelen, 1989). *B. juncea* originated in the Middle East where its parent species (*B. rapa* and *B. nigra*) originally coexisted. Because of extensive early trade in black mustard, secondary centers of *B. juncea* origin are found in China, India, and the Caucasus. *B. carinata* arose in northeast Africa (Ethiopia, formerly Abyssinia).

The Brassica inflorescence is a corymbiform raceme of indeterminate flowering, beginning at the lowest bud on the main raceme (Downey *et al.*, 1980). Flowers on secondary branches open 3-5 days after the first flowers on the main raceme. The total flowering period is 2-3 weeks (Downey and Röbbelen, 1989). The regular and radial hypogenous flowers consist of four relatively large, erect and free yellow petals which alternate with four sepals in characteristic cross shape (cruciform) (Blackmore and Tootill, 1984). The floral formula is K2+2, C4, A2+4, G(2) (Downey and Röbbelen, 1989). The stamens are comprised of one lateral pair with shorter filaments and four median stamens with longer filaments. Sutures of the anthers are introrse at the bud stage. Typically, the four long stamens (almost level to the height of the stigma) extrorse after flowering and dehisce outward, whereas the two short stamens (below the stigma) dehisce inward. An exception is yellow sarson, a self-fertile Indian form of *B. rapa*, in which all six stamens remain introrse, dehiscing inward (Downey and Rakow, 1987). Four nectaries are equally spaced between the two groups of stamens—two functional nectaries at the base of the outer, short

stamens, and two nonfunctional nectaries at the base of the pairs of the long stamens.

Flowers are protogynous with the stigma normally receptive three days before to three days after flower opening. Fertilization is usually complete within 24 hours after pollination. The ovary is syncarpous, normally bearing two carpels, although three- and four-valved species exist. After fertilization, the ovary elongates to form a bivalved fruit pod (silique or siliqua) with a longitudinal false septum. Thus, two loculi are formed each bearing a single row of seeds. Since each pod may bear in excess of 25 seeds, the generational multiplication rate of Brassica species is greater than 1000 to 1.

Developing seeds lose their initial green colour, becoming yellow when the embryo is physiologically mature. Depending on genotype, the seed coat (testa) turns black, reddish brown, or remains translucent. Reticulations on the seed coat surface, resulting from different lengths of the palisade cells, are characteristics at the species level. At maturity, seeds are predominantly embryc tissue (85% of seed weight), with a thin endosperm layer immediately under the seed coat.

Fertilization is well adapted for insect vectors although this mode appears ancillary to wind pollination. Autopollination is the primary mode of fertilization in *B. napus* and *B. juncea*, which typically have only 30% outcrossing under western Canadian field conditions (Rakow and Woods, 1987). Sporophytic self-incompatibility, controlled by a series of S alleles, exists in most *B. rapa* strains.

In general, Brassicas follow a similar growth pattern (Downey *et al.*, 1975). Cotyledons are conduplicate (having the larger outer cotyledon folded over the inner one), enclosing a small plumule and are attached to a short radicle. After cotyledon emergence, a rosette of broad leaves is quickly established. Winter forms require long vernalization (40 days of near freezing temperatures). Summer forms continue development from floral primordia without vernalization. The various Brassica species are reliably distinguished by their upper leaf morphology—*B. rapa*, completely clasping; *B. napus*, half clasping; and *B. juncea*, termination of the leaf blade on the petiole.

So-called rapid-cycling Brassicas have been developed (Williams and Hill, 1986; Hill and Williams, 1988). These plants display petit habit with short generation periods (35-50 days).

Bailey's bafflement arose from the fact that similar plant forms exist in more than one Brassica species, which promoted considerable confusion and misclassification by early botanists. This confusion was slowly elucidated by determination of Brassica cytogenetics starting with the pioneering work of Karpechenko of the former USSR and, more often cited in the west, Morinaga and U of Japan (Prakash and Hinata, 1980). Based on cytological evidence, Morinaga (1934)

found that *B. carinata* (n=17,BCbbcc), *B. juncea* (n=18,ABaabb), and *B. napus* (n=19,ACaacc) were amphidiploids arising from crosses of monogenomic diploid species, viz., *B. nigra* (n=8,Bbb), *B. oleracea* (n=9,Ccc), and *B. rapa* (n=10,Aaa) (where n denotes the haploid number of chromosomes, upper case denotes cytoplasm type, and lower case denotes genome type, as in the style of Rimmer and van den Berg, 1992). U (1935) confirmed this proposal by artificially synthesizing *B. napus* via crossing its putative diploid parents, *B. rapa* and *B. oleracea*. Since then, the other amphidiploid species have also been synthesized (Downey *et al.*, 1975). The taxonomic relationship of the Brassicas is often depicted by the classical 'triangle of U', where the diploid elementary species are assigned to apicies of an equilateral triangle and the amphidiploids are assigned between their appropriate parents. Other proofs of the amphidiploid origin have been found based on anatomy, DNA hybridization, and protein profiles (Prakash and Hinata, 1980). The use of random amplified polymorphic DNA (RAPD) technology has recently confirmed the classic triangle description (Demeke *et al.*, 1992). RAPD analysis shows that amphidiploids are located at intermediate positions on the 'triangle of U' relative to their parents, and not exactly at the centre of each side, as is often depicted. In 1950, Mizushima deduced, with subsequent substantiation by Röbbelen in 1960, that the three monogenomic ancestors are secondary polyploid in nature and originated from a now extinct progenitor (basic chromosome number, x=6) (Attia and Röbbelen, 1986).

In addition to production of synthetic amphidiploids, interspecific hybridization of Brassicas is also possible, allowing advantageous incorporation of useful agronomic characteristics. For example, resistance to blackleg from *B. juncea* was transferred to a *B. napus* background via interspecific hybridization (Roy, 1984). The first low erucic acid, low glucosinolate strains of *B. rapa* were derived from interspecific crosses (Downey *et al.*, 1975). Partial homology has been found among the genomes of *Brassica*, *Sinapis*, *Diplotaxis*, *Eruca*, and *Raphanus* (Downey and Rakow, 1987), although RAPD analysis has indicated that *Raphanus* and *Sinapis* are distinct taxa from *Brassica* (Demeke *et al.*, 1992). Previously, merging of these two genera with *Brassica* was suggested based on mutual relationships of chloroplast DNA.

It is interesting to note that the a-genome is common to three economically important oilseed Brassica species (*B. napus*, *B.rapa*, and *B.juncea*) (Downey and Rakow, 1987). Homeologous pairing in amphidiploids (Attia and Röbbellen, 1986) and in trigenomic hybrids ("trigenomic test") (Attia *et al.*, 1987; Busso *et al.*, 1987) has revealed insight into the nature of auto- and allosyndetic pairing between the three monogenomes. Generally, the a- and c-genomes tend to display high allosyndetic pairing (Attia *et al.*, 1987), whereas the b-genome gives indication of less extensive homeology. The b-genome is thus regarded as being more distinct from the other two, despite a common ancestor (Attia and Röbbelen, 1986). Additional evidence for greater

distance of the b-genome has been provided by RAPD analysis (Quiros *et al.*, 1991; Demeke *et al.*, 1992). No genetic suppression factor influencing allosyndesis was found in the b-genome (Attia *et al.*, 1987; Busso *et al.*, 1987), nor was cytoplasmic regulation of a- and c-genome homeologous pairing observed (Busso *et al.*, 1987). This lower level of homeologous pairing for the b-genome impinges upon attempts to transfer characteristics resident on the b-genome via interspecific crosses. Nevertheless, blackleg resistance from *B. nigra* was transferred into *B. napus* by interspecific hybridization with *B. juncea* (Sacristán and Gerdemann, 1986).

1.3.2. Brassica Resistance of Blackleg.

Before incorporation of disease resistance into a plant breeding programme or strategy, extant sources must be identified via a survey of the species and related germplasm. Subsequently, the identified resistance must be evaluated for suitability. Sources of blackleg resistance within the Brassica species have been identified and evaluated (Gabrielson, 1983; Thompson, 1983; Kolte, 1985; Rimmer and van den Berg, 1992).

Among summer cultivars of *B. napus*, good field resistance was found in cultivars from Japan, Australia, and Europe (Rimmer and van den Berg, 1992), as well as the French cultivars Bienvenue (Rouxel *et al.*, 1990b) and Crésor (Sawatsky, 1989; Gugel *et al.*, 1990). Among winter cultivars of *B. napus*, the French cultivar Jet Neuf has consistently displayed good field resistance at the adult stage. Other European sources are cultivars Rafal, Ramses, Major, and Tamara (Petrie, 1975; Roy and Reeves, 1975). Intermediate resistance has been found in cultivars Primor and Kapora. Generally, Australian winter cultivars show good resistance to endemic blackleg isolates.

No genotypes of *B. rapa* display high levels of resistance. Often these cultivars are more susceptible to blackleg than are *B. napus* cultivars (Rimmer and van den Berg, 1992). No resistance has been found among *B. oleracea* cultivars (Mithen *et al.*, 1987; Sjödin and Glimelius, 1988).

Other species comprising the 'triangle of U' have also been surveyed for resistance. Generally, Brassica species with the b-genome possess a hypersensitive type response to blackleg (Rimmer and van den Berg, 1992). *B. nigra* is highly resistant (Sacristán and Gerdemann, 1986). The amphidiploid species with the b-genome (*B. carinata* and *B. juncea*) have been stated to have absolute and stable resistance that is effective from the seedling stage onwards (Sacristán

and Gerdemann, 1986). However, examples of b-genome susceptible species have been found (Williams, 1992).

Resistant germplasm from wild Brassica relatives has also been identified. For example, a Sardinian accession of *B. insularis* Morris (2n=18), a wild relative found in the Mediterranean basin, gives a hypersensitive response, in both seedling and adult stages, to highly aggressive *L. maculans* isolates (Mithen and Lewis, 1988). This resistance may be a different type than that associated with the b-genome Brassica species, as *B. insularis* has the c-genome (Mithen *et al.*, 1987). In a cross of the *B. insularis* accession with the highly susceptible *B. oleracea* var. *alboglabra* (Chinese kale), F_1 and F_2 progeny analysis indicated two dominant, independently segregating genes (Mithen and Lewis, 1988).

Blackleg resistance is a heritable and a selectable character (Rimmer and van den Berg, 1992). For example, Jet Neuf was developed through a French pedigree breeding programme initiated after a blackleg epidemic in France (1966-1967) (Thompson, 1983). The genetic basis of resistance in Jet Neuf, which has Lemke material in its pedigree, may have a common component to the partial resistance observed in cultivars Garant, Norli, and Quinta (Newman, 1984). In host differential tests of English *L. maculans* isolates, the latter three cultivars (developed by Hans-George Lemke K.G., Post Holtsee, Germany) scored comparably to a breeding line derived from Jet Neuf. Between 1980 and 1987, over 80% of oilseed Brassica acreage in Europe was seeded to Jet Neuf (Newman, 1980; Badawy *et al.*, 1991).

Consistent exposure of this resistance, derived from a single source, has raised the concern that such pressure may affect changes in the pathotype of *L. maculans* through inadvertent selection. Badawy *et al.* (1991) found a pathogenicity group of isolates, A2, which produced high disease scores on Jet Neuf hypocotyls. Thus, selection for A2 strains in areas where Jet Neuf is extensively grown is a distinct concern. However, the efficacy of Jet Neuf resistance in Europe remains undiminished to date (Rimmer and van den Berg, 1992). Furthermore, although virulent blackleg has been active in Canada for twenty years, all isolates remain within one pathogenicity group (Stringam *et al.*, 1992). Nevertheless, it is important to develop a broad genetic base of resistance in oil quality Brassicas.

The inheritance of resistance from several sources has been studied. Cargeeg and Thurling (1980a) found continuous variation in seedling resistance between and within *B. napus* cultivars. Thus, they concluded that at least a major portion of the resistance was under polygenic control. However, although polygenic control is often associated with horizontal resistance, they stated that the *B. napus–L. maculans* relationship was not truly horizontal. This was based on the great variability they observed in the severity of disease damage to host Brassicas by different *L.*

maculans isolates. Cargeeg (1980) performed diallel analysis on 17 lines of *B. napus* that showed response variation to infection when grown in the greenhouse and the field. From this study, she conceded that major genes could be operating in the resistance tested. Delwiche (1980) found two different dominant genes operating in B. napus seedling resistance. One (*Lm1*) was found in a French breeding line, while the other (*Lm2*), was found in cultivar Girta. Recombination was 22.9 ± 2.3%, indicating linkage.

In 1984, Roy referred to the available resistance in *B. napus* at that time, as a gene complex conferring only partial resistance, and that it was most likely under polygenic control. This source was European and Japanese in origin. Previously, Roy and Reeves (1975) found a single major resistance gene in cultivar Major, which Roy incorporated into the Australian cultivar Wesreo (Roy, 1978b).

Sawatsky (1989) determined the cotyledon resistance of two summer *napus* lines was controlled by a single recessive gene. On the other hand, Sawatsky (1989) determined that resistance of adult progeny was controlled by two dominant genes, *Bl-1* and *Bl-2*, by crossing two other summer rape lines with the susceptible cultivar, Regent. Dominant alleles at both loci resulted in high resistance whereas intermediate resistance was expressed when one locus was occupied by a dominant gene and the other locus recessive. Stringam *et al.* (1992) found cotyledon resistance of Australian cultivars Shiralee and Maluka to be controlled by a single dominant gene, based on results of F_1 derived doubled haploid progeny from crosses of resistant parent (either Shiralee or Maluka) with a susceptible parent (University of Alberta germplasm). Rimmer and Mengistu (unpublished data, cited in Rimmer and van den Berg, 1992) determined that two dominant genes were operating in the *B. napus* hosts tested in differential screening of *L. maculans* isolates. They concluded that cultivar Glacier may have one R gene in common with either of the two R genes in cultivar Quinta. Using synthetic lines of *B. napus* developed by crossing *B. rapa* with several wild members of the *B. oleracea* complex (all with the c-genome) followed by scoring lesion size in F_2 progeny, Mithen and Magrath (1992) suggested polygenic control of this resistance to foliar infection.

The resistance of *B. juncea* was reported to be absolute and durable (Roy, 1978a; Sacristán and Gerdemann, 1986), although Sjödin and Glimelius (1988) found several *juncea* accessions to be susceptible. Roy (1984) transferred *juncea*-type resistance into a *B. napus* background via interspecific crosses. Based on progeny analysis to F_3, he reported that the *juncea*-type resistance behaved like major gene(s), with simple inheritance. Keri (1991) found good seedling resistance in *B. juncea*, which was determined to be controlled by two genes with dominant recessive epistatic action (Rimmer and van den Berg, 1992), thus indicating *B. juncea*-type resistance was not absolute.

B. nigra displays a high level of resistance effective at all developmental stages (Zhu *et al.*, 1993). Using *B. napus-B. nigra* addition lines, Zhu *et al.* (1993) studied the effect of single b-genome chromosomes on resistance. They concluded that at least three different chromosomes contribute to b-genome *B. nigra* resistance, indicative of polygenic control.

In efforts to introduce blackleg resistance from related species into oil quality Brassica cultivars, hybrids from interspecific crosses (*B. juncea* x *B. napus* and *B. carinata* x *B. napus*) have been developed and used in breeding strategies. To overcome infertility in F_1 and later generations, Roy (1978a) either carefully selected compatible genotypes or (1984), used embryo rescue after fertilization. Roy (1978a) observed that the F_1 population showed blackleg lesions on cotyledons, but all survived, and all adults were canker free. In 80-90% of the F_1 progeny of *B. juncea* x *B. napus*, tumors on the roots and crown region were observed. This condition was diagnosed as "clubroot" (*Plasmodiophora brassicae*) infection, although clubroot was not endemic to Australia at that time. Based on progeny analysis of F_2 and F_3, he concluded that complete resistance (expressed in seedling and adult) was carried on the b-genome while adult resistance was associated with the a-genome. Although Roy reported normal chromosome numbers (2n=38) in his advanced lines (F_6-F_7), they were still segregating for disease resistance, suggestive of aneuploidy. Subsequently, Rimmer and van den Berg (1992) found most of this material to have 39 or 40 chromosomes.

Sacristán and Gerdemann (1986) have also used *B. juncea* and *B. carinata* as sources of resistance in interspecific hybridizations, which were further developed through a compact programme of backcrossing and single parent selection. The *B. juncea* hybrids transferred resistance to about 50% of the progeny of the first BC, some with higher level resistance than Jet Neuf (only with *B. juncea* donors). The *B. carinata* hybrids lost resistance after the initial BC. This suggests either some of the resistance genes in *B. juncea* were resident in the a-genome, or that chromosome pairing had occurred, with introgression of the b-genome resistance elements more favourable in *B. juncea* crosses (aabc), as opposed to *B. carinata* crosses (abcc). That is, the b-genome may recombine more frequently with the c-genome than with the a-genome. Also, Sacristán and Gerdemann (1986) observed tumors in roots and crowns of *B. juncea* interhybrids. These tumors were determined to be genetic in origin and not a result of clubroot infection.

Euploid lines derived from the BC_5 generation after *B. napus* x *B. nigra* with recurrent backcrossing to *B. napus* cultivar Andor, were found to have the same level of resistance as Jet Neuf, based on cotyledon assay and mature plant response to hypocotyl inoculation (Zhu *et al.*, 1993). Meiotic analysis of metaphase I pollen mother cells (PMCs) revealed that 98% of the PMCs had 19 bivalents. This lack of univalents suggests that recombination or translocation between the b-genome of *B. nigra* and the ac-genome of *B. napus* had occurred soon after hybridization. Partial

pairing of the b-genome with the a- and c-genomes has been cytologically substantiated elsewhere (Attia and Röbbelen, 1986).

Another strategy to introduce interspecific resistant material into *B. napus* was through the development of somatic hybrids (Glimelius *et al.*, 1991). Somatic hybridization is a method whereby infertility and lack of seed set often encountered in sexual crosses between related species may be partially overcome. The fusion of *B. napus* and *B. nigra* protoplasts has produced a novel somatic hybrid, called *B. naponigra* (Sjödin and Glimelius, 1989). Regenerated hybrid plants that had the anticipated 54 chromosomes were reported to have resistance to blackleg, as assessed in adult leaves and stem bases. Asymmetric somatic hybridization may prove useful to transfer portions of donor genomes into target species. Through asymmetric hybridization, hygromycin resistance incorporated into *B. nigra* was transferred to *B. napus* (Sacristán *et al.*, 1989). Thus, introduction of traits from a b-genome donor into a *napus* background is possible through this method.

The exact mechanisms of resistance have not yet been determined. Environmental conditions readily influence resistance, making assessment difficult. For example, in the greenhouse, temperature influences resistance, whereas in the field, resistance comes under the influence of the rate of plant development, which in turn, is subject to the climate of the particular growing season. However, general mechanisms of resistance have been elucidated.

Brunin (1972) found that resistance was associated with the rapid formation of a thick, corky layer around resistant stem, as well as the differentiation of lignified fibres outside the vascular cylinder. These lignified vessels impeded fungal invasion into the medullary rays, thus offering a non-specific "mechanical resistance". This morphological barrier appeared to be more effective in older seedlings. In very young seedlings, the lesions were often progressive and lethal. Hammond and Lewis (1987) also noted lignification as the first observable event associated with mechanical confinement of fungal ingress. With some host-pathogen combinations, they observed that the accumulation of lignin polymers was associated with the gradual slowing of lesion spread, leading eventually to lesion containment. This phenomenon has been observed in many other host-pathogen interactions. It may also be related to the inherent antifungal properties of polyphenols. When containment occurred, Hammond and Lewis (1987) always observed the development of new cambium with concomitant callose deposition at the periphery of the lignified zone. In addition, they detected the accumulation of calcium in association with containment.

