GENETICS AND HISTOLOGY OF

RESISTANCE AND SUSCEPTIBILITY TO *ALBUGO CANDIDA* IN *BRASSICA NAPUS*

by

Qi Liu

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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FORWARD

The format of this thesis follows the manuscript style which has been outlined by the council of the Faculty of Graduate Studies and the Department of Plant Science of the University of Manitoba. Three manuscripts are presented, including abstract, introduction, materials and methods, results and discussion. A general abstract, a general introduction and a literature review precede the manuscripts. A general discussion, a literature cited and an appendix terminate the thesis.

Some of the work presented in these manuscripts has previously been presented at the 1986 Meeting of the Canadian Phytopathological Society (the best student paper award) and at the 7th International Rapeseed Congress held in Posnan, Poland, 1987.

GENERAL ABSTRACT

LIU, QI. M.Sc., The University of Manitoba, June, 1987. Genetics and histology of resistance and susceptibility to Albugo candida in Brassica napus. Major Professor; S.R. Rimmer.

Progenies derived from the backcross [Regent (Resistant) x 2282-9 (susceptible)] x 2282-9 were obtained to test the hypothesis that the inheritance of resistance to Albugo candida race 7 in the Brassica napus cultivar Regent is conditioned by two independent dominant genes designated Ac7-1 and Ac7-2 denoted as R_1 and R_2. Resistance was determined by inoculating the cotyledons with a zoospore suspension of A. candida race 7.

Nine accessions of genotype $R_1R_1r_2r_2$ or $r_1r_1R_2R_2$ were established through selfing the resistant F_1BC_1 plants for two generations. One accession was assumed to be of genotype $R_1R_1r_2r_2$ and used as a parent for test crosses with the other accessions. The resulting F_1 progenies were all resistant. F_1 plants were selfed and backcrossed to the susceptible line 2282-9. Progenies from four of the accessions x tester segregated in 15:1 (F_2) and 3:1 (BC_1) ratios. This indicates that the four accessions were of genotype $r_1r_1R_2R_2$. Progenies of the other accessions x tester were all resistant, indicating that these accessions had the same genotype as the tester. The study confirms that R_1 and R_2 are two independent dominant genes conferring resistance to A. candida race 7 in B. napus.

Two susceptible Chinese lines 2282-9 and GCL (B. napus) and one susceptible Canadian cultivar Torch (B. campestris) were tested for variation in disease reaction by inoculating the seedlings at the second and fifth leaf stage. Statistical analyses indicated that the line/cultivar, inoculum concentration, incubation temperature and their interactions had significant effects on white rust development. Day/night temperatures of 22/17°C resulted in higher infection levels than those of 15/10°C. The infection level increased with inoculum concentration on all of the line/cultivar investigated. The study revealed a marked difference in disease reaction between B. napus and B. campestris and provided support for the contention that genes with minor effects are present to modify the degree of susceptibility in the two susceptible B. napus lines.

Cotyledons of one resistant and three susceptible lines/cultivars of Brassica napus and B. campestris were inoculated with zoospores of A. candida race 7 to determine the earliest events which could distinguish genetically distinct combinations between the host and parasite. Samples of whole cotyledons were collected at regular intervals after inoculation and prepared for observation with a Zeiss Standard Microscope equipped with a Differential Interference Contrast objective. The time course of the infection process was followed histologically. The sequence of pre- and early post-penetration events were similar in compatible and incompatible interactions. Germination of zoospore cysts occurred 2-3 h after inoculation. Infection was initiated with germ tubes penetrating through stomata. Haustorium formation was first observed in the palisade mesophyll cells adjacent to the substomatal chambers 8 h after inoculation.

Only after the establishment of the first haustorium did compatible and incompatible interactions begin to differentiate. In the resistant cultivar, most primary hyphae produced single haustoria. Necrosis of the invaded host cells was first observed 12 h after inoculation followed by cessation of fungal growth. The death of host cells was largely restricted to the penetration site where the host cell had contacted with a fungal hypha bearing an apical haustorium; the adjacent non-penetrated cells remained apparently unaffected. In the susceptible line/cultivar, necrosis of infected cells occurred only infrequently and hyphal growth continued unabated, resulting in mycelial ramification into several layers of the mesophyll. Numerous haustoria were produced. Sporulating pustules formed within 5-6 days after inoculation.

Histological comparisons of the sequence and timing of early events of the infection process of A. candida race 7 on the susceptible and resistant rape lines/cultivars revealed that the earliest event distinguishing a compatible from an incompatible interaction occurred after formation of the first haustorium and that resistance was not expressed until the host mesophyll cell had come into contact with the first haustorium. The distinction between compatibility and incompatibility was substantiated by quantitative analysis of white rust development on these lines/cultivars.

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Chapter I

GENERAL INTRODUCTION

White rust caused by Albugo candida (Pers. ex Hook.) Kuntze is a common disease found on a wide range of cruciferous species and some species of Capparidaceae. It has been reported that 241 species in 63 genera of Cruciferae are attacked by the fungus (Biga, 1955). To date, eight biological races have been identified and classified on the basis of host specialization, of which five races (race 1, 2, 3, 7 and 8) are of economic importance (Pound and Williams, 1963; Verma et al., 1975; Delwiche and Williams, 1977). They attack Raphanus sativus L., Brassica juncea (L.) Coss., Armoracia rusticana Gaertn., Mey., and Scherb., Brassica campestris L. and Brassica nigra (L.) Koch., respectively. The isolate which predominantly occurs on B. campestris can also infect susceptible lines/cultivars of Brassica napus L. (Fan et al., 1983; Pidskalny and Rimmer, 1985).

In western Canada, white rust is a major disease on turnip rape (B. campestris) and brown mustard (B. juncea). Considerable yield losses of turnip rape were reported. In Manitoba, yield reductions in heavily infected fields ranged from 30 to 60% in 1971 (Bernier, 1972). In Saskatchewan, yield losses in 1970, 1971 and 1972 were approximated at 3, 6 and 9%, respectively (Petrie, 1973). In central and northern Alberta, yield losses were estimated at 1.2% in 1971 (Berkenkamp, 1972). Canadian cultivars of B. napus have been shown to be highly resistant to A.

candida in both field and laboratory studies (Petrie, 1975b; Petrie and Dueck, 1979). Also, in spite of having been exposed to the isolates of A. candida from B. campestris and B. juncea for over 40 years, the resistance in B. napus has still remained effective. In central and eastern China, however, some B. napus cultivars are very susceptible. Stagheads occur frequently in the field every year, and heavy yield losses have been reported in different localities.

In their pioneer study on the inheritance of resistance to A. candida in B. napus, using the resistant Canadian cultivar Regent and two susceptible Chinese lines 2282-9 and Green Cup Leaf (GCL), Fan et al. (1983) proposed a genetic model in which resistance is conditioned by the three independent dominant genes R_1, R_2 and R_3. The first two resistance genes are homogeneous in the cultivar Regent. As a single dominant allele at any one of the three loci is sufficient to confer resistance, the heterozygosity or heterogeneity of the third locus can be masked. This information can be useful in resistance breeding programs provided the proposed model has been confirmed.

The recessive genes for susceptibility to A. candida in 2282-9 and GCL were suggested to be allelic. However, genes with minor effects might be present to modify the degree of susceptibility as variation in sporulation was observed between these two lines as well as within their F_1 and F_2 progenies (Fan et al., 1983). Phenotypic expression on the host is a result of the interactions between host-parasite genotypes. Variation in susceptibility can result from genetic variation in the host and parasite, and/or changes of environment. More information is needed to determine whether there are genes with minor effects to modify the degree of susceptibility.

As an obligate parasite, A. candida grows in intimate association with the living cells of its host. Little is known of the mechanisms responsible for establishing and maintaining such a relationship, and how an incompatible reaction occurs when the fungus confronts host cultivars carrying resistance genes. Histological studies of the pathogen on both resistant and susceptible cultivars may reveal the earliest events in the infection process, which could distinguish genetically distinct combinations of host cultivar and fungal race. This information is essential for interpreting results of biochemical and physiological research on mechanisms of resistance.

Verma et al. (1975) studied the infection of A. candida on four Brassica species and found that the early events of the infection process on hosts and non-hosts were similar and resistance was not expressed until a hyphal tip had come into contact with a host mesophyll cell. Pidskalny (1984) also described some aspects of the infection process of the fungus on two B. campestris cultivars. However, in both of these studies, observations on the fungal pathogenesis were not started until 24 h after inoculation. In fact, the initial 24 h can be a crucial stage in the life history of an obligate parasite. A series of host-parasite interactions including structural and probably biochemical changes may occur during this period of time. As the length of time that elapses between inoculation and when microscopic examinations are made can greatly affect the interpretations that can be made, it is logical to begin comparing the histological events in resistant and susceptible rape lines/cultivars at the first hour after inoculation so as to detect the earliest events which distinguish a compatible from an incompatible interaction.

With these facts in mind, the objectives of this study were a) to test the hypothesis that resistance in Regent is conditioned by two independent dominant genes, b) to determine whether genes with minor effects exist to modify the degree of susceptibility, c) to compare the histological events of fungal pathogenesis in both resistant and susceptible lines/cultivars, and d) to determine the earliest events which can distinguish genetically distinct types of host-parasite interactions.

Chapter II

LITERATURE REVIEW

2.1 THE HOST (OILSEED RAPE)

Oilseed rape, one of the most important oil crops in the world, is cultivated predominantly in western Europe, Canada, China and the Indian subcontinent. The seed contains 33 to 50% oil on a dry weight basis, which is extracted for edible as well as for industrial uses. The remaining meal, containing 38 to 41% protein, is a feedstuff of high nutritional value. The commodity oilseed rape can be cultivars of three distinct species, Brassica napus L., Brassica campestris L. and Brassica juncea (L.) Coss.. Each species is further divided into subspecies, forms and varieties or cultivars. Winter and spring forms occur in both B. napus and B. campestris species, which differ significantly in morphology and in physiology as well. There are also other Brassica species cultivated as vegetable and condiment, etc..

2.1.1 Botanical Relationships

The botanical relationships among the cultivated Brassica species have long been studied, but it was not well understood until the mid-1930s when Morinaga (1934) provided cytological evidence showing that the higher chromosome Brassica species B. napus (n = 19, AC), B. juncea (n = 18, AB) and Brassica carinata Braun (n = 17, BC) were amphidiploids

originally derived from natural interspecific hybridization between lower chromosome species Brassica nigra (L.) Koch. (n = 8, B), Brassica oleracea L. (n = 9, C) and B. campestris (n = 10, A). This finding (Figure 1) was later confirmed by U (1935) who succeeded in artificial synthesis of B. napus from crosses between diploid types of B. campestris and B. oleracea. Useful hybrids of B. juncea and B. carinata were also obtained from the interspecific crosses between B. nigra and B. campestris or B. oleracea (Downey et al., 1975; Olsson and Ellerström, 1980).

This understanding of the relationship among Brassica species has encouraged plant breeders to create new synthetic materials and to transfer desirable agronomic characteristics from species to species through interspecific hybridization. Although to date no cultivars have been released as a direct result of reconstitution of a species through interspecific crosses, some desirable characteristics have been successfully transferred from one species to another through artificially synthesized amphidiploids which function as a "bridge". At the Agriculture Canada Research Station, Saskatoon, Saskatchewan, the first doublelow (low erucic acid in the oil and low glucosinolate in the meal) strains of turnip rape were developed from interspecific crosses among turnip rape (B. campestris), rape (B. napus) and oriental mustard (B. juncea) (Downey, et al., 1975). In China and Japan, interspecific crosses between B. campestris and B. napus have been used as a routine method to transfer such characteristics as early maturity, cytoplasmic male sterility, self-incompatibility and yellow seed-coat from the former to the latter, and to broaden the genetic basis of B. napus through genome replacement (Liu, 1985).

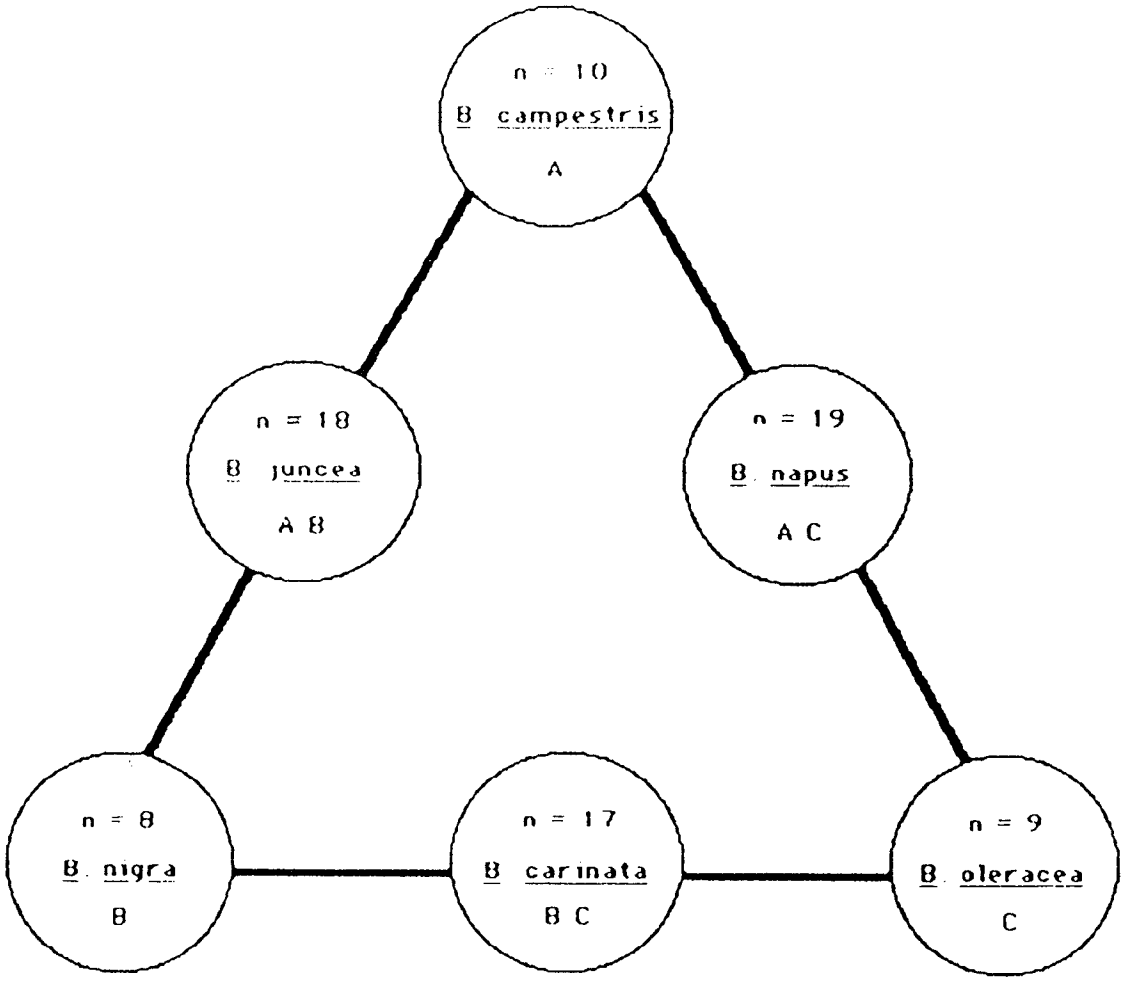

Figure 1. The derivation of the higher chromosome Brassica species according to U (from Frandsen, 1943)

2.1.2 Centre of Origin and History of Oilseed Rape Cultivation

Europe and Asia are generally considered as the centre of origin for B. campestris and B. juncea. B. napus, together with B. nigra and B. oleracea, is thought to have originated in the Mediterranean region and in western or northern Europe (Tsunoda, 1980). From these centres of origin, oilseed rape is believed to have spread into America and Oceania.

Oilseed rape has a long history of cultivation. The earliest records of rape cultivation are found in the Indian Sanskrit writings from 2000 to 1500 BC. Yellow Sarson (B. campestris) was considered to be the oldest of various rapes or mustards grown in India (Appelqvist, 1972).

China is believed to have been the centre of origin for B. campestris var. oleifera DC. (Liu, 1985). Although the ancient agricultural literature indicates that rape cultivation occurred about 2500 years ago, carbonized seeds of B. juncea and B. rapa of 6000-7000 years old have been unearthed in the site of a neolithic village called Banpo. As recorded, rape plants were first cultivated and consumed as a vegetable, and later rapeseed oil was used for illumination, in foods and as a cooking oil. B. napus was introduced to China from Europe in the late nineteen century, but commercial production did not begin until 1950s. Now, it has been distributed throughout China and has almost totally replaced B. campestris in the southern regions.

In Europe, turnip rape and rape are considered to be among the oldest and most widespread of various Brassica species. Turnip rape from the Bronze Age was found in Zurich, northeast Switzerland. Rape cultivation is thought to have commenced in the thirteen century. It used to be an

important source of lamp oil until replaced by petroleum oil (Appelqvist, 1972).

