HOST-PATHOGEN INTERACTIONS IN THE WHEAT-*MYCOSPHAERELLA GRAMINICOLA* PATHOSYSTEM

BY

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A Thesis
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FORWARD

The format of this thesis is presented as two manuscripts, which include materials, methods, results and discussion. Preceding the manuscripts is a general review of the literature, a general discussion and bibliography follow.

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1. GENERAL ABSTRACT

Mycosphaerella graminicola (Fückel) Schröeter (anamorph: *Septoria tritici* Rob.ex Desm.), the causal agent of Septoria leaf blotch of wheat, has increased in prevalence in Western Canada in recent years. Physiologic variation has been reported for this pathogen in other countries. Isolates collected from bread wheat growing in Manitoba and Saskatchewan were tested for virulence on a set of tetraploid and hexaploid wheat differential lines. Seedlings were inoculated with a conidial suspension and assessed three weeks later for the presence of necrosis, chlorosis and pycnidia to identify virulence patterns. Reactions of the differential host lines to different isolates ranged from susceptible to near-immune. Differences in virulence patterns indicated that two races were present in the pathogen population of Western Canada, suggesting that physiological specialization does occur. Cytological observations of resistant and near-immune reactions suggested that a hypersensitive response occurred in both tetraploid and hexaploid wheat lines as early as two to three days post-inoculation. In these lines, mesophyll cell collapse occurred in areas surrounding the stomatal penetration sites. These cells fluoresced when stained with KOH-aniline blue. There was no evidence of direct penetration of the mesophyll cells. Limited intercellular growth of hyphae did occur in both resistant lines. In the susceptible lines, both tetraploid and hexaploid, extensive intercellular growth of hyphae was consistently observed in the mesophyll tissue. Mesophyll cell collapse and pycnidia formation in the substomatal chambers of infected stomates began eight to ten days post-inoculation. No fluorescence was evident in the compatible reaction, at any of the sampling periods.

2. GENERAL INTRODUCTION

Mycosphaerella graminicola (Fückel) Schröeter (anamorph: *Septoria tritici* Rob.ex Desm.), the causal agent of Septoria leaf blotch of wheat, is a pathogen of worldwide occurrence (Eyal et al., 1985; Leath et al., 1993). The disease has caused considerable losses in both bread (*T. aestivum*) and durum (*T. durum*) wheats, resulting in a decrease in both yield and quality (Eyal and Ziv, 1974). The implementation of conservation tillage methods played a major role in the increase of the disease, due to the stubble-borne nature of the pathogen (Brokenshire, 1975b). As well, the wide use of semi-dwarf wheat cultivars is believed to have contributed to an increase in disease levels, as many of these cultivars are susceptible (Camacho-Casas et al., 1995).

Genetic variation, within the pathogen population, has been studied in most areas were the disease poses a serious threat to wheat production (Eyal et al., 1985; Kema et al., 1996a; Kema et al., 1996b). In Israel, were the pathogen has been present for many decades genetic variability, based on nuclear and mitochondrial DNA polymorphisms, was found to be greater (McDonald et al., 1995) and, once partially resistant cultivars have eventually succumbed to the disease (Eyal et al., 1973). The discovery of the teleomophic state of the pathogen was thought explain this genetic diversity in many of these populations (Hunter et al., 1999; McDonald et al., 1995; Zhan et al., 1998).

Physiological specialization occurs within the pathosystem but controversy as to the extent of this specialization exists. Certain authors suggested primarily that host species-specialization (hexaploid vs. tetraploid) occurred (Kema et al., 1996a; Van Ginkel and Scharen, 1988), while others suggested that host cultivar-specialization

occurred (Ahmed et al., 1996; Kema et al., 1996b). The presence of pathogenic races has also been documented (Eyal et al., 1973; Saadaoui, 1987).

Although cultivar resistance has been reported (Rosielle, 1972), previous studies looking at the infection process between host and pathogen have not determined the mode of host resistance (Cohen and Eyal, 1993, Kema et al., 1996c). A limited number of reactions and cultivars were studied and it was determined that the intercellular growth of the pathogen was possibly limited by compounds, which precluded fungal colonization (Kema et al., 1996c). However, others suggested pycnidium arrestment accounted for host resistance (Cohen and Eyal, 1993).

Control of the pathogen would benefit from the development of new resistant cultivars. An understanding of host-pathogen interactions and genetic diversity within the pathogen population is a prerequisite for developing such cultivars. The objectives of this study are 1) to assess the variability in virulence of the pathogen population from Western Canada and 2) to characterize, at the cytological level, the nature of the resistant and near-immune reactions exhibited by some wheat genotypes in response to infection by *M. graminicola*.

3. REVIEW OF THE LITERATURE

3.0 The Host

The Ascomycete fungus, *M. graminicola*, is an important pathogen of bread (*Triticum aestivum*) and durum (*T. durum*) wheats, and was reported to have a great impact on wheat production in several countries (Rosielle, 1972). Although *T. spelta*, *T. compactum* and the tetraploid wheat, *T. dicoccum*, were also found to exhibit susceptibility to the pathogen (Brokenshire, 1976), this has less of an impact on worldwide wheat production. Wild *Triticum* species represent an accessible source of resistant germplasm to *M. graminicola* (Yechilevich-Auster et al. 1983). The wild wheat species, *T. longissimum*, *T. speltoides*, *T. turgidum dicoccoides*, *T. monococcum boeoticum*, *T. tauschii* and *T. searsii*, all exhibited resistance to one of seven *M. graminicola* isolates tested by Yechilevich-Auster and co-workers (1983). Inoculation of wild diploid and tetraploid species suggested that pathogenicity patterns were unique when compared to those observed on cultivated wheats and that nine resistance genes were hypothesized to exist among 44 wheat lines (Yechilevich-Auster et al., 1983). In septoria afflicted areas, *Triticale* is also considered to be an alternative source of usable germplasm because of its high disease resistance (Brokenshire, 1976; Eyal et al., 1985).