Plant development and morphology may account for field resistance. Cultivars with rapid leaf development may reduce the incidence of infection (Rimmer and van den Berg, 1992), particularly when the cultivar is known to possess leaf resistance. Using two winter *B. napus* cultivars, Elvira (susceptible) and Jet Neuf (resistant), Hammond and Lewis (1986) studied field

resistance. The difference in field performance was a result of lower incidence of leaf infection in Jet Neuf, ultimately manifested as less stem canker development. Thus, in this study, field resistance was associated with the formation of morphological barriers that restricted systemic infection, in this study.

In the field, *L. maculans* has a prolonged period of latent infection on the resistant *B. napus* cultivar, Crésor (Xi *et al.*, 1991). Therefore, field resistance may be promoted by delayed disease development resulting in reduced disease severity at the end of the season. In this study, host resistance did not affect the incidence of infection.

Resistance may be associated with plant natural products, either constitutive or induced upon pathogenic challenge. Glucosinolates have been suspected as having a role in resistance. However, they do not appear to be directly involved in resistance to *L. maculans*, but rather, are more likely involved in Brassica defense of animal pests (Mithen and Magrath, 1992). Phytoalexins (section 1.3.3.) and defense-response proteins (section 1.4.1) are both pathogenically induced.

In summation, several different resistance sources are available to exploit. The resistance operative in seedlings may differ from that of mature plants. Resistance has been described from each of the three monogenome types. Using various technologies, cultivar development may access resistance identified in other species and potentially, other genera.

1.3.3. Phytoalexins.

Phytoalexins are antimicrobial compounds of low molecular weight synthesized and/or accumulated by plants after exposure to microorganisms (Paxton, 1981). Absent in healthy plants, phytoalexins accumulate at the site of infection (Darvill and Albersheim, 1984). The rate of *de novo* synthesis of phytoalexins is under transcriptional control of the induced mRNAs which encode the enzymes leading to phytoalexin synthesis (Darvill and Albersheim, 1984). The majority of plants from which phytoalexins have been isolated are dicots, although some monocots (oats, sugarcane, and sorghum) form various phenols with antimicrobial properties (Nicholson and Hammerdschmidt, 1992). Phytoalexins are extensively reviewed (for example, Darvill and Albersheim, 1984; Keen, 1990b).

Phytoalexins are indiscriminate in their action, requiring almost millimolar concentrations to inhibit microbes (Darvill and Albersheim, 1984). However, they are concentrated at the site of infection, the site of their greatest efficacy. At physiological concentrations, phytoalexin action on

microbes is often static rather than toxic. It has been proposed that phytoalexins act by a general mechanism of "membrane perturbation", causing dysfunction of all cell types (Darvill and Albersheim, 1984). Although phytoalexins are important in plant disease resistance, they are not always effective plant protectants. Furthermore, although a pathogen may be able to tolerate a phytoalexin, this ability alone may not be sufficient to render it pathogenic. For example, some isolates of *Nectria haematococca* are able to demethylate pisatin, a phytoalexin of pea (*Phaseolus vulgaris* L.). Though highly tolerant of pisatin, these isolates are unable to cause significant disease symptoms in peas (Darvill and Albersheim, 1984).

Molecules that signal plants to synthesize phytoalexins, descriptively referred to as elicitors, may be of biotic or abiotic origin (Darvill and Albersheim, 1984). Biotic elicitors are generally constituents derived from microbial sources. Several types have been identified and include glycoproteins, water-soluble glucans, eicosapentanoic and arachadonic acids, chitosan (a nonacetylated β-4-linked D-glucosamine polymer), and microbial enzymes (e.g., endopolygalacturonase and endopolygalacturonic acid lyase) (Darvill and Albersheim, 1984). The most often encountered examples of abiotic elicitors are various heavy metals, e.g., $AgNO_3$ and $CuCl_2$. Others include UV light, autoclaved ribonuclease, chloroform, and detergents.

Phytoalexins have been identified from cruciferous sources (reviewed by Pedras and Séguin-Swartz, 1992; Gross, 1993). Challenge of crucifers by either phytopathogens or abiotic elicitors results in the induction of "biogenetically-related" phytoalexins, which are characteristic of the crucifer source species (Gross, 1993). Typically, these compounds are comprised of an indole ring, variously altered at positions 3 and/or 2 with nitrogen- or sulphur-containing substituents (Gross, 1993). Indeed, the first reported source of sulphur-containing indole phytoalexins (namely brassinin, methoxybrassinin, and cyclobrassinin) was from a Brassica source (*Brassica pekiniensis*) (Takasugi *et al.*, 1988). Elicitation of phytoalexins by fungal challenge of crucifers was first reported by Conn *et al.* (1988).

Brassica phytoalexins may have a role in protecting plants from invasion by *L. maculans*. For example, when present in culture medium, brassinin inhibits the biosynthesis of sirodesmins (Pedras and Taylor, 1993a).

Dahiya and Rimmer (1989) found that resistant germlines of Brassica species accumulated more phytoalexins than susceptible lines. Using various Brassica species (*B. napus*, *B. rapa*, and *B. juncea*), they found that aggressive isolates of *L. maculans* induced only cyclobrassinin whereas non-aggressive isolates induced both methoxybrassinin and cyclobrassinin. Results obtained by co-inoculation with aggressive and non-aggressive isolates indicate that aggressive isolates are apparently able to suppress induction of methoxybrassinin synthesis (Dahiya and Rimmer, 1988a). The level of cyclobrassinin in inoculated *B. napus* leaf and

stems increased, commencing six days after inoculation, to a maximum at twelve days (Dahiya and Rimmer, 1988b). Declining levels of cyclobrassinin after twelve days were attributed to metabolism by the host. In the same study, methoxybrassinin attained a constant maximal level ten days after inoculation. In addition, Dahiya and Rimmer (1989) found effective phytoalexin elicitation in leaves by $AgNO_3$ (10^{-3} M) and with $HgCl_2$ in stems. These treatments resulted in phytoalexin profiles comparable to infection with *L. maculans*. The same workers described an HPLC-based method for rapid purification and quantification of methoxybrassinin and cyclobrassinin with greater sensitivity (limit 0.1 µg) than that attainable through TLC-based *Cladosporium* bioassays (Dahiya and Rimmer, 1988b).

The hypersensitive response of *B. juncea* to *L. maculans* was proposed to be related to the synthesis of brassilexin, a sulphur-containing indole compound isolated from elicited leaves (30 mg per kg fresh weight), which is absent in healthy leaves (Devys *et al.*, 1988; Rouxel *et al.*, 1989; Rouxel *et al.*, 1990b). Accumulation of brassilexin was strongly inhibited by treatment with a protein synthesis inhibitor (cycloheximide) prior to elicitation. Thus, brassilexin production appears to depend upon *de novo* synthesis of enzymes in response to elicitor challenge (Rouxel *et al.*, 1989). Both *B. napus* (cv. Brutor) and *B. juncea* (cv. Aurea) produced brassilexin in response to non-specific elicitation by $CuCl_2$. However, the *B. juncea* cultivar (hypersensitive) responded faster and produced more brassilexin than the *B. napus* cultivar (susceptible). Rouxel *et al.* (1991) found a high correlation between the ability to accumulate brassilexin and disease resistance, and additionally, a high correlation between those two variables and the presence of the b-genome in the host species. No susceptible plant accumulated brassilexin to levels comparable to the majority of lines possessing the b-genome. However, Rouxel *et al.* (1991) noted some exceptions, namely (1), some lines having the b-genome showed a hypersensitive response yet accumulated brassilexin only to low levels, and (2), a *B. rapa* line (*i.e.*, no b-genome) that gave consistent hypersensitive response upon $CuCl_2$ treatment but accumulated low amounts of brassilexin. Thus, Rouxel *et al.* (1991) concluded that the ability to accumulate phytoalexins was not the main determinant for resistance and that the relationship between the b-genome and hypersensitivity to *L. maculans* was not absolute. Since the *B. rapa* line in question was found to segregate for resistance, they concluded that the genetic factors determining its resistance lay outside the b-genome, possibly on the a-genome.

The major phytoalexin(s) produced by a Brassica species is indicative of its genome constitution. Those with the c-genome produce methoxybrassinin and cyclobrassinin. whereas the latter is the major phytoalexin in a-genome species (Rouxel *et al.*, 1991). When the b-genome is present, brassilexin and cyclobrassinin sulphoxide are the main phytoalexins produced. Thus, in terms of phytoalexin formation, genomic interrelationship between the a- and c-genomes appears

closer than either to the b-genome. This concurs with conclusions based from cytological analysis (Prakash and Hinata, 1980). *Sinapis alba* and *Raphanus sativa*, very close Brassica relatives, developed hypersensitive responses that resembled those of resistant Brassicas (Rouxel *et al.*, 1991). However, none of the Brassica indole phytoalexins were elicited. Compounds closely related to the indole phytoalexins were found in elicited *Camelina sativa* and *Capsella bursa-pastoris*, both of which are weed relatives of Brassica (Conn *et al.*, 1988) .

Field resistance of Brassica oilseed cultivars does not appear to be expressed via higher levels of brassilexin production (Rouxel *et al.*, 1990b) since *B. napus* cultivars Jet Neuf (high adult resistance) and Bienvenue (moderate adult resistance) both accumulate the equivalent amount of brassilexin as the susceptible cultivars, Brutor and Primor.

In summation, both resistant and susceptible plants have the ability to synthesize phytoalexins although the timing and rate of production differs. Also, the accumulation of phytoalexins is probably not the major factor determining resistance of Brassica species to *L. maculans* (Rouxel *et al.*, 1991).

1.4. Plant Defense Responses.

There is an extensive literature regarding plant defense responses. Because this body of knowledge is continually expanding, reviews appear frequently (for example, Collinge and Slusarenko, 1987; Dixon and Harrison, 1990; Keen, 1992). Responses fall into two broad categories, static and dynamic (Oku, 1992). Static responses are related to characteristics present in healthy, uninfected plants. Examples include hardness and thickness of cuticle and the level of constitutive expression of inhibitors to cell wall degrading enzymes. Usually, these preformed characteristics are governed by horizontal resistance genes. On the other hand, dynamic plant responses are activated by pathogenic challenge. As such, they are mediated by the activation of vertical resistance genes not normally expressed in healthy tissue. Examples of dynamic resistance are formation of papillae, cell wall modification, hypersensitive response, induction of phytoalexins by exogenous or endogenous elicitors (Dixon and Harrison, 1990), rapid lignification (Ride, 1983), and formation of a suite of *de novo* proteins (see section 1.4.2.).

In order for pathogens to successfully infect their hosts, two general strategies are observed. Necrotrophic pathogens are unspecialized "thugs" (terminology of Keen, 1986) that

induce extensive host damage through brute force, mediated by enzymes or toxins. Since pathogens using this strategy cause extensive host response, they rely on mechanisms that avoid or overcome host defenses. Biotrophs, or "con men", are susceptible to host defense responses, hence, they stealthily elude host recognition mechanisms while parasitizing their host.

Differential responses of flax (*Linum usitatissimum*) cultivars to distinct pathogen races of the rust (*Melampsora lini*) led to the "gene-for gene" hypothesis, proposed by Flor in 1947 (reviewed by Ellingboe, 1981; Keen, 1990a). This hypothesis predicts the outcome of interaction based on the nature of "corresponding" genes in the host and the pathogen. An incompatible interaction (pathogen avirulent, host resistant, both determined by dominant genes) results in active resistance of gene-encoded functions in the host that suppresses or contains virulence gene-encoded products arising from the pathogen. This result is proposed to be epistatic for any gene combination when multiple resistance and virulence genes are involved. The compatible interaction (pathogen virulent, host susceptible, both recessive genes) predicts that the pathogen overcomes the host defense response, with ensuing disease development. Rimmer and Mengistu (unpublished, cited in Rimmer and van den Berg, 1992) found preliminary evidence that supported a gene-for-gene model consistent with two avirulence genes in *L. maculans* and two resistance genes in *B. napus*.

Host recognition factors are postulated to be constitutively produced surface molecules (de Wit, 1986). Lectins have been proposed to be likely candidates, particularly in bacteria-host interactions. However, few indications exist for a role of lectins in fungus-host interactions. Conidia of *P. lingam* isolates from western Canada (all PG2) failed to agglutinate when suspended in lectin solutions (Lange, 1993). Thus, lectins are likely not involved in Brassica recognition of the blackleg fungus.

1.4.1. Study of Defense Responses.

As host defense responses have been detected within minutes of elicitation, signal transduction has been a major focus of research (reviewed by Lamb *et al.*, 1989; Dixon and Lamb, 1990). Proposed signals include pectic fragments from cell walls, electrical potentials (Dixon and Lamb, 1990), plant hormones (jasmonic acid and abscisic acid), systemin and enzymes that process the systemin precursor (Gundlach *et al.*, 1992; McGurl *et al.*, 1992; Pautot *et al.*, 1993). Signal transduction may involve the action of protein kinases (Oku, 1992). For example, the *Pto* gene in tomato confers resistance to the bacterial pathogen, *Pseudomonas syringae* pathovar

tomato (Martin *et al.*, 1993). The deduced amino acid sequence of the *Pto* gene product bears similarity to published sequences of plant serine-threonine protein kinases. Results of pulse-labelling experiments using ^{32}P-phosphate, with and without protein kinase inhibitors, showed that kinases are essential for signal transduction in elicitor-treated plant cells (Felix *et al.*, 1991). Furthermore, continuous phosphorylation of proteins by kinases is required to maintain the elicited state.

Molecular biology techniques have been applied to several host-pathogen systems in efforts to determine the molecular genetic basis of plant resistance. Several strategies have been proposed to achieve this goal—for example, shotgun gene cloning, transposon tagging, and linkage to genetic maps derived from DNA polymorphic markers (such as restriction fragment length polymorphic, or RFLP, markers) (reviewed by Ellis *et al.*, 1988; Dixon and Harrison, 1990; Michelmore *et al.*, 1992). Each of these strategies has inherent drawbacks and complications, all of which are related to the fact that the exact nature and mechanism of resistance is unknown. Nevertheless, the *HM1* gene in maize (*Zea mays* L.) was identified as a disease resistance gene, through combined transposon tagging and linkage to RFLP markers (Johal and Briggs, 1992). *HM1* encodes an NADP-dependent reductase that inactivates HC toxin, a compatibility factor that permits infection of maize by the fungus *Cochliobolus carbonum* Nelson race 1.

Other approaches identify genes with expression related to plant defense responses, but not necessarily resistance *per se*. Further characterization is required to determine the function of the genes identified. For example, Fristensky *et al.* (1985) identified three groups of defense related genes in pea (*Pisum sativum* L.), categorized according to response kinetics. Another common initial strategy is to construct cDNA libraries from mRNA populations after infection or elicitation, and screen with antibodies raised to known enzymes or proteins (Dixon and Harrison, 1990).

The cruciferous weed, *Arabidopsis thaliana*, has several characteristics that render it suitable as a model for molecular dissection (Dixon and Harrison, 1990; Dean, 1993). It has one of the smallest genome sizes of any angiosperm (n = about 100 Mb), and has very low content of repetitive DNA. Transformation procedures for Arabidopsis are well established. Also, RFLP maps have been published. Cloning of genes from Arabidopsis is further facilitated by well-characterized mutants. For example, via a genomic subtraction technique utilizing a gibberelin-responsive mutant, the Arabidopsis *GA1* locus was identified (Sun *et al.*, 1992). Subtractive techniques rely on populations expressing strong positive/negative response to a stimulus. As some Arabidopsis genotypes are susceptible to *L. maculans* (Sjödin and Glimelius, 1988) and other pathogens (Davis, 1992), it is possible that subtractive techniques could reveal

genes conferring disease resistance. Furthermore, some Arabidopsis genotypes have disease resistance loci that are potentially functional homologues of bean and pea (Dangl *et al.*, 1992). Therefore, disease resistance genes identified in Arabidopsis may be conserved in other more agronomically important plants. Evidence supporting the conservation in Arabidopsis of elicitor-responsive genes from parsley (*Petroselinum crispum*) has been reported (Trezzini *et al.*, 1993).

Arabidopsis mutants have been used in an effort to delineate phytoalexin biosynthesis (Tsuji *et al.*, 1993). The Brassica phytoalexin, camalexin, has been isolated from elicitor treated Arabidopsis (Tsuji *et al.*, 1992). Camalexin is an indole glucosinolate, initially isolated from *Camelina sativa* and is potentially related to the Brassica phytoalexins (Gross, 1993). Although camalexin is structurally similar to tryptophan, evidence supports anthranilate as the intermediate.

1.4.2. Defense Response Proteins.

Pathogenesis-related (PR or b-) proteins are a group of host-encoded, inducible proteins whose synthesis is often associated with resistance to pathogens (Carr and Klessig, 1989). The nomenclature was developed from tobacco, where PR proteins were first detected in response to tobacco mosaic virus (Van Loon *et al.*, 1985). They were operationally classified as acid-soluble proteins with low molecular weight, extreme isoelectric points, extracellular localization, and resistance to proteolytic degradation (Sommisch *et al.*, 1986). Different families share common serological cross-reactivity and amino acid sequence homology. PR proteins have been found in several other plant species and were categorized according to similarity to tobacco PR proteins. Some PR protein groups have been functionally identified—PR2 proteins are β-1,3-glucanases, while PR3 proteins are group I and II chitinases. PR proteins have been well reviewed (Carr and Klessig, 1989; Linthorst, 1991; Ohashi and Ohshima, 1992).

PR proteins are sometimes referred to by other names which more accurately portray their nature, for example, disease resistance response proteins (Fristensky *et al.*, 1988). cDNA clones for genes of many plant defense response proteins have been found (Dixon, 1992).

Some defense related proteins display interesting and unusual characteristics. A 17 kDa inducible protein, similar to PR1, but lacking the extracellular signal peptides of other PR1 proteins (Walter *et al.*, 1990) has been found in potato (*Solanum tuberosum*) (Brisson *et al.*, 1994). Protein

homologues have been found in bean (Walter *et al.*, 1990), pea (Fristensky *et al.*, 1988), parsley (Somissich *et al.*, 1986), tomato, cowpea, maize, and barley (Walter *et al.*, 1990). In addition, a major pollen allergen from birch was also found to share significant homology (Walter *et al.*, 1990). Although the function of this ubiquitously conserved protein remains undetermined, evidence suggests it plays a role in signal transduction (Brisson *et al.*, 1994).

The early determination of the PR2 family as chitinases has led to extensive investigation regarding the role of this enzyme in plant defense response, in particular to fungal pathogens. Various isoforms of chitinase exist which display differential induction and compartmentalization (Rasmussen *et al.*, 1992a,b; reviewed by Broglie and Broglie, 1993). Plant endochitinases hydrolyze the internal β-1,4-glycosidic linkage of chitin (a homopolymer of *N*-acetyl-D-glucosamine) (Iseli *et al.*, 1993), thereby limiting fungal growth. Chitin is not found in higher plants but is a major constituent of the cell walls of most filamentous fungi except the Oomycetes (Broglie *et al.*, 1991). Because groups I and II chitinases are localized in the vacuole, they are thought to be a mechanism of 'last line of defense' and not involved in pathogen recognition (Edington *et al.*, 1991). However, glycosidic fragments released through chitinase degradation can act as elicitors.

Induction of chitinase in bean suspension cultured cell has been detected within five minutes of treatment with fungal elicitor (Hedrick *et al.*, 1988). Chitinase activity was strongly induced in cotyledons of *B. napus* cultivar Bienvenu inoculated with *P. lingam* conidia (Rasmussen *et al.*, 1992a). In contrast, sensitive cultivars had increases in chitinase activity delayed by 24 hours post-treatment. The cDNA isolated in this study was used to probe Northern blots of RNA. One day after inoculation, the chitinase mRNA transcript was expressed at a level three-fold greater in resistant tissue (cultivar Libraska) than in sensitive tissue (cultivar Cobra). This difference was diminished after eight days. The chitinase cDNA was found to have low homology with group I and II chitinases but high homology and serological cross-reactivity to an acidic isoform found in sugar beet (*Beta vulgaris* L.). Southern hybridization analysis revealed that this chitinase isoform is encoded by a small family of genes. Peterka and Schlösser (1991) found increased chitinase activity in cotyledons of resistant Brassica species (*B. napus* and *B. juncea*) in response to inoculation with a nonaggressive *P. lingam* isolate. When the same species were treated with an aggressive isolate, chitinase activity was suppressed.