Commercial production of oilseed rape in western Canada was started in 1942 with the primary aim of providing a domestic supply of lubricants for marine engines during the war time embargo. The two distinct species of rape grown in Canada are spring turnip rape and spring rape. Rapeseed oil was first extracted for edible use in Canada in 1956-57. In the past 20 years, extensive research has been conducted to improve the quality of rapeseed oil and meal. The successful production of doublelow cultivars has increased the economic value of oilseed rape and expanded markets for rapeseed products. Canada is today the world's largest exporter of rapeseed.

2.2 THE PATHOGEN

Albugo, the only genus in the family Albuginaceae, consists of about 30 species of obligate parasites causing diseases known as white rusts (Walker, 1969; Webster, 1980). Of these, Albugo candida (Pers. ex Hook.) Kuntze is the one of economic importance. It attacks at least 241 species in 63 genera of Cruciferae (Biga, 1955) and some species of Capparidaceae.

Albugo candida has several synonyms: Aecidium candidum Pers., Uredo candida thlaspeus Pers., Uredo cheiranthi Pers., Uredo candida (Pers.) Fr., Albugo cruciferarum S.F. Gray, and Cystopus candidus (Pers.) Lev..

Species of less economic importance are A. ipomoeae-panduraneae (Schw.) Swing. or A. minor (Speg.) Cif. on sweet potato; A. occidentalis

G. W. Wilson on spinach; and A. tragopogonis (DC.) S. F. Gray on salsify (Tragopogon porrifolius), goatbeard (T. pratensis) and Senecio squalidus (Webster, 1980).

2.2.1 Symptoms

A. candida causes two types of infection, local and systemic. The local infection is characterized by raised white pustules which are commonly developed on the abaxial surface of leaves. These pustules vary considerably in size. They may arise individually, ranging from 1 to 2 mm in diameter, or coalesce to form large patches (Walker, 1969). When the host epidermis ruptures, zoosporangia are released as a white powdery mass (Petrie, 1975a). Secondary pustules may later develop around the original pustules, forming successive concentric rings (Endo and Linn, 1960). The host tissues which surround the invading fungus usually become chlorotic more rapidly than the tissues at infection sites, forming small so-called "green islands". These remain visible on the adaxial surface of senescing leaves, and directly underneath are sporulating pustules (Martens et al., 1984).

Frequently the fungus becomes systemic in young stems or inflorescences, stimulating hypertrophy and hyperplasia (Walker, 1969). This is the most conspicuous stage of the disease. The degree of deformation varies. Sometimes on a given inflorescence, parts of the individual flowers or pods become enlarged and distorted, whereas others remain normal. Sometimes the entire inflorescence is swollen and replaced by a spiny sterile structure called staghead (Petrie, 1975a). The young stagheads are green due to formation of chloroplasts, and white pustules

similar to those on leaves may appear on the surface. At maturity, stagheads turn brown and brittle. They are almost entirely composed of thick-walled resting spores called oospores.

The hypertrophies of host tissues caused by A. candida appear to offer favorable sites for the spore production and dissemination of secondary microorganisms, and stem blisters may serve as an important avenue for invasion of the plant by other fungi. Over 20 species of fungi, including several pathogens of crucifers, have been observed in association with hypertrophied inflorescences as well as stem and pod blisters (Petrie and Vanterpool, 1974). Of these, Peronospora parasitica has the most intimate relationship with A. candida. In nature, these two fungi can cause simultaneous infection on rape plants. However, the mutual interaction of A. candida and associated microorganisms is open to question.

2.2.2 Disease Cycle

A. candida overwinters mainly by means of oospores either in the soil or mixed with seeds. In perennial hosts such as horseradish, mycelium may also function as an overwintering form in the crowns and occasionally in lateral roots (Kandow and Anderson, 1940; Takeshita and Linn, 1953). Oospores may survive adverse conditions and have been shown to remain viable for 20 years in dry storage (Verma and Petrie, 1975).

Oospores are considered to be an important primary source of inoculum. According to the survey by Vanterpool in 1959, infection was usually 3 to 4 times higher when rape had been continuously cropped on the

same land than when there was a rotation of summer fallow or cereal crops. In addition, seed samples of B. campestris in western Canada were found to be heavily contaminated by oospores (Petrie, 1975b). This is not surprising because oospores are formed in great abundance in hypertrophied inflorescences of rape plants and are chiefly released during threshing.

The mode of oospore germination is well documented (DeBary, 1887; Vanterpool, 1959; Petrie and Verma, 1974; Verma and Petrie, 1975). Oospores germinate 1) by forming a sessile vesicle in which zoospores are formed and from which they escape by rupturing the thin evanescent membrane, 2) by producing a discharge tube with a terminal or lateral zoosporangium, or 3) by producing 1 or 2 germ tubes which give rise directly to mycelium. The most common type of oospore germination is by the production of a sessile vesicle. The presence of the other two less common types of germination may be indicative of the high adaptability of the pathogen to the environmental stress. Petrie and Verma (1974) found that a slow leaching of oospores by spring rain could stimulate germination.

Zoospores produced by oospores appear to be the primary inoculum for the infection of Brassica species. The invasion takes place when the germ tubes of zoospores penetrate the tissue of cotyledons or true leaves through stomata. The germ tube elongates and the first haustorium is formed at the tip of the primary hypha. The mycelium continues to grow intercellularly into the mesophyll layer. Short, club-shaped sporangiophores are later produced from the tips of hyphal branches beneath the abaxial host epidermis. The ultrastructure of sporangiophores and

the process of sporangial production was described in detail by Khan (1977). Sporangia are produced in basipetal chains at the apices of the sporangiophores and secede one by one as they are delimited by the septa. The mature, detached sporangia are accumulated in the space between the sporangiophores and the host epidermis. The first sign of infection is shiny, smooth, white pustules on the abaxial surface of cotyledons and leaves. The formation of numerous sporangia gradually exerts a great pressure on the host epidermis, causing it to bulge and eventually to rupture. Consequently huge numbers of sporangia are released into the air. Disseminated by wind, water or perhaps other agents, sporangia act as an effective secondary inoculum and are ready to germinate on the leaf surface of appropriate hosts, which is covered with a film of water.

As early as 1911, Melhus emphasized the importance of chilling treatment for sporangium germination. After chilling, sporangia could germinate within the range of 1 to 28°C. Napper (1933) found that germination required dehydration of the water content of the sporangia to approximately 30%. When a sporangium germinates, it gives rise to 4 to 12 biflagellate zoospores. The infection process thereafter is the same as that caused by zoospores released from oospores, and the secondary infection can cycle several times during a single growing season, spreading the disease from leaf to leaf and plant to plant. Ultimately, the disease becomes systemic with formation of stagheads. It is within these hypertrophied inflorescences that sexual reproduction of A. candida takes place. Multinucleate sexual organs, oogonia and antheridia, are formed within the tissue of the host (Alexopoulos and Mims, 1979).

Meiosis has been shown to occur in these gametangia (Sansome and Sansome, 1974). When gametangial contact is effected, a fertilization tube is formed by the antheridium. During fertilization, a single male nucleus migrates through the fertilization tube into the oosphere and fuses with the egg nucleus. The resulting zygote nucleus divides mitotically for several times, forming a mature oospore with a thick ornamental wall (Alexopoulos and Mims, 1979). At harvest, crushing of stagheads returns oospores to the soil or distributes them among seeds.

2.2.3 Biological Races

Biological specialization in A. candida has long been noted. As early as 1904, Eberhardt recognized two specialized forms of the fungus occurring on turnips. Since then, the existence of distinct biological races has been confirmed by many workers (Melhus, 1911; Hiura, 1930; Napper, 1933; Togashi and Shibasaki, 1934). However, the extent of specialization has never been fully explored, and several different classifications of the fungal races have been proposed.

On the basis of host specialization, Pound and Williams (1963) have described six North American races of the pathogen, of which the first three are of some economic importance. Race 1 is restricted to radish. Race 2 is largely limited to brown mustard, but may also be found on collards, Chinese cabbage, turnip and several seed mustards. Race 3 is strictly confined to horseradish. Races 4, 5 and 6 can attack only the weed hosts from which they are collected. This classification is subsequently enlarged to include the isolate occurring predominantly on turnip rape in western Canada (Verma et al., 1975; Pidskalny and Rimmer,

1985) and the isolate attacking B. nigra (Delwiche and Williams, 1977). They are designated race 7 and race 8, respectively.

The above classification has been further verified by Pidskalny and Rimmer (1985). They tested all the differential hosts except Sisymbrium officinale (susceptible to race 5) with zoosporangia of A. candida collected from B. campestris and B. juncea, and found that these two isolates were not cross pathogenic. Therefore, they were race 7 and race 2, respectively.

2.3 HISTOPATHOLOGY

2.3.1 Infection Process

The infection process of A. candida has been investigated in the cotyledons of Raphanus sativus L. (Williams and Pound, 1963) and a few Brassica species (Verma et al., 1975; Pidskalny, 1984).

When cotyledons are inoculated with a suspension of zoospores, the biflagellate zoospores will swim for a while and then become encysted. The encysted zoospores germinate by forming germ tubes which penetrate through stomata and thus initiate the infection. The time required for cyst germination has been reported by several workers, but results are inconsistent. According to Napper (1933), zoospores could germinate and enter the host within a few hours. Whereas Pidskalny (1984), working with A. candida race 7 on B. campestris, reported that at 24 h after inoculation only cysts were observed and very few germ tubes were produced even at 48 h following inoculation. The failure to detect the germinated cysts at the earlier stage could be due to the inoculation method and/or the microscopic technique he employed.

After penetration, germ tubes elongate to form primary hyphae. They grow intercellularly, and small spherical haustoria, ranging from 2.0 to 4.0 μm in diameter, develop in the palisade mesophyll cells close to the substomatal chambers. The haustorium is connected to a larger intercellular haustorial mother cell by a narrow neck about 1.8 μm long and 0.4 μm wide (Coffey, 1975), and continuously surrounded by an intact host plasma membrane as well as a layer of host cytoplasm (Berlin and Bowen, 1964). The host cell wall invaginates at the point of haustorial penetration to form a penetration jacket (sheath) around the proximal portion of the haustorial neck.

With the aid of electron microscopy, Berlin and Bowen (1963) were the first to describe the host-parasite interface involving the haustorial apparatus of A. candida in the mesophyll cell of Raphanus. They found the absence of the fungal cell wall in the distal portion of the haustorial neck and a layer of an amorphous, moderately electron-dense material lying between the haustorium wall and the host plasma membrane and extending into the penetration region to join the penetration jacket. However, their microscopic evidence was not considered convincing. Later, Coffey (1975) has demonstrated that the fungal cell wall is continuous throughout the haustorium and that the layer of electron-dense material surrounding the haustorium is a structure distinct from the penetration jacket. Because of its position immediately adjacent to the fungal wall, this layer is termed an extrahaustorial matrix and is covered with the extrahaustorial membrane which is an extension of the host plasma membrane. A recent cytochemical analysis concerning the nature of the haustorial apparatus of Albugo has revealed that the extrahausto-

rial matrix and penetration jacket are cytochemically different (Coffey, 1983). Similar fine-structural features of the host-parasite interface have been described for other Oomycetes, e.g. Phytophthora infestans in Solanum tuberosum (Hohl and Stössel, 1976; Hohl and Suter, 1976) and Peronospora pisi in Pisum sativum (Hickey and Coffey, 1977). However, the origin and composition of extrahaustorial matrix are still uncertain, let alone its function.

After the formation of the first haustorium, hyphal growth usually increases dramatically. The primary hyphae continue to grow intercellularly into different layers of mesophyll with production of more haustoria. As many as 14 haustoria were observed in a single host cell of a B. juncea leaf (Verma et al., 1975). The dimension of the individual hyphae appears to conform to the intercellular space. Hyphae were very narrow where the intercellular spaces were small, but could swell or branch profusely to form thick, compact mycelial masses when the spaces were large (Fraymouth, 1956). Generally, about 3 or 4 days after inoculation, almost all the available intercellular spaces are occupied by the mycelium. Numerous club-shaped sporangiophores later arise from dense mycelial masses beneath the epidermis (Webster, 1980). Lemon-shaped sporangia, about 19-22 x 14-17 µm, are produced in basipetal chains at the tips of the sporangiophores (Khan, 1977). Depending on hosts, pustules become visible 4-7 days after inoculation.

2.3.2 Histology of Compatibilty and Incompatibilty between Host-Parasite

The sequence of events brought about by histological studies can distinguish genetically distinct combinations of host and parasite. Species effect on the infection process of A. candida was investigated by Verma et al. (1975) in the cotyledons of four Brassica species: B. campestris (susceptible to race 7), B. juncea (susceptible to race 2), B. hirta (moderately resistant) and B. napus (resistant). No marked differences between the susceptible and resistant species were detected in the initial stage of infection from zoospore encystment to the formation of the first haustorium. Encysted zoospores germinated in resistant B. napus as readily as in susceptible species, and the primary hypha usually produced only one haustorium. But fungal growth was checked at approximately 72 h after inoculation because encasements (encapsulations) had formed around each haustorium, and subsequently only remnants of dead hyphae could be seen. In contrast, hyphal growth in susceptible hosts increased rapidly after the first haustorium had been formed. Hyphae grew around mesophyll cells, penetrating individual cells with various numbers of haustoria. These haustoria were seldom surrounded by encasements. Therefore, it is likely that resistance was not expressed unless contact had been made between a hyphal tip and a host cell. Later, similar results were obtained by Pidskalny (1984) who studied the infection process in two B. campestris varieties, Torch (susceptible) and Tobin (moderately resistant).

The deposition of a thick electron-lucent encasement around the haustorium has also been observed in other host-parasite interaction involv-

ing haustoria-forming biotrophic fungi. The encasement consists of an electron-lucent matrix embeded with fibrillar material and irregularly shaped, electron-dense deposits. With P. infestans in potato tubers, encasements occurred infrequently in the compatible interaction (Allen and Friend, 1983; Hohl and Stössel, 1976), whereas with Phytophthora parasitica in tobacco roots, encasements were a common feature of the compatible interaction (Hanchey and Wheeler, 1971). More detailed cytochemical and biochemical analysis will be required before the possible role of encasement in defense mechanism can be determined.

The histology of host resistance to A. candida race 1 was studied by Williams and Pound (1963) in R. sativus, and two types of resistant responses were discovered. In China Rose Winter (CRW), a hypersensitive response was expressed. The host epidermal cells as well as several layers of mesophyll cells in the vicinity died and suberized in response to the invading fungus while in Round Black Spanish (RBS), there was no sign of fungal invasion. Resistance could be attributed to the morphological feature of and/or toxic diffusates from the host.

2.4 GENETIC STUDIES OF HOST RESISTANCE TO A. CANDIDA

2.4.1 Resistance to Race 1

The inheritance of resistance to different races of A. candida has been investigated in a number of cruciferous hosts. In a screening test of 283 radish (R. sativus) accessions and 14 commercial varieties, two varieties of radish, CRW and RBS, were found to be resistant to A. candida race 1 (Williams and Pound, 1963). Genetic studies indicated that the resistance was governed by a single dominant gene in both CRW and RBS.

However, their resistant reactions were somewhat different. Normally a hypersensitive reaction occurred on CRW after the contact had been established between the host and parasite. Under certain environmental conditions, however, host response shifted from resistance to tolerance with production of discrete white pustules on the adaxial surface of the cotyledons. The sensitivity of CRW to environment suggested the presence of genes with minor effects to modify the resistant reaction conditioned by the major gene. Unlike CRW, RBS showed no hypersensitive reaction to the invading fungus, nor sensitivity to environmental factors.

The monogenic dominant resistance in CRW was later confirmed by Humaydan and Williams (1976). The resistance gene designated Ac1 was found to be closely linked with the gene (Pi) conditioning the expression of pink pigmentation in the radish plant. This finding has created the possibility of selecting for root colour and resistance to white rust simultaneously.

Bonnet (1981) described a monogenic resistance in two varieties of small radish: "Biser", white Bulgarian radish, and "Rubiso", round scarlet radish. Homozygous resistant lines were obtained through selfing resistant plants of the "Rubiso" variety, and the resistance gene is being transferred into various radish material.

2.4.2 Resistance to Race 2

A. candida race 2 mainly infects B. juncea (Pound and Williams, 1963; Pidskalny and Rimmer, 1985). Sources of resistance appear to be very limited. Parui and Bandyopadhyay (1973) found that a strain, Yellow rai

T4, was virtually immune to natural infection by A. candida race 2. Ebrahimi et al. (1976) described the inheritance of resistance to white rust in the accession P.I.347618. F_1 progenies from the crosses between resistant and susceptible plants gave a disease reaction similar to that of the resistant parent. However, no F_2 data have ever been reported. During 1977 and 1978, Bains and Jhooty screened 150 lines/cultivars of B. juncea against mixed infections caused by A. candida and P. parasitica, but no resistant line/cultivar was found. Failure to locate the source of resistance to A. candida race 2 in B. juncea was also reported by other workers (Delwiche and Williams, 1974).