The wide host range of the pathogen was evident when 8 of 22 inoculated cultivated and weed graminaceous species were found to support pycnidial development. These included *Arrhenatherum elatius, Hordeum vulgare, Hordeum murinum, Poa annua*, and *Vulpia bromoides* (Brokenshire, 1975a). The incubation and latent periods of the pathogen were found to be longer in these species than in cultivated wheats;

nevertheless sporulation was detected indicating that these plants could act as alternative hosts for the pathogen (Brokenshire, 1975a).

3.1 The Pathogen

3.1.1. The anamorph

Before the identification of the teleomorphic state (Sanderson, 1972), the pathogen was most commonly thought to propagate through asexual pycnidiospores that disperse via free water on the plant leaf surface (Fig. 1). Rain-splashed pycnidiospores are released during periods of high humidity and can cause vertical movement up the crop canopy, to ultimately cause infection at the flag leaf stage (Shaner and Buechley, 1995). The hyaline, filiform, pycnidiospores may be present as either single celled micropycnidiospores, or as two to seven celled macropycnidiospores that may measure 98 μm at their greatest length (Eyal et al., 1987; Shipton et al., 1971). However, the development of microspores was not evident when certain isolates were tested (Kema et al., 1996c).

Pycnidia are formed directly below the stomatal aperture of the host plant and are embedded in the epidermal and mesophyll tissue on both the abaxial and adaxial leaf surfaces (Cohen and Eyal, 1993; Eyal et al., 1987; Kema at al., 1996c). The pycnidium body, including the ostiole, is restricted by the contour of the stoma as fungal structural elements, such as paraphyses, are not formed (Kema et al., 1996c).

Pycnidiospores were found to maintain viability five to six months on remaining crop debris following harvest. However, environmental conditions and host-cultivar may have a bearing on the infectious period (Brokenshire, 1975b; Hilu and Bever, 1957). As

well, crop debris may support many generations of sporulating pycnidia, as cirrhi can be produced successively from the same pycnidium (Brokenshire, 1975b). Liberation of pycnidiospores occurs as a cirrhus of spores is released 30 minutes after exposure to moisture (Eyal et al., 1987). However, occurrence of rain seven days prior to sampling was negatively correlated with isolation of the pathogen from wheat fields sampled in Manitoba (Gilbert et al., 1998). The asexual state was found to maintain stability in culture and through re-isolation, without loss of virulence (Eyal et al., 1973).

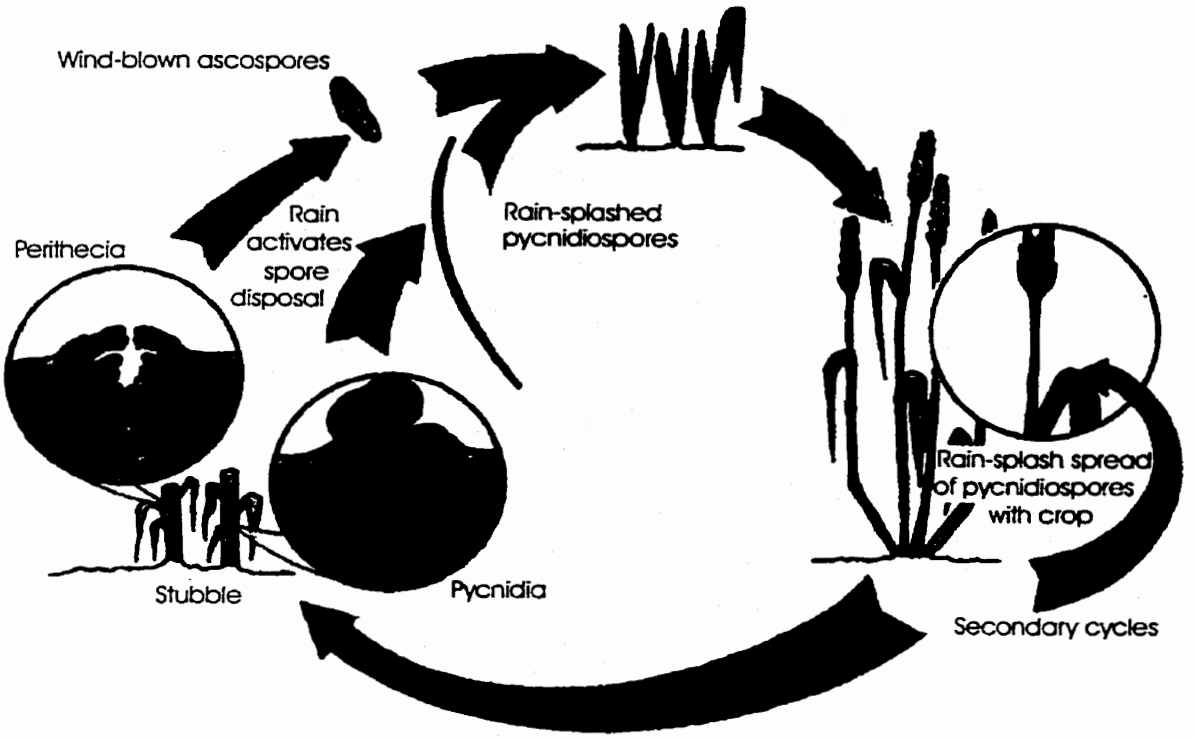
3.1.2. Teleomorph

Identification and classification of the sexual state revealed the possibility that ascospores could play a major role in the initial infection process by *M. graminicola* (Sanderson, 1972). Infection from pycnidiospores is limited by short-range, water-splashed dispersal within the crop canopy (Shaner and Buechley, 1995). Conversely, ascospores may be disseminated over long distances due to their airborne nature (Eyal et al., 1987; Zhan et al., 1998). Therefore, the pathogen is not confined to single fields and may be disseminated over an entire area of wheat production.

Pseudothecia, dark brown in color, develop on leaf blades and more prominently on leaf sheaths (Scott et al., 1988). Pear-shaped asci are bitunicate, containing eight ascospores in random arrangement (Sanderson, 1976; Scott et al., 1988). Ascospores, 10-18 μm in length, are conspicuous due to a single septum, producing two unequally shaped cells (Eyal et al., 1987; Sanderson, 1976; Scott et al., 1988).