Further evidence to support a role for chitinase in Brassica disease resistance comes from results of experiments using transgenic canola (cultivar Westar) plants treated with the soil-inhabiting fungus, *Rhizoctonia solani* (causative agent of root rot) (Broglie and Broglie, 1993). When transformed with bean endochitinase *CH5B* gene under control of a strong promoter (cauliflower mosaic virus 35S), transformants expressing high levels of chimeric chitinase activity

were found to have more vigorous germination and double the survival rate in infested soil, as well as greater resistance to root rot, relative to controls (Broglie *et al.*, 1991). Infested transgenic canola was also found to contain less fungal biomass than control plants (Broglie and Broglie, 1993). Additionally, a greater extent of hyphal alterations (lysis and chitin breakdown) was observed in the transgenic plants when compared to control plants.

In addition to encoding degradative enzymes, cDNAs of defense response genes encode for diverse systems. As cell wall modifications are frequently observed in host-pathogen interactions (Heath, 1992), it is not unusual that genes relating this event have been determined (section 1.4.2.). Other examples include a highly anionic peroxidase associated with suberization (Kolattukudy *et al.*, 1989), thionins (Bohlmann *et al.*, 1988), hydroxyproline-rich glycoproteins (HRGPs) and extensins (Showalter *et al.*, 1991). Many defense genes are strongly expressed during floral development—phenylpropanoid genes are involved in pigmentation and other floral organs; chitinase, glucanase, and HRGPs in styles, and other functionally unidentified genes are induced by flowering (Dixon and Lamb, 1990).

1.4.3. Phenylpropanoids and Lignification.

Activated host responses to pathogens include structural alterations within plant cells. For example, results from electron microscopy and cytochemical studies indicate the formation of papilla-like structures (appositions) under spores or penetrating hyphae (Bolwell, 1988). These structures are progressively encrusted with a callose material that is glycoproteinaceous and lignin-like. Host cells also respond by accumulating phenols (benzoic acids and phenylpropanoids) and by the formation of new lignin. The hypersensitive response is often associated with lignification (de Wit, 1986).

Lignin is the second most abundant organic compound, after cellulose. The biosynthesis of lignin is complex (reviewed by Higuchi, 1990). Lignin is a heterogenous polymer of monolignols with a variable three-dimensional structure. The ultimate step in lignin biosynthesis is the uncoordinated coalesence of free radical precursors. The inherent insolubility of lignin complicates the analysis of lignin composition. Results obtained with different histochemical reagents must be substantiated by other techniques, especially when evaluating changes in composition (Nicholson and Hammerschmidt, 1992). Lignin is categorized into three broad groups, determined by the

predominant constituents (Higuchi, 1990). Thus, normal angiosperm dicots have lignin comprised of approximately equivalent amounts of guaicyl- and syringylpropane units.

Lignin induced in diseased and wounded tissue has a different composition than healthy lignin (Ride, 1983). The disturbance of normal lignin formation by *P. lingam* in resistant rape was established in early histological studies (Brunin, 1972). The observed "anarchic" lignin was related to inhibited fungal development in xylem and woody tissues.

In the role of plant defense, lignification may function in several capacities (Ride, 1983). Ingress of pathogens and pathogenic phytotoxins may be mechanically restricted, while egress of nutrients from the host may be impeded. Chemical modification of plant cell walls may render them more resistant to degradative enzymes released by invading pathogens. Precursors of lignin are known to be toxic. Also, the formation of lignin involves free radicals. The presence of these conditions may make the plant-pathogen interface an environment deleterious to pathogen growth. In addition, lignification and physical entrapment of the pathogen has been observed. Mechanical isolation of a pathogen through lignification may be a more important determinant of resistance than production of phytoalexins for some host-pathogen interactions, for example, tomato (*Lycopersicon esculentum*) and the wilt pathogens (*Verticillium* and *Fusarium*) (Nicholson and Hammerschmidt, 1992).

For most plants, the initial host response is the formation of low molecular weight phenols, in particular, phenylpropanoids (reviewed by Nicholson and Hammerschmidt, 1992). Cross-linking of phenylpropanoid esters leads to the formation of lignin-like polymers.

Defense mechanisms are associated with changes in enzyme populations as a result of *de novo* protein synthesis. The most studied system is the response of French bean (*Phaseolus vulgaris* L.) cell suspension cultures to challenge with elicitors derived from the anthracnose fungus, *Colletotrichum lindemuthianum* (Robbins *et al.*, 1985, Dixon *et al.*, 1986). In this system, the induction of the phenylpropanoid pathway, leading to several products including lignin monomers and isoflavonoid phytoalexins, is reflected by increased levels of both the *de novo* synthesis of mRNA and the transcriptional activity of key enzymes (Dixon and Lamb, 1979; Dixon *et al.*, 1986; reviewed by Hahlbrock and Scheel, 1989). Similar induction of the same enzymes has been noted in parsley (*Petroselinum crispum*) (Hahlbrock *et al.*, 1986), potato (*Solanum tuberosum*) (Hahlbrock and Scheel, 1989), pea (*Pisum sativum*) (Loschke *et al.*, 1983), *Arabidopsis thaliana* (Dong *et al.*, 1991), and most higher plants.

Rapid elicitor-responsive induction of the major phenylpropanoid pathway enzymes has been reported—within five minutes for phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and chalcone synthase (CHS), and within ten minutes for chalcone isomerase (Dixon and Lamb, 1979;

Lawton and Lamb, 1987; Hedrick *et al.*, 1988). Levels of mature mRNA for these enzymes rise rapidly after elicitation but resume original levels after several hours. This increased activity may reflect a general rise in host metabolism, necessary for the accumulation of precursors essential in host resistance, as not all products are phytoalexins (Keen, 1990b). Accumulated phenolics may serve as a metabolic pool for diversion into the "browning process" (Nicholson and Hammerschmidt, 1992). The accumulation of the branch point phenylpropanoid product, *trans*-cinnamic acid, controls the key enzymes of the pathway through inhibitory regulation (Bolwell *et al.*, 1988). Additionally, cinnamic acid has been found to be a precursor of salicylic acid in healthy and pathogen-inoculated tobacco (Yalpani *et al.*, 1993). Salicylic acid is a putative endogenous regulator of localized and systemic acquired disease resistance in plants.

1.4.4. Enzymology of Lignification.

PAL and CHS are encoded by small gene families—for example (in the case of PAL), three genes in bean, (Cramer *et al.*, 1989), four in parsley (Lois *et al.*, 1989), and one in Arabidopsis (Ohl *et al.*, 1990). Conserved motifs and regulatory elements in the promoter regions of phenylpropanoid pathway genes have been reported. However, distinct functional specializations may exist (Ohl *et al.*, 1990). Chromatofocusing studies reveal that the induced isoforms of PAL and CHS differ from those observed in unelicited cells (Hamadan and Dixon, 1987). Also, cultivar differences in isogene expression have been observed (Ellis *et al.*, 1989). Although PAL is generally considered to be representative of initiation of phenylpropanoid synthesis in response to infection, some increase in its activity may accrue from its wound-inducibility (Nicholson and Hammerschmidt, 1992).

Cinnamoyl alcohol dehydrogenase (CAD) is the regulatory enzyme of lignin formation (Higuchi, 1990). CAD exhibits broad substrate specificity for *p*-hydroxycinnamoyl aldehydes, reducing them to alcohol lignin-precursors, via an NADPH-dependent reaction. Induction by elicitation of CAD is rapid, for example, 1.5 to 2 hours after treatment of cultured bean cells (Walter *et al.*, 1988). Induction of CAD after elicitor treatment has been reported to correspond with a switch from healthy lignin to disease lignin (Mitchell *et al.*, 1994). A "causal relationship between lignin precursors and resistance of wheat to stem rust" has been established through the use of competitive inhibitors of CAD (Moersbacher *et al.*, 1990). A lower frequency of lignification in CAD-

and PAL-inhibited treatments resulted in increased fungal growth. CAD inhibitors were more effective than were PAL inhibitors, in this regard.

Lignin-specific marker enzymes have been cited (Dixon and Harrison, 1991) to include caffeic acid *O*-methyl transferase, lignin-specific peroxidase, and cinnamoyl alcohol dehydrogenase (Walter *et al.*, 1988).

1.5. Discussion of the Proposed Model System.

Simplified plant model systems are often used to analyze complex problems. However, evidence or indications obtained *in vitro* may not correspond to actual mechanisms *in planta*. For example, inducibility of disease-like symptoms by culture filtrates and filtrate constituents constitutes a "phenomenological approach" to understanding plant-pathogen interaction (Dickman and Mitra, 1992). *In vivo* roles for these factors must be established through additional, more detailed analysis.

The response of Brassica species cotyledons to culture filtrate from highly virulent *Leptosphaeria maculans* (anamorph: *Phoma lingam*) is the proposed model for this study. Therefore, the suitability of culture filtrate as representative of the pathogen and cotyledons as representative of host tissue must be considered.

1.5.1. Use of Culture Filtrate as a Fungus Model.

Host wound responses to invading fungi may interfere with evaluation of defense responses. For example, key enzymes of the phenylpropanoid pathway are wound-inducible (Ohashi and Ohshima, 1992). Also, the presence of fungal biomass in host material inoculated by the pathogen may introduce a source of error by contributing to extractable nucleic acids. Additional strategies and assays are required to evaluate the extent of fungal contribution (Xue *et al.*, 1992; Talbot *et al.*, 1993). Therefore, elimination of inoculation and culture of the fungal organism would avoid these problems.

On the other hand, culture conditions are known to affect the fungal growth and development as well as influencing phytotoxin production (Yoder, 1980). For example, novel metabolites are produced by weakly virulent blackleg isolates under various conditions (Pedras and Taylor, 1993a). The developmental stage of cultured fungi is reflected by the secondary metabolites synthesized. Phomalide is produced by 24-60 hour-old virulent blackleg isolates (Pedras and Taylor, 1993b) whereas sirodesmins are found in staling cultures (Pedras and Séguin-Swartz, 1992). This fact may correspond to the *in planta* change from biotrophic to necrotrophic stages of *L. maculans*. Thus, media conditions which optimize qualities such as maximal conidia production may not favour cultural replication of virulence or pathogenicity factors.

Both non-host specific and host-specific toxins can be produced *in vitro*. Non-host specific toxins are usually virulence factors (Mitchell, 1984). That is, they increase the extent of the disease rather than cause the disease. As such, they play a role in the disease cycle after the initial recognition event between plant and host. Culture filtrates have been successfully used in several programmes. For example, alfalfa plants with increased disease resistance have been obtained through the use of non-host specific culture filtrates of *Verticillium albo-atrum* (Frame *et al.*, 1991). De March *et al.* (1986) suggest that, through the use of non-host specific toxins, plants with greater tolerance to blackleg fungus may be selected. In the field, higher tolerance may be as effective as resistance. Culture filtrates of *L. maculans*, harvested after 12 or more days of culture, are non-host specific (De March *et al.*, 1986). The virulence of eight isolates, including isolate Leroy, correlates with the phytotoxicity of the culture filtrates they produce when assayed on cotyledons of *B. napus* (De March *et al.*, 1986). No similar correlation is found when seedling roots or hypocotyls are assayed.

Host-specific toxins have been used as *in vitro* selective agents in several host-pathogen systems (Bains and Tewari, 1987; Buiatti and Ingram, 1991; Löffler and Mouris, 1992). Phomalide or analogues of phomalide may prove to be useful as *in vitro* selective host-specific agents for the Brassica species–*L. maculans* interaction.

1.5.2. Use of Cotyledons as a Plant Model.

The relationship between cotyledons and leaves has long been debated. Leaves grow from organized apical meristems (Meinke 1992). In contrast, cotyledons are initiated in

embryogenesis well before the shoot apex is established. Furthermore, cotyledons differ from leaves in morphology, ultrastructure and patterns of gene expression. Typically, Brassica cotyledons have simple vascularization. The cotyledon is divided into several chambers whereas the vascularization of Brassica leaves is more reticulated. However, study of a homeotic Arabidopsis mutant for leafy cotyledons (*lec* mutant) suggests that a single regulatory gene controls the differences between leaves and cotyledons (Meinke, 1992). Thus, cotyledons and leaves may be closely related structures. The general consensus is that cotyledons arose from specialized leaves.

At seed maturity, the Brassica embryo comprises about 85% of the seed weight (Downey and Rakow, 1987), with a very large proportion consisting of conduplicate cotyledons (Downey, 1983). Major changes in a developing Brassica embryo are increased cell volume, a decreased volume fraction of the cytosol and plastids, and increased lipid and vacuole volume fractions (Mansfield and Briarty, 1992). The latter phenomenon is attributable to the formation of lipid storage bodies (Mansfield and Briarty, 1992) and protein storage bodies by the cisternae of rough endoplasmic reticulum (Höglund *et al.*, 1992). These bodies are transported to the vacuole for further processing and storage. Storage protein bodies are restricted to one developmental stage (Crouch and Sussex, 1981). They are neither organ- nor tissue-specific in that stage. Additionally, at maturity, Brassica embryos have no chlorophyll and have acquired desiccation tolerance through the formation of dehydrins and other related late-embryogenic proteins (Close and Lammers, 1993).

Post-germinative growth ensues from the mobilization of both storage lipids, through increased lipase activity (Lin and Huang, 1983; Lin and Huang, 1984; Murphy *et al.*, 1989a), and storage proteins (Murphy et al., 1989b). Lipase activity peaks at day 4 of seedling growth in *B. napus* and *B. juncea* (Lin and Huang, 1983). The major storage proteins of *B. napus* are the 12S globulin, cruciferin, and the 2S albumin, napin, at 60% and 20% of total mature embryo protein, respectively (Höglund *et al.*, 1992). The hydrophobic polypeptide, oleosin, may comprise up to 20% of total seed protein (Murphy *et al.*, 1989b). The major storage proteins are rapidly degraded as sources of nitrogen and carbon beginning two days after germination (Murphy *et al.*, 1989b). Antibodies raised to cruciferin do not detect antigens in either cotyledons or axes five days after germination (Crouch and Sussex, 1981). Expressed in terms of mean radicle length of a germinated *B. napus* population, cruciferin antibodies fail to detect antigens in radicles between 3-5 mm (Nykiforuk, personal communication). The catabolism of napin follows a similar time line (Nykiforuk, personal communication).

Brassica seedlings are epigeal. Thus, the elongating hypocotyl raises the cotyledons above the soil. As a result of a far-red biophysical signal, stimulated changes in seedling

development include the formation of the photosynthetic apparatus as well as other physiological changes associated with the transition from saprophytic to auxotrophic growth (Attridge, 1990). In photosynthetically active Brassica cotyledons, post-germinative growth is marked by the conversion of glyoxysomes into "leaf-type" peroxisomes (Zhang *et al.*, 1994). Glyoxysomes contain the glyoxylate cycle and β-oxidation enzymes that catalyze the net conversion of fatty acids into succinate, ultimately permitting the use of lipids as a carbon source in the synthesis of carbohydrates.

Other proteins may have role at later stages of cotyledon development. Translocation of nitrogen in senescing radish (*Raphanus satvia* cultivar Comet) cotyledons to growing parts appears to be enhanced by an increased expression of nuclear-encoded genes and their protein products (Ziho, 1991). The mRNA and active protein of the cytolsolic isoform of glutamate synthetase increases in senescing cotyledons while the choroplastic isoform and other nuclear-encoded chloroplast proteins are down-regulated.

Although the main function of Brassica cotyledons is that of a storage organ, this role appears to be complete by 5-6 days after germination. After that time, cotyledons contribute to seedling development through their photosynthetic activity and remain viable on the young plant several days after emergence of true leaves.

Few studies have addressed the inheritance of cotyledon characteristics. A single partially dominant gene was found to control the chlorotic cotyledon trait in the M_3 generation of mutagenized seed (Stringam, 1969). However, it has been established that maternal genotype determines the total aliphatic glucosinolate content of cotyledons in *B. napus* (Magrath and Mithen, 1993) and *A. thaliana* (Haughn *et al.*, 1991). The glucosinolate content of leaves is determined by the zygotic genotype. Thus, caution must be exercised when extrapolating conclusions to mature plant characteristics from assessments that are based on cotyledons.

Nevertheless, Brassica cotyledons are often used to screen plant material for resistance to several diseases, including *L. maculans* (Williams, 1985; section 1.2.3.). Cotyledon inoculation with conidia from isolate Leroy is routinely used to screen Brassica genotypes prior to advancement in breeding programmes (Gugel *et al.*, 1990). Cotyledon rating after cotyledon inoculation with conidia is well correlated with field ratings of the individual plants and their progeny (McNabb *et al.*, 1993; Bansal *et al.*, 1994). In addition, cotyledon inoculation and rating more precisely predicts field ratings than do other inoculation techniques. Resistance selected at the cotyledon stage persists to the mature plant, although mature resistance is not always indicative of cotyledon resistance (Stringam *et al.*, 1992). Therefore, screening of mature plant tissue may be required to thoroughly assess all resistance factors.

1.6. Objective of the thesis.

The objective of this thesis was to assess and analyze a simplified model system of host-pathogen interaction, specifically, cotyledonary tissue of Brassica species host plants in response to filtrate derived from cultured *Leptosphaeria maculans* (anamorph: *Phoma lingam*), highly virulent isolate Leroy. Assessment and analysis was based on phenomenological response of treated Brassica cotyledons, comparison of inducible proteins in response to treatment, and evaluation of the potential role of lignification in cotyledonary response.

2. CHAPTER 2. Materials and Methods.

Reagents. All reagents were purchased from Sigma (St. Louis, MO) except for those noted below.

2.1. Experiment 1. Phytotoxicity screening and treatment procedure.

2.1.1. Plant material. Five Brassica species were chosen for screening based on the reported results of Gugel *et al.* (1990): *Brassica napus* (canola cultivar, Westar), *B. juncea* (L.) Czern. (oriental mustard, cultivar Domo), *B. carinata* Braun (Ethiopian mustard, cultivars S67 and Dodola), *B. nigra* L. (a population of black mustard, Type I, selected at Agriculture Canada, Saskatoon). Seed for these five species was kindly donated by Dr. G. Rakow, Agriculture Canada, Saskatoon, Saskatchewan. Additionally, *B. napus* var. *biennis* (winter oilseed rape, cultivar Jet Neuf) was also chosen for study.

2.1.2. Fungal culture. The highly virulent isolate of *Leptosphaeria maculans*, Leroy, was selected for use. A single ascospore culture of isolate Leroy was kindly donated by Dr. G. A. Petrie (Agriculture Canada, Saskatoon, SK) and subcultured as reported by DeMarch *et al.* (1986). Growth for production of conidia was carried out on 20% V8-juice agar (200 mL V8-juice, 800 mL distilled water, 0.75 g $CaCO_3$, 15 g Difco agar) supplemented with Rose Bengal (40 mg L^{-1}) and streptomycin sulphate (100 mg L^{-1}) in 9-cm petri dishes for 10 days (approximately 21°C, 12 h photoperiod, under two tubes each of Sylvania cool white, 40 watts, and Sylvania supersaver warm white, 34 watts). Spore suspensions were obtained by flooding with sterile water. The colonies were gently rubbed with a glass rod to promote displacement. After filtration through cheesecloth under sterile conditions, inoculum was prepared for liquid still culture (10^6 spores L^{-1}, monitored by hemacytometer), by appropriate dilutions with sterile water. Liquid still culture was with minimal medium (Tinline *et al.*, 1960) supplemented with thiamine (100 µg L^{-1}). After 28 days of stationary culture (approximately 20°C, in a dark box), the medium was filtered through filter paper (Whatman No. 3), a filter membrane (0.45 µm, Millipore), and stored in a dark bottle at 4°C. Glucose concentration of the culture filtrate was measured (Diastix, Miles Laboratories, Etobicoke, ON).