In addition to B. juncea, A. candida race 2 also attacks B. campestris, B. nigra and B. carinata (Pound and Williams, 1963). As reaction to A. candida race 2 in B. campestris varies among individual plants, ranging from low to high infection type, resistance has been suggested to be governed by both major and minor genes and quantitatively inherited (Edwards and Williams, 1982; 1987). With a rapid-cycling experimental population of B. campestris, CrGC-1, they found that the quantitative resistance conditioned by minor genes could be effectively enhanced by means of mass selection or half-sib family selection. In B. nigra and B. carinata, resistance to race 2 has been reported to be conferred by a single dominant gene (Delwiche and Williams, 1974, 1976, 1977).

2.4.3 Resistance to Race 3

Incorporation of white rust resistance into desirable cultivars is no doubt an economic measure for controlling this disease. However, this seemed to be inapplicable to horseradish because the Common horseradish

is male sterile. The discovery that certain clones of Bohemian horseradish resistant to A. candida race 3 could provide some functional pollen has made it possible to cross between the Common and Bohemian varieties of radish. The reaction of F_1 progenies segregated into three categories: highly resistant without sporulation, resistant with limited sporulation and highly susceptible with abundant sporulation (Hougas et al., 1952).

2.4.4 Resistance to Race 7

The inheritance of resistance to A. candida in B. napus was investigated by Fan et al. (1983) using one resistant Canadian variety, Regent and two susceptible Chinese lines, 2282-9 and GCL. F_2 progenies from both crosses, (2282-9 x Regent) and (GCL x Regent), and their reciprocals segregated in the ratio of 15 resistant to 1 susceptible, suggesting that resistance was governed by two independent dominant genes. Resistant plants would result from the presence of a dominant allele at either of the two loci, and susceptibility would be expressed when the alleles at both loci were homogeneous recessive. In addition to the 15:1 ratio, some of the F_2 progenies from the GCL x Regent cross segregated in the ratio of 63 resistant to 1 susceptible. This indicated the presence of a third dominant resistance gene. These results showed that all Regent plants were homozygous and homogeneous for alleles conferring resistance to white rust at two loci and, some plants also had a resistance allele at a third locus. As the presence of a dominant allele at any one of the three loci would confer resistance, variability at the third locus could not be detected until the progenies of appropriate

crosses had been examined. The three resistance genes were designated Ac7-1, Ac7-2 and Ac7-3 according to the proposal of Humaydan and Williams (1976).

F_2 plants from 2282-9 x GCL and the reciprocal were all susceptible to white rust. This suggested that the recessive genes carried by 2282-9 and GCL were allelic. These two Chinese lines were found to be somewhat different in their reaction to white rust (Fan et al., 1983). Pustules on 2282-9 frequently coalesced to form large patches and occasionally were associated with chlorotic rings, while pustules on GCL were small and discrete, and seldom merged into patches. However, the difference was not very conspicuous, and variation occurred within the parental lines, their F_1 and F_2 progenies, and even among the leaves of the same plant. It is uncertain whether genes with minor effects exist to modify the degree of susceptibility.

Chapter III

INHERITANCE OF RESISTANCE TO *ALBUGO CANDIDA* RACE 7 IN *BRASSICA NAPUS*

3.1 ABSTRACT

Progenies derived from the backcross [Regent (resistant) x 2282-9 (susceptible)] x 2282-9 were obtained and tested to confirm the genetic model according to which resistance in the *Brassica napus* cultivar Regent is controlled by two independent dominant genes designated Ac7-1 and Ac7-2 denoted as R_1 and R_2. Resistance was determined by inoculating the cotyledons with a zoospore suspension of *Albugo candida* race 7.

Nine accessions of genotype $R_1R_1r_2r_2$ or $r_1r_1R_2R_2$ were established through selfing the resistant F_1BC_1 plants for two generations. One accession was assumed to be of genotype $R_1R_1r_2r_2$ and used as a parent for test crosses with the other accessions. The resulting F_1 progenies were all resistant. F_1 plants were selfed and backcrossed to the susceptible line 2282-9. Progenies from four of the accessions x tester segregated in 15:1 (F_2) and 3:1 (BC_1) ratios. This indicates that the four accessions were of genotype $r_1r_1R_2R_2$. Progenies of the other accessions x tester were all resistant, indicating that these accessions had the same genotype as the tester. The study confirms that R_1 and R_2 are two independent dominant genes conferring resistance to *A. candida* race 7 in *B. napus*.

3.2 INTRODUCTION

White rust, caused by Albugo candida (Pers. ex Hook.) Kuntze, is a major hazard to the production of turnip rape (Brassica campestris L.) and of brown mustard [Brassica juncea (L.) Coss.] in western Canada and other regions of the world. Average yield losses of turnip rape in Alberta and Saskatchewan due to white rust were reported to be between 1.2 and 9.0% (Berkenkamp, 1972; Petrie, 1973). In Manitoba, yield reduction ranging from 30 to 60% occurred in heavily infected fields (Bernier, 1972).

Although it can attack a wide range of cruciferous species, A. candida is considered to be composed of specialized races, each of which is largely restricted to certain species or varieties of the host. To date, eight biological races have been identified and classified on the basis of host specialization. The isolate predominantly found on B. campestris could also infect susceptible lines/cultivars of Brassica napus L. (Fan et al., 1983; Pidskalny and Rimmer, 1985).

In western Canada, commercial varieties of rape (B. napus) are highly resistant to A. candida. Susceptible plants have never been identified after artificial inoculation in the greenhouse or under natural conditions (Petrie, 1973, 1975b; Verma et al., 1975; Petrie and Dueck, 1979; Pidskalny and Rimmer, 1985). Moreover, resistance in B. napus has remained effective after over 40 years of continuous cultivation in certain areas of high disease intensity. In central and eastern China, however, some of the cultivars are quite susceptible. The epidemics of white rust occurred frequently during 1960s and early 1970s. In a sur-

vey conducted in the Shanghai region in 1973, it was found that on an average 72.6% of the plants in field had systemically infected inflorescences (stagheads), causing yield losses amounting to between 20 and 30%.

A model of the inheritance of resistance to white rust caused by A. candida in B. napus has been proposed by Fan et al. (1983). In this model resistance is conditioned by three independent dominant genes designated Ac7-1, Ac7-2 and Ac7-3. The first two genes are homogeneous in the cultivar Regent. As a single dominant allele at any one of the three loci is sufficient to confer resistance, the heterozygosity or heterogeneity of the third locus tends to be masked.

In the present study, F_1BC_1 plants from [Regent (resistant) x 2282-9 (susceptible)] x 2282-9 were obtained and tested to confirm the digenic model with dominant resistance conferred by Ac7-1 and Ac7-2.[1]

3.3 MATERIALS AND METHODS

3.3.1 Confirmation of the Digenic Model

F_1BC_1 seeds from (Regent x 2282-9) x 2282-9 were supplied by Fan. The experiment was conducted in the growth cabinet and greenhouse during 1985-86. The test procedure is outlined in Figure 2. One hundred and five F_1BC_1 seedlings were tested for resistance to A. candida race 7 by inoculating the cotyledons with a suspension of zoospores (see below). Resistant F_1BC_1 plants were selected and grown to flower. While one

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[1] For the sake of convenience, the two resistance genes are denoted as R_1 and R_2, respectively in the following text, i.e. Ac7-1 = R_1 and Ac7-2 = R_2.

inflorescence of each selected plant was self-pollinated, another was backcrossed to the susceptible line 2282-9. The purpose of obtaining backcross progenies was to increase the precision in selecting for the desirable genotypes.

The genotype of each resistant F_1BC_1 plant at the loci involved in white rust resistance was determined by inoculating the cotyledons of F_2BC_1 and F_1BC_2 plants with A. candida race 7. Resistant plants were selected from the F_2BC_1 progenies which segregated in the ratio of 3 resistant to 1 susceptible. They were self-pollinated and backcrossed to 2282-9 in the same way as mentioned above. Plants of genotype $R_1R_1r_2r_2$ and $r_1r_1R_2R_2$ could be obtained when F_3BC_1 and F_2BC_2 populations were tested for white rust resistance at cotyledon stage. Non-segregating resistant progenies were considered to be derived from the F_2BC_1 parent of homozygous dominance and selected, whereas segregating progenies were assumed to be derived from the F_2BC_1 parent of heterozygous dominance and discarded.

To determine the genotype of each selected F_3BC_1 accession, one established accession was assumed to be of genotype $R_1R_1r_2r_2$ and used as a tester to cross with the others. Every F_3BC_1 accession was divided into two subunits, each of which consisted of 5 plants. As they were derived from the same F_3BC_1 plant, the paired subunits were of the same genetic make-up and expected to give the identical results in crossing with the tester. Also, when the paired subunits were sib-mated and then self-pollinated, the subsequent progenies would not segregate unless mutation had occurred. The objective of employing two subunits for each F_3BC_1 accession in the investigation was to ensure the validity of the experimental results.

The F_1 progenies from the crosses (Accessions 1, 2, ...8 x tester) were self-pollinated and backcrossed to 2282-9 once more. The genotype of each accession could then be determined according to the segregation ratios of the F_2 progenies and validated by the corresponding backcross data. The digenic model could be confirmed if some of the progenies segregated into 15:1 (F_2) ratio and 3:1 (BC_1) ratio.

The population size of each generation was calculated on the basis of the frequency at which the desirable genotype might appear by using the formula developed by Mainland (1951).

3.3.2 Inoculation and Assessment

Mature zoosporangia of A. candida race 7 used as inoculum in the study were collected from the susceptible cultivar, Torch (B. campestris). They were stored in #00 gelatin capsules in glass screw-cap vials at about -10°C.

Suspensions of zoospores were prepared according to the methods described by Fox and Williams (1984). Zoosporangia were placed in an Erlenmeyer flask containing an appropriate amount of double distilled water. The flask was covered with "parafilm" and shaken gently for even distribution of zoosporangia. The resulting suspension was incubated at 10-12°C for approximately 3.5 h to induce zoosporogenesis.

A hemocytometer was used to quantify the number of zoospores per ml of suspension. Before the slide was covered with the cover slip, a cotton swab soaked in formaldehyde solution was passed over the drop of the suspension for a few seconds to immobilize zoospores. The suspension was then placed in an ice bucket to prevent zoospore encystment.

For each generation, seeds were sown about 0.5 cm deep in Jiffypots with Metro-Mix. Test for white rust resistance was made when cotyledons had expanded for 24-48 h, approximately 6 days after seeding. A 10 µl droplet containing 1-1.5 x 10^5 zoospores ml^{-1} was delivered on the adaxial surface of each half cotyledon using an Eppendorf micropipette. Inoculum was agitated frequently during the inoculation to maintain zoospore mobility. The inoculated plants were incubated in a mist chamber programmed at 100% RH and 20°C for 12 h in dark followed by 12-14 h in light. They were then moved to the growth cabinet and grown under 18 h illumination, and 6 h darkness. The day/night temperatures were 22/17°C.

On day 7 after inoculation, seedlings were assessed for the intensity of sporulation. Cotyledons which showed no symptom or small necrotic flecks on adaxial surfaces without sporulation were recorded as resistant. Susceptibility was assigned to those which showed medium to large coalescing pustules on abaxial surfaces, sometimes associated with many scattered pustules on adaxial surfaces. After disease assessment, selected plants were vernalized in the 4°C room for three weeks and then transplanted into pots containing a 2:1:1 (V/V/V) mixture of soil, sand and peat.

3.3.3 Data Analysis

The Chi-square test was used to analyze the data from the segregating progenies.

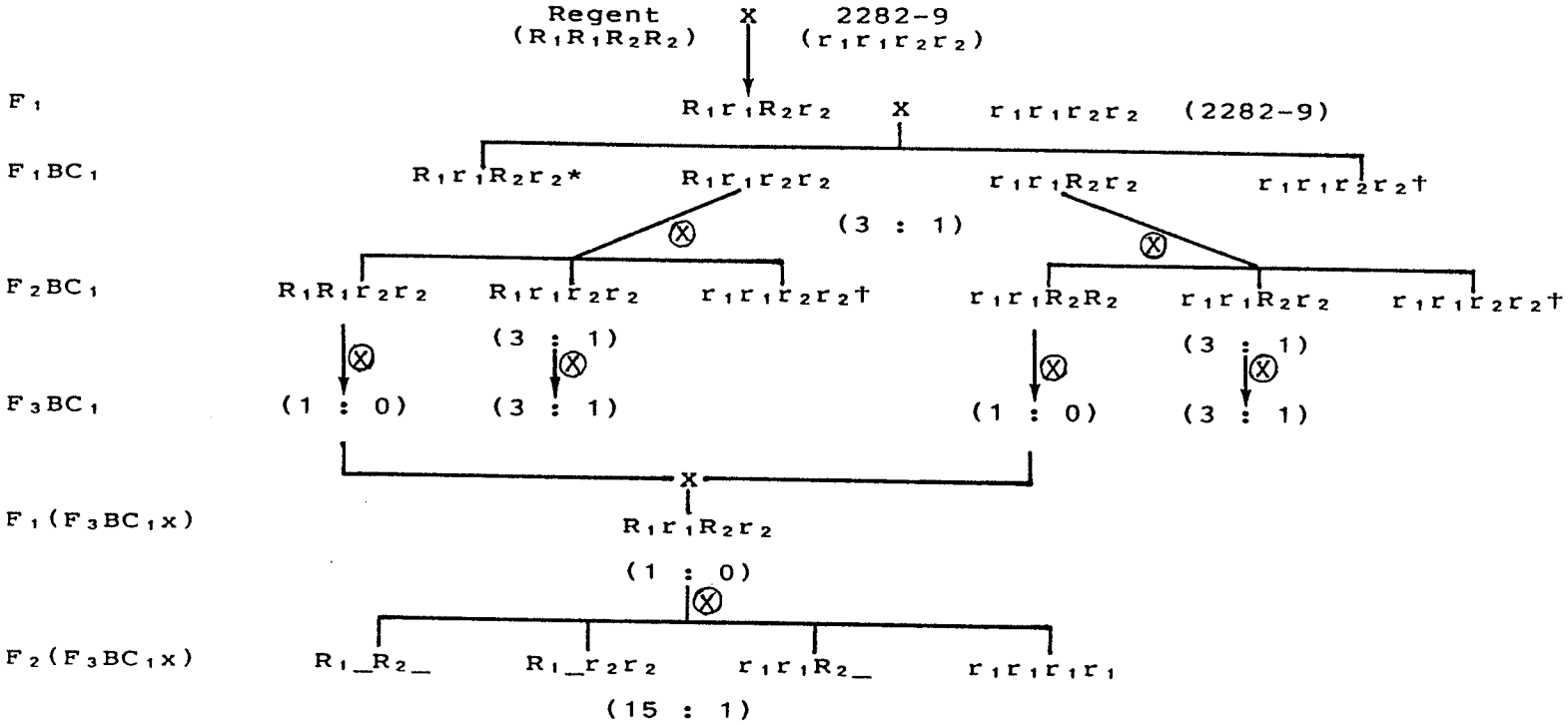

Figure 2. Simplified diagram of the test procedure used to confirm the digenic model with dominant resistance. The backscross procedure is not illustrated.

* Selfed progenies segregated in the ratio of 15 : 1, discarded.

† Susceptible to Albugo candida race 7, discarded.

3.4 RESULTS AND DISCUSSION

Progeny from the backcross (Regent x 2282-9) x 2282-9 segregated with the ratio of 3 resistant to 1 susceptible (Table 1). The data were taken to indicate that resistance in Regent to A. candida race 7 was conditioned by two independent dominant genes, and dominance at either of the two loci would confer resistance whereas digenic recessives were susceptible. Consequently, the genotypes of the F_1BC_1 plants were considered as $R_1r_1R_2r_2$, $R_1r_1r_2r_2$, $r_1r_1R_2r_2$ and $r_1r_1r_2r_2$.

When the resistant F_1BC_1 plants were self-pollinated and backcrossed to the susceptible line 2282-9, some resulting progenies segregated into 15:1 self-pollination ratio and 3:1 backcross ratio, while others segregated into 3:1 self-pollination ratio and 1:1 backcross ratio. This can be explained by assuming that the former were derived from the F_1BC_1 parent of genotype $R_1r_1R_2r_2$, whereas the latter were derived from the F_1BC_1 parent of genotype $R_1r_1r_2r_2$ or $r_1r_1R_2r_2$. Resistant plants were selected from the F_2BC_1 progenies (Accession numbers 1 to 9) which segregated for white rust resistance in the ratio of 3 resistant to 1 susceptible and advanced to F_3BC_1. The Chi-square tests for these F_2BC_1 accessions indicate a good fit of the observed ratio to the expected ratio except for Accession 3 (X^2=3.415, P=0.05-0.10) (Table 2). This could be due to the possession of a third dominant resistance gene. The data from the corresponding backcross progenies (F_1BC_2) are given in Table 3.