The existence of the teleomophic state has been verified in a number of wheat producing countries including Australia (Brown et al., 1978), the Netherlands (Kema et

Figure 1. Life cycle of ***Mycosphaerella graminicola*** (Eyal et al., 1987).


Wind-blown ascospores
Perithecia
Rain activates spore disposal
Rain-splashed pycnidiospores
Rain-splash spread of pycnidiospores with crop
Stubble
Pycnidia
Secondary cycles

al., 1996d), New Zealand (Sanderson, 1972), the United Kingdom (Hunter et al., 1999; Scott et al., 1988; Shaw and Royle, 1989) and the United States (Garcia and Marshall, 1992). Ascospores have been found to provide a source of initial inoculum and are able to cause infection throughout the year in various wheat producing regions (Hunter et al., 1999; Shaw and Royle, 1989). Initially, pseudothecia found on wheat stubble from the previous cropping season were seen to discharge ascospores during periods of leaf wetness, most notably in late autumn and declining in early spring (Brown et al., 1978). However, most recent investigations indicated that ascospores are produced throughout the year and are dispersed from pseudothecia formed within the present crop canopy (Kema et al., 1996d). The ability of ascospores to infect the flag leaf means that the entire crop canopy is vulnerable to airborne infections (Hunter et al., 1999).

Viability of pseudothecia was maintained for as long as one year on stubble (Brown et al., 1978). However, pseudothecia from crop stubble may be depleted by midwinter in other locations (Hunter et al., 1999). Ascospores were reported to be discharged under both light and dark conditions, three to four hour after rainfall (Brown et al., 1978). Viability of ascospores was retained for one to two weeks after discharge, provided they were not exposed to direct sunlight (Brown et al., 1978). Pycnidia derived from ascospores were required for infection of the host, as direct ascospore infection was not observed (Garcia and Marshall, 1992).

Despite the continuous production of ascospores throughout the season (Kema et al., 1996d), the most prominent time of pseudothecia development occurred from December to January following a decrease in pycnidia formation in the United Kingdom (Hunter et al., 1999, Scott et al., 1988). Initial inoculum for autumn sown winter wheat

crops was shown to be composed of evenly dispersed, airborne infections of ascospores (Shaw and Royle, 1989).

Recently, the pathogen was reported to have a bipolar heterothallic mating system (Kema et al., 1996d), which would explain the high degree of genetic variation found within the pathogen population (McDonald and Martinez, 1990a). The ability of *M. graminicola* to complete many sexual cycles within a single season may also lead to both airborne infections on different leaf layers within the crop canopy and extensive genetic variation within a single crop (Hunter et al., 1999; Kema et al, 1996d). Sexual reproduction, as compared to asexual reproduction, was demonstrated to have a greater impact on the genetic structure of the pathogen as determined through gametic disequilibrium (McDonald et al., 1995).

The use of restriction fragment length polymorphisms suggested that a high degree of genetic variation occurred within a single geographical area (MacDonald and Martinez, 1990b). This was evident on such microgeographic level that different genotypes were present within the same lesion (MacDonald et al., 1995). The authors also suggested that variation in the *M. graminicola* population may lead to the selection of individuals that may conceivably overcome host-specific resistance or become fungicide resistant (MacDonald and Martinez, 1990a).

Gene flow between populations was suspected to account for similar levels of genetic diversity in populations separated by as much as 750 km (Boeger et al., 1993). It was estimated that as many as 12 individuals migrated between the two populations in Oregon and California. Immigration of genotypes had a greater impact on the genetic

structure of the population in the early rather than in the late growing season (MacDonald and Martinez, 1990a).

3.2 Epidemiology

Phenotypic traits associated with certain wheat cultivars are thought to influence progression of the disease. Host resistance is often found in tall, late-maturing cultivars rather than in short, early-maturing cultivars (Eyal, 1981; Eyal and Blum, 1989; Shaner et al., 1975), although this type of resistance is believed to be an escape mechanism and not truly genetically inherited (Camacho-Casas et al., 1995). However, even on semi-dwarf wheats, disease progression was seen to differ between cultivars as certain cultivars developed higher disease levels more rapidly (Eyal and Ziv, 1974). Nonetheless, plant height and heading date were found to be negatively associated with disease severity, which may impose limitations to breeding short, early-maturing cultivars resistant to the disease. No resistant progeny of short plant height or early maturity were found in a selection for disease resistance when a susceptible, short, and early-maturing parent was crossed with a resistant, tall, and late-maturing parent (Camacho-Casas et al., 1995). Conversely, Arama et al. (1999) suggested that resistance was not dependent on heading date but on resistance level and that late-heading cultivars are not genetically more resistant than early-heading cultivars.

Adult plant inoculations are necessary to ensure that disease reaction does not change with the plant growth stage. Expression of plant reactions does not seem to be affected by plant age (Shipton et al., 1971) and corresponding seedling and adult plant inoculations provided evidence of consistent disease reactions regardless of plant growth

stage (Eyal et al., 1973). Conversely, Kema and van Silfhout (1997) found a good correlation between seedling and adult plant infections with two isolates, but not with a third isolate. Thus, they suggested that adult plant inoculations were pertinent for a true analysis of disease reactions.

Although phenotypic characteristics may influence disease progression, environmental factors, such as temperature, were shown to have a much greater influence on level of disease than the growth stage at the time of plant inoculation (Wainshilbaum and Lipps, 1991). Increases in both post-inoculation moisture and temperature were positively correlated with disease development, as temperatures of 25 °C and moisture periods of 96 hours were shown to produce the greatest disease severity in susceptible cultivars (Hess and Shaner, 1987). Temperatures of 18 to 25 °C were reported to be optimal for disease development (Hess and Shaner, 1987; Wainshilbaum and Lipps, 1991). Suppression of pathogen growth was thought to occur at temperatures $\geq$ 25 °C (Magboul et al., 1992). However, Wainshilbaum and Lipps (1991) determined that growth was suppressed at 29 °C. Pathogen growth was also limited at 10 °C (Magboul et al., 1992).