2.1.3. Screening Brassica species with culture filtrate. Seeds were sown in Metro Mix 220 (W. R. Grace & Co., Edmonton, AB) in flats (about 36-48 seeds per 60 cm^2). In some cases, seeds were pregerminated overnight on moistened filter paper. The flats were placed in a growth cabinet (20/15°C, 16/8 h photoperiod). White light (intensity 250 ± 25 µmol m^{-2} sec^{-1}) was provided by 215 watt VHO fluorescent bulbs supplemented with 40 watt incandescent bulbs. The seedlings were fertilized (20:20:20, approximately 250 mg L^{-1}) 7-8 days after sowing, to ensure luxuriant growth. Seedlings were watered daily with tap water, until treatment.

Seedlings and excised cotyledons of 10-day old plants were assessed in a preliminary screening of treatment protocols. Both cotyledonary lobes of a seedling were inoculated with 10 µL of either culture filtrate or sterile water (control). Culture filtrate was serially diluted in 20% steps with sterile water. Inoculation was either with or without wounding. Wounding was performed by gently rubbing the cotyledon surface with the pipette tip. The treated tissue was maintained on 9-cm petri plates lined with moistened filter paper on a light tray (ambient room temperature, 12 h photoperiod, with a light source of two Westinghouse 40 watt F40/Agro tubes and two Sylvania cool white 40 watt tubes (F40-CW) per shelf, about 140 µm m^{-2} s^{-1}). The tissue was visually examined at 24 hour intervals for four days. The experiment was repeated once.

2.1.4. Seedling treatment. Subsequent inoculation was performed as follows. Ten-day old seedlings were sprayed with a fine mist (using an autoclaved chromatography sprayer) of either sterile water or full strength culture filtrate. Flats of treated seedlings were placed in a growth cabinet (constant 18°C, 16 h photoperiod, light intensity described earlier), in an unperforated liner and covered with a clear plastic dome. Enough water was added to the flat to ensure high humidity, while care was taken to ensure against waterlogging the seedlings. Cotyledons were harvested at twenty four hour intervals, placed in RNAase-free plastic bags, and quickly frozen by immersion in liquid nitrogen. Culled samples were kept at -80°C until processing.

2.2. Experiment 2. Electrophoresis of aqueous proteins.

2.2.1. Aqueous protein extraction. Aqueous proteins of treated cotyledons were extracted with either a modified 1X SDS gel-loading buffer of Sambrook *et al.* (1989) (50 mM Tris-Cl, 100 mM dithiothreitol (DTT), 1.0 mM PMSF, and 10% glycerol) or soluble protein extraction buffer (SPEB) of Balass *et al.* (1992). SPEB is 25 mM Tris base, 272 mM sucrose, 2 mM ethylenediaminetetraacetate·$2H_2O$ (EDTA), 10 mM DTT, and 0.5 mM PMSF, all adjusted to pH

7.8 before adding 1% insoluble polyvinylpyrolidone (PVP). The former method was used for one-dimension sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Experiment 2), the latter for gels subjected to fluorography (Experiment 3).

Frozen plant tissue (100 mg) was ground for 3 minutes on ice with sand in a chilled mortar in the presence of the appropriate extraction buffer (1.0 mL). DTT was added just prior to use. The mixture was transferred to a microcentrifuge tube (1.5 mL) and centrifuged in a benchtop microcentrifuge (Biofuge A, Canlab, Mississauga, ON) to pellet cellular debris (15 minutes at 10,000 *g*). The supernatant was carefully removed to another 1.5 mL tube and kept at -20°C. Samples were denatured for 60 seconds in a boiling water bath just prior to loading on electrophoretic gels.

2.2.2. One-dimension discontinuous electrophoresis—SDS-PAGE. Protein concentrations of the extracted samples were determined by the Bradford dye-binding assay using bovine serum albumin (BSA) as the standard (Bradford, 1976). Ten micrograms per sample were electrophoresed on a Mini-PROTEAN II apparatus (Bio-Rad, Mississauga, ON) at 180 volts (constant voltage) per two gels. Prior to electrophoresis, tracking dye (bromophenol blue, final concentration 0.1% w/v) and SDS (final concentration 2% w/v) were added to each sample. Samples were then held in a bath of boiling water for 60 seconds followed by quickly chilling on ice. Low range molecular weight standards (10 µg per lane, Bio-Rad, Mississauga, ON) were run concurrently .

Stacking and resolving gels were prepared according to the formulations of Sambrook *et al.* (1989, Table 18.3). Typically, resolving (or separating) gels were either constant, 12%, or linear gradient, 8-15%. Resolving gels (per 10 mL) were formulated from the following—**12%**: 3.3 mL deionized, distilled water (ddH_2O), 4.0 mL 30% acrylamide mix (stock solution: 29% (w/v) electrophoresis grade acrylamide (BDH, Toronto, ON) and 1% N,N'-methylenebisacrylamide (bis) (w/v) in ddH_2O), 2.5 mL 1.5 M Tris (pH 8.8), 0.1 mL 10% SDS, 0.1 mL 10% ammonium persulphate (freshly prepared), and 4.0 µL N,N,N',N'-tetramethylenediamine (TEMED); **8%**: 4.6 mL ddH_2O, 2.7 mL 30% acrylamide mix, 2.5 mL 1.5 M Tris (pH 8.8),0.1 mL 10% SDS, 0.1 mL 10% ammonium persulphate, and 6.0 µL TEMED; **15%**: 2.3 mL ddH_2O, 5.0 mL 30% acrylamide mix, 2.5 mL 1.5 M Tris (pH 8.8), 0.1 mL 10% SDS, 0.1 mL 10% ammonium persulphate, and 4.0 µL TEMED. The formulation for the 5% stacking gel (per mL) was 0.68 mL ddH_2O, 0.17 mL 30% acrylamide mix, 0.1 mL Tris (pH 6.8), 0.01 mL 10% SDS, 0.01 mL 10% ammonium persulphate, and 1.0 µL TEMED.

Tris-glycine electrophoresis buffer was prepared according to the manufacturer's

directions (Bio-Rad, Mississauga, ON) as a 5X Running Buffer (L^{-1}): 15 g Tris base, 72 g glycine, and 15 g SDS in ddH_2O (pH adjusted to 8.3).

Gels were developed either by Coommassie Brilliant Blue (CBB) stain (as described by Bio-Rad manual, Mississauga, ON) or silver salts stain (Morrissey, 1981). For CBB stain, the gel was immersed for at least 30 minutes in 0.1% Coommassie Brilliant Blue R-250 in fixative (40% methanol, 10% glacial acetic acid), followed by destaining with fixative (1 h, minimum) and a small piece of foam. For staining with silver salts, gels were fixed for 30 minutes in each of the following (based as v/v): (1) 50% methanol, 10% glacial acetic acid, (2) 5% methanol, 7% glacial acetic acid; and (3) 10% glutaraldehyde. After rinsing overnight in water, gels were given a 30 minute soaking in fresh water, 30 minutes in 5 μg mL^{-1} DTT, and 30 minutes in 0.1% $AgNO_3$ (added without rinsing). Gels were then rinsed several times with small volumes of water and twice with a small volume of developer (50 μL of 37% formaldehyde in 100 mL of 3% Na_2CO_3 (w/v)). The reaction was stopped by adding 5 mL of 2.3 M citric acid per 100 mL of developer and agitating for 10 minutes. Gels were washed several times with water, followed by a 10 minute soaking in 0.03% Na_2CO_3 (w/v).

Prior to drying down, gels were soaked in glycerol (minimum of 10%) to deter cracking. Gels were dryed over night between two water-soaked sheets of stretched-out cellophane clamped firmly in a plastic, homemade drying frame. Alternatively, a gel drying apparatus (Model SE540, Hoefer, San Francisco, CA) was used (2h minimum, 65°C, under vacuum).

2.2.3. One-dimension discontinuous electrophoresis—SDS-PAGE with urea. Soluble aqueous protein one-dimension profiles were also produced in the presence of 6M urea (Batista Fontes *et al.*, 1984). Soluble aqueous protein extractions were as described above. Electrophoresis was carried out on a Mini-PROTEAN II (Bio-Rad, Mississauga, ON) apparatus at 100 volts per gel, or 180 volts per two gels.

Linear gradient gels (10-18%) were prepared according to Batista Fontes *et al.* (1984). The formulations for the resolving gels were based on variable concentrations of solutions A (29.2 g acrylamide (BDH, Toronto, ON), 0.8 g bis, 36.0 g urea in a final volume of 100 mL) and B (18.15 g Tris base, 0.2 g SDS, 36.0 g urea, and 24.0 mL 1.0 M hydrochloric acid)—**10%**: 8.0 mL solution A, 7.0 mL solution B, 1.2 mL of freshly prepared 1.4% ammonium persulphate, and 20.0 μL TEMED (final volume adjusted to 24.0 mL with ddH_2O); **18%**: 14.8 mL solution A, 7.0 mL solution B, 1.2 mL 1.4% ammonium persulphate, and 20.0 μL TEMED (final volume adjusted to 24.0 mL). The formulation of the stacking gel was 4.0 mL solution A, 7.0 mL solution B, 2.0 mL 1.4% ammonium persulphate, and 20.0 μL TEMED (final volume adjusted to 24.0 mL).

The gels were developed with Coommassie Brilliant Blue stain and dried as previously described.

2.3. Experiment 3. *In vivo* protein determination.

2.3.1. *In vivo* labelling procedure. Cotyledons of 10-day old seedlings (*B. juncea* cultivar Domo and *B. carinata* cultivar S67) were treated with culture filtrate as above (section 2.1.4.), permitted to dry for 30 minutes and then excised with the petiole submersed under water. One cotyledon from each seedling was placed in a small petri plate (6 cm) containing labelling solution (100.0 µL of 2% L-[^{35}S]-methionine (v/v) (*in vivo* cell labelling grade, Amersham, Oakville, ON) and 2% Tween-20 (v/v) in an aqueous solution). The labelling solution contained 20 µCi (740 000 Bq) per 100.0 µL. The other cotyledon was used as a control by identical handling but with ddH_2O as required (that is, 100.0 µL). The first sample was collected one hour after labelling. The other samples were collected after two hours of labelling. Labelling time led into sample collection times—for example, the four hour time sample was labelled between hour 2 and hour 4 after the commencement of the experiment). A small amount of ddH_2O (~75 µL) was added, if required, during the labelling procedure to prevent desiccation. The experiment was duplicated.

Duplicate 5 µL aliquots of each protein sample were assayed for radiolabel on a liquid scintillation counter (Searle, DesPlaines, IL) using ECONOFLUOR (NEN, Boston, MA) as the fluor. Samples were stored at -20°C until analyzed by SDS-PAGE.

Samples of 50,000 counts per minute were loaded on SDS-PAGE modified according to S. Dunn (personal communication)—**13%** resolving gel: 4.3 mL 30% acrylamide mix (29.2 % acrylamide (w/v), 0.8 % bis (w/v)), 3.3 mL 1.5 M Tris-HCl (pH 8.8), 0.1 mL 10% SDS, 2.25 mL ddH_2O, 0.04 mL 10% ammonium persulphate, and 3.0 µL TEMED; 5% stacking gel: 1.0 mL 30% acrylamide mix, 0.4 mL 1.0 M Tris-HCl (pH 6.8), 0.06 mL 10% SDS, 4.47 mL ddH_2O, 0.04 mL 10% ammonium persulphate, and 3.0 µL TEMED; and electrophoresis running buffer (per L): 6.4 g Tris base (53mM), 4.0 g glycine (53 mM), and 1.0 g SDS (0.1%).

SDS-PAGE was performed on the Mini-PROTEAN II (Bio-Rad, Mississauga, ON) apparatus at 180 volts (constant voltage) per two gels. Non-radioactive low molecular weight protein standards (Bio-Rad, Mississauga, ON) were also run on the same gel.

When electrophoresis was completed, the gels were prepared for fluorography by soaking in fluor (1h, EN^{3}HANCE, NEN, Boston, MA), followed by fixative (30% methanol with 6% glycerol for 1h). Gels were dried under vacuum (2h at 65°C). In a darkroom, the dried gels were placed on Fuji RX medical X-ray film inside a light-proof cassette. The cassette was stored at -80°C. Different

exposure times were assessed for suitability (up to 4 days).

2.4. Experiment 4. Northern analysis.

2.4.1. Isolation of RNA. Two methods were used to isolate total cytoplasmic RNA from Brassica cotyledons, (1) phenol-chloroform ($CHCl_3$) and, (2) guanidinium isothiocyanate (GITC). The GITC method was found to consistently provide high quality RNA while decreasing the extent of personal exposure to toxic reagents (in particular, phenol). RNA isolated by the phenol-$CHCl_3$ method was transferred to PhotoGene membrane (Gibco BRL, Burlington, ON), whereas RNA isolated by the GITC method was transferred to Zeta-Probe membrane (Bio-Rad, Mississauga, ON).

Glassware was thoroughly cleaned and either baked (4h minimum, 180°C) or autoclaved (30 minutes minimum, 15 psi). Bags of disposable plasticware, reagents, and an electrophoresis gel apparatus (Taylor Research, Edmonton, AB) were assigned exclusively for use in RNA procedures. Prepared solutions and reagents were treated with diethyl pyrocarbonate (DEPC, 0.1% final volume) before autoclaving (except for unautoclavable solutions). The gel box was soaked in 3% H_2O_2 (10 minutes) followed with several rinses with DEPC-treated ddH_2O. Disposable gloves were worn and frequently changed during all RNA manipulations.

2.4.2. RNA extraction by phenol-chloroform. Frozen plant tissue (1 g) was ground to a powder in liquid nitrogen using a mortar and pestle with sand. The powder was transferred to a 30 mL Corex tube (Fisher, Ottawa, ON) with 3-5 volumes (per gram of plant tissue) of extraction buffer (EB) (0.6 M sorbitol, 200.0 mM Tris (9.0), 200.0 mM KCl, 35.0 mM $MgCl_2 \cdot 6H_2O$, 12.5 mM EGTA, and 15.0 mM DTT) (mL gfw^{-1}), an equal volume of equilibrated phenol-chloroform ($CHCl_3$) (1:1), SDS (final concentration 1%), and EDTA (final concentration 50mM). After vigorously shaking by hand for 10 minutes, the mixture was spun in a Sorvall SS34 rotor (10 minutes at 4° and 10,000 *g*). The supernatant was decanted to a fresh tube, SDS and EDTA (adjusted to final volumes of 1% and 50.0 mM, respectively), and an equal volume of equilibrated phenol-$CHCl_3$ added. After thorough agitation (up to 20 minutes), the phases were separated on a Sorvall SS34 (15 minutes at 4°C and 10,000 *g*).The aqueous phase was carefully removed and extracted with an equal volume of phenol-$CHCl_3$ until there was no interface after the phases were separated (typically 4-6

times). The aqueous phase was then extracted with an equal volume of $CHCl_3$ until no interface appeared (typically, 2-4 times). RNA was precipitated overnight at -20°C with 0.1 volume of 3.0 M sodium acetate (pH 5.2) and 2-2.5 volumes of cold double distilled 95% ethanol (EtOH). After centrifugation in a Sorvall SS34 (20 minutes at 4°C and 10,000 *g*), the RNA pellet was washed with 70% EtOH and repelleted. The EtOH was removed. When the final traces of EtOH had evaporated, the RNA pellet was suspended in DEPC-treated ddH_2O and quantitated spectrophotometrically (at 230, 260,and 280 nm). 1 A_{260} single-stranded RNA = ~40 µg mL^{-1} (Ausubel *et al.*, 1992).

2.4.3. RNA extraction by guanidinium isothiocyanate (GITC). GITC extraction of RNA was based on the method of Chomczynski and Sacchi (1987). Frozen plant tissue (1 g) was ground to a powder in liquid nitrogen using sand in a mortar and pestle. The powder was transferred to a 30 mL Corex tube (Fisher, Ottawa, ON), to which was added GITC buffer (5 mL gfw^{-1}), 0.1 volume 2.0 M sodium acetate (pH 4.0), 0.2 volume 10% SDS, an equal volume of equilibrated phenol and 0.5 volume of $CHCl_3$/isoamyl alcohol ($CHCl_3$/IAA, 24:1 v/v). GITC buffer is 100 g GITC (ICN, Mississauga, ON), 21 mL 20% sodium lauryl sarcosine, 10 mL 1.0 M Tris (pH 8.0), 10 mL 0.2 M EDTA (pH 8.0), 2.1 mL 2-mercaptoethanol, brought to a final volume of 212 mL with DEPC-treated ddH_2O. The tube was shaken vigorously by hand for ten minutes, then incubated on ice for 15 minutes. After centrifugation in a Sorvall SS34 rotor (20 minutes, 10,000 *g*, 4°C), nucleic acids were precipitated from the aqueous phase by an equal volume of ice-cold isopropanol (15-30 minutes on ice). After centrifugation in a Sorvall SS34 rotor (30 minutes, 10,000 g, 4°C), the pellet was taken up in a small volume (50-500 µL) of DEPC-TE buffer (TE is 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0) in DEPC-treated ddH_2O). The dissolved pellet and subsequent aqueous phases were repeatedly extracted with (1) an equal volume of phenol and 0.5 volume of $CHCl_3$/IAA followed by, (2) an equal volume of $CHCl_3$/IAA until no interface was visible when the phases separated, typically, 4-6 times for (1) and 2-4 times for (2). Phase separation was facilitated by brief centrifugation on a benchtop microfuge (1-2 minutes at 4°C and 10,000 *g*). RNA was precipitated from the aqueous phase by the addition of 0.1 volume 3.0 M sodium acetate and 2.5 volumes of double distilled 95% EtOH. After pelleting the RNA on a benchtop microfuge (30 minutes at 4°C and 10,000 *g*), the pellet was washed with 70% EtOH and either stored under 80% EtOH at -70°C or dissolved in a minimal volume of DEPC-treated water for quantitation (described above).

2.4.4. Northern blotting of RNA. Northern blots were prepared as described by Fourney

et al. (1990). RNA (30 µg) was adjusted to a volume of 5 µL. Twenty five microlitres of electrophoresis sample buffer was added. Electrophoresis sample buffer is 0.75 mL deionized formamide, 0.15 mL 10X MOPS, 0.24 mL 37% formaldehyde, 0.1 mL DEPC-treated ddH_2O, 0.1 mL glycerol, 0.08 mL 10% (w/v) bromophenol blue. 10X MOPS is 0.2 M MOPS 3-(N-morpholino)propanesulphonic acid, 50 mM sodium acetate, 10 mM EDTA, all adjusted to pH 7.0, DEPC treated, and autoclaved. Samples and RNA molecular weight markers (Gibco BRL, Burlington, ON) were held at 65°C for 15 minutes after which 1 µL of ethidium bromide solution (10 mg mL^{-1}) was added and mixed. Samples were then loaded on a denaturing gel (1.5-1.7 g RNAase-free high grade agarose (UltraPure, Gibco BRL, Burlington, ON), 10 mL 10X MOPS, and 87 mL DEPC-treated ddH_2O, heated in a microwave oven, to which 5.1 mL 37% formaldehyde was added upon cooling). The loaded gel was electrophoresed at room temperature in a fumehood either overnight at 30 volts (constant voltage) or for 2 hours at 100 volts (constant voltage). Separated RNA was transferred by capillary action exactly as described by Fourney *et al.* (1990), either to PhotoGene membrane (Gibco BRL, Burlington, ON) or to Zeta-Probe membrane (Bio-Rad). RNA was fixed to the membrane by (1) a 2.5 minute exposure to a short UV wavelength (254 nm) source (2 tubes each of UV-C fluorescent 15 watt bulbs, Xymotech, Mt. Royal, PQ), with the RNA-face about 30 cm from the light source, followed by (2) baking in a vacuum oven for 2 hours at 80°C. The molecular marker lane was cut from the fixed membrane and stained with methylene blue (Herrin and Schmidt, 1988).