Approximately one third of the resistant plants in each selected F_2BC_1 progeny were homozygous at either of the two loci (R_1 or R_2) con-

ditioning resistance to white rust. Accessions of these two genotypes were obtained by inoculating F_3BC_1 and F_2BC_2 plants. The progenies which segregated into 3:1 self-pollination ratio and 1:1 backcross ratio were thought to indicate that the F_2BC_1 parent possessed $R_1r_1r_2r_2$ or $r_1r_1R_2r_2$, and the non-segregating progenies indicated that the F_2BC_1 parent possessed $R_1R_1r_2r_2$ or $r_1r_1R_2R_2$. Table 4 shows 9 F_3BC_1 accessions carrying a pair of homozygous resistance alleles at either of the two loci conditioning white rust resistance. The selection for the F_3BC_1 progenies of the desirable genotypes was assisted by referring to the data (Table 4) from the backcross progenies (F_2BC_2).

Accession 9 (A) was assumed to carry $R_1R_1r_2r_2$ and used as a parent for test crosses with the other accessions. The resulting F_1 progenies from the crosses (Accessions 1, 2, 3, ... 8 x Tester) were all resistant (Table 5), as were the progenies from the sib-mating between paired subunits (F_3BC_1) (Table 6).

The F_2 progenies from the crosses of the tester with Accessions 1, 2, 3 and 4 segregated for white rust resistance in the ratio of 15 resistant to 1 susceptible. The results are a good fit ($P>0.05$) to the ratio expected for a segregation of two independent dominant genes (Table 7). These data indicated that resistance in the four accessions was conferred by a pair of homozygous dominant alleles at the second locus ($r_1r_1R_2R_2$). It has, therefore, been proved that resistance genes R_1 and R_2 are inherited at two discrete loci.

Backcrosses of F_1 from the crosses (Accessions 1, 2, 3 and 4 x Tester) to the susceptible line 2282-9 produced the predicted 3:1 ratio of

resistance to susceptibility ($P>0.05$) (Table 8), thus confirming the non-allelism of genes R_1 and R_2.

The F_2 progenies from the crosses of the tester with Accessions 5, 6, 7 and 8 were all resistant (Table 9), indicating that resistance in these four accessions was conditioned by a pair of homozygous dominant alleles at the first locus ($R_1R_1r_2r_2$). In other words, these accessions had the same genotype as the tester. This was confirmed by the data from the backcross (Accessions 5, 6, 7 and 8 x Tester) x 2282-9 (Table 9).

Table 10 provides further evidence that the objective of ensuring the precision in selecting for the desirable genotypes had been achieved. The nine F_3BC_1 accessions selected must be homogeneous at either locus R_1 or R_2, otherwise homozygous recessive susceptible plants would have appeared in the population when paired subunits (F_3BC_1) were sib-mated and then self-pollinated or test-crossed with the homozygous recessive line 2282-9.

From these data, a digenic model with dominant resistance conferred by R_1 and R_2 has been confirmed. Presence of a dominant allele at either of the two loci will confer resistance to a plant, whereas homozygous recessives at the both loci will give a susceptible phenotypical expression.

In Canada, resistance in B. napus cultivars to A. candida has remained effective even after over 40 years of exposure to the isolates of A. candida which can attack B. campestris and B. juncea. This can be ascribed to the number of resistance genes carried by B. napus cultivars

and the low capacity of the pathogen to adapt to the resistance genes in B. napus. Even so, rapeseed breeders should be cautious not to lose the resistance through introducing susceptibility from oriental cultivars or through interspecific crosses between B. napus and B. campestris.

The susceptibility of Chinese B. napus cultivars to A. candida has been considered as being transferred from interspecific crosses between B. napus and B. campestris (Fan, 1983). Unfortunately, no evidence has been provided to support this assumption. White rust is, in fact, one of the first diseases of B. napus in China, dating back to 1950s when commercial production of B. napus commenced, and most varieties grown then had been selected from introduced stocks or from hybrid offsprings of introductions crossed with adapted local varieties. Moreover, although the exact number of resistance genes in B. napus cultivars is unknown, at least three independent dominant resistance genes have been identified in the cultivar Regent, and the mode of inheritance of the first two genes has been proven in the present study. The uncommon use of interspecific hybridization during the period of 1950-60 and the number of genes involved in white rust resistance have made Fan's interpretation concerning the source of susceptibility in Chinese B. napus cultivars rather questionable.

It would be useful to trace the susceptibility of B. napus cultivars to the primary species making up the amphidiploid, as both B. oleracea and B. campestris have strains susceptible to A. candida. Since the infection type is a result of interactions between host-parasite genotypes under the influence of environmental conditions, it would be instructive to investigate biological races of A. candida in China and to test an

adequate collection of representative Chinese and introduced cultivars of B. napus under similar environmental conditions, and it would be also useful to cross B. napus (resistant) with B. campestris (susceptible) for the genotype of B. napus such that a better understanding of the resistance and susceptibility of B. napus cultivars to A. candida can be obtained.

TABLE 1. Observed segregation and Chi-square test for backcross data from (Regent x 2282-9) x 2282-9 involving resistance and susceptibility to *Albugo candida* race 7

	Observed	Expected	Corrected (O-E)	$(O-E)^2/E$
Resistant	78	78.75	-0.25	0.00079
Susceptible	27	26.25	0.25	0.00238
Total	105	105.00		X^2=0.00317
				P=0.95

TABLE 2. Observed segregation and Chi-square tests for F_2BC_1 data from Regent x 2282-9 involving resistance (R) and susceptibility (S) to Albugo candida race 7

Accession*	Reaction R	Reaction S	Ratio	X^2	P
1	20	6	3 : 1	0.051	.75-.90
2	44	11	3 : 1	0.733	.25-.50
3	52	9	3 : 1	3.415	.05-.10
4	65	15	3 : 1	1.667	.10-.25
5	30	9	3 : 1	0.077	.75-.90
6	41	12	3 : 1	0.157	.50-.75
7	16	4	3 : 1	0.267	.50-.75
8	25	5	3 : 1	1.111	.25-.50
9	69	20	3 : 1	0.303	.50-.75
Total	362	91	3 : 1	7.781	
Deviation X^2				5.829	.01-.03
Heterogeneity X^2				1.952	.97-.99

* Each F_2BC_1 accession was derived from a single identified F_1BC_1 plant.

TABLE 3. Observed segregation and Chi-square tests for F_1BC_2 data from [(Regent x 2282-9) x 2282-9] x 2282-9 involving resistance (R) and susceptibility (S) to Albugo candida race 7

Accession*	Reaction		Ratio	X^2	P
	R	S			
1	19	19	1 : 1	0.000	>.99
2	15	16	1 : 1	0.032	.75-.90
3	19	15	1 : 1	0.735	.25-.50
4	22	22	1 : 1	0.000	>.99
5	19	18	1 : 1	0.027	.75-.90
6	26	20	1 : 1	0.783	.25-.50
7	17	25	1 : 1	1.524	.10-.25
8	16	11	1 : 1	0.926	.25-.50
9	17	17	1 : 1	0.000	>.99
Total	170	163	1 : 1	4.315	
Deviation X^2				0.147	.50-.70
Heterogeneity X^2				4.168	.75-.90

* The accession numbers correspond to those in F_2BC_1, indicating that both populations were derived from the same maternal parent.

TABLE 4. Reactions of selected F_2BC_2 and F_3BC_1 progenies from Regent x 2282-9 to Albugo candida race 7

Accession*		F_2BC_2 Reaction		F_3BC_1 Reaction		Accession*		F_2BC_2 Reaction		F_3BC_1 Reaction	
		R	S	R	S			R	S	R	S
1	(A)	20	0	40	0	1	(B)	18	0	40	0
2	(A)	21	0	30	0	3	(B)	17	0	30	0
3	(A)	19	0	36	0	4	(B)	20	0	30	0
4	(A)	20	0	40	0	5	(B)	19	0	40	0
5	(A)	17	0	30	0	6	(B)	16	0	30	0
6	(A)	19	0	30	0	7	(B)	18	0	30	0
7	(A)	14	0	30	0	8	(B)	16	0	40	0
8	(A)	20	0	26	0	9	(B)	14	0	30	0
9	(A)	19	0	40	0	9	(B)	18	0	30	0

* Each accession consists of two subunits (named A and B, respectively) which were derived from a single maternal F_2BC_1 parent.

TABLE 5. Reactions of progenies from crosses F_3BC_1 x Tester [Accession 9 (A)] to Albugo candida race 7

Accession*	Reaction R	Reaction S	Accession*	Reaction R	Reaction S
1 (A) X Tester	23	0	1 (B) X Tester	28	0
2 (A) X Tester	25	0	2 (B) X Tester	29	0
3 (A) X Tester	30	0	3 (B) X Tester	29	0
4 (A) X Tester	28	0	4 (B) X Tester	29	0
5 (A) X Tester	29	0	5 (B) X Tester	27	0
6 (A) X Tester	22	0	6 (B) X Tester	23	0
7 (A) X Tester	27	0	7 (B) X Tester	25	0
8 (A) X Tester	30	0	8 (B) X Tester	22	0

* The accession numbers correspond to those in F_3BC_1.

TABLE 6. Reactions of progenies from sib-matings between paired subunits to Albugo candida race 7

Accession* (Sib-mating)			Reaction R	Reaction S
1 (A)	X	1 (B)	28	0
2 (A)	X	2 (B)	21	0
3 (A)	X	3 (B)	28	0
4 (A)	X	4 (B)	24	0
5 (A)	X	5 (B)	25	0
6 (A)	X	6 (B)	26	0
7 (A)	X	7 (B)	28	0
8 (A)	X	8 (B)	29	0

* The accession numbers correspond to those in F_3BC_1.

TABLE 7. Observed segregation and Chi-square tests for F_2 data from crosses F_3BC_1 x Tester [Accession 9 (A)] involving resistance (R) and susceptibility (S) to Albugo candida race 7

Accession	Reaction		Ratio	X^2	P
	R	S			
1 (A)	94	5	15 : 1	0.244	.50-.75
1 (B)	140	10	15 : 1	0.044	.75-.90
2 (A)	276	19	15 : 1	0.018	.75-.90
2 (B)	272	16	15 : 1	0.237	.50-.75
3 (A)	208	10	15 : 1	1.031	.25-.50
3 (B)	210	11	15 : 1	0.610	.25-.50
4 (A)	83	5	15 : 1	0.048	.75-.90
4 (B)	142	8	15 : 1	0.217	.50-.75
Total	1425	84	15 : 1	2.449	
Deviation X^2				1.202	.25-.50
Heterogeneity X^2				1.247	.95-.99

TABLE 8. Observed segregation and Chi-square tests for backcross data from (F_3BC_1 x Tester) x 2282-9 involving resistance (R) and susceptibility (S) to Albugo candida race 7

Accession*	Reaction R	Reaction S	Ratio	X^2	P
1 (A)	26	4	3 : 1	2.178	.10-.25
1 (B)	25	7	3 : 1	0.044	.75-.90
2 (A)	40	10	3 : 1	0.667	.25-.50
2 (B)	31	8	3 : 1	0.419	.50-.75
3 (A)	43	11	3 : 1	0.617	.25-.50
3 (B)	28	6	3 : 1	0.980	.25-.50
4 (A)	47	14	3 : 1	0.137	.50-.75
4 (B)	64	19	3 : 1	0.197	.50-.75
Total	304	79	3 : 1	5.239	
Deviation X^2				3.907	.03-.05
Heterogeneity X^2				1.332	.95-.99

* The accession numbers correspond to those in F_2, indicating that both populations were derived from the same maternal parent.

TABLE 9. Reactions of F_2 and F_1BC_1 from crosses F_3BC_1 x Tester [Accession 9 (A)] to Albugo candida race 7

	Reaction			
	F_2		F_1BC_1	
Accession	R	S	R	S
5 (A)	140	0	30	0
5 (B)	138	0	55	0
6 (A)	145	0	34	0
6 (B)	128	0	35	0
7 (A)	191	0	45	0
7 (B)	152	0	39	0
8 (A)	149	0	35	0
8 (B)	141	0	35	0

TABLE 10. Reactions of self-pollinated and test-crossed progenies from sib-matings between paired subunits derived from the individual F_3BC_1 plants to Albugo candida race 7

Accession (Sib-mating)	Self-pollination Reaction R	Self-pollination Reaction S	test-cross Reaction R	test-cross Reaction S
1 (A) x 1 (B)	20	0	21	0
2 (A) x 2 (B)	20	0	20	0
3 (A) x 3 (B)	35	0	21	0
4 (A) x 4 (B)	24	0	24	0
5 (A) x 5 (B)	21	0	29	0
6 (A) x 6 (B)	25	0	26	0
7 (A) x 7 (B)	64	0	36	0
8 (A) x 8 (B)	25	0	25	0

Chapter IV

CAUSE OF VARIABILITY IN THE DEGREE OF SUSCEPTIBILITY TO *ALBUGO CANDIDA* IN OILSEED RAPE

4.1 ABSTRACT

Although the recessive genes for susceptibility carried by the two susceptible Chinese lines 2282-9 and GCL (*Brassica napus*) were reported to be allelic, genes with minor effects were shown to exist to modify the degree of susceptibility.

The two susceptible Chinese lines and one susceptible Canadian cultivar Torch (*B. campestris*) were tested for variation in disease reaction by inoculating the seedlings at the second and fifth leaf stage. Statistical analyses indicated that the line/cultivar, inoculum concentration, incubation temperature and their interactions had significant effects on white rust development. Day/night temperatures of 22/17°C resulted in higher infection levels than those of 15/10°C. The infection level increased with inoculum concentration on all of the line/cultivar investigated. The study revealed a marked difference in disease reaction between *B. napus* and *B. campestris* and provided support for the contention that genes with minor effects are present to modify the degree of susceptibility in the two susceptible *B. napus* lines.

4.2 INTRODUCTION

Genetic studies on the inheritance of resistance to Albugo candida in Brassica napus, involving the resistant Canadian cultivar Regent and two susceptible Chinese lines 2282-9 and Green Cup Leaf (GCL), indicated that variation in disease reaction existed between 2282-9 and GCL although the recessive genes for susceptibility carried by these two lines were allelic (Fan et al., 1983). Pustules on 2282-9 frequently coalesced to form large patches whereas those on GCL were usually scattered and often surrounded by chlorotic rings. Such variation in disease reaction was also observed among the F_1 and F_2 plants derived from the cross 2282-9 x GCL and its reciprocal when seedlings were inoculated at the 2-3 leaf stage (growth stage 2.2-2.3) (Harper and Berkenkamp, 1975). In general, the F_2 plants were more susceptible than the F_1 and their parents. Therefore, Fan et al. (1983) suggested that genes with minor effects might exist to modify the degree of susceptibility. However, no quantitative evaluations were made nor was variation in disease reaction found between 2282-9 and GCL in the preliminary experiment in which seedlings were inoculated at cotyledon stage.

Phenotypic expression on the host is a result of the interactions between host-parasite genotypes under different environmental conditions. From their experiment regarding white rust development in detatched leaves of Brassica campestris L., Verma et al. (1983) concluded that temporal progression of white rust was significantly influenced by temperature, leaf age, and their interactions. Since variability in disease reaction can result from genetic variation in the host and parasite, and/or changes of environment, more information is needed to determine

whether genes with minor effects are present to modify the degree of susceptibility.

In the present study, lines 2282-9 and GCL as well as a susceptible Canadian cultivar Torch (B. campestris)[2] were tested with A. candida race 7 to investigate the effect of inoculum concentration and incubation temperature on white rust infection so as to learn if variability in disease reaction between the susceptible lines is caused by the possession of genes with minor effects.

4.3 MATERIALS AND METHODS

4.3.1 Inoculation and Assessment

Two susceptible Chinese lines 2282-9 and GCL, and one susceptible Canadian cultivar Torch were tested with zoospores of A. candida produced from germinating zoosporangia. The procedures for raising plants and preparing a zoospore suspension were as mentioned in the previous chapter (see Section 3.3.2 Inoculation and Assessment).

Seedlings at growth stage 2.2 and 2.5 (the second and fifth leaf stage) were allocated to two experiments. They were spray-inoculated with suspensions of zoospores at three concentrations: 1×10^4, 1×10^5 and 5×10^5 zoospores ml^{-1} and incubated in a mist chamber for 72 h at 20°C. They were then transferred to two growth cabinets programmed for day/night temperatures of 22/17 and 15/10°C, respectively.

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[2] For simplicity, the term "line" will be used to refer to both line and cultivar.

Except for an initial 24 h dark period in the mist chamber, the seedlings were placed under 18 h illumination and 6 h darkness both in a mist chamber and in the growth cabinets throughout the experiments. The infection level defined as percent leaf area covered with white pustules (Figure 3) was estimated daily on day 7 to 10 inclusive after inoculation. Since white pustules on the seedlings treated with the lower temperatures were formed 2-3 days later than those on the seedlings treated with the higher temperatures, only the data indicating maximum sporulation were used in quantitative evaluations. Both experiments were conducted in a split-split-plot design with temperature treatments assigned to the main plot, inoculum concentrations to the subplots and lines to the sub-subplots. Both were repeated for four times. For statistical analyses, repetitions were considered as replicates.