Magboul et al. (1992) suggested that the optimal temperature for host infection was independent of the leaf wetness period. Conversely, Hess and Shaner (1987) suggested that compensation effects existed when short moisture periods were followed by warm temperatures or when long moisture periods were followed by a cool incubation period. A post-inoculation leaf wetness period of 96 hours, rather than 24 hours, was optimal to increase disease severity on susceptible cultivars (Hess and Shaner, 1987;

Magboul et al., 1992; Shipton et al., 1971). A leaf wetness period of 96 hr was also required for ascospore-generated pycnidia to be produced (Garcia and Marshall, 1992).

Lesion size, as affected by spore germination, germ tube elongation and penetration rate, was shown to increase with an increasing leaf wetness period, irrespective of the temperature (Magboul et al., 1992). Senescence was also determined to be higher at 25 °C. However, disease severity was greater at 20 °C (Magboul et al., 1992). Temperature and moisture affected development of necrotic tissue more in the susceptible cultivars than in the resistant cultivars (Hess and Shaner, 1987).

Microclimatic conditions are also important when considering environmental factors critical to disease development. An increase in crop canopy temperature was reported to occur with an increase in disease severity, resulting in a decrease in green leaf tissue (Eyal and Blum, 1989). Eyal and Blum (1989) suggested that infrared thermometry may be useful in determining high residual green leaf area. However, cultivar differences in maturity and natural leaf senescence may limit evaluation of disease severity using this method.

The latent period of the pathogen differed depending on the cultivar infected (Brokenshire, 1976; Shaw, 1990). Shaw (1990) also found that high temperatures prolonged the latent period and that minimum latent periods occurred at 17 °C, in agreement with Hess and Shaner (1987). The latent period was more affected by temperature in the resistant than in the susceptible cultivars (Hess and Shaner, 1987). Inoculum density and leaf wetness did not seem to affect the length of the latent period (Shaw, 1990).

Although winter wheat cultivars may appear asymptomatic as autumn seedlings, fall infections are likely to initiate disease development during spring growth (Shaner and Buechley, 1995). Wainshilbaum and Lipps (1991) suggested that the cooler temperatures of spring favored the infection of wheat by *M. graminicola*.

3.3 Disease Control

Control of the pathogen can be practically implemented by eliminating wheat trash and stubble from infected wheat fields. Tillage systems that bury trash below the soil surface have been shown to greatly reduce the viability of pycnidiospores and consequently, their pathogenicity (Brokenshire, 1975b). As both the sexual and asexual states of the pathogen are sources of initial inoculum, three to five year crop rotations (Eyal, 1981) and elimination of crop debris from the previous season would greatly reduce the viability of the pathogen in the field. Conversely, epidemics occurred during periods of heavy plowing, suggesting that stubble-borne inoculum could not always be destroyed (Shaner and Beuchley, 1995) or that infections may be initiated by ascospores from other fields.

In wheat growing areas where disease pressure from Septoria leaf blotch is high, chemical control measures are required to prevent yield and quality loss, due to damage from the disease (Eyal, 1981; Shaner and Buechley, 1995). Determining the appropriate time to apply such control measures may, however, be difficult due to spatial patterns of disease development (Shaner and Buechley, 1995) and environmental conditions influencing disease progression (Eyal, 1981). Shaner and Buechley (1995) suggested that

depending on environmental conditions, foliar fungicides would be of benefit when applied at the time of spike emergence.

The introduction of disease tolerant wheat genotypes could aid in disease control, as these cultivars are able to sustain high disease levels without incurring substantial yield losses and without exerting selective pressure on the pathogen population (Ziv and Eyal, 1978; Zuckerman et al., 1997). This type of "generalized resistance" could also be of benefit, considering the high genetic variability leading to new virulence patterns in the pathogen population. Disease severity may also be decreased, in areas where the pathogen is known to be present, by implementing later seeding dates (Shaner et al., 1975).

Breeding for resistance to *M. graminicola* is thought to be the most economically suitable (Eyal, 1981; Shipton et al., 1971) and environmentally sustainable means of disease control. Resistant germplasm does exist among *T. aesitivum* and *T. durum* varieties (Rosielle, 1972). However, they are often difficult to detect. The stability of this resistance may also be of concern in situations where resistant cultivars have succumbed to the disease (Eyal et al., 1973), stressing the need for sustained breeding programs to control the disease.

3.4 Genetic Variation in the Pathogen Population

Debate as to the extent of host specialization and the existence of races is pertinent to the study of interactions in the wheat-*M. graminicola* pathosystem. Host species-specialization is suggested to exist within the pathogen population as Kema and co-workers (1996a) found that isolates specialized to either bread or durum wheats. Van

Ginkel and Scharen (1988) agreed, stating that cultivar X isolate interactions were insignificant and that host-species specialization, rather than race differentiation should be considered because of significant differences due to cultivars and isolates.

Kema et al. (1996b) did, however, find cultivar-specificity among each of the bread and durum wheat isolates, indicating that specific factors for resistance and virulence may exist within host and pathogen genotypes, respectively. In a study incorporating both diploid and tetraploid wild *Triticum* species, Yechilevich-Auster et al. (1983) determined that population X isolate interactions were considerable, regardless of species, genomes or environmental factors. Nevertheless, environmental variables optimal for disease development are considered by many to be an important aspect in determining disease reaction and thus host specificity (Eyal and Levy, 1987; Kema et al., 1996a).