2.4.5. Preparation of cDNA probes. Several cDNA clones were obtained from gracious donors, although only two were ultimately used to probe Northerns—phenylalanine ammonia-lyase (pPAL5) from bean (*Phaseolus vulgaris* L.) generously donated by Dr. C. J. Lamb (Salk Institute, San Diego, CA) and cinnamyl alcohol dehydrogenase (pTCAD4) from tobacco (*Nicotiana tabaccum* cv. Samsun) generously donated by Dr. W. Schuch (ICI Seeds, Bracknell, UK).

DH5α or XL-1 Blue was transformed, cultured, and cDNA was isolated by standard procedures (Sambrook *et al.*, 1989) with the following exceptions: (1) preparation and transformation of competent cells was according to Ausubel *et al.* (1992); (2) miniscale screening of *E. coli* colonies was the rapid plasmid procedure of Birnboim and Doly (1979).

cDNA was excised from polyethylene glycol (PEG)-purified supercoiled plasmid DNA by digestion with the appropriate restriction enzyme (Gibco BRL, Burlington, ON)—PstI for pPAL5, EcoRI and HindIII (simultaneous digestion in Gibco Buffer 3) for pTCAD4. Digestion products were confirmed by visualization on a gel of 0.8% agarose (SeaKem, FMC, Mandel, Guelph, ON) containing ethidium bromide (EtBr) (3 µL of EtBr stock per 100 mL). Gel bands of the expected

size (1.5 kb for pPAL5, 0.95 kb for pTCAD4) were excised from the gel. DNA was extracted from the gel bands either by electrophoresis in dialysis tubing followed by salt/alcohol precipitation or by the GeneClean™ DNA isolation kit (Bio 101, La Jolla, CA). DNA size was determined by comparison to migration of DNA molecular weight standards (typically, 1 kb DNA Ladder, Gibco BRL, Burlington, ON) concomitantly electrophoresed with putative cDNA. DNA was quantitated spectrophotometrically. 1 A_{260} double-stranded DNA = ~50 µg mL^{-1} (Ausubel *et al.*, 1992).

Purified cDNA (100 ng) was labelled with non-radioactive label by the PhotoGene system (Gibco BRL, Burlington, ON) (pPAL5 only) or with [α-^{32}P] dCTP using the Multiprime DNA labelling system (Amersham, Oakville,ON) (both pPAL5 and pTCAD4). Radiolabelled probes were cleaned prior to use by spinning through a Sephadex G-50 (Bio-Rad, Mississauga, ON) column (prepared according to Sambrook *et al.*, 1989). Prehybridization, hybridization, and washes were followed according to the manufacturer's instruction booklets (PhotoGene, or for radiolabelled probes, Zeta-Probe). Additionally, low stringency wash conditions (twice with 2X SSC/0.1% SDS, 15 minutes each, at room temperature) were used with radiolabelled hybridizations. 1X SSC is 0.15 M NaCl and 0.015M sodium citrate. Radiolabels were stripped from membranes according to manufacturer's directions. RNA status after stripping was checked by reversible staining with 0.02% methylene blue (Herrin and Schmidt,1988).

Autoradiography of hybridized membranes were produced by exposure to Fuji RX medical X-ray film in a lightproof cassette (up to 4 days, -80°C). Development of X-ray film was by standard protocol (Kodak).

3. CHAPTER 3. Results.

3.1. Experiment 1. Phytotoxicity screening and treatment procedure.

3.1.1. Screening Brassica species with culture filtrate.

Seedling and excised cotyledons of Brassica species were screened with 10 µL of culture filtrate from 28 day-old still culture of *Leptosphaeria maculans* (isolate Leroy). Glucose consumption by the fungus during culture was found to be 0.125 g as evaluated with Diastix.

Results indicated that only undiluted culture filtrate elicited response on the tissue tested. Application of undiluted filtrates and sterile water (10 µL) as a control was either with or without concomitant abrasion (wounding). The results are given in Table 3.1. The overall results indicate that cotyledons respond to culture filtrate by the formation of necrotic lesions. When accompanied with wounding, larger lesions are produced earlier than when wounding is absent. In appearance, lesions varied from brown or black to colourless dead rings around the site of filtrate application. Some lesions appeared as wet spots. Chlorosis was often displayed by samples scored at later incubation times (72 and 96 hours after treatment).

When wounding was absent, the seedlings and excised cotyledons of the *B. napus* cultivars, Jet Neuf and Westar, had no or slow response to culture filtrate. However, when wounding was concurrent with filtrate treatment, both cultivars displayed high sensitivity that increased over time. The other Brassica species all displayed a similar trend in that more cotyledon area was damaged (necrotic and chlorotic) when inoculation of culture filtrate was with wounding.

3.1.2. Brassica seedling treatment.

Typical cotyledon responses of treated Brassica 10 day-old seedling populations are shown in Figures 3.1.1 to 3.1.6. Both *B. napus* cultivars (Figure 3.1.1., *B. napus* var. *biennis* cultivar Jet Neuf and Figure 3.1.2., *B. napus* var. *annua* cultivar Westar) displayed response to culture filtrate treatment within 24 hours. The severity of this response increased with time. The response was characterized by the formation of water-soaked lesions on the upper epidermis of the cotyledons. A general chlorotic condition increased with time.

Within the first 24 hours, the upper epidermis surface of treated *B. nigra* (population Type I) cotyledons (Figure 3.1.3.) developed a rough texture that was dimpled at several points. By 48 hours after treatment, most treated Type I cotyledons showed necrotic lesions and slight chlorosis. Both necrosis and chlorosis became more extensive at 72 and 96 hours after treatment. By 96 hours after treatment, most *B. nigra* seedlings had perished. Cotyledons of control *B. nigra* seedlings displayed a lower level of chlorosis at 72 and 96 hours than did treated cotyledons.

Both *B. carinata* cultivars, Dodola and S67 (Figures 3.1.4. and 3.1.5., respectively), responded to culture filtrate treatment by severe curling of the cotyledons. The curling of S67 was more extensive than that of Dodola. Slight cotyledon curling was noted in control seedlings of both cultivars. The cotyledon upper epidermis of both cultivars became very rough and pitted within the

first 24 hours after treatment. Some pits were black in appearance. With increased time, pitting and darkening of the pits became more extensive. General chlorosis of the cotyledon lamina became evident in the later time samples of treated seedlings. Control seedlings remained healthy and green through 96 hours.

Cotyledons of treated *B. juncea* cultivar Domo (Figure 3.1.6.) became rough textured within 24 hours. By 48 hours after treatment, pitting of the upper epidermis was visible. The pits were dry and light brown. General laminar chlorosis became evident as time after treatment increased. A few cotyledons developed dark bifacial necrotic lesions after 72 hours. Slight chlorosis of cotyledon veins was observed in the 72 and 96 hour samples of Dodola, S67, and Domo.

3.2. Experiment 2. Aqueous protein profiles.

Results of one-dimensional SDS-PAGE of aqueous proteins extracted from cotyledons of Domo (*B. juncea*) and S67 (*B. carinata*) are given in Figures 3.2.1. and 3.2.3., respectively. A prominent protein low molecular weight band was detected at approximately 14.5 kDa in treated cotyledons of Domo (lanes 2, 4, and 6) that was present at a lower level in control cotyledons (lanes 1, 3, and 5) (Figure 3.2.1.). On other independently run gels of the same sample as well as corresponding samples from other replicates of treatment, the band in question consistently displayed a relative molecular mass (M_r) of 16 to 16.5 kDa (not shown). Silver staining did not reveal any further banding patterns (not shown). The prominent band at M_r 55 kDa represents the large subunit of ribulose bisphosphate carboxylase (LSU). Lower levels of staining intensity for LSU in control tissue (lanes 1 and 3) indicates possible variation in the amount of protein present. Thus, the level of the putative filtrate-induced band in control cotyledon samples (lanes 1 and 3) may be underrepresented. However, the 72 hour samples display approximately equivalent staining intensity for LSU rubisco yet, the staining level for the putative filtrate-induced protein in the control sample (lane 5) is less than that for the treated sample (lane 6). When electrophoresis was carried out using a urea protocol, a single band at approximately 16 kDa was found to be more prominent in treated tissue (Figure 3.2.2., lanes 2 and 4) than in control tissue (lanes 1 and 3). By 72 hours after treatment, there is no difference in the level of protein content between control (lane 5) and treated (lane 6) samples.

For aqueous proteins of S67 (Figure 3.2.3.), no difference in band intensity is displayed by a protein staining at about 16 kDa when treated and control samples are compared. The protein

content of the 96 hour control sample (lane 7) appears to be below that of all other samples, based on staining intensity of LSU. When S67 aqueous proteins are analyzed on gels incorporating urea (Figure 3.2.4), the same level of staining intensity is observed for the 16 kDa band in question in control (lanes 1, 3, and 5) and treated samples (lanes 2, 4, and 6).

3.3. Experiment 3. *In vivo* protein determination.

Photographic reproductions of autoradiographs obtained by *in vivo* protein labelling of treated and control cotyledons are given in Figures 3.3.1. (*B. juncea* cultivar Domo) and 3.3.2. (*B. carinata* cultivar S67). More protein bands are discernable in the S67 fluorograph than in that from Domo material.

Samples were loaded on the basis equivalent counts (50,000 cpm). A prominent band at about 16 kDa is evident in treated Domo cotyledons after one hour (lane 2). This band is detected at a lower level of intensity and with no apparent difference in intensity between treated (lanes 4 and 6) and control (lanes 3 and 5), 4 and 12 hours after treatment.

Patterns for separated in vivo labelled aqueous proteins of S67 are essentially similar for treated and control tissue over the times analyzed (Figure 3.3.2.). Some variation in loading is evident.

3.4. Northern analysis.

Extracted total Domo (*B. juncea*) RNA, separated on agarose gels under denaturing conditions (Northerns), was visualized via UV fluoresence of ethidium bromide (Figure 3.4.1., panel A). Transfer of RNA from the gel to a nylon membrane (Zeta Probe) via capillary action was confirmed by examination of the membrane under a UV light source (Figure 3.4.1., panel B). Degradation of the 24 hour RNA sample of treated Domo tissue is evident (lane 3). Also, overloading of the 72 hour RNA sample of treated Domo tissue is apparent lane 7). The other samples indicate good RNA integrity (indicated by strong banding patterns of the various ribosomal RNA species) with equivalent intensity.

The products of restriction enzyme digestion by PstI of purified plasmid DNA (transformed

with the cDNA from bean phenylalanine ammonia-lyase, PAL) are shown in Figure 3.4.2. (lanes, 1-4, panel A). The 1.5 kilobase (kb) band (panel A, indicated by an arrow) was used to probe Northern blots. The upper band is the linearized vector plasmid pAT153 (3.6 kb), free of inserted DNA.

Approximately 1 µg of cDNA of pTCAD4 from tobacco (0.95 kb) which had been excised from the vector, pBluescript SK$^-$, isolated on an agarose gel, and cleaned with GeneClean™ is shown is Figure 3.4.2 (lane 1, indicated in panel B by an arrow). Aliquots from the same pTCAD4 stock were used to probe Northerns.

The autoradiographs produced by probing the Northern blot of Domo total RNA with pPAL5 and pTCAD4 are depicted in Figures 3.4.3. and 3.4.4., respectively. As an internal control, 1 µg of denatured pPAL was simultaneously electrophoresed on the same RNA gel. Probing with radiolabelled pPAL revealed hybridization to the pPAL control (lane C). No hybridizable bands were detected with Domo RNA. Background noise is visible in Figure 3.4.3. Similar results were obtained with RNA blotted on to PhotoGene membrane and probed with non-radioactive labelled pPAL. In this case, biotinylated pPAL hybridized to control pPAL.

The Northern blot of Domo total RNA (Figure 3.4.1., panel B) was probed with radiolabelled pTCAD4 and the resulting autoradiograph is given in Figure 3.4.4. RNA from controls indicate an equivalent degree of hybridizable bands, represented as a doublet (lanes 3, 5, and 7). Interpretation of the hybridization patterns of pTCAD4 with RNA from culture filtrate-treated cotyledons is difficult as a result of underloading (48 hour sample, lane 4) and overloading (72 hour sample, lane 6). However, the hybridized signal in the 72 hour sample (lane 6) appeared at a lower apparent molecular weight than the other detected signals. The downward shift in the apparent weight of the 72 treated RNA sample is also discernable on the unprobed membrane (Figure 3.4.1., lane 7). Both control and treated 24 hour samples (lanes 1 and 2, respectively) appear to strongly hybridize to the pTCAD4 probe. Homology exists between tobacco and Brassica CAD as a detectable signal at low stringency was produced.

TABLE 3.1. Responses of 10 day-old Brassica seedlings and excised cotyledons to treatment with culture filtrate of *Leptosphaeria maculans* (isolate Leroy). Filtrate (10 µL) was applied with (+) or without (−) wounding. Cotyledons treated with 10 µL sterile H_2O displayed damage limited to the wound site or no reaction in the absence of wounding, for all species and times. Symptoms were scored according to the following scale: 0 = no reaction; 1 = superficial response at application site; 2 = tissue death within 1 mm radius of application site; 3 = tissue death within 1-2 mm radius of application site; 4 = tissue death within 3-4 mm radius of application site; ϕ = not done. The results shown indicate the average score and the standard error for four replicates per treatment and time.

		Excised cotyledons Hours after treatment				Seedlings Hours after treatment		
		24	48	72	96	24	48	72
B. napus	−	$0_{\pm 0.0}$	$0.8_{\pm 0.3}$	$0.8_{\pm 0.3}$	$0.8_{\pm 0.3}$	$0_{\pm 0.0}$	$0_{\pm 0.0}$	$0_{\pm 0.0}$
cv. Jet Neuf	+	$2.0_{\pm 0.0}$	$2.3_{\pm 0.3}$	$2.8_{\pm 0.3}$	ϕ	$1.8_{\pm 0.0}$	$2.3_{\pm 0.0}$	$2.8_{\pm 0.3}$
B. napus	−	$1.0_{\pm 0.0}$	$1.0_{\pm 0.0}$	$1.0_{\pm 0.0}$	$2.3_{\pm 0.3}$	$0.5_{\pm 0.3}$	$1.5_{\pm 0.3}$	$1.8_{\pm 0.3}$
cv. Westar	+	$2.3_{\pm 0.3}$	$2.8_{\pm 0.3}$	$3.8_{\pm 0.3}$	ϕ	$1.8_{\pm 0.3}$	$2.8_{\pm 0.3}$	$3.5_{\pm 0.3}$
B. nigra	−	$1.0_{\pm 0.6}$	$1.5_{\pm 0.3}$	$1.8_{\pm 0.3}$	$2.3_{\pm 0.3}$	$0.8_{\pm 0.3}$	$1.3_{\pm 0.3}$	$2.8_{\pm 0.3}$
Type I	+	$1.0_{\pm 0.0}$	$1.5_{\pm 0.5}$	$2.0_{\pm 0.6}$	ϕ	$1.0_{\pm 0.4}$	$1.5_{\pm 0.5}$	$2.5_{\pm 0.3}$
B. carinata	−	$0.5_{\pm 0.3}$	$0.8_{\pm 0.5}$	$1.0_{\pm 0.6}$	$1.0_{\pm 0.6}$	$0.3_{\pm 0.3}$	$0.5_{\pm 0.3}$	$2.0_{\pm 0.0}$
cv. Dodola	+	$1.5_{\pm 0.3}$	$1.8_{\pm 0.3}$	$2.3_{\pm 0.5}$	ϕ	$1.0_{\pm 0.0}$	$2.0_{\pm 0.0}$	$2.0_{\pm 0.0}$
B. carinata	−	$0_{\pm 0.0}$	$0.5_{\pm 0.5}$	$0.8_{\pm 0.5}$	$1.3_{\pm 0.3}$	$0_{\pm 0.0}$	$0.3_{\pm 0.3}$	$1.3_{\pm 0.3}$
cv. S67	+	$1.0_{\pm 0.6}$	$1.5_{\pm 0.9}$	$2.3_{\pm 0.9}$	ϕ	$1.3_{\pm 0.3}$	$2.5_{\pm 0.7}$	$3.3_{\pm 0.5}$
B. juncea	−	$0.8_{\pm 0.3}$	$1.0_{\pm 0.0}$	$1.3_{\pm 0.3}$	$1.8_{\pm 0.5}$	$0.8_{\pm 0.3}$	$1.3_{\pm 0.3}$	$1.3_{\pm 0.3}$
cv. Domo	+	$1.0_{\pm 0.0}$	$2.3_{\pm 0.3}$	$2.3_{\pm 0.3}$	ϕ	$1.0_{\pm 0.0}$	$1.5_{\pm 0.3}$	$2.0_{\pm 0.0}$

Figure 3.1.1. Response of 10 day-old seedlings of *Brassica napus* var. *biennis* (cultivar Jet Neuf) sprayed with either sterile ddH_2O as control (A–D) or culture filtrate of *Leptosphaeria maculans* (isolate Leroy) (E–H). Seedlings are shown at 24 hours (A, E), 48 hours (B, F), 72 hours (C, G), and 96 hours (D, H) after treatment.


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Figure 3.1.2. Response of 10 day-old seedlings of *Brassica napus* var. *annua* (cultivar Westar) sprayed with either sterile ddH_2O as control (A,B, and D) or culture filtrate of *Leptosphaeria maculans* (isolate Leroy) (E–H). Seedlings are shown at 24 hours (A, E), 48 hours (B, F), 72 hours (G), and 96 hours (D, H) after treatment.

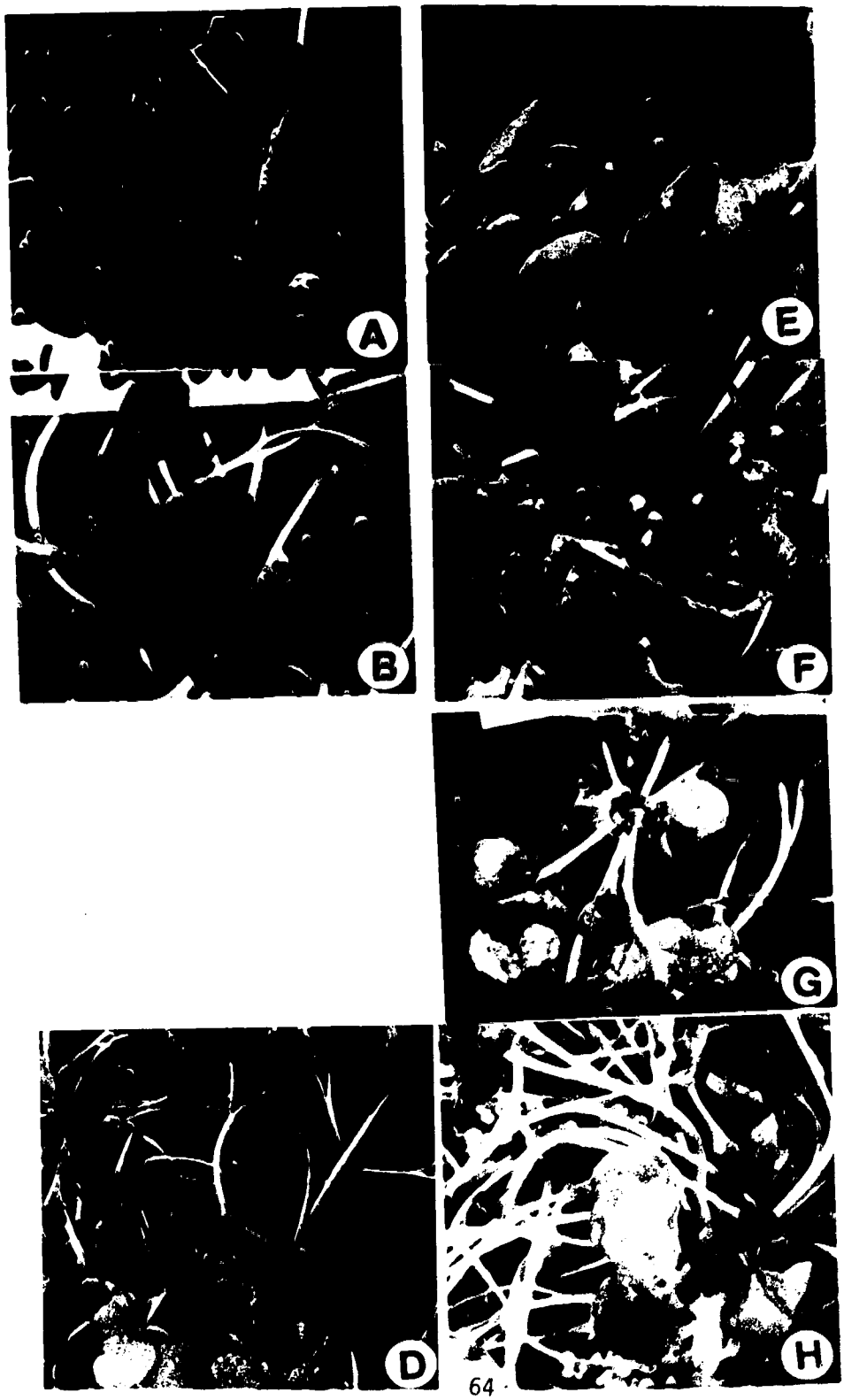
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Figure 3.1.3. Response of 10 day-old seedlings of *Brassica nigra* (population Type I) sprayed with either sterile ddH_2O as control (A–C) or culture filtrate of *Leptosphaeria maculans* (isolate Leroy) (E–H). Seedlings are shown at 24 hours (A, E), 48 hours (B, F), 72 hours (C, G), and 96 hours (H) after treatment.