4.3.2 Data Analysis

Data from the experiments of infection level were transformed to square roots and analyzed in a split-split-plot design. Each replicate consisted of five leaves per temperature-concentration-line combination. Statistical analyses were performed using SAS package on the University of Manitoba AMDAHL 580 mainframe computer.

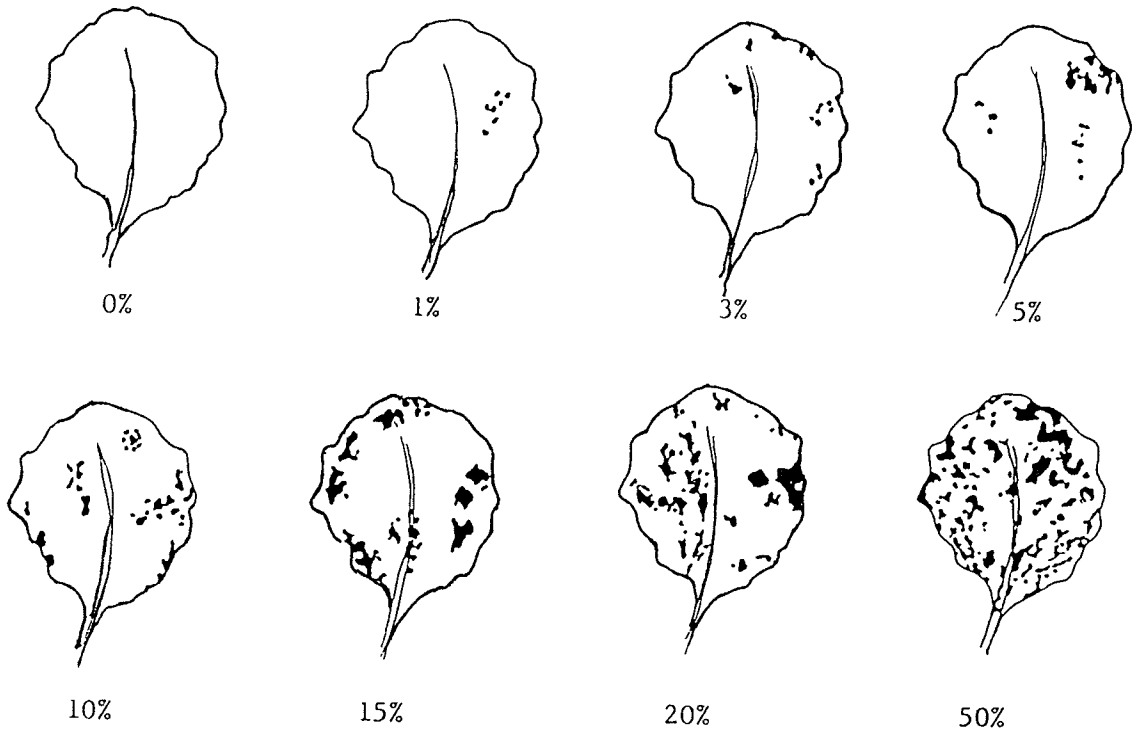


Figure 3. Diagram for estimating proportion of leaf area covered with white pustules

4.4 RESULTS AND DISCUSSION

4.4.1 Seedlings Inoculated at Growth Stage 2.2

The interaction of incubation temperature and inoculum concentration was significant ($P<0.05$). When seedlings were incubated at 22/17°C, the mean infection levels increased consistently with inoculum concentration. At 15/10°C, however, a relatively fast increase in infection level occurred after the inoculum concentration reached 1 x 10^5 zoospores ml^{-1} (Figure 4). In general, the day/night temperature of 22/17°C appeared to be more favourable to the fungal growth and sporulation than the day/night temperature of 15/10°C at all three levels of inoculum concentration tested. This is in agreement with Verma et al. (1983), who reported that the optimum temperature for white rust development was around 18-21°C.

The infection level was generally higher at higher inoculum concentration for all three lines investigated (Figure 5). The interaction of inoculum concentration and line was highly significant ($P<0.01$) with the effect of inoculum concentration being particularly noticeable on Torch. Apparent differences in size of pustules and formation of secondary pustules were also observed among the three susceptible lines. Pustules occurring on Torch were usually tiny, ranging from 0.5 to 1.6 mm in diameter. When the leaves were inoculated with the zoospore suspension containing 5 x 10^5 zoospores ml^{-1}, the whole leaf could be covered with numerous individual pustules. Secondary pustules were seldom found when disease was assessed at day 7 to 10 inclusive after inoculation. Instead of being evenly distributed on the whole leaf, pustules produced on 2282-9 and GCL were usually located at the edge of the leaf or in the

proximity of the central vein. These pustules frequently merged to form large patches varying from 1.5 to 8.5 mm in diameter. Although variation in pustule production appeared on different leaves of the same plant, 2282-9 generally had larger pustule patches and more intensive production of secondary pustules.

Analysis of variance indicated highly significant differences ($P<0.01$) between incubation temperatures, among inoculum concentrations and among lines investigated (Appendix Table 1). Irrespective of the interactions, infection level on the second leaves generally increased with incubation temperature and inoculum concentration (Tables 11 and 12). Torch was most susceptible, followed by 2282-9 and GCL (Table 13).

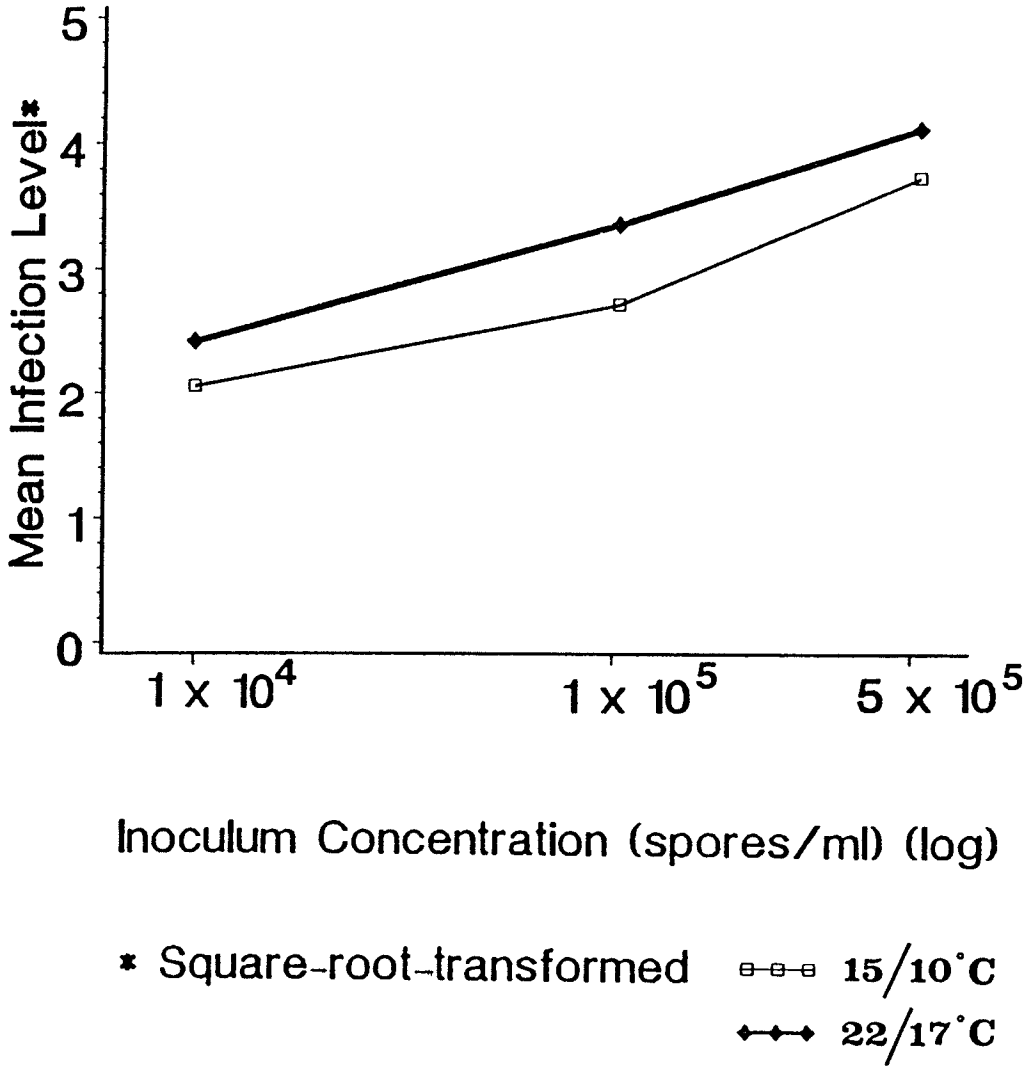


Figure 4. The effect of inoculum concentration on infection levels of Albugo candida on Brassica napus lines 2282-9 and GCL, and B. campestris cultivar Torch at growth stage 2.2 as influenced by incubation temperatures

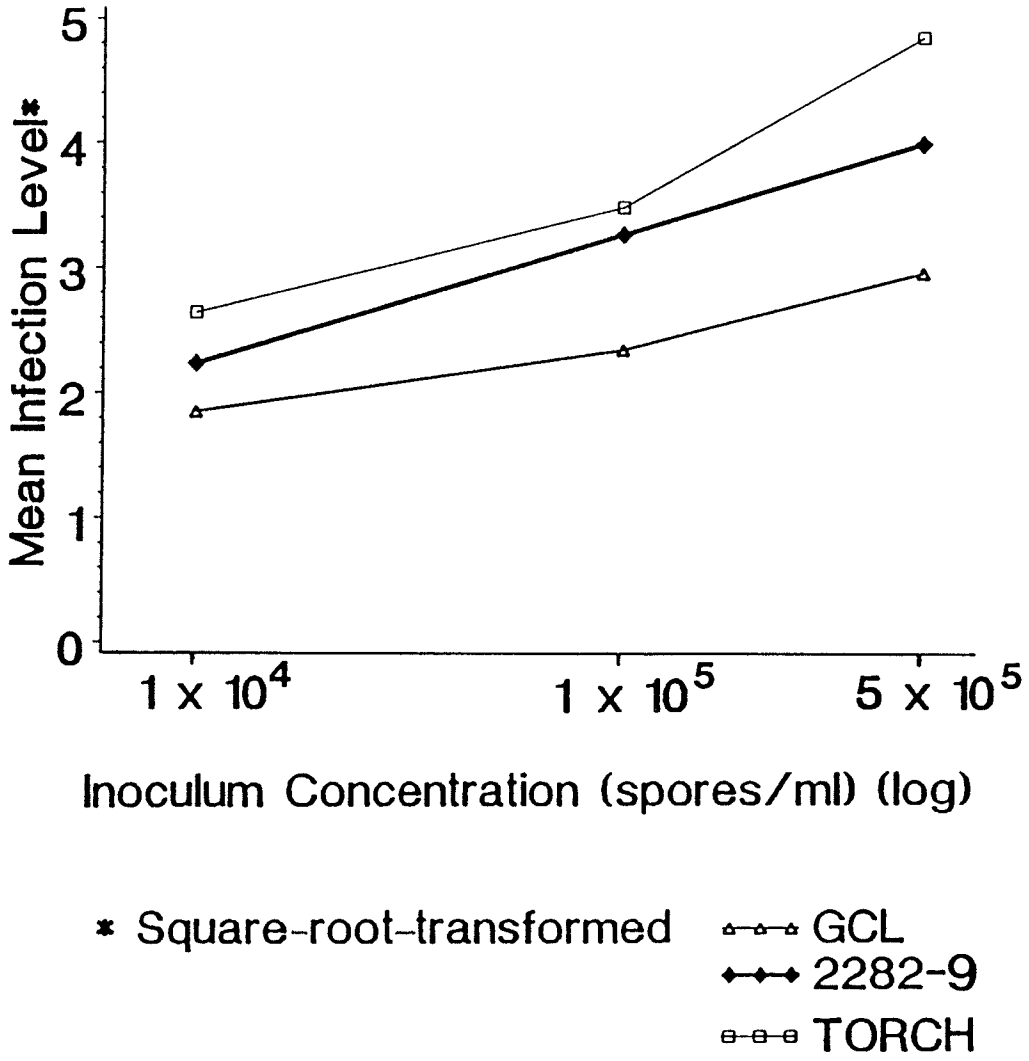


Figure 5. Mean infection levels of Albugo candida on rape plants at growth stage 2.2 as influenced by inoculation concentrations and types of host

TABLE 11. Effect of incubation temperature on combined infection levels of *Albugo candida* on *Brassica napus* lines 2282-9 and GCL, and *B. campestris* cultivar Torch at inoculum concentrations of 1×10^4, 1×10^5 and 5×10^5 zoospores ml^{-1}

Incubation Temperature	Mean Infection Level*	
	Growth Stage 2.2	Growth Stage 2.5
22/17°C	3.30 a†	3.12 a
15/10°C	2.83 b	2.63 b
Standard Error of a Mean ($S\bar{x}$)	0.05	0.03

* Square-root-transformed values.

† Values in a column followed by different letters differ significantly according to Duncan's Multiple Range Test (DMRT), P = 0.05.

TABLE 12. Effect of inoculum concentration on combined infection levels of *Albugo candida* on *Brassica napus* lines 2282-9 and GC1, and *B. campestris* cultivar Torch at day/night temperatures of 22/17°C and 15/10°C

Inoculum Concentration	Mean Infection Level*	
	Growth Stage 2.2	Growth Stage 2.5
5×10^5	3.93 a†	3.66 a
1×10^5	3.03 b	3.01 b
1×10^4	2.24 c	1.97 c
Standard Error of a Mean ($S\bar{x}$)	0.04	0.06

* Square-root-transformed values.

† Values in column followed by different letters differ significantly according to DMRT, $p = 0.05$.

TABLE 13. Combined infection levels of Albugo candida on Brassica napus lines 2282-9 and GCl, and B. campestris cultivar Torch at day/night temperatures of 22/17°C and 15/10°C and at the three inoculum concentrations

Line/Cultivar	Mean Infection Level*	
	Growth Stage 2.2	Growth Stage 2.5
Torch	3.65 a†	3.63 a
2282-9	3.16 b	2.92 b
GCL	2.38 c	2.09 c
Standard error of a mean ($S\bar{x}$)	0.04	0.05

* Square-root-transformed values.

† Values in column followed by different letters differ significantly according to DMRT, p = 0.05.

4.4.2 Seedlings Inoculated at Growth Stage 2.5

Results from the experiment with seedlings inoculated at growth stage 2.5 were similar to those obtained in the experiment described above (Tables 11, 12 and 13).

Analysis of variance indicated that the interaction of incubation temperature and inoculum concentration, the interaction of concentration and line, as well as the effect of incubation temperature, inoculum concentration and line were either significant ($P<0.05$) or highly significant ($P<0.01$) (Appendix Table 2). The response curves for the two temperature treatments over inoculum concentration were similar to those obtained in the experiment with seedlings inoculated at growth stage 2.2. It is interesting to note that the higher day/night temperature was not significantly better for disease development than the lower day/night temperature at the lowest level of the inoculum concentration investigated (Figure 6). It is possible that visual assessment of percent leaf area covered with white rust pustules is not precise enough to detect all of the existing differences.

It is clear from Figure 7 that infection level on each line was generally higher at higher inoculum concentration, but the different response curves for the three lines over inoculum concentration implied a significant interaction between the inoculum concentration and line. The infection level on GCL and 2282-9 increased in a regular and constant manner with inoculum concentration, but at a different rate, whereas infection level on Torch increased with inoculum concentration to 1×10^5 zoospores ml^{-1} and then showed a tendency to level off.

The growth stage of rape plants may also influence the infection level. The mean infection levels on the second leaves were slightly higher than those on the fifth leaves (Tables 13, Figures 5 and 7). Results from the preliminary experiment showed that infection levels on all three lines were even higher when cotyledons were inoculated. It is envisaged that percent leaf area infected decreases as a plant ages.