Evidence of differential interactions between host cultivars and *M. graminicola* isolates suggested the presence of physiological specialization within the pathosystem (Ahmed et al., 1995; Ahmed et al., 1996; Eyal et al., 1973; Saadaoui, 1987). Five isolates, originating from the wheat growing regions of Israel, were seen to differ in their virulence patterns on a host differential set containing many indigenous cultivars (Eyal et al., 1973). Physiological specialization was also suggested by Saadaoui (1987) who distinguished three races in 19 *M. graminicola* isolates collected from various areas of Morocco. Although the host genotype was unknown at the time of isolation, isolates were seen to differentiate on both *T. aestivum* and *T. durum* species of the host. However, virulence patterns on bread and durum wheats were distinct, suggesting that specific resistance genes were more likely to be present in either species. Isolates

collected from tetraploid cultivars in Israel were more virulent on tetraploid differentials even though significant cultivar X isolate interactions were evident (Eyal et al., 1985).

Selection for cultivar-specific virulence was shown to exist among isolates obtained from winter wheats (Ahmed et al., 1996). Disease levels were greater when isolates were tested on cultivars from which they were isolated. An increase in aggressiveness is also hypothesized to occur, as isolates from susceptible cultivars induced higher disease levels than isolates from either moderately resistant or resistant cultivars (Ahmed et al., 1996). Geographical adaptation of isolates was also observed when both isolates and cultivars were tested from Oregon and California (Ahmed et al., 1995). Higher disease levels were obtained when cultivars were inoculated with isolates recovered from the same region. Notably, virulence frequencies were seen to vary from region to region with high virulence frequencies occurring in Brazil, Mexico, Uruguay, Oregon, Israel, Tunisia, Turkey and Ethiopia and low virulence frequencies occurring in Chile (Eyal et al., 1985).

It is suspected that the gene-for-gene operates within the pathosystem due to cultivar specificity (Eyal et al., 1985; Kema et al., 1996a). However, statistical analysis could not conclusively provide evidence of this model. Kema and co-workers (2000) determined that avirulence of *M. graminicola* was controlled by a single locus, and suggested that the wheat-*M. graminicola* pathosystem followed the gene-for-gene model. Segregation of avirulence in this heterothallic fungus was seen to occur in a 1:1 ratio in the F_1 and F_2 generations in the absence of any recombinant phenotype.

3.5 Infection of the Host Tissue

Few studies have been reported that investigated the infection process of *M. graminicola* and the resistant mechanism(s) employed by the host. The same cultivars, Shafir (susceptible) and Kavkaz/K4500 1.6.a.4 (resistant) were studied using isolates from Israel by Cohen and Eyal (1993), and by Kema and co-workers (1996a; 1996b) with isolates from Turkey and the Netherlands. Pycnidiospore germination was similar on both the susceptible and resistant cultivars. Stomatal penetration and intercellular hyphal growth in the mesophyll tissue were characteristic of the infection process of the pathogen (Cohen and Eyal, 1993; Kema et al., 1996c). Although Kema and co-workers (1996c) indicated that host penetration was strictly stomatal, Cohen and Eyal (1993) suggested that direct penetration might be a secondary method of infection. Host penetration did occur at the junction of epidermal cell walls in some cultivar-isolate interactions (Rohel et al., 2001).

Arrested development of pycnidia and pycnidial initials was suggested as a possible resistance mechanism, which ultimately prevented the formation of mature pycnidia (Cohen and Eyal, 1993). Arrested developmental stages of pycnidial formation were not seen in the incompatible interaction described by Kema et al. (1996c). However, a lack of colonization and limited hyphal development in the intercellular spaces of the mesophyll tissue were typical of the incompatible response of cultivars inoculated with isolates from Turkey and the Netherlands. Due to this type of reaction, Kema and co-workers (1996c) suggested that production of compounds might be responsible for the limitation of fungal growth within the host tissue.

3.6 Symptomology

Necrosis, the production of pycnidia, and often chlorosis on the plant leaf tissue are characteristic symptoms of Septoria leaf blotch (Eyal et al., 1987). Chlorosis is commonly the first expression of infection, occurring five to six days after inoculation (Eyal et al., 1987). Subsequently, necrotic tissue develops in these areas nine to 12 days post-inoculation followed by the production of pycnidia 15 days after the initial infection. Necrotic tissues, containing light to dark brown pycnidia, are distinct characteristics of the disease. Lesions are often elongated, running parallel to the vascular tissue (Eyal et al., 1987). *M. graminicola* was also reported to cause disease symptoms on glumes, as black streaks can form along the vascular tissue of the glume, under optimal post-inoculation conditions (Wainshilbaum and Lipps, 1991). Rarely have seed head infections been observed (Brokenshire, 1976).

Eyal and Brown (1976) found that the density of pycnidia present on the leaf was related to the size of pycnidia that developed. Hess and Shaner (1987) determined that both necrosis and pycnidia density increased, with an increase in temperature. However, host cultivar had the most effect on pycnidia density (Hess and Shaner, 1987; Shaner and Finney, 1982) when compared to temperature or growth stage (Wainshilbaum and Lipps, 1991). The number of pycnidiospores per pycnidium was 2.0 - 2.5 times greater in susceptible and moderately resistant cultivars than in resistant cultivars, regardless of incubation conditions. However, twice as many pycnidiospores per pycnidium were produced when plants were incubated in a growth chamber rather than the greenhouse, regardless of the cultivar used (Gough, 1978).

Although the production of necrosis and pycnidia seem to be closely related in both space and time of development, each has been seen to form independently of the other. Pycnidia have been seen to form on green tissue in the absence of necrosis (Cohen and Eyal, 1993). As well, extensive necrosis was seen to occur in response to the infecting pathogen, often in the absence of pycnidial formation or in the presence of severely restricted pycnidial formation (Rosielle, 1972). Kema et al. (1996a) suggested that the production of pycnidia and necrosis may be under different genetic control.

Symptoms produced by the ascospores were found to be similar to those produced by pycnidiospores. However, a longer incubation period was required and fewer pycnidia were produced when plants were inoculated with ascospores. This was due to the decreased number of infection units and the time required for ascospores to generate pycnidiospores for infection (Garcia and Marshall, 1992). Hunter and co-workers (1999) suggested that lower disease severities might result from ascospore infection.