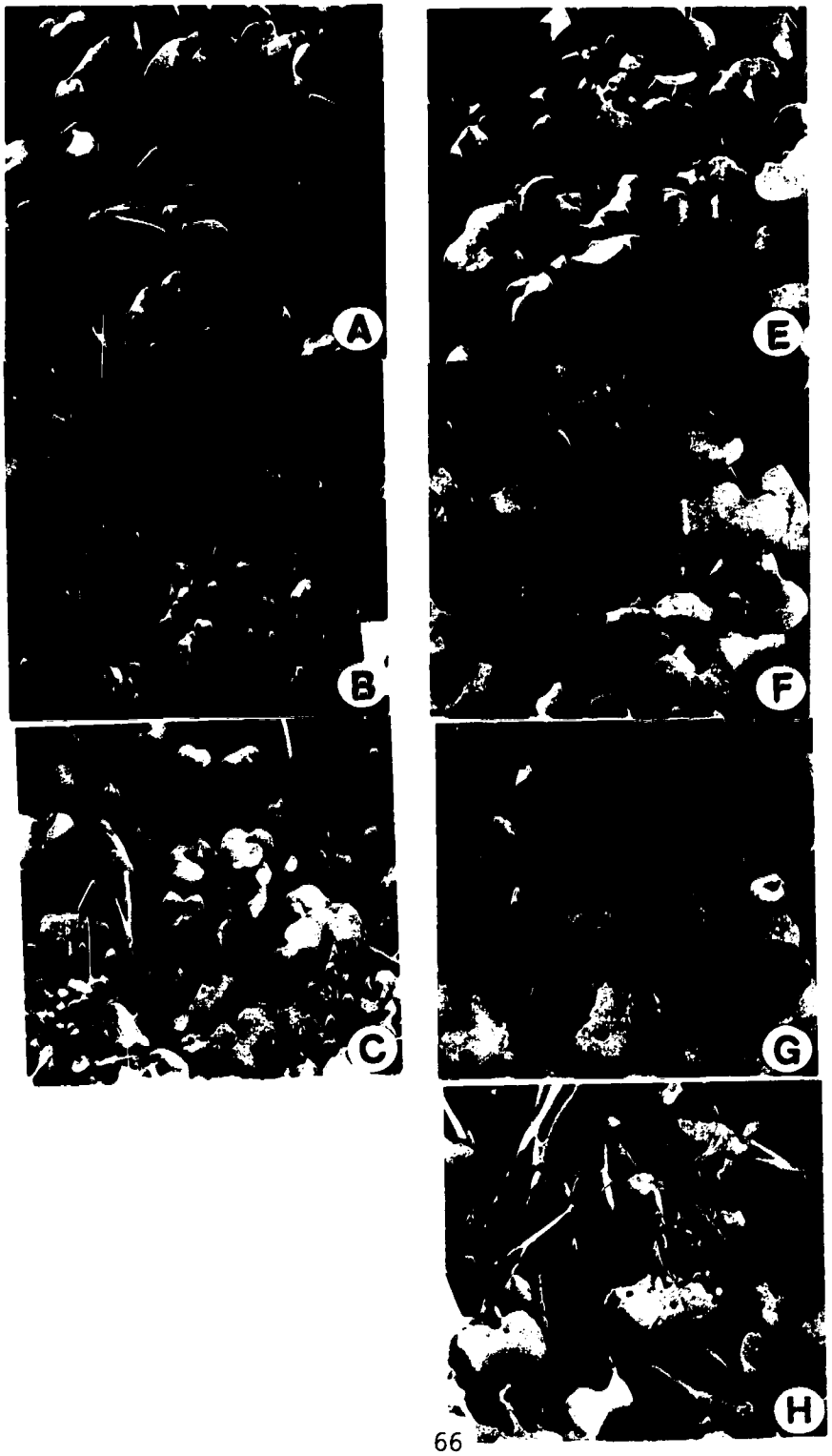
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Figure 3.1.4. Response of 10 day-old seedlings of *Brassica carinata* (cultivar Dodola) sprayed with either sterile ddH_2O as control (A–D) or culture filtrate of *Leptosphaeria maculans* (isolate Leroy) (E–H). Seedlings are shown at 24 hours (A, E), 48 hours (B, F), 72 hours (C, G), and 96 hours (D, H) after treatment.

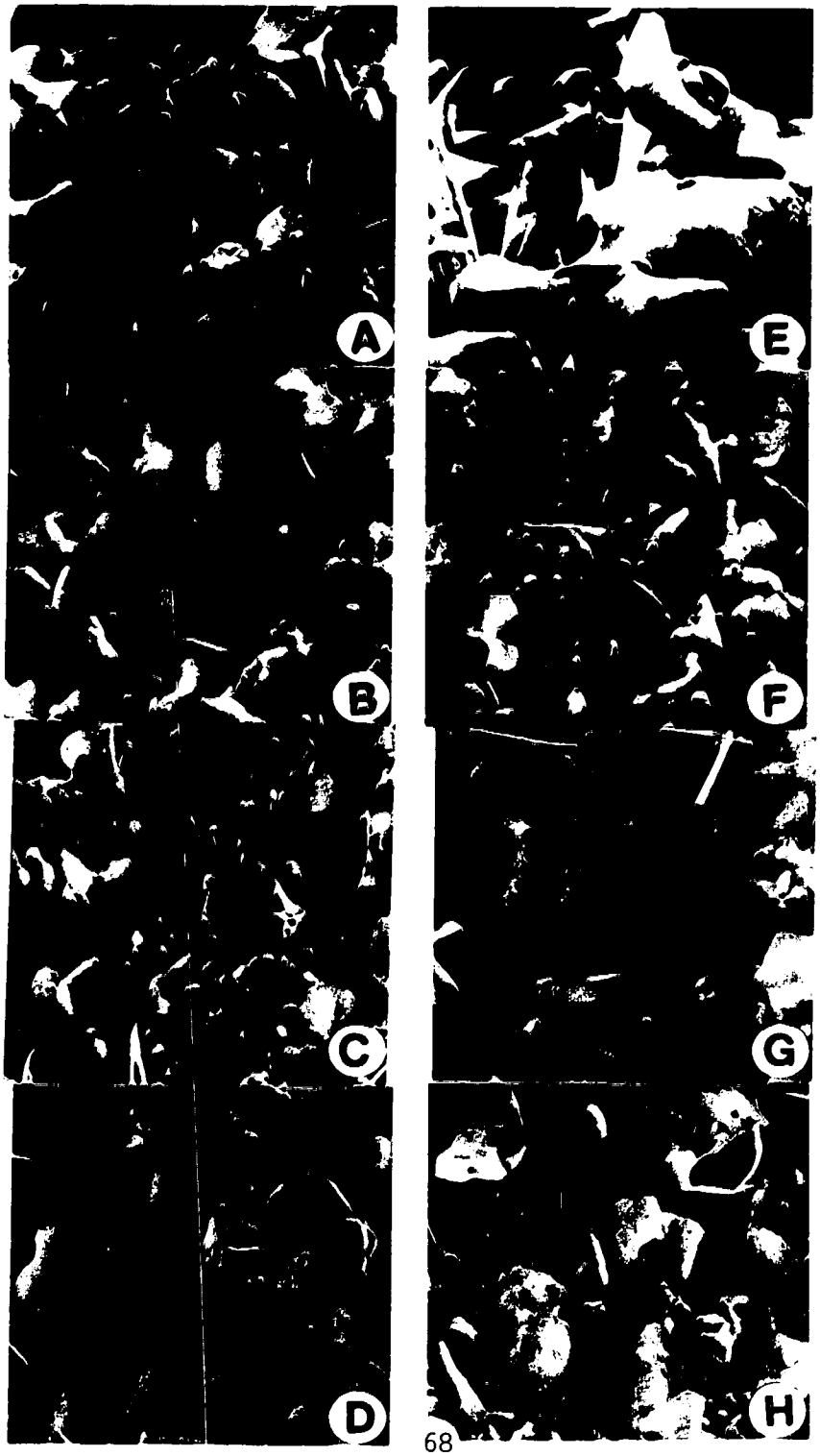
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Figure 3.1.5. Response of 10 day-old seedlings of *Brassica carinata* (S67) sprayed with either sterile ddH_2O as control (A–D) or culture filtrate of *Leptosphaeria maculans* (isolate Leroy) (E–H). Seedlings are shown at 24 hours (A, E), 48 hours (B, F), 72 hours (C, G), and 96 hours (D, H) after treatment.

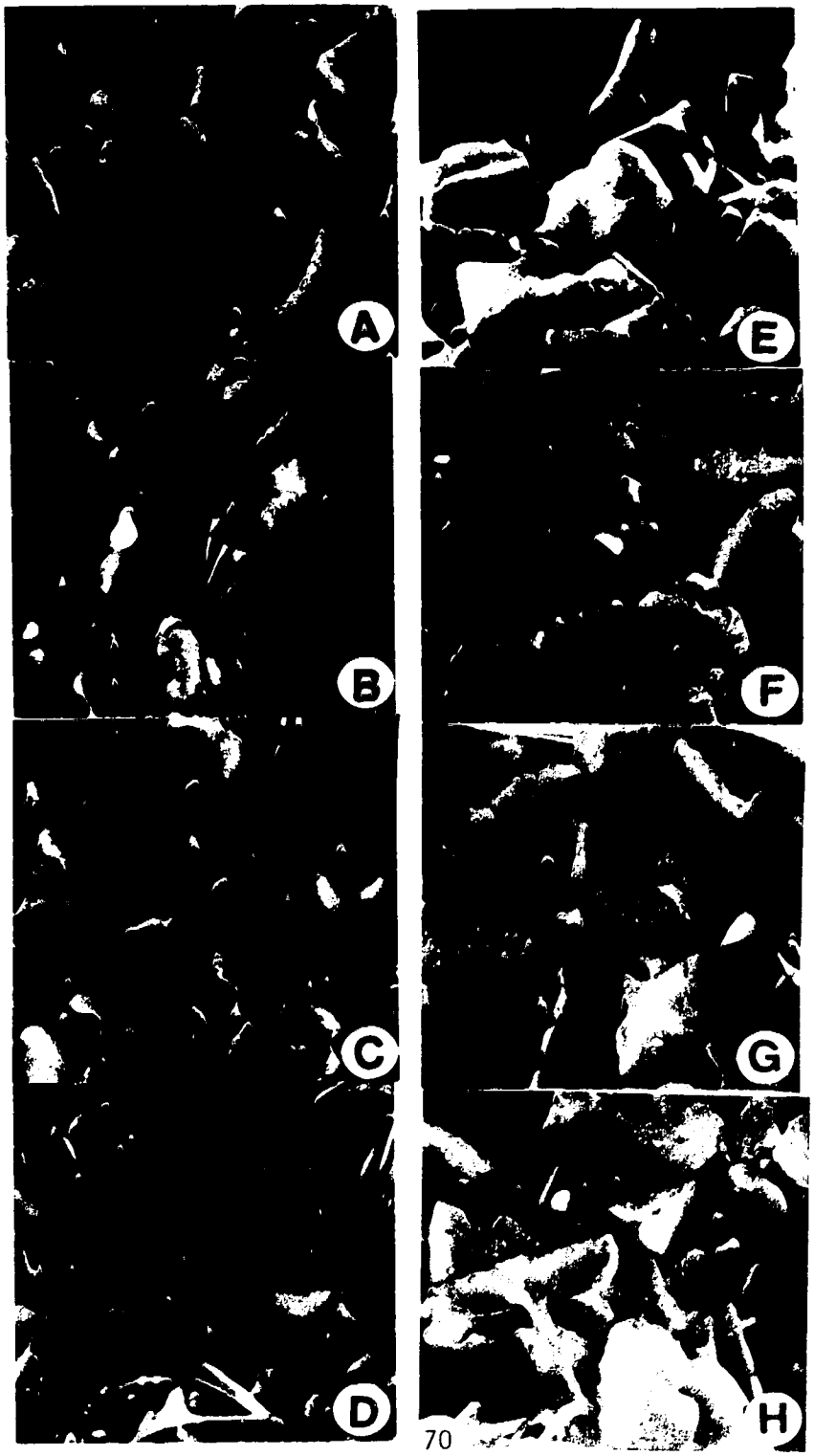
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Figure 3.1.6. Response of 10 day-old seedlings of *Brassica juncea* (cultivar Domo) sprayed with either sterile ddH_2O as control (A–D) or culture filtrate of *Leptosphaeria maculans* (isolate Leroy) (E–H). Seedlings are shown at 24 hours (A, E), 48 hours (B, F), 72 hours (C, G), and 96 hours (D, H) after treatment.

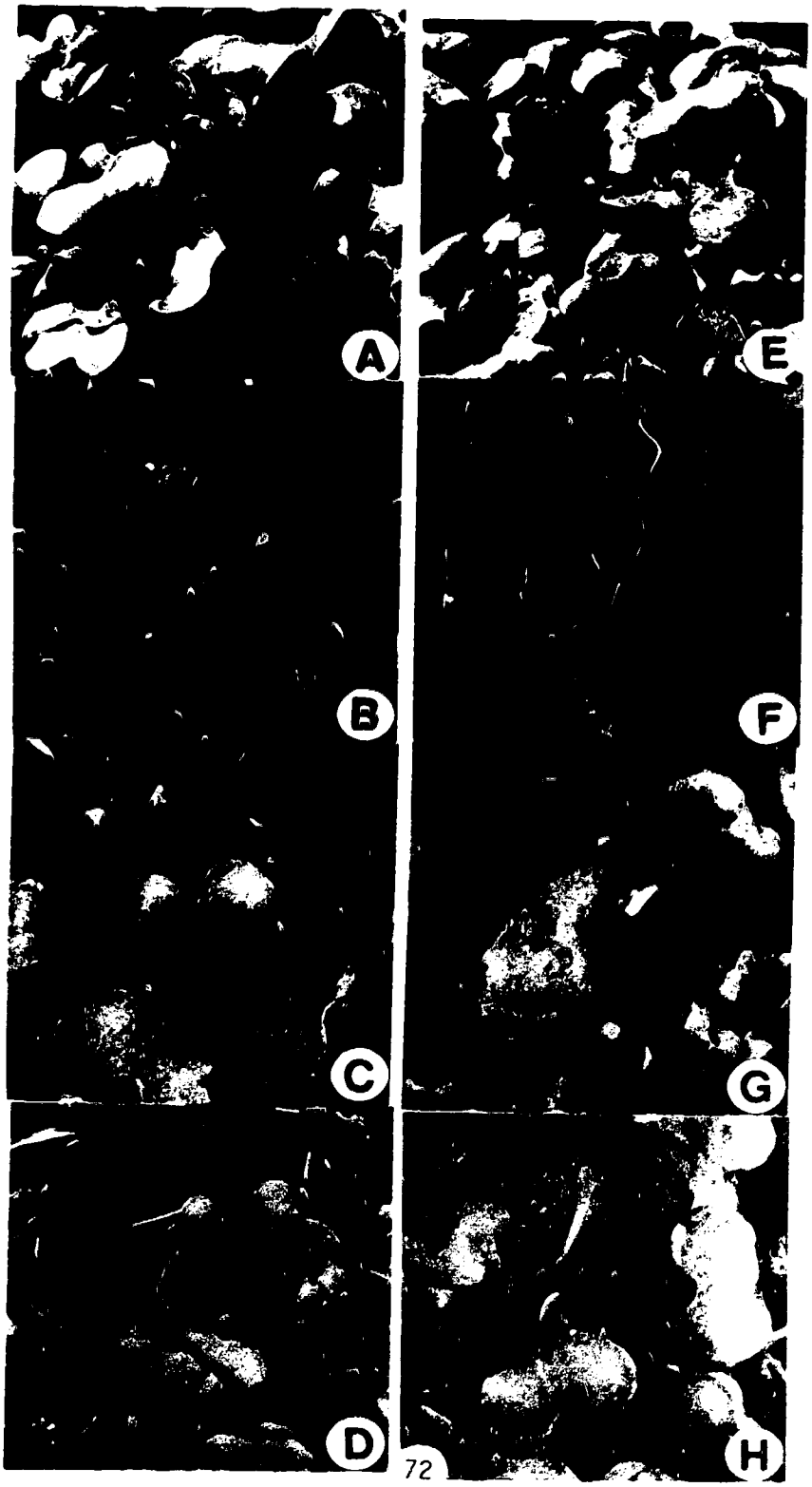
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Figure 3.2.1. SDS-PAGE (8-15 % linear gradient) profile of aqueous proteins from 10 day-old cotyledons of *Brassica juncea* (cultivar Domo), sprayed with sterile ddH_2O as control (lanes 1, 3, and 5) or with filtrate from cultured *Leptosphaeria maculans* (lanes 2, 4, and 6). Proteins were extracted from treated cotyledons at 24 hours (1, 2), 48 hours (3, 4), and 72 hours (5, 6) after treatment. Proteins were detected with Coommassie Brilliant Blue. Positions of concurrently run molecular weight standards are represented by the bars at the left (MW, kDa): phosphorylase b (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), trypsin inhibitor (21.5), and lysozyme (14.4) (from top to bottom). A prominent protein band at about 14.5 kDa (lanes 2 and 4) was apparent in treated cotyledons when compared to control cotyledons (lanes 1 and 3).