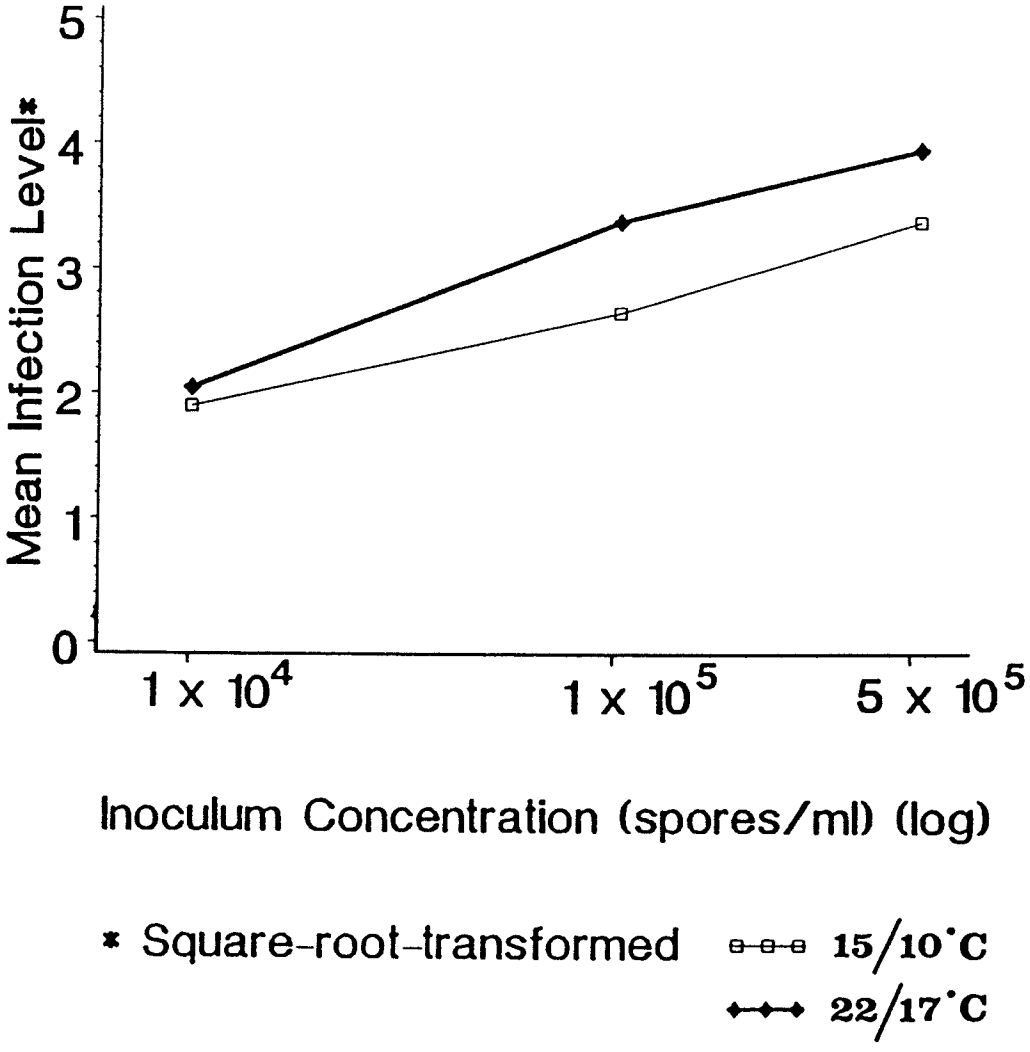


Figure 6. The effect of inoculum concentration on infection levels of Albugo candida on Brassica napus lines 2282-9 and GCL, and B. campestris cultivar Torch at growth stage 2.5 as influenced by incubation temperatures

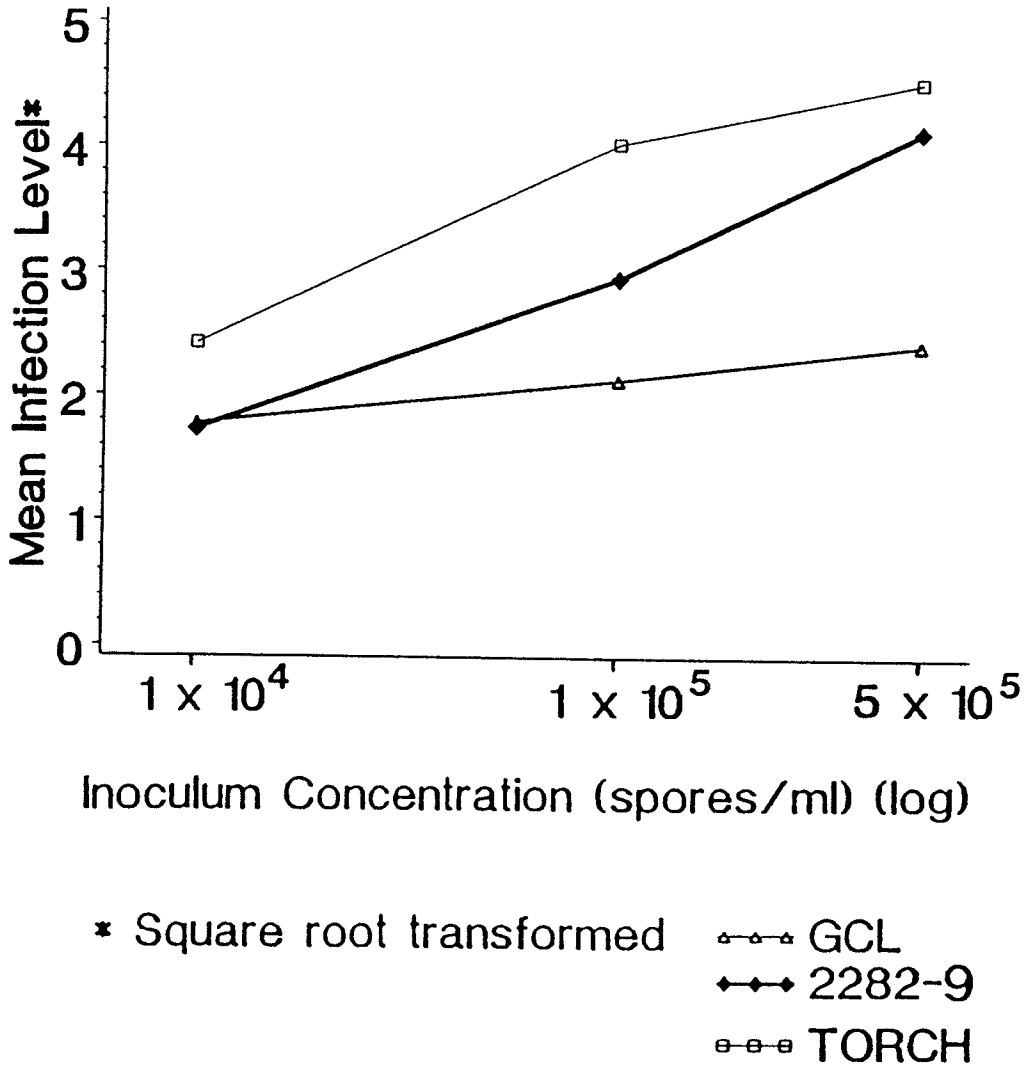


Figure 7. Mean infection levels of Albugo candida on rape plants at growth stage 2.5 as influenced by inoculation concentrations and types of host

The present study has shown the differences in a compatible interaction between the two Brassica species. These differences could result from different host-parasite genotype combinations. However, it is not possible to determine from these data whether the differences are caused by genuine genetic variation between the B. campestris cultivar Torch and the two susceptible B. napus lines. One factor which may partially account for the differences in leaf area infected is the amount of leaf wax deposits which can reduce the adhesion of inoculum to the leaf surface as Tewari and Skoropad (1976) have reported that epidermal cells of Torch have a very limited amount of cylindrical and plate-like wax crystals and subsequently low water repellence.

Variation in disease reaction between the two B. napus lines 2282-9 and GCL has been reported (Fan et al., 1983) but no data to substantiate this was provided. The present study has indicated that although the line effect on infection levels was influenced by "environmental" factors such as temperature and inoculum concentration, 2282-9 was overall more susceptible than GCL since the mean infection level on 2282-9 was invariably higher than that on GCL (Figures 5 and 7). These data have provided reasonable indications that genes with minor effects are present to differentiate the degree of susceptibility. These genes may be sensitive to changes of environment, and their expression as variation in sporulation intensity is obvious when true leaves are inoculated with a zoospore suspension. Influence of plant age on gene expression was also described in other host-parasite systems such as in the interactions between wheat and the powdery mildew fungus (Ellingboe, 1972).

Chapter V

HISTOPATHOLOGY OF COMPATIBILITY AND INCOMPATIBILITY BETWEEN OILSEED RAPE AND *ALBUGO CANDIDA*

5.1 ABSTRACT

Cotyledons of one resistant and three susceptible lines/cultivars of *Brassica napus* and *B. campestris* were inoculated with zoospores of *Albugo candida* race 7 to seek the earliest events which could distinguish genetically distinct combinations between the host and parasite. Samples of whole cotyledons were collected at regular intervals after inoculation and prepared for observation with a Zeiss Standard Microscope equipped with a Differential Interference Contrast objective. The time course of the infection process was followed histologically. The sequence of pre- and early post-penetration events were similar in compatible and incompatible interactions. Germination of zoospore cysts occurred 2-3 h after inoculation. Infection was initiated with germ tubes penetrating through stomata. Haustorium formation was first observed in the palisade mesophyll cells adjacent to the substomatal chambers 8 h after inoculation.

Only after the establishment of the first haustorium did compatible and incompatible interactions begin to differentiate. In the resistant cultivar, most primary hyphae produced single haustoria. Necrosis of the invaded host cells was first observed 12 h after inoculation followed by cessation of fungal growth. The death of host cells was large-

ly restricted to the penetration site where the host cell had contacted with a fungal hypha bearing an apical haustorium; the adjacent non-penetrated cells remained apparently unaffected. In the susceptible line/cultivar, necrosis of infected cells occurred only infrequently and hyphal growth continued unabated, resulting in mycelial ramification into several layers of the mesophyll. Numerous haustoria were produced. Sporulating pustules formed within 5-6 days after inoculation.

Histological comparisons of the sequence and timing of early events of the infection process of A. candida race 7 on resistant and susceptible lines/cultivars revealed that the earliest event distinguishing a compatible from an incompatible interaction occurred after formation of the first haustorium and that resistance was not manifested until the host mesophyll cell had come into contact with the first haustorium. The distinction between compatibility and incompatibility was substantiated by quantitative analysis of white rust development on these lines/cultivars.

5.2 INTRODUCTION

Albugo candida (Pers. ex Hook.) Kuntze, an obligate parasite, grows in intimate association with the living cells of its host. Little is known of the mechanisms responsible for establishing and maintaining such a relationship, and how an incompatible interaction occurs when the fungus confronts host cultivars carrying resistance genes. Histological studies of the pathogen on both resistant and susceptible cultivars may reveal the earliest events in the infection process, which could distinguish genetically distinct combinations of host cultivar and fungal race. This information is essential for interpreting results of biochemical and physiological research on mechanisms of resistance.

Verma et al. (1975) studied the infection of four Brassica species (B. campestris, susceptible; B. napus, resistant; B. hirta, moderately susceptible and B. juncea, susceptible) by A. candida. Also, Pidskalny (1984) described some aspects of the infection process in a compatible interaction between B. campestris cultivars and A. candida race 7. No marked differences in the early events prior to formation of the first haustorium were observed among the different hosts investigated. Zoospore cysts germinated on the cotyledon surface of the resistant species as readily as on that of susceptible species to form germ tubes. Infection was initiated by germ tubes penetrating stomata. Compatible and incompatible interactions began to differentiate after the first haustorium had been produced at the tip of a primary hypha. In resistant B. napus, the young haustoria were soon surrounded by encasements and cessation of hyphal growth occurred within 48-72 h after inoculation. In contrast, the fungal growth in susceptible hosts increased rapidly after

formation of the first haustorium. Mycelia ramified and branched profusely in the intercellular space and eventually produced sporulating pustules. The similar sequence of the initial events in both compatible and incompatible interactions up to formation of the first haustorium implies that resistance is not expressed until contact is made between a hyphal tip and a host mesophyll cell.

In both of the above studies, observations on the infection process were not started until 24 h after inoculation. In fact, the initial 24 h can be a crucial stage in the life history of an obligate parasite. A series of host-parasite interactions including structural and probably biochemical changes may occur during this period of time. As the time between inoculation and the start of microscopic examination can greatly affect the interpretations that can be made, it is logical to begin the examination at the first hour after inoculation and observe the earliest host-parasite interactions until a functional relationship between the host and parasite is established or in the case of an incompatible interaction, the host reacts to reject the invading fungus. Also, no-one has observed the infection process of A. candida on susceptible lines of B. napus.

The present study was undertaken to describe the sequence and timing of early events in the infection process beginning when zoospores of A. candida race 7 are inoculated on cotyledons of rape plants and to determine when and how compatible and incompatible interactions can be differentiated. Emphasis was placed on observing early post-penetration phenomena, and histological changes were substantiated by the quantitative analysis of white rust infection stages on both resistant and susceptible lines/cultivars.

5.3 MATERIALS AND METHODS

5.3.1 Pathogen and Host

The pathogen used in this study was A. candida race 7 obtained from naturally occurring infections on the B. campestris cultivar Torch. The maintenance of the isolate and preparation of inoculum were as described in Section 3.3.2 Inoculation and Assessment.

The host material consisted of a B. napus cultivar, Regent carrying at least two independent dominant genes (R_1 and R_2) for white rust resistance and two B. napus lines, 2282-9 and Green Cup Leaf (GCL) with homozygous recessive genes for susceptibility. Also included was a B. campestris cultivar, Torch which was highly susceptible to A. candida race 7. For convenience, the term "line" will be used to refer to the hosts investigated.

5.3.2 Inoculation and Preparation of Host Tissue for DIC Microscopy

Seedlings were grown in a growth cabinet at day/night temperatures of 22/17°C with a 16 h photoperiod. Inoculation was performed 6 days after seeding. A 10 µl droplet of zoospore suspension containing 1×5^5 zoospores ml^{-1} was placed on the centre of the adaxial surface of each half cotyledon. The inoculated seedlings were incubated in a computerized mist chamber, programmed at 20°C and 100% RH, for 24 h with initial 12 h in dark. They were then moved to the growth cabinet and grown under 18 h illumination, and 6 h darkness. The day/night temperatures were 22/17°C.

Samples of whole cotyledons were collected at 1, 2, 3, 4, 8, 12, 24, 36, 48, 60, 72, 84 and 96 h after inoculation. They were cleared and stained according to the method of Bruzzese and Hasan (1983). The cleared specimens were mounted in lactophenol and examined with a Zeiss Standard Microscope equipped with a x40 Neofluar Differential Interference Contrast (DIC) objective and x10 kpl ocular.

5.3.3 Microscopic Examination

The time course of the infection process was followed histologically. For quantitative determination of white rust development on both resistant and susceptible lines, the following data were obtained at each sampling time: the length of germ tubes, the length of primary hyphae, the number of haustoria per infection site, and the size of fungal colonies. Only isolated infection sites were examined.

The length of germ tube was determined by measuring from its tip to the centre of the stoma through which it had penetrated into the host tissue. The primary hypha was classified as a distinct individual elongating from a germ tube, not a short branch of another hypha. Regardless of the irregular, complex, three-dimensional structure of colonies, the colony size was estimated by measuring its length and width between 60 and 96 h after inoculation. All measurements were made on 5 infection sites on each of 4 (or 6) cotyledons in each of 4 lines tested except for the data of colony size which were obtained from 3 susceptible lines. By 96 h, extensive growth of the pathogen in the susceptible lines made quantitative measurements difficult, thus reducing accuracy. Percentage of necrotic host cells [(number of necrotic host cells/number

of infected host cells observed) x 100] was recorded on the resistant line Regent. A cell was considered to be necrotic when its outline was visibly distorted and the cytoplasm stained intensely with aniline blue.

5.3.4 Data Analysis

Statistical analyses were performed using the SAS package on the University of Manitoba AMDAHL 580 mainframe computer. Log-transformation was applied to the measurements of colony size. Analysis of variance was made on the data which had been tested and shown to be normally distributed. Because the data of numbers of haustoria per infection site could not be assumed to be normally distributed, mean number of haustoria per infection site was calculated for each line at each sampling time and plotted against hours after inoculation.

5.4 RESULTS AND DISCUSSION

A number of methods of host tissue preparation was tried in preliminary experiments, but as the method of Bruzzese and Hasan was found to be most satisfactory, it was used in this experiment. DIC microscopy allows the examination of the entire fungal colony. Even in cotyledons collected at 96 h after inoculation, when extensive fungal colonies had developed, it was possible to trace the newer parts of the mycelium to the oldest part near the stoma through which the germ tube had penetrated into the host tissue.

5.4.1 A Summary of the Time Course of the Infection Process of A. candida Race 7 on Susceptible Lines

The sequence of the events of the infection process of A. candida on susceptible lines was: accumulation and encystment of zoospores, cyst germination and germ tube elongation, stomatal penetration, primary hypha formation and elongation, haustorium development, secondary hypha formation, mycelium ramification, sporangiophore formation, and production of sporangia.

At 1 h after inoculation, the majority of zoospore cysts were found in stomata on the upper epidermal surface (Figure 8). Probably when a droplet of zoospore suspension was delivered on the cotyledon surface, the motile zoospores tended to move towards the nearest stoma, and after which they became encysted in the stomatal chambers. Zoospore cysts were observed to germinate with single germ tubes within 2-3 h after inoculation (Figure 9). Infection was soon initiated by penetration of germ tubes through stomata (Figure 10). Occasionally single, narrow

germ tubes formed directly from zoosporangia, but these appeared to collapse before reaching a stoma, thus failing to initiate infection.