Hastened leaf senescence, accelerated decrease of green leaf area, slowed leaf expansion and size reduction of new leaves, were caused by the disease (Gaunt et al., 1986). Yield reduction, due to the effects of the disease on plant development both pre-anthesis and post-anthesis, was explained by a reduction in the number of grains per spikelet and a reduction in grain weight (Eyal and Ziv, 1974; Thomson and Gaunt, 1986).

3.7 Rating System

Disease response to infection by the pathogen has been reported to be variable, as Yechilevich-Auster et al. (1983) observed plants on which interactions were expressed as either susceptible or resistant, or as a gradient of responses from susceptible to resistant.

Disease rating for Septoria tritici blotch has most commonly been conducted using quantitative methods of disease assessment (Ahmed et al., 1995; Eyal and Brown, 1976; Eyal et al., 1985; Saadaoui, 1987). Due to the influence of environmental variables on disease production, consistent quantitative results may be difficult to achieve. As well, other variables may influence disease reaction. Plant inoculations conducted using a mixture of isolates, particularly those grown together in culture, produced significantly less pycnidia than when each isolate was used in individual inoculations (Zelikovitch and Eyal, 1991). Suppression of pycnidia development also occurred when sequential inoculations were conducted first using an avirulent isolate followed by a virulent isolate (Halperin et al., 1996).

The presence of pycnidia was suggested to be the most appropriate parameter of disease rating, as necrosis could be associated with resistance rather than susceptibility (Kema et al., 1996a). Density of pycnidia is often the exclusive criterion used for assessing cultivar response to the disease (Eyal et al., 1985). However, this inherently leads to quantitative results.

Rosielle (1972), who classified inoculated wheat cultivars into six classes based on reaction type, introduced a qualitative assessment of disease. Immune to very susceptible reactions were identified among 7,500 wheat varieties including both hexaploid and tetraploid wheats. A number of early maturing bread wheat cultivars exhibited a highly resistant response when only pycnidial development was assessed. However, extensive leaf necrosis developed.

Shaner and colleagues (1975) suggested that two types of resistance were evident among wheat cultivars evaluated for the disease. The first was characterized by a

reduction in the number of pycnidia produced and the second characterized by a reduction in the rate of disease development. The latter reaction was not associated with fewer pycnidia per lesion but was associated with late-maturing wheat cultivars. This type of resistance, seen in late-maturing cultivars, may be an escape mechanism (Camacho-Casas et al., 1995).

Kema and co-workers (1996d) suggest that disease reactions may only appear quantitative due to the presence of many pathogen genotypes producing qualitative reactions on the same host. Genetic variation identified within single lesions (MacDonald et al., 1995) is consistent with this hypothesis.

4. RESULTS OF RESEARCH

4.0 Host-pathogen interactions in the wheat-*Mycosphaerella graminicola* pathosystem

4.0.0 Abstract

Mycosphaerella graminicola (Fückel) Schröeter (anamorph: *Septoria tritici* Rob.ex Desm.), the causal agent of Septoria leaf blotch of wheat, has increased in prevalence in the prairie region of Canada in recent years. Physiologic variation has been reported for this pathogen in other countries. Seventy-four pathogen isolates were collected from bread wheat fields in Manitoba and Saskatchewan and tested for virulence on a set of differential cultivars, including four bread and two durum wheat lines/cultivars. Seedlings were inoculated with a conidial suspension and assessed three weeks later for the presence of necrosis, chlorosis and pycnidia to identify virulence patterns. Reactions of the differential host lines to different isolates ranged from susceptible to near-immune. Differences in virulence patterns suggested that physiological specialization occurs and that two races of *M. graminicola* are present in the pathogen population of western Canada.

4.0.1 Introduction

Septoria leaf blotch of wheat, caused by the fungal pathogen *Mycosphaerella graminicola* (Fückel) Schröeter (anamorph: *Septoria tritici* Rob. ex Desm.), is a serious disease of wheat worldwide (Leath et al., 1993). The pathogen, able to infect both common (*Triticum aestivum*) and durum (*T. durum*) wheat (Rosielle, 1972), has been reported to have devastating effects in a number of countries, including Australia (Murry et al., 1990; Rosielle, 1972), Canada (Gilbert et al., 1998), Israel (Eyal et al., 1973), Morocco (Saadaoui, 1987), the Netherlands (Kema et al., 1996a), the United Kingdom (Scott et al., 1988), the United States (Shaner and Buechley, 1995), and a number of countries in Asia and Africa (Saari and Wilcoxson, 1974). The pathogen causes significant yield losses in its wheat host (Shipton et al., 1971). Eyal et al. (1987) reported yield losses as great as 50%.

Growth of the pathogen and infection of the host are favored by moist, temperate conditions. The severity of the disease and the rate of epidemic development are dependent on the temperature and the leaf wetness period following inoculation (Magboul et al., 1992). A post-inoculation moisture period of 96 hours and a post-inoculation temperature of 25 °C were found to produce the highest disease development, as assessed by pycnidial production (Hess and Shaner, 1987). Similarly, high levels of disease were found to occur at 19 and 24 °C. However, symptom development decreased at temperatures of 29 °C (Wainshilbaum and Lipps, 1991). According to Magboul et al. (1992) temperatures greater than 25 °C inhibit pathogen growth.

The pathogen overwinters on crop debris to produce inoculum for the following cropping season. In areas where decomposition of the crop residues occurs rapidly,

disease levels remain generally low (Saari and Wilcoxson, 1974). Previously, pycnidiospores were primarily thought to provide initial inoculum, as rain splashed conidia would begin the infection process (Brokenshire, 1975a). Recently, the teleomophic state has been found as the overwintering form of the pathogen in many countries, and was shown to contribute most of the initial (Garcia and Marshall, 1992; Scott et al., 1988; Shaw and Royle, 1989) and much of the secondary inoculum (Hunter et al., 1999). Wind-blown ascospores can be carried over great distances to infect developing crops, even when the asexual state of the pathogen is not locally present (Shaner and Buechley, 1995). Symptoms developing from ascospore-generated conidia were similar to those caused by pycnidiospores (Garcia and Marshall, 1992).