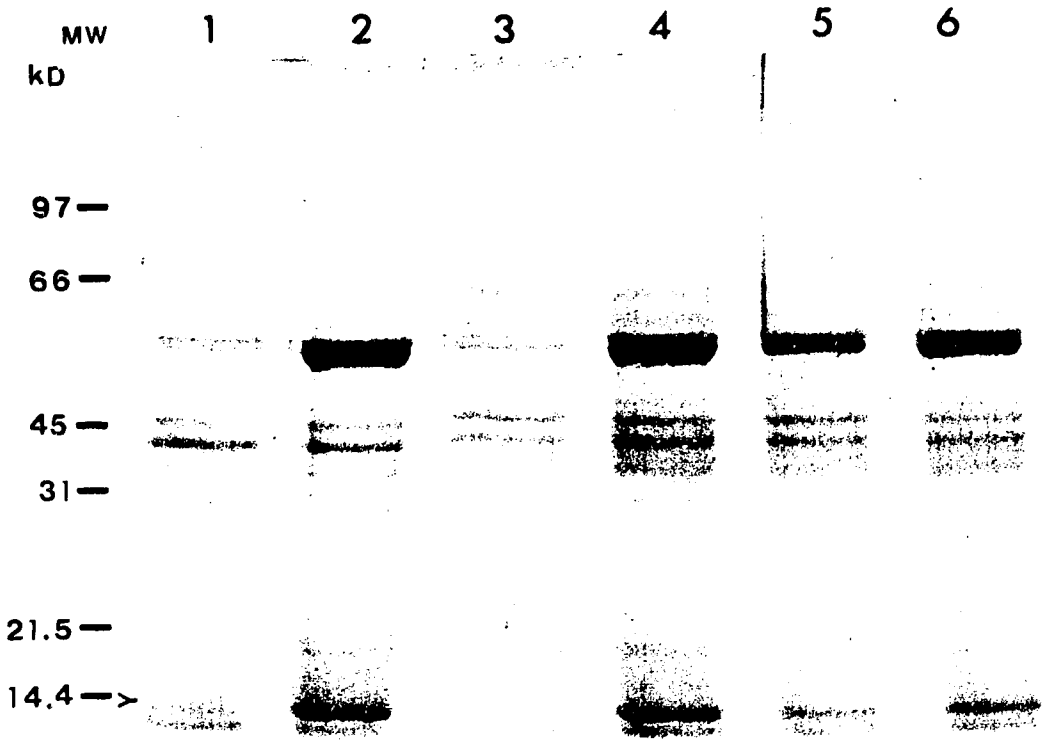
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Figure 3.2.2. SDS-PAGE incorporating 6M urea (10-18 % linear gradient) profile of aqueous proteins from 10 day-old cotyledons of *Brassica juncea* (cultivar Domo), sprayed with sterile ddH_2O as control (lanes 1, 3, and 5) or with filtrate from cultured *Leptosphaeria maculans* (lanes 2, 4, and 6). Proteins were extracted from treated cotyledons at 24 hours (1, 2), 48 hours (3, 4), and 72 hours (5, 6) after treatment. Proteins were detected with Coommassie Brilliant Blue. Positions of concurrently run molecular weight standards are represented by the bars at the left (MW, kDa): phosphorylase b (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), trypsin inhibitor (21.5), and lysozyme (14.4) (from top to bottom). A prominent protein band at about 16.0 kDa (lanes 2 and 4) is seen in treated cotyledons when compared to control (lanes 1 and 3) cotyledons.

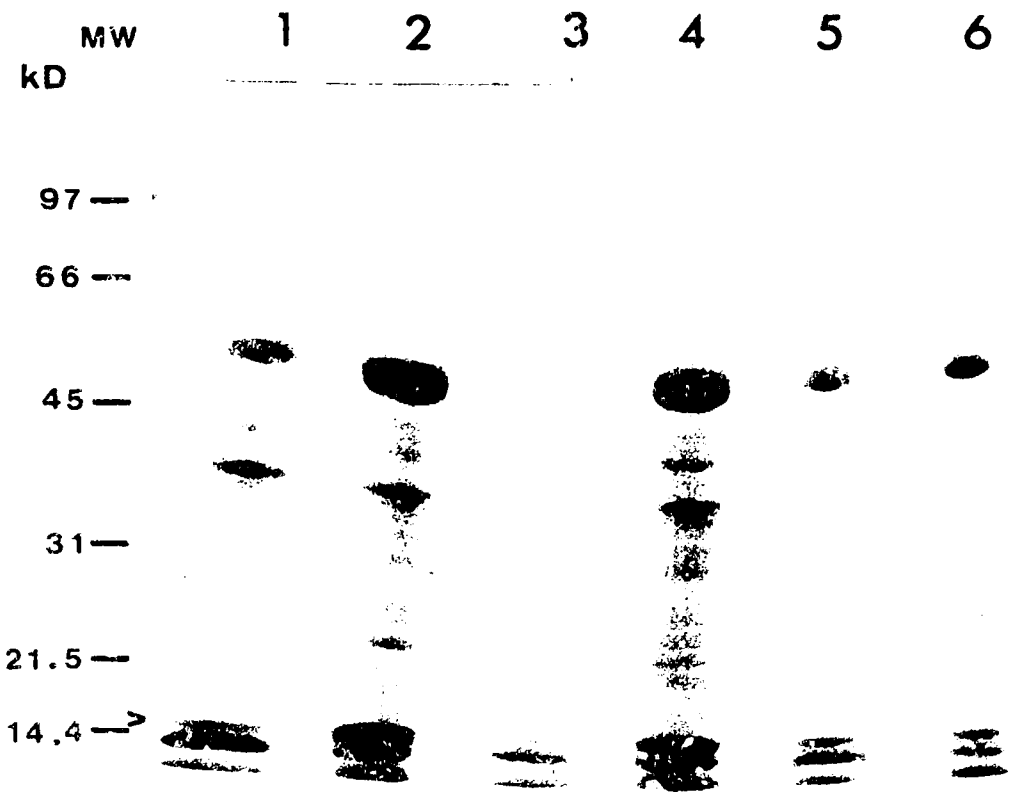
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Figure 3.2.3. SDS-PAGE (8-15 % linear gradient) profile of aqueous proteins from 10 day-old cotyledons of *Brassica carinata* (cultivar S67), sprayed with sterile ddH_2O as control (lanes 1, 3, 5, and 7) or with filtrate from cultured *Leptosphaeria maculans* (lanes 2, 4, 6, and 8). Proteins were extracted from treated cotyledons at 24 hours (1, 2), 48 hours (3, 4), 72 hours (5, 6), and 96 hours (7, 8) after treatment. Proteins were detected with Coommassie Brilliant Blue. Positions of concurrently run molecular weight standards are represented by the bars at the left (MW, kDa): phosphorylase b (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), trypsin inhibitor (21.5), and lysozyme (14.4) (from top to bottom). No differences in protein banding patterns are detected between control and treated cotyledons.

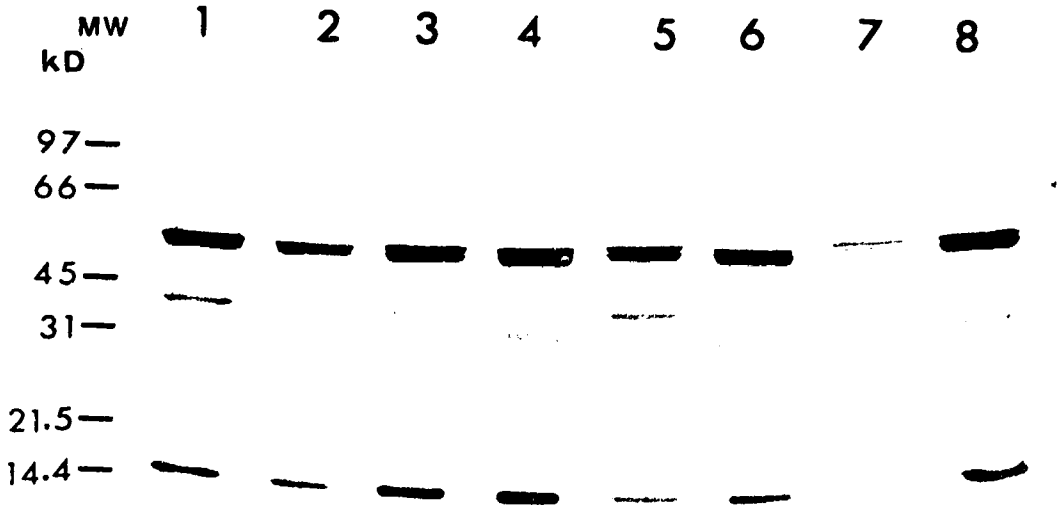
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Figure 3.2.4. SDS-PAGE incorporating 6M urea (10-18% linear gradient) profile of aqueous proteins from 10 day-old cotyledons of *Brassica carinata* cultivar S67, sprayed with sterile ddH_2O as control (lanes 1, 3, and 5) or with filtrate from cultured *Leptosphaeria maculans* (lanes 2, 4, and 6). Proteins were extracted from treated cotyledons at 24 hours (1, 2), 48 hours (3, 4), and 72 hours (5, 6) after treatment. Proteins were detected with Coommassie Brilliant Blue. Positions of concurrently run molecular weight standards are represented by the bars at the left (MW, kDa): phosphorylase b (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), trypsin inhibitor (21.5), and lysozyme (14.4) (from top to bottom). No differences in protein banding patterns are detected between control and treated cotyledons.

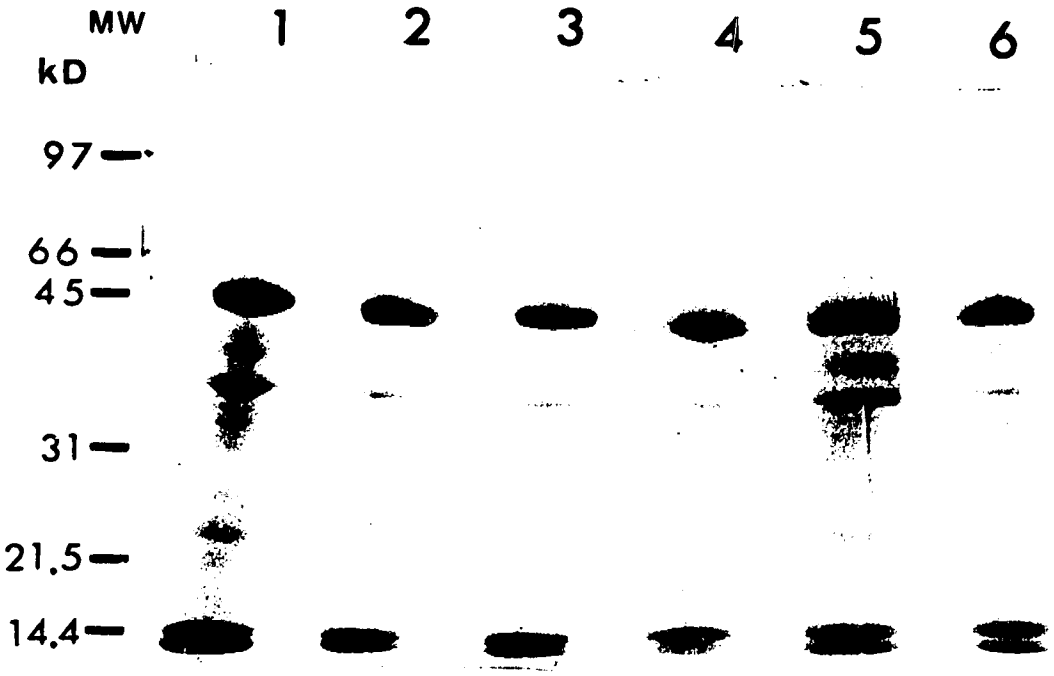
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Figure 3.3.1. Fluorograph of aqueous proteins from 10 day-old cotyledons of *Brassica juncea* cultivar Domo that were excised and *in vivo* labelled with ^{35}S-methionine. SDS-PAGE (13%) was modified as described (section 2.). Proteins were loaded on the basis of equivalent detectable radiolabel (50,000 cpm). Lanes 1, 3, and 5 represent proteins from control cotyledons (sprayed with sterile ddH_2O), at 1, 4, and 12 hours after treatment, respectively. Lanes 2, 4, and 6 represent proteins from filtrate-treated cotyledons (sprayed with filtrate of cultured *Leptosphaeria maculans*), at 1, 4, and 12 hours after treatment, respectively. Positions of concurrently run molecular weight standards are represented by the bars at the right (MW, kDa): phosphorylase b (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), trypsin inhibitor (21.5), and lysozyme (14.4) (from top to bottom). The 1-hour control is not discerned (lane 1). A prominent protein band at about 16 kDa is detected one hour after filtrate treatment (lane 2). This band is detected at lower levels of intensity in both control and treated cotyledons as treatment times were increased.

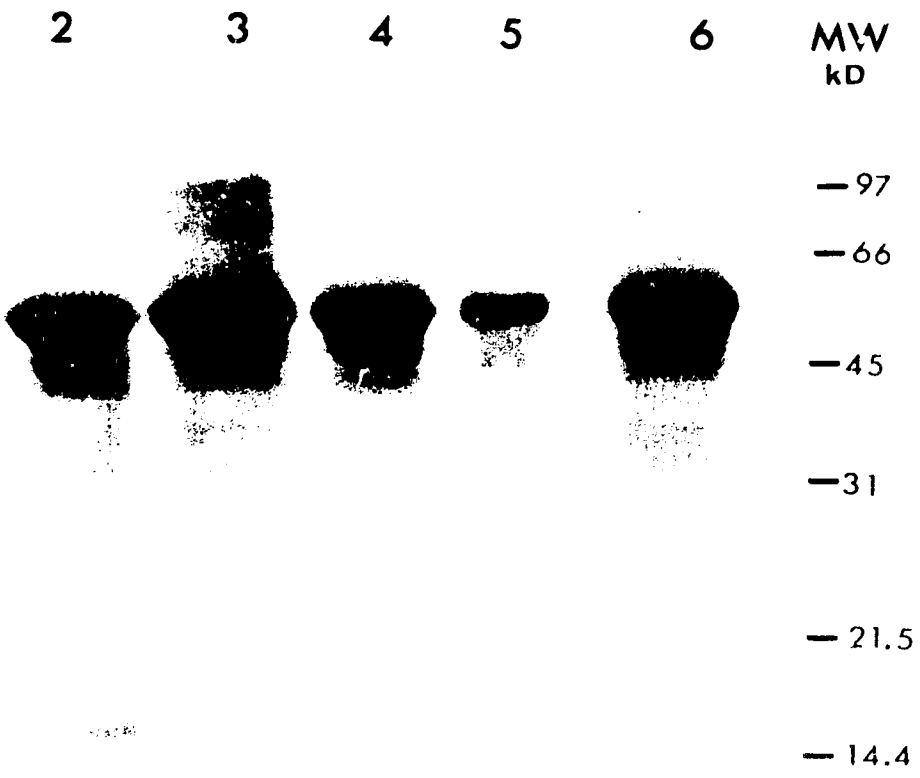
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Figure 3.3.2. Fluorograph of aqueous proteins from 10 day-old excised cotyledons of *Brassica carinata* cultivar S67 that were *in vivo* labelled with ^{35}S-methionine. SDS-PAGE (13%) was modified as described (section 2.). Proteins were loaded on the basis of equivalent detectable radiolabel (50,000 cpm). Lanes 1, 3, and 5 represent proteins from control cotyledons (sprayed with sterile ddH_2O), at 1, 4, and 12 hours after treatment, respectively. Lanes 2, 4, and 6 represent proteins from filtrate-treated cotyledons (sprayed with filtrate of cultured *Leptosphaeria maculans*), at 1, 4, and 12 hours after treatment, respectively. Positions of concurrently run molecular weight standards are represented by the bars at the right (MW, kDa): phosphorylase b (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), trypsin inhibitor (21.5), and lysozyme (14.4) (from top to bottom). Protein banding patterns between control and treated cotyledons are equivalent when variations in protein loading are considered.


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MW
kD
— 97
— 66
— 45
— 31
— 21.5
— 14.4

Figure 3.4.1 RNA from 10 day-old cotyledons of *Brassica juncea* cultivar Domo electrophoresed under denaturing conditions (A) and blotted to ZetaProbe nylon membrane (B), as described (section 2.). Lane 1: RNA molecular weight ladder (Gibco BRL, Burlington, ON); lanes 2, 4, 6, and 8: RNA extracted from control cotyledons sprayed with sterile ddH_2O at 24, 48, 72, and 96 hours after treatment; lanes 3, 5, and 7: RNA extracted from cotyledons sprayed with filtrate from cultured *Leptosphaeria maculans* at 24, 48, and 72 hours after treatment.

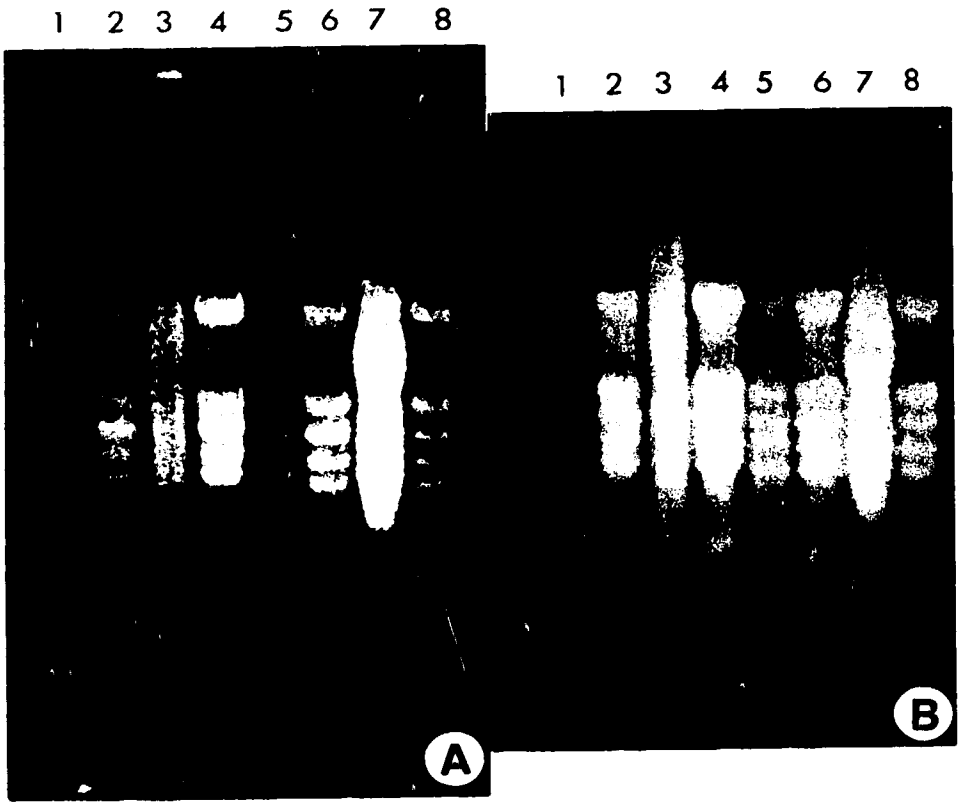

1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
A
B

Figure 3.4.2. **A.** PstI digest products (lanes 1-4) of PEG-purified plasmid DNA that was carrying the pPAL5 cDNA insert. The arrow indicates excised pPAL5 at 1.5 kb. 1 kb DNA ladder (Gibco BRL, Burlington, ON) is shown on the left (MW). **B.** pTCAD4 cDNA (approximately 1.0 µg) following purification of agarose gel-separated restriction enzyme digest products via the GeneClean™ kit (Bio101, La Jolla, CA). The arrow indicates the expected insert size for pTCAD4 cDNA (0.95 kb). 1 kb DNA ladder (Gibco BRL, Burlington, ON) is shown on the left (MW).