Within the substomatal chamber of the host, the germ tube swelled to form a substomatal vesicle and then elongated to become the primary hypha, which extended to the palisade mesophyll. The first haustorium usually formed at the tip of the primary hypha and was initially detected in the palisade mesophyll cell adjacent to the substomatal chamber 8 h after inoculation (Figure 11). The haustorium was spherical in shape and connected to the hypha with a narrow neck. Within 24 h after inoculation, secondary hyphae formed as side branches from the primary hypha at the site which had given rise to the first haustorium.

On the susceptible lines, the formation of the first haustorium followed by successful development of the secondary hyphae seems to be a manifestation of the establishment of a functional relationship between the host and A. candida. Thereafter, the fungus colonized the host tissue extensively, resulting in ramification of hyphae as spirals into several layers of the mesophyll. By 48 h, the hyphal length in the three susceptible lines averaged 67 µm with 1-8 haustoria per infection site (Figure 12). Intercellular hyphal growth continued unabated with production of more haustoria within mesophyll cells (Figure 13). From 84 h onwards, some of the fungal colonies became so extensive that they overlapped to form large compact mycelial masses. By 96 h, almost all the intercellular spaces of the inoculated cotyledon were occupied by mycelia (Figure 14). Even at this stage, the host cells did not seem to be disrupted by the fungus to any degree. Numerous club-shaped sporangiophores later developed from the dense mycelial mat beneath the lower

epidermis. White pustules were macroscopically visible within 5-6 days after inoculation.

In the present study, a series of early events in the infection process such as germination of zoospore cysts, penetration by germ tubes, formation of the first haustorium, necrosis of the infected host cells of the resistant line and cessation of hyphal growth (see below) were found to occur within the initial 24 h. In the literature, however, the time for cyst germination varied from a few hours (Napper, 1933) to about two days (Pidskalny, 1984), and no haustorium was observed in B. campestris leaves until 48 h after inoculation (Verma et al., 1975). Although the onset of infection may vary with incubation conditions (e.g. temperature, moisture and photoperiod, etc.), it is most likely that the time between inoculation and the start of observation, as well as the method by which the infected host tissues are prepared and examined, are responsible for the discrepancy in the timing of early events in the infection process. As the growth of an obligate parasite relies on obtaining nutrients from its compatible host, or in an incompatible interaction the invading fungus would trigger a series of host responses including physiological, biochemical and morphological changes which eventually lead to a resistant reaction, the pre- and post-penetration phenomena reported here are believed to be consistent with those occurring in nature. It has been reported that 2-4 h is enough to initiate infection so along as the leaf surface is humid from rain or dew (Endo and Linn, 1960; Hougas et al., 1952; Raabe and Pound, 1952).

Figure 8. Torch 1 h. Zoospore (arrowed) in stoma. X 380.

Figure 9. GCL 3 h. Germinated cysts (arrowed). X 420.

Figure 10. 2282-9 8 h. Germ tube elongation and stomatal penetration. X 420.

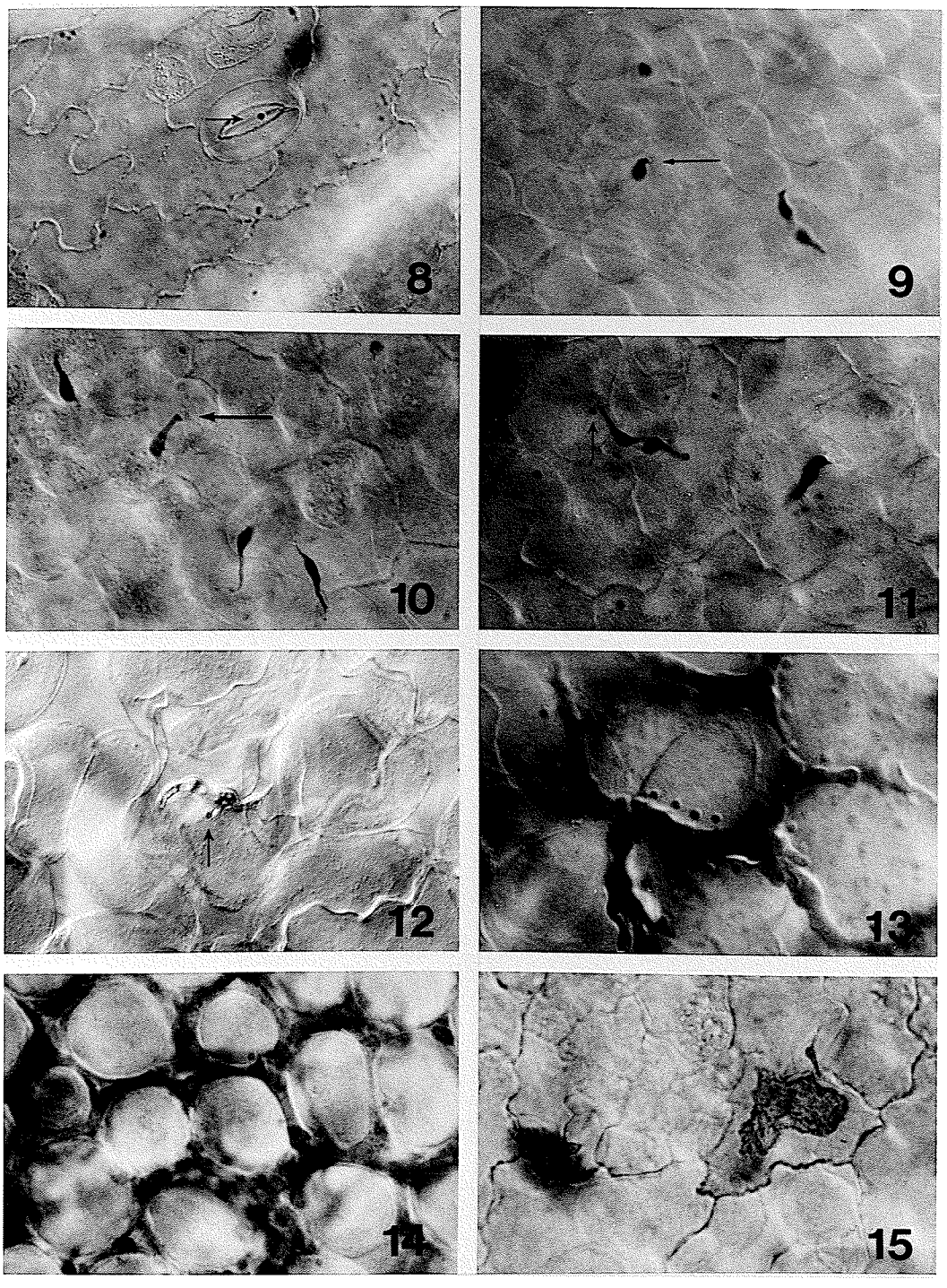
Figure 11. GCL 8 h. Penetration of stoma by germ tube and formation of the first haustorium arrowed. X 420.

Figure 12. 2282-9 24 h. Primary hypha bearing a mature haustorium (arrowed) with a narrow neck. X 480.

Figure 13. Torch 72 h. Note a number of haustoria per infection site. X 480.

Figure 14. GCL 96 h. Note intercellular fungal thallus. X 380.

Figure 15. Regent 12 h. Host cell necrosis due to penetration by Albugo candida race 7. Cells adjacent to the infected cell are apparently healthy. X 420.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5.4.2 Quantitative Determination of White Rust Development

Germination of zoospore cysts, their subsequent penetration of host tissues by growth of germ tubes through stomata, and formation of the first haustorium were almost synchronous in the four lines tested. This allowed a quantitative determination of the infection process.

5.4.2.1 The Length of Germ Tubes. Germination of cysts was defined as the production of a single germ tube from a zoospore cyst. The average germ tube lengths on 2282-9, GCL, Regent and Torch at 4 h after inoculation were 18.3, 17.4, 18.9 and 18.7 μm, respectively. The analysis of variance indicated no significant difference in the germ tube length among the 4 rape lines investigated (Appendix Table 3). This implied that the resistant line possessed no morphological or physiological features which could prevent cyst germination or subsequent elongation of germ tubes.

5.4.2.2 The Length of Primary Hyphae. The average length of primary hyphae for the lines at each sampling time are illustrated in Figure 16. The mean length of primary hyphae in the three susceptible lines increased uniformly to the time after inoculation. The greatest increase occurred between 12-36 h, indicating that the fungal growth was stimulated by formation of the first haustorium. In contrast, the resistant line supported little fungal growth. Rapid necrosis of the penetrated host cells (Figure 15), which stained differently and more intensely with aniline blue, was first observed at 12 h after inoculation followed

by cessation of hyphal growth occurring between 12 and 48 h (Figure 16). Analysis of variance indicated a significant interaction ($P<0.01$) of lines and time after inoculation (Appendix Table 4). Difference in mean primary hyphal length in resistant vs. susceptible lines was significant at 24, 36 and 48 h following inoculation.

5.4.2.3 The Number of Haustoria per Infection Site. Haustoria were first developed in both resistant and susceptible lines at 8 h after inoculation. The average and maximum number of haustoria produced on each line at each sampling time is shown in Table 14. Most fungal hyphae in the resistant line produced only one haustorium, and host cell necrosis became evident 12 h after inoculation. From then, the number of infection sites with cell necrosis increased rapidly, and by 48 h over 95% of the haustorium-containing cells were necrotic (Table 14). At this point, it was no longer possible to make an accurate measurement of the incompatible interaction because of the collapse of hyphae and haustoria, and no further fungal growth was observed.

While hyphal growth in the resistant line ceased after the first-formed haustorium was encased within the necrotic host cell, hyphal growth in the susceptible lines was remarkably increased after formation of the first haustorium. Necrosis of infected cells was only detected infrequently in GCL. Hyphae ramified intercellularly, giving rise to an extensive mycelium with numerous haustoria (Figures 13 and 17). As many as 44 haustoria per infection site were observed within a mesophyll cell of a Torch cotyledon at 84 h after inoculation (Table 14). More haustoria were formd at 96 h or later, but the coalescence or inter-mingling

of fungal thalli made it impossible to obtain accurate counts of the number of haustoria per infection site.

5.4.2.4 The Size of Fungal Colonies. Hyphae grew between the cells of the host tissue, branched in all directions and finally took the shape imposed by the intercellular spaces in which they occupied. The mean colony size in all three susceptible lines increased significantly with time after inoculation (Figure 18), but there was no significant interaction of susceptible lines with time after inoculation (Appendix Table 5). The average colony size in Torch was significantly different from that of GCL, but not from that of 2282-9 [Duncan's Multiple Range Test ($p=0.05$)]. There was no significant difference in mean colony size between the two susceptible B. napus lines, 2282-9 and GCL.

TABLE 14. Mean and maximum number of haustoria per infection site in cotyledons of susceptible and resistant lines/cultivars of *Brassica napus* and *B. campestris*†

Hours after inoculation	Line/Cultivar								
	Torch		2282-9		GCL		Regent		
	Mean	Max.	Mean	Max.	Mean	Max.	Mean	Max.	Nec.*
8	0.65	1	0.65	1	0.50	1	0.55	1	0 %
12	0.95	1	0.80	1	0.65	1	0.65	1	26 %
24	1.05	2	0.93	2	0.90	1	0.26	1	53 %
36	1.60	3	1.45	3	1.30	3	0.20	1	80 %
48	3.10	8	1.70	3	1.45	3	0.20	2	>95 %
60	6.30	16	4.80	9	4.85	10	--	--	--
72	5.95	18	5.75	12	5.60	12	--	--	--
84	22.80	44	19.85	38	13.45	31	--	--	--

† Observations made on 5 infection sites on each of 4 cotyledons in each of 4 lines/cultivars investigated.

* Percentage of necrotic host cells.

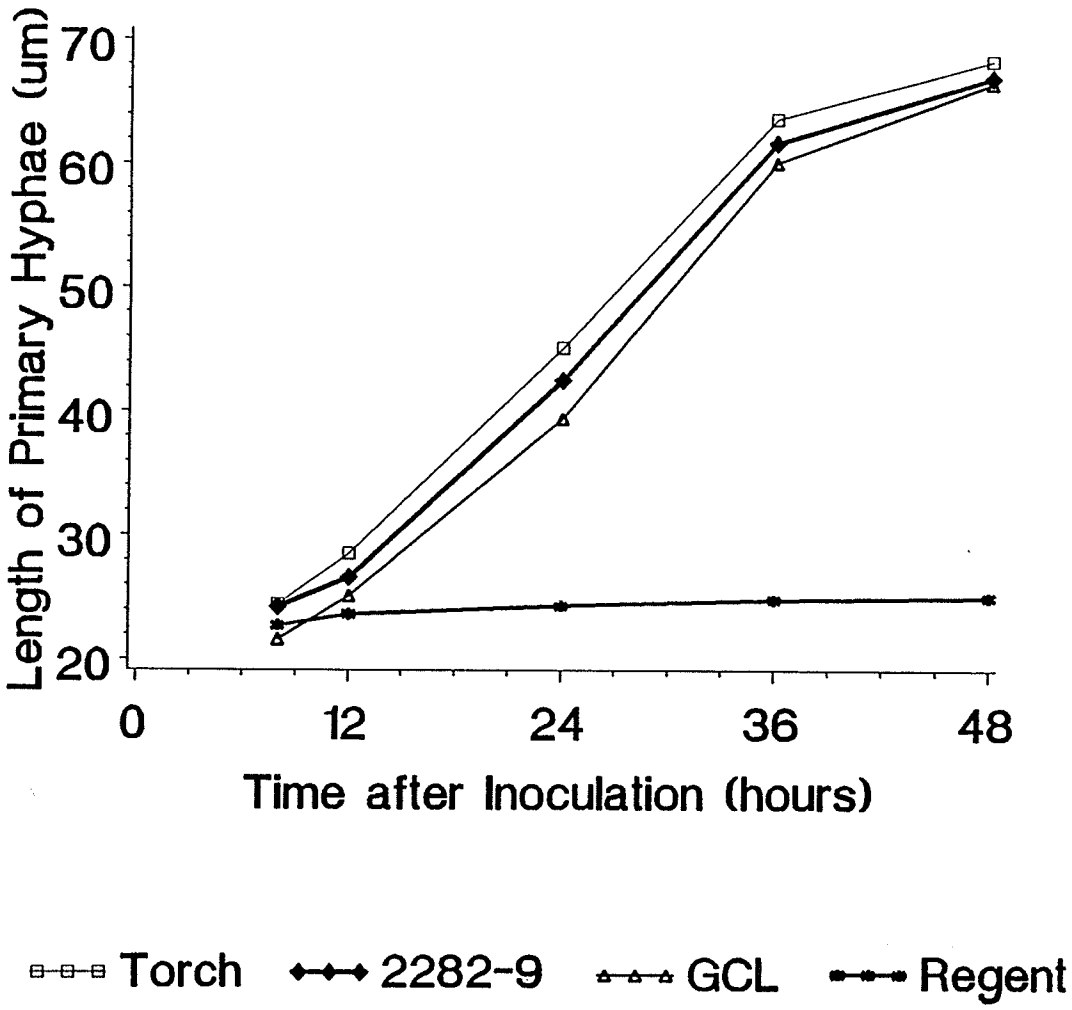

Figure 16. Mean primary hyphal length in cotyledons of susceptible and resistant lines/cultivars of Brassica napus and B. campestris from 8 to 48 h after inoculation with zoospores of Albugo candida race 7

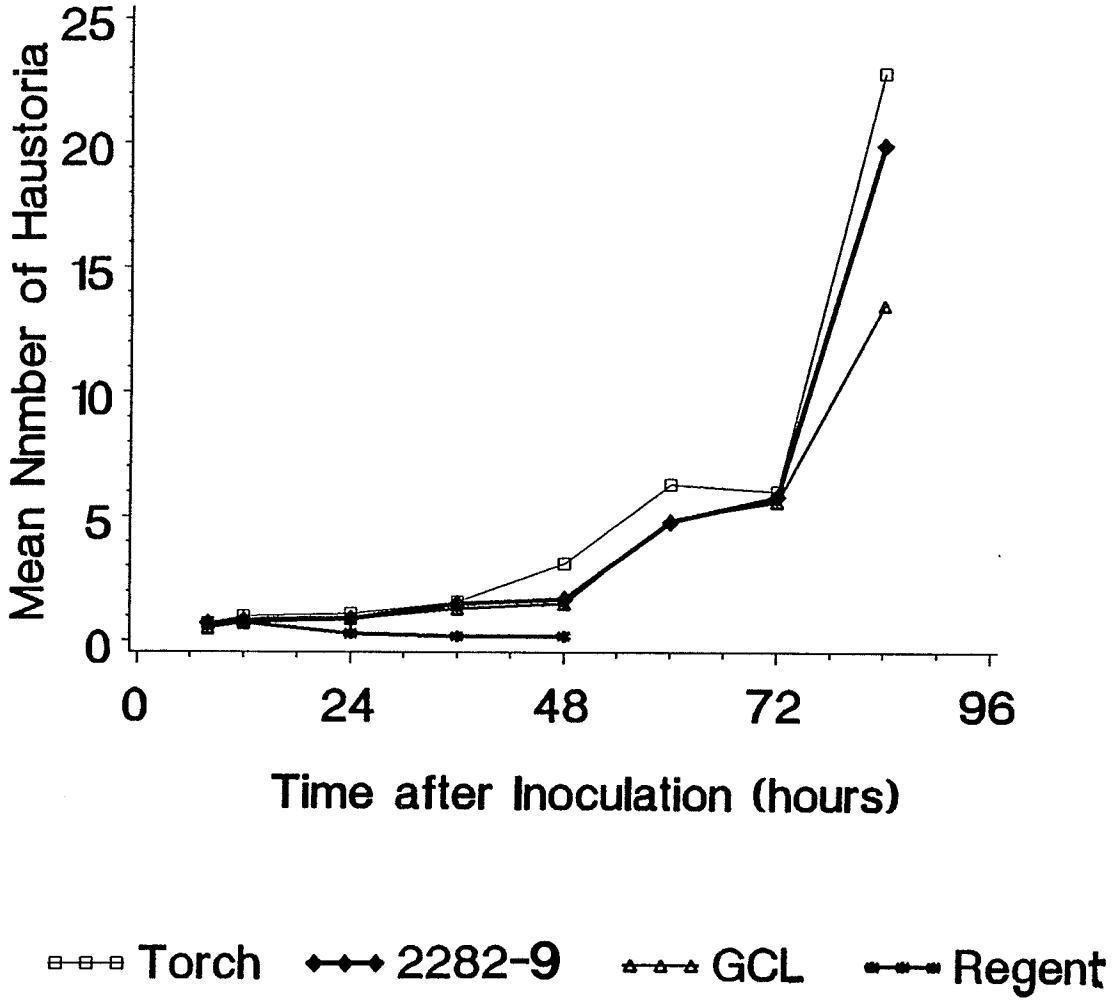

Figure 17. Mean number of haustoria per infection site in cotyledons of susceptible and resistant lines/cultivars of Brassica napus and B. campestris. A total of 20 infection sites were examined for each point.