The presence of the teleomorphic state is significant, as McDonald et al. (1995) found that sexual reproduction had a greater impact on genetic diversity when compared with asexual reproduction. Immigration of isolates between populations of *M. graminicola* was found to have a large impact in the early to mid-seasons of crop growth (Zhan et al., 1998). Consequently, gene-flow between populations could prove detrimental to currently resistant cultivars, as it was shown to occur between populations over hundreds of kilometers (Boeger et al., 1993).

Because of the pathogen's life cycle, management practices which incorporate minimum tillage often bring about high disease levels the following cropping season (Shaner and Buechley, 1995). As well, the relative susceptibility of wheat cultivars grown regionally the previous season, also affects the severity of epidemics the subsequent year (Murray et al., 1990). The introduction of semi-dwarf cultivars appeared to encourage disease, as many of these cultivars were susceptible to the pathogen (Eyal

et al., 1973). Saari and Wilcoxson (1974) suggested that the presence of highly susceptible semi-dwarf cultivars emphasized the need for resistance, if they were to be intensely cultivated.

Fungicides are used to control the disease, particularly in areas that experience severe epidemics and heavy yield losses (Shaner and Buechley, 1995). However, the need to implement chemical control measures is often difficult to ascertain, due to fluctuating precipitation and the economic feasibility of such spray programs (Eyal, 1981). Some suggested that minimum disease resistance standards be incorporated for agronomically used cultivars in areas that are plagued with severe epidemics of Septoria leaf blotch (Murray et al., 1990). Ziv and Eyal (1978) determined that the introduction of tolerant wheat genotypes was a viable means of plant protection, as they tend to decrease the selective pressure on the pathogen to increase in virulence. However, breeding for disease resistance may be the best method for disease control (Rosielle, 1972). A comprehensive knowledge of host-pathogen interactions, such as the presence of physiological specialization and host resistance, is required in order to implement an effective breeding program, aimed at incorporating resistance to *M. graminicola* into commercially grown wheat cultivars (Eyal et al., 1973).

Controversy over physiological specialization and the presence of races within the pathosystem exists. The presence of physiological races, within the pathogen population from Israel, was demonstrated when five isolates were shown to differ in their degree of virulence on a differential host set (Eyal et al., 1973). Similarly, the presence of three distinct pathogen races was also reported in the pathogen population from Morocco (Saadaoui, 1987). Different virulence patterns were distinguished on both durum and

bread wheat differential cultivars and highly significant cultivar X isolate interactions were detected (Saadaoui, 1987). These studies assessing physiological variation within the *M. graminicola* population were largely based on quantitative disease evaluations.

Ahmed et al. (1996) found significant pathogen, cultivar and pathogen population X cultivar effects in a study of pathogenic variation among winter wheats. Isolates exhibited a greater degree of virulence on cultivars from which they were isolated, indicating an obvious cultivar-specific selection. As well, a location-specific adaptation of the pathogen was observed (Ahmed et al., 1995). Isolates, tested for pathogenic variability on a set of geographically diverse wheat differentials, were found to produce significantly more disease in cultivars from the same geographical region.

In addition to variation in virulence to individual cultivars, isolates of *M. graminicola* were found to be specific to hexaploid and tetraploid wheats, leading Kema and coworkers (1996b) to propose a designation of *M. graminicola* into "varieties" based on the ploidy level of the host of origin. Van Ginkel and Scharen (1988) also reported host-species specialization in the pathosystem. The authors concluded that cultivar X isolate interactions were not significant and that the differential interactions were due to differences in aggressiveness rather than virulence, thereby suggesting that true physiological races do not exist within the *M. graminicola* population (Van Ginkel and Scharen, 1988).

To date, no studies of host-pathogen interactions in the wheat-*Mycosphaerella graminicola* pathosystem have been conducted using isolates of the pathogen and host populations from Western Canada. Although not the primary disease threat to wheat producers of the prairies, Septoria leaf blotch has been found to be an important

component of the leaf-spotting complex of wheat across the Manitoba prairie region (Gilbert et al, 1998). The objectives of the present study were to assess the extent of pathogenicity in the *M. graminicola* population from the Canadian prairies and to determine if races of the pathogen could be identified.

4.0.2 Methods and Materials

Wheat Differential Lines

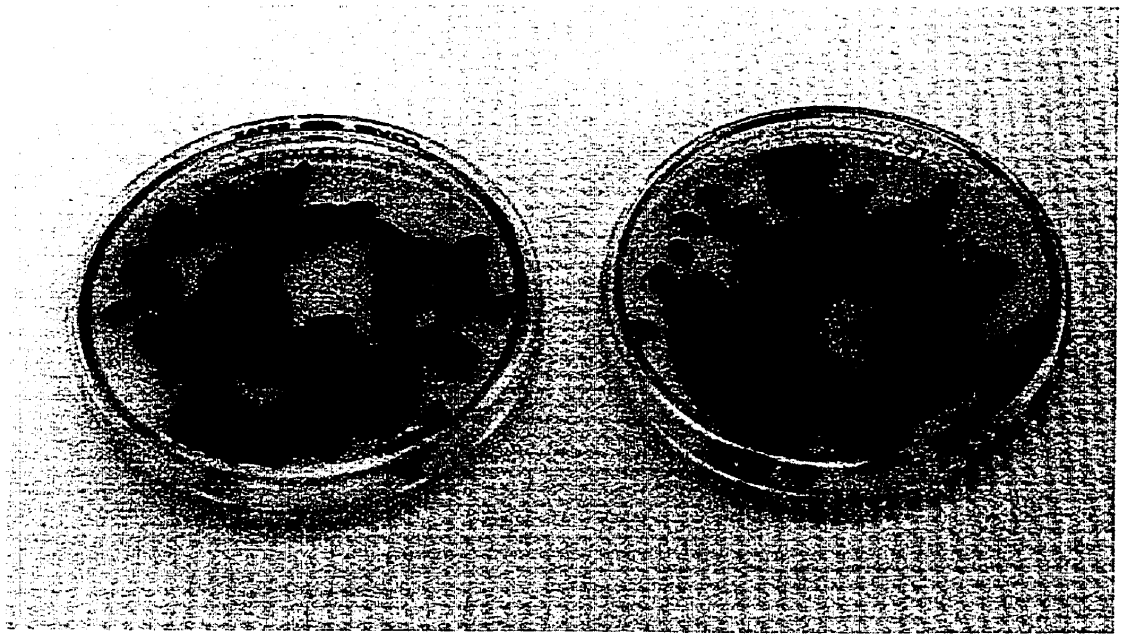
Six wheat lines, including both bread and durum wheat, were used for isolate screening. Four hexaploid lines were used; Erik, an early maturing cultivar; Salamouni, a tall, late-maturing accession from Lebanon, ST 6 from Uruguay; and Amazon, a recently registered tan spot resistant cultivar in Manitoba. The two tetraploid lines, Coulter (Canadian cultivar) and 4B1149 wheat accession (CIMMYT, Mexico), are not currently grown in the area as there is very limited durum wheat production. Seeds were pre-germinated on moist filter paper in petri dishes and place in the dark for 48 hours at 4 °C, followed by 48 hours at 22 °C (Fig. 2A). Four seeds of each of the six lines were planted in 10-cm-diameter clay pots. Two wheat lines, typically a susceptible and a resistant line, were sown as two separate clumps in the same pot. A soil, sand and peat mixture (2:1:1) amended with 11-53-00 fertilizer was used for planting. Seedlings were allowed to grow in either a growth chamber or growth room set at 22/18 °C (day/night) and a 16-hour photoperiod under fluorescent light at 181 $\mu E.m^{-2}.s^{-1}$. Seedlings were inoculated at the 2–3 leaf stage, approximately 12-14 days after planting (Fig. 2B).