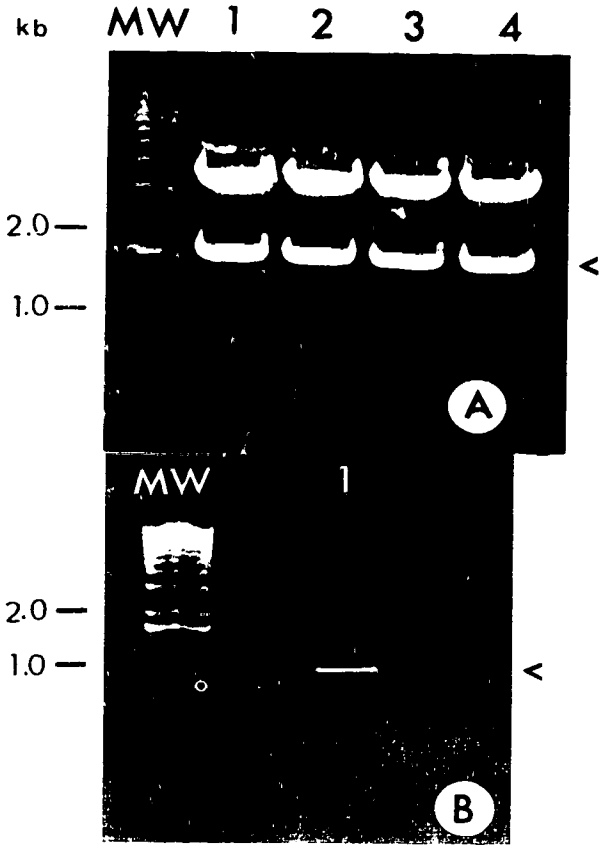
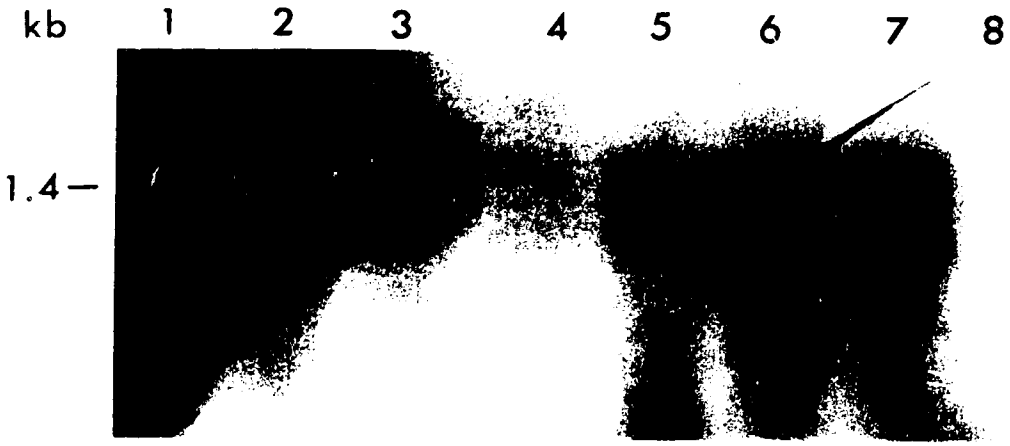

kb
MW
1
2
3
4
2.0
1.0
A
MW
1
2.0
1.0
B

Figure 3.4.3. Northern of RNA from 10 day-old cotyledons of *Brassica juncea* cultivar Domo that was probed with ^{32}P-labelled bean pPAL5 cDNA. Lane C represents denatured pPAL5 cDNA (1.0 µg) included in the Northern as an internal control. Bean pPAL5 hybridizes only to the internal control. No Brassica RNA species are recognized by pPAL5 (blank area to the right of lane C).


C

Figure 3.4.4. Northern of RNA from 10 day-old cotyledons of *Brassica juncea* cultivar Domo that was probed with ^{32}P-labelled tobacco pTCAD4 cDNA. Lanes 1, 3, 5, and 7 represent RNA from control cotyledons sprayed with sterile ddH_2O, at 24, 48, 72, and 96 hours after treatment, respectively. Lanes 2, 4, 6, and 8 represent RNA from cotyledons sprayed with filtrate from culture of *Leptosphaeria maculans*, at 24, 48, 72, and 96 hours after treatment, respectively. The position of the 1.4 kb band from an RNA molecular weight standard (Gibco BRL, Burlington, ON) is shown at the left. At low stringency, tobacco pTCAD4 recognizes a doublet Brassica RNA species, at approximately 1.4 kb.


kb
1
2
3
4
5
6
7
8
1.4—

4. CHAPTER 4. Discussion and Conclusions.

Canola has emerged from an ancient and humble origin to become a commodity of international significance (Downey and Röbbelen, 1989). As a result of the efforts of Canadian plant breeders, oilseed Brassicas have been transformed into canola, a crop that satisfies the demands of our modern, health-conscious society. The name "canola" was chosen to connote both health-associated characteristics and its Canadian origin. The agronomic performance of canola (*Brassica napus* and *B. rapa*) is continuously undergoing improvement through active plant breeding programmes. In addition to tailoring traits such as consistent yield of defined oil and quality within an achievable growing season, breeding programmes incorporate improved agronomic performance of cultivars via increased resistance or tolerance to stresses such as low temperatures, at either end of the growing term, and plant diseases. Canola producers on the Canadian prairies are restricted in their option to select canola for cultivation, not merely by prevailing and forecasted economic factors, but by the current history of their land use. As canola is susceptible to the blackleg fungus, *Leptosphaeria maculans* (teleomorph: *Phoma lingam*), crop rotation has been the most effective method available for control of this disease (Hall, 1992). To reduce economic losses to blackleg fungus, field resistance has been deemed the ultimate long-term solution (Rimmer and van den Berg, 1992).

Resistance to blackleg fungus has been identified in various Brassica species. Resistance to blackleg fungus operates at different stages of plant development. The general consensus is that whole plant resistance is associated with factors carried by the b-genome of Brassica species (Rimmer and van den Berg, 1992).

Incorporation of whole plant resistance into canola cultivars requires techniques that overcome natural barriers between species. Such methods are time-consuming and labour-intensive. To abbreviate the selection process, methods for early detection of resistant germplasm are sought. It has been standard practice to screen for disease resistance via fully expanded cotyledons (Williams, 1985), despite the fact that cotyledon screening involves only limited plant material. Cotyledon assay is routinely carried out with the highly aggressive *L. maculans* isolate (Leroy) (Gugel *et al.*, 1990). Of several inoculation–rating combinations studied, both McNabb *et al.* (1993) and Bansal *et al.* (1994) found that rating of inoculated cotyledons held the highest precision for predicting field performance. Resistance detected at the cotyledon stage has been found to persist to the mature plant stage (Stringam *et al.*, 1992). Thus, use of cotyledon tissue appears to be expeditious and, within certain limits, efficacious for assessing some

components of Brassica resistance.

L. maculans has both biotrophic and saprophytic stages (Williams, 1992). Because of the latter characteristic, *L. maculans* is readily cultured *in vitro*. Early studies focused on selection of Brassica resistance through the use of co-cultivation with *L. maculans*, or more simply, through the use of culture filtrate and purified phytotoxins from aggressive strains (Sacristán, 1882, 1985; Sjödin and Glimelius, 1988). Culture conditions and pathogen growth stage influence the constituents identified. Thus there are contrasting results and opinions regarding efficacy of the method (Pedras and Séguin-Swartz, 1992). Most identified phytotoxins are non-host-specific, and so, are more likely to be virulence factors rather than pathogenic ones (De March *et al.*, 1986). Recently, a host-specific phytotoxin called phomalide been identified from *L. maculans* (Pedras and Taylor, 1993b). Further, it is possible that minor components of *L. maculans* culture filtrates remain unidentified or that filtrate constituents act in a synergistic fashion not yet understood.

There is a paucity of inforrnation about the molecular mechanisms underlying Brassica cotyledon response to *L. maculans* or filtrate from its culture. The present study was devised to address this issue. Reports in the literature regarding induced or elicited Brassica response to *L. maculans* are limited to either phytoalexins (Rouxel *et al.*, 1991; Pedras and Séguin-Swartz, 1992) or chitinase (Rasmussen *et al.*, 1992a,b). Cotyledons of resistant Brassicas were found to display rapid induction of chitinase activity restricted to the site of pathogen attack.

As plant organs, the relationship between cotyledons and leaves has long been a source of controversy (section 1.5.2.). A single gene may regulate the differences between cotyledons and leaves of Arabidopsis (Meinke, 1992). Although initially a storage organ for germination and early seedling growth, by 10 days Brassica cotyledons possess operational photosynthetic apparatus and contribute as a source to the growing seedling. Storage proteins are metabolized well before the 10 day stage (Crouch and Sussex, 1981; Murphy *et al.*, 1989b; Höglund *et al.*, 1992). Lipase activity, indicative of metabolism of triacylglycerols, continues past the 10 day stage after having peaked at day 4 (Lin and Huang, 1983). Furthermore, glyoxysomes become converted to "leaf-type" peroxisomes in photosynthetically active Brassica cotyledons (Zhang *et al.*, 1994).

Nevertheless, cotyledons are not leaves. Although blackleg resistance observed at the cotyledon stage persists to the mature plant stage (Stringam *et al.*, 1992), certain other Brassica cotyledon characteristics have been established as being indicative of maternal genotype, not zygotic genotype—for example, glucosinolate profile (Magrath and Mithen, 1993). The zygotic genotype may affect Brassica cotyledon resistance to the blackleg fungus. Using winter oilseed rape varieties (*B. napus* var. *biennis*) as female parents in interspecific crosses with *B. juncea*,

Sacristán and Gerdemann (1986) found that some R_3 plants displayed higher resistance against *L. maculans* than did Jet Neuf. They determined that the average cotyledon lesion rating for these crosses (3.5 to 4.7) was greater or equivalent to that found for Jet Neuf (3.5).

The use of live pathogens in the study of host defense responses offers several complications. Inoculation of several dozen cotyledons of different species is time-consuming and tedious. Often abrasion of cotyledon laminae is performed to enhance inoculation. Even if abrasion does not accompany inoculation, fungal ingress induces wound responses in the host. Also, quarantir.es and extra precautions relating to pathogen containment are required when handling aggressive pathogens. The presence of fungal biomass may contribute to any extractable proteins or nucleic acids. In an effort to reduce these factors, a model system involving noninvasive application to Brassica species cotyledons of filtrate derived from the culture of an aggressive *L. maculans* strain (isolate Leroy) was proposed for study. A correlation between level of virulence of *L. maculans* and phytotoxicity of its untreated culture filtrate has been established (De March *et al.*, 1986). The relevance of simplifying the pathogen to treatment by its culture filtrate and the host to an immature growth stage has been previously discussed (section 1.5). It is necessary to reiterate that conclusions based on a reductionistic model are limited by the methodology.

Response of seedlings and excised cotyledons of six Brassicas to noninvasive culture filtrate treatment generally mimicked, albeit more slowly, the response when treatment was concurrent with wounding (Table 3.1). The bifacial lesions (affecting upper epidermis and underlying tissue) produced by Leroy culture filtrate in this study are similar to the findings of De March *et al.* (1986). Except for the case of unwounded Jet Neuf seedlings, all screened Brassicas revealed cotyledon lesion development following filtrate treatment. Again, this result was similar to that obtained by De March *et al.* (1986) for those five species.

Jet Neuf is known to be blackleg-susceptible up to the nine-leaf stage (Rouxel *et al.*, 1990a). Preliminary screening results of this study(Table 3.1) indicates that Jet Neuf performed better than resistant cultivars, particularly when no wounding accompanied inoculation. During treatments rounds to dervie plant material for protein and RNA analyses, Jet Neuf sedlings (Figure 3.1.1.) responded similar to Westar (Figure 3.1.2.). Thus, the cotyledon assay for Jet Neuf resistance to culture filtrate was variable.

When small populations of plant material were treated en masse, the lesions varied from darkened, dry spots to dark green, water-soaked areas (Figures 3.1.1. to 3.1.6.). Thus, plasmolysis may occur in response to Leroy filtrate. Electrolyte leakage assays or microscopic examinations would be required to determine and evaluate plasmolysis.

Selection of the appropriate control is a concern when using culture filtrate. In the present study, ddH_2O was used as the control. Sterile water is the standard control for cotyledon assays

(Williams, 1985). My results suggest that culture medium may be more appropriate as a control for some species. For example, both *B. carinata* species, which are considered resistant (Gugel *et al.*, 1990), displayed extensive curling in response to filtrate treatment. Perhaps the use of appropriately modified liquid medium might provide insight into the cause of the curling. Alteration of culture medium through fungal metabolism would be anticipated. Thus, additional chemical analysis would be required to allow correct adjustment.

The identity of the putative filtrate-induced protein in treated Domo (*B. juncea*) (Figure 3.2.1. and Figure 3.2.2.) requires further substantiation and analysis to conclusively establish correlation with resistance to blackleg fungus. Culture filtrate of purified phytotoxins may merely be inducing or selecting for responses to those treatments and not for disease resistance or disease responses. Analysis of proteins from conidia-inoculated cotyledons could confirm whether the protein in question is induced by live fungus, or if it is a filtrate-induced artifact. However, appropriate controls for fungal proteins would be difficult to accurately ascertain as protein profiles from mycelia cultured on defined media may not be similar to those derived from *in planta*. A number of proteins have been identified. For example, *L. maculans* is known to express cell-wall degrading enzymes (Easton and Rossall, 1985) and surface proteins of *L. maculans* mycelia have been analyzed (Hassan *et al.*, 1991). Comparison of results reported by that group to results obtained with conidia-inoculated cotyledons may offer insight into the relationship between *in vitro* versus *in planta* mycelial protein profiles.

The putative filtrate-induced protein appears to be a single polypeptide, based on results of protein analysis in the presence of 6M urea (Figure 3.2.2.). Isoelectricfocusing and electrophoresis in two dimensions (2D) would conclusively establish the fact that it is a single polypeptide. Furthermore, additional differences may be determined by 2D gels. Microsequencing of proteins from 2D gels has been used to derive degenerate primers subsequently used in gene analysis.

Evidence for induction within 1 hour of the putative filtrate-induced protein is partially indicated by *in vivo* labelling (Figure 3.3.1.). Evidence is partial as a result of the absence of one hour control (lane 1) from the fluorograph. Also, levels of intensity for the protein band are similar for control and treated cotyledons at later sample times. As, defense responses have been reported within minutes of elicitor treatment of cell suspension cultures (Hedrick *et al.*, 1988), these results are in agreement with the work of others. Additionally, the response may have been transient and so, was not as strongly expressed at later labelling times.

In vivo labelling of excised S67 cotyledons resulted in more protein bands discerned by fluorography (Figure 3.3.2) than for excised Domo cotyledons (Figure 3.3.1.). *B. carinata* cotyledons are thicker and waxier than those of *B. juncea*. Thus, S67 cotyledons may have a

greater capacity to withstand the labelling procedure used in this sudy. Alternatively, the differences in labelled protein profiles may simply reflect differences in metabolic rates between Domo and S67. *In vivo* labelling by Brassica seedling uptake may offer improvement in future studies. This procedure obviates wound response imposed by excising cotyledons and has been shown to produce acceptable levels of incorporation.

The relative molecular weight (M_r) of the putative filtrate-induced protein was frequently determined to be 16.0–16.5 kDa, but varied from 14.5 kDa to 17.0 kDa. Load variation, evident in Figure 3.2.1. and Figure 3.2.2., may account for some apparent difference between treated and control samples. One strategy to identify this protein, without having to sequence its amino acid constituents, would be to search a plant protein databank for cytosolic proteins of 14.5 to 17 kDa. Comparison to published *B. napus* cotyledon protein profiles indicates that the putative filtrate-induced protein is not cruciferin (31 and 22 kDa) nor napin (13 and 11 kDa) (Crouch and Sussex, 1981; Höglund *et al.*, 1992). Additionally, a prominent band in the 16 to 16.5 kDa region is not evident in total proteins from 7.8 day-old *B. napus* cotyledons (Murphy *et al.*, 1989b). Comparison with those studies is not absolutely conclusive as Domo is a different species (*B. juncea*). The rubisco small subunit precursor of radish has been reported as 23 kDa (Meza-Basso *et al.*, 1986). The size of the processed Brassica rubisco small subunit is about 20 kDa (Nykiforuk, personal communication). Rubisco small subunit precursor may be evident in both control and treated cotyledons (Figures 3.2.1 to 3.2.4.).

Both Domo and S67 have been reported as very resistant to Leroy isolate of *L. maculans* based on disease ratings of inoculated cotyledons (Gugel *et al.*, 1990). The presence of a putative filtrate-induced protein in Domo (Figures 3.2.1 and 3.2.2.) but not S67 (Figures 3.2.3. and 3.3.4.) may be related to genotypic differences, assuming that filtrate-treatment is equally effective for both species. Domo has both a- and b-genome components whereas S67 has b- and c-genome components. Resistance to blackleg fungus effective at all Brassica growth stages has been associated with the elements found on the b-genome (Rimmer and van den Berg, 1992). Within the model used in this study, it is not conclusive whether the putative filtrate-induced protein resides on the a-genome of Domo (*B. juncea*), and hence, is absent from treated S67 (*B. carinata*) cotyledons, or if the b-genome of Domo responds to filtrate differently than the b-genome of S67. The curling of filtrate-treated *B. carinata* cotyledons may argue in favour of the latter. Although the b-genome of both species originated from the same source (*B. nigra*), they are not considered to be completely identical. Therefore, it is possible that elements responsive to Leroy filtrate exist in *B. juncea* but are absent in *B. carinata*. Without detailed information at the molecular level or appropriate mutants, firm conclusions are not possible in this regard. If the difference observed

in this study is correlated with the a-genome, the observed difference relates to filtrate resistance and not disease resistance. Thus, a-genome resistance may not operate at the seedling stage.

General induction of the phenylpropanoid pathway and lignin precursors indicated by *de novo* synthesis of mRNA and transcriptional activity of key phenylpropanoid enzymes have been observed host-pathogen interactions (Hahlbrock and Scheel, 1989). Northern analysis of Domo RNA with a probe derived from bean for phenylalanine ammonia-lyase (PAL), the first committed and major regulatory enzyme of the phenylpropanoid pathway, failed to discern any signal (Figure 3.4.4.). Results were similar for both radiolabelled and biotinylated pPAL5. Since the internal control of denatured pPAL5 cDNA hybridized with the probe and was visualized with both detection systems at low wash stringency, it is likely that homology between bean pPAL5 and Brassica PAL is too low for detection. Use of a homologous probe for PAL is therefore warranted.

Northern analysis of Domo RNA with tobacco cDNA for cinnamoyl alcohol dehydrogenase (CAD) (Figure 3.4.3.) detected a doublet in both control and treated samples. CAD probes are used to demonstrate activity of the lignin-biosynthetic pathway. Both PAL and CAD are known to exist as small gene families in other plant species. As a shift in apparent MW was observed in the 72 hour sample (lane 6), it is possible that pTCAD4 cDNA hybridized to a Brassica isoform that is defense-related or filtrate-inducible. Further, the lignification observed in Brassica response to *L. maculans* was termed "anarchic" in deference to the fact that it was different to lignin formed in healthy, unchallenged cotyledons (Brunin, 1972). Histochemical staining of filtrate-treated cotyledons by stains specific for lignin or performing enzyme activity assays would assist the analysis of lignin induction by filtrate treatment. Nevertheless, it is apparent that lignification occurs in Domo 10 to 14 day-old cotyledons.

Protein analysis of *in vitro* translated RNA products would have greatly benefitted the present study. Substantiation of the putative filtrate-induced protein as well as valuable information regarding *de novo* induced responses could be gained.

A more accurate indication of *in planta* response would be obtained by analysis of conidia-inoculated cotyledons, provided appropriate controls were considered. Since the inception of this study, Leroy specific PCR primers have been reported (Xue *et al.*, 1992). A quantification scheme taking advantage of these primers could be devised to assess fungal donation to nucleic acids extracted from conidia-inoculated cotyledons. For example, a standard curve could be established with known concentrations of Leroy DNA. Comparision of PCR products of amplified DNA from infected plant material to a standard curve has been reported to provide an estimation of the extent of fungal presence in an infected host (Moukhamedov *et al.*, 1994). Further, a single-protocol DNA extraction method of plant tissue inoculated with an Ascomycete fungus has been reported to be quantitatively mass dependent (Talbot *et al.*, 1993). A similar assay could further

determine the amount of fungal biomass in conidia-inoculated cotyledons.

In summary, a simplified model system was applied to study the *Leptosphaeria*-Brassica interaction. Cotyledon response to treatment with filtrate from culture of the aggressive strain Leroy broadly mimicked reported results for the same Brassica entries inoculated with Leroy conidia (Gugel *et al.*, 1990). Observed response differences between susceptible and resistant Brassica were subtle. A filtrate-induced protein (M_r 16-16.5 kDa) was observed within 24 hours after treatment in *B. juncea* cultivar Domo but not *B. carinata* cultivar S67. Further analysis is necessary to establish correlation of this protein to disease response. *In vivo* labelling of Domo cotyledons provides partial and tentative evidence that the putative filtrate-induced protein may be synthesized *de novo* within one hour of treatment. Northern analysis of Domo failed to conclusively indicate general induction of the phenylpropanoid pathway; however, lignification may have been implicated in response to treatment with fungal filtrate. Overall, the suitability of the proposed model has not been substantiated and would require modification to yield applicable information.

5. CHAPTER 5. Literature Cited.

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