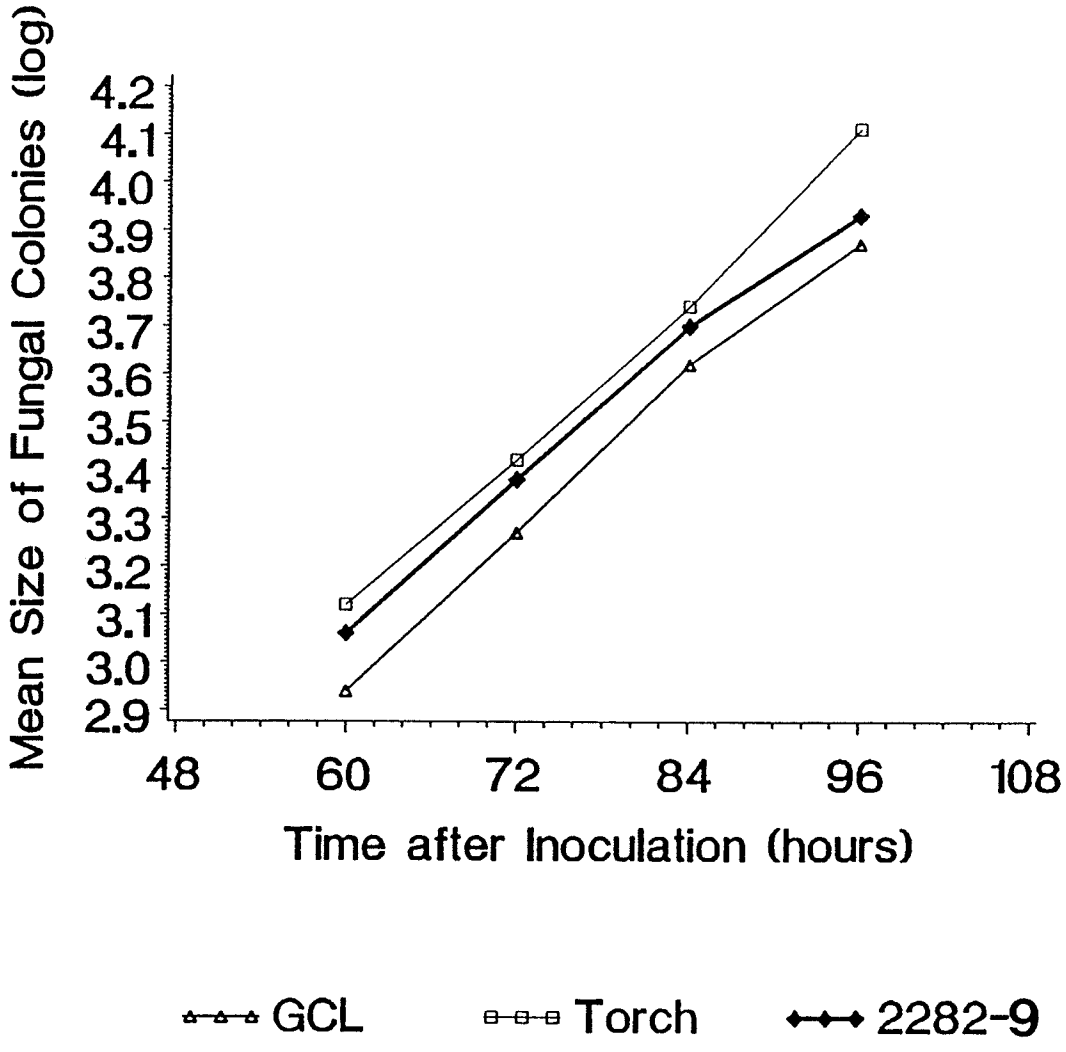

Figure 18. Mean size of fungal colonies in susceptible Brassica napus lines 2282-9 and GCL and B. campestris cultivar Torch from 60 to 96 h after inoculation with zoospores of Albugo candida race 7

Irrespective of whether the tested line is resistant or susceptible, the initial stages of infection, so far as DIC microscopy can reveal, are similar for cyst germination, stomatal penetration, primary hyphal growth and subsequent haustorium formation. Differences between resistant and susceptible reactions are not apparent until the establishment of the first haustorium in the host palisade mesophyll cell. This suggests that the successful formation of the first haustorium is essential for the establishment and maintenance of a compatible relationship between A. candida and its hosts.

In this investigation, rapid necrosis of invaded host cells was observed in the resistant cultivar Regent after formation of the first haustorium. The death of host cells seemed to be quite localized because only the invaded cells became necrotic; the adjacent non-penetrated cells and the mesophyll cells below the dead cells remained apparently healthy. There are several ways in which that host necrosis could be involved in resistance. First, necrosis might starve the fungus by impeding the passage of nutrients to it. Second, toxic substances produced by the fungus or generated as a result of host-parasite interaction could disrupt host cells and consequently cease the fungal growth. Third, elicitors released from dying host cells or from the fungus might induce the accumulation of phytoalexins produced by neighboring healthy cells in necrotic host cells, which could act further to restrict the development of the fungus.

However, the present studies have provided no information on the cause of disruption of the biotrophic relationship between A. candida race 7 and its incompatible cultivar. Although the rapid necrosis of

the invaded host cells appears to be the earliest event which distinguishes an incompatible from a compatible interaction, it is hard to conclude that such a host response is the primary determinant in the restriction of fungal growth because it is unknown when cell necrosis begins and how impaired functioning of haustoria affects further fungal growth. Even if it is, the observable cell necrosis may be preceded by a series of previous interactions between the host and parasite. In a number of host-parasite systems, a wide array of changes in cell ultrastructure has been reported to occur prior to the death of host cells (Harder et al., 1979; Heath, 1972, 1980). Also, there are reasonable indications that resistant plants may possess a number of types of defense mechanism against a single fungus. Verma et al. (1975) described the formation of encasements around each haustorium produced in a resistant host, which could kill the haustoria and consequently arrest the fungal growth. Compared with the rapid death of host cells, this type of host response may be equally effective against fungal infection.

Histological studies made with whole cotyledon preparations can provide a simple but effective means of quantitative determination of fungal development, can reveal the earliest stage at which compatible and incompatible interactions are differentiated, and can be used not only as a supplemental means of screening for disease resistance but also as an adjunct to biochemical and physiological studies. The information provided here could be useful in guiding biochemical and physiological researches on mechanisms of resistance using A. candida and Brassica species as a model system.

Chapter VI

GENERAL DISCUSSION

In the investigation of the progeny from the cross involving Regent and GCL, Fan et al. (1983) observed that some F_2 progenies segregated in a 15:1 ratio while others segregated in a 63:1 ratio. Since both F_1 and F_2 progenies from the cross and its reciprocal of 2282-9 x GCL were all susceptible, they postulated that the population of Regent was homogeneous at the two loci and heterogeneous at one locus for the genes conditioning white rust resistance.

Further evidence has been provided in the present study to confirm the digenic model with dominant resistance controlled by two non-allelic genes Ac7-1 and Ac7-2 denoted as R_1 and R_2. With this model, resistance will result from the presence of a dominant allele at either of the two loci, and susceptibility will be expressed when the alleles at both loci are homozygous recessive.

Difference in the disease reaction between and within 2282-9 and GCL, as well as their F_1 and F_2 progenies were reported by Fan et al. (1983) but no data to substantiate it was provided. The present study has revealed that although the infection types may be influenced by such environmental factors as temperature and inoculum concentration, 2282-9 is overall more susceptible than GCL. This implies that genes with minor effects, whose expression is subject to environmental changes, are present to modify the degree of susceptibility.

Depending on the success or failure of the parasite to establish a biotrophic relation with the host, the host cultivars are frequently classified as being compatible and incompatible with respect to their specific genotypes. Histological studies of events in the infection process on both susceptible and resistant lines of oilseed rape have indicated that differences in compatible and incompatible interactions would not be differentiated until the formation of the first haustorium. Resistance in Regent was found to be associated with the rapid death of the host cells in the infection court followed by cessation of fungal growth. The phenomena observed in the study seem to agree with the traditional view of "hypersensitive" reaction to biotrophic parasites, which states that the primary events determining an incompatible interaction occur after formation of the first haustorium and subsequent host cell necrosis inhibits the further growth of incompatible races of the fungus (Maclean et al., 1974; Skipp and Samborski, 1974; Samborski et al., 1977; Maclean and Tommerup, 1979).

However, the validity of this traditional view of hypersensitivity has been questioned by some plant pathologists, e.g. Brown et al., (1966), Ogle and Brown (1971) and Király et al. (1972) who have suggested that in diseases caused by Phytophthora infestans, Puccinia graminis tritici and Uromyces phaseoli, it is the death of the pathogen that results in host cell necrosis. Whether hypersensitivity is the cause or effect of plant resistance to infection has been fervently debated among plant pathologists, and detailed investigations of several host-parasite hypersensitive reactions have been reported (e.g. Littlefield, 1973; Tani et al., 1975; Harder et al., 1979; Mayama et al., 1982; Rohringer and Heitefuss, 1984).

Heath (1976, 1980, 1981) and Ingram (1978) have discussed the role of host cell necrosis in the resistance of plants to fungal infection and have pointed out that host cell death is not a uniform process but differs ultrastructurally and probably biochemically in different host-parasite interactions. Depending on the particular host-parasite system under study, host cell necrosis can be either the cause or a consequence of plant resistance to infection, or even an event irrelevant to the resistant reaction. Also, host cell necrosis may play a different role in different host response. Thus, they consider that the all-embracing term "hypersensitivity" is misleading and should be abandoned.

The histological studies have revealed that compatible and incompatible interactions do not diverge until the first haustoria are formed, but have not provided any information on host defense mechanisms in relation to changes in cell ultrastructure or in metabolic process following fungal infection for this was not attempted in this study. Therefore, although the rapid necrosis of infected host cells is shown to be the earliest event determining incompatible interaction it is difficult to generalize that this type of host response is the primary determinant in restriction of fungal growth. It is likely that in a particular host-parasite system more than one resistance mechanism operates in incompatible interactions. A similar study comparing the B. napus lines containing only R_1 and R_2 derived in the inheritance study may be useful in clarifying how resistance is operative in the cultivar Regent.

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Appendix A

TABLES OF ANALYSIS OF VARIANCE

TABLE 1. Analysis of variance for white rust infection on susceptible Brassica napus lines 2282-9 and GCL, and B. campestris cultivar Torch at growth stage 2.2 in relation to incubation temperatures and inoculum concentrations¬

Source	DF	SS	MS	F†
Replication	3	4.34392143		
Temperature (A)	1	19.64167838	19.64167838	41.89**
Error (a)	3	1.40652246	0.46884082	
Conc. (B)	2	170.68499537	85.34249770	558.32**
A x B	2	1.34455944	0.67227972	4.40*
Error (b)	12	1.83425446	0.15285454	
Line (C)	2	98.53514018	49.26757009	205.32**
A x C	2	1.05925259	0.52962630	2.21ns
B x C	4	14.50242785	3.62560696	15.11**
A x B x C	4	3.65349565	0.91337391	3.81*
Error (c)	36	8.63817912	0.23994942	
Sampling error	288	95.84535296	0.33279636	
Total	359	421.48977990		

¬ Experimental design was a split-split plot with four replications, each consisting of five leaves. Analysis of variance is based on square-root-transformed data. CV (a) = 22.36%, CV (b) = 12.77%, CV (c) = 15.99%.

† ** = Significant at 1% level, * = Significant at 5% level, ns = not significant.

TABLE 2. Analysis of variance for white rust infection on susceptible Brassica napus lines 2282-9 and GCL, and B. campestris cultivar Torch at growth stage 2.5 in relation to incubation temperatures and inoculum concentrations¬

Source	DF	SS	MS	F†
Replication	3	4.68629156		
Temperature (A)	1	21.50366897	21.50366897	125.76**
Error (a)	3	0.51296903	0.17098968	
Conc. (B)	2	174.35708047	87.17854020	215.12**
A x B	2	5.21532513	2.60766257	6.43*
Error (b)	12	4.86308258	0.40525688	
Line (C)	2	143.24371159	71.62185575	240.58**
A x C	2	1.18244339	0.59122170	1.99ns
B x C	4	40.40763838	10.10190960	33.93**
A x B x C	4	2.57698971	0.64424743	2.16ns
Error (c)	36	10.71742337	0.29770620	
Sampling error	288	99.87217870	0.34677840	
Total	359	509.13880288		

¬ Experimental design was a split-split plot with four replications, each consisting of five leaves. Analysis of variance is based on square-root-transformed data. CV (a) = 14.37%, CV (b) = 22.11%, CV (c) = 18.97%.

† ** = Significant at 1% level, * = Significant at 5% level, ns = not significant.

TABLE 3. Analysis of variance for germ tube length in cotyledons of susceptible and resistant lines/cultivars of Brassica napus and B. campestris at 4 hours after inoculation¬

Source	DF	SS	MS	F†
Lines	3	40.29892620	13.43297540	0.41ns
Cotyledons within lines (Exp. error)	20	657.52692407	32.87634621	3.24**
Observation within cotyledons (Sampling error)	96	937.37406360	10.13931316	
Total	119	1671.19991387		

¬ CV = 17.37%.

† ** = Significant at 1% level, ns = not significant.

TABLE 4. Analysis of variance for primary hyphal length in cotyledons of susceptible and resistant lines/cultivars of Brassica napus and B. campestris in relation to hours after inoculation¬

Source	DF	SS	MS	F
Replications	3	62.53123442		
Line (A)	3	11142.83377995	3714.27793	28.87**
Error (a)	9	1157.97234824	128.66359	
Hour (B)	4	53955.11772490	13488.77943	136.64**
A x B	12	12515.28917416	1042.94076	10.56**
Error (b)	45	4442.28809601	98.71751	
Sampling error	302	9505.72448948	31.47591	
Total	378	136512.21035843		

¬ Experimental design was a split-plot with four replications, each consisting of five cotyledons. CV (a) = 28.34%, CV (b) = 24.83%.

** = Significant at 1% level.

TABLE 5. Analysis of variance for colony size in cotyledons of susceptible *Brassica napus* lines 2282-9 and GCL, and *B. campestris* cultivar Torch in relation to hours after inoculation¬

Source	DF	SS	MS	F†
Replications	3	0.25207698	0.08402566	
Line (A)	2	1.17550550	0.58775275	5.78*
Error (a)	6	0.60982328	0.10163721	
Hour (B)	3	29.37608709	9.79202903	148.14**
A x B	6	0.15587696	0.02597949	0.39ns
Error (b)	27	1.78464267	0.06609788	
Sampling error	192	6.12671387	0.03190997	
Total	239	39.48072636		

¬ Experimental design was a split-plot with four replications, each consisting of five cotyledons. Analysis of variance was based on log-transformed data. CV (a) = 9.07%, CV (b) = 7.32%.

† ** = Significant at 1% level, * = significant at 5% level, ns = not significant.