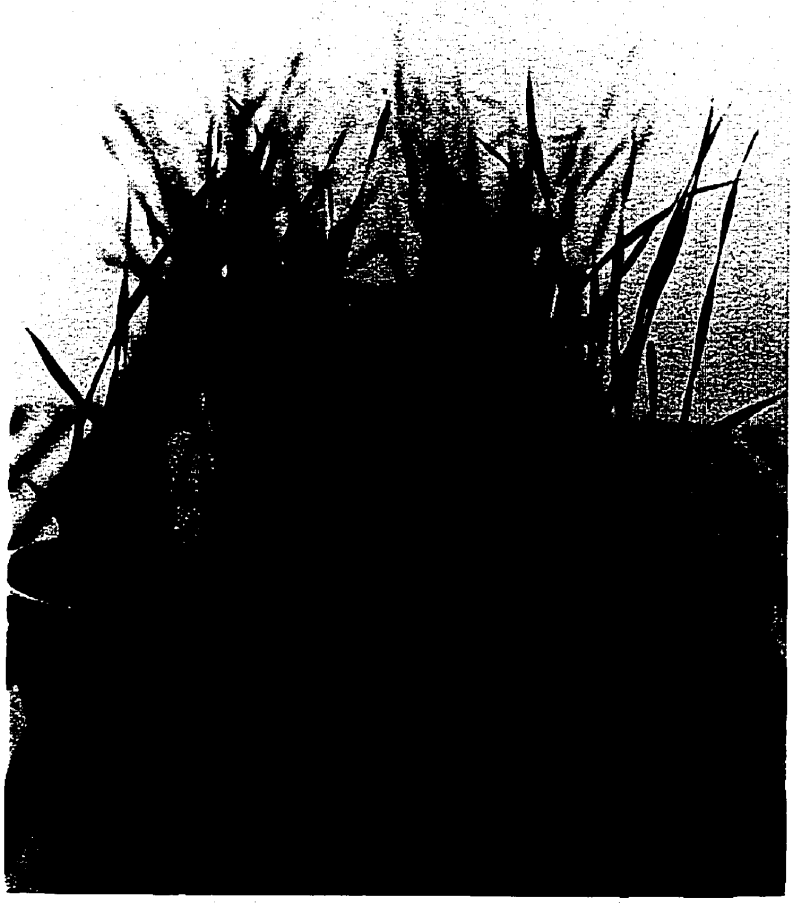
Isolates of *M. graminicola*

Isolates of the pathogen were collected from the wheat growing regions of the Canadian prairies during the summers of 1996 to 1999. Diseased wheat leaf samples were extensively collected from randomly selected fields throughout southern and central Manitoba, and to lesser extent in areas of southern Saskatchewan, from plants at the flag

Figure 2A) Pre-germinated seeds of the two durum cultivars, Coulter and 4B1149 (L-R).

Figure 2B) Seedlings of wheat differentials, at the two-three leaf stage, to be inoculated.





leaf stage. All fields sampled in this study were bread wheats, as durum wheats are rarely grown in this region. The samples were air dried for one week on a laboratory bench and kept in cool storage (5 °C) until isolation of the pathogen was required for inoculation.

Following rehydration of the desiccated leaf samples, a single cirrhus of conidia was transferred to Yeast Malt Agar containing 0.25% chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$ / Sigma) to prevent bacterial contamination, as leaf surface sterilization was omitted at the time of isolation (Appendix 1). Plates were kept in an incubator (20 °C) in the dark, for two to three weeks until cultures were subcultured to Yeast Malt Agar plates without chloramphenicol. These subcultures were then used in inoculation experiments and stored for further use. To maintain viability and virulence of cultures in long-term storage, pycnidiospores were suspended in sterile water, combined with glycerol (1:4) and frozen at –70 °C. Inoculation of host differential lines was often conducted using isolates that had been previously kept at –70 °C.

Seedling inoculation

Inoculum was harvested from Yeast Malt Agar plates containing 5 to 7 day old streaked cultures of pycnidiospores that had been placed under a fluorescent light source to ensure yeast-like, conidial growth of the pathogen. The spore concentration of the suspension was measured using a hemocytometer and adjusted to 1.0×10^7 pycnidiospores/ml. Two drops of Tween 20 (polyoxyethylene-sorbitan monolaurate /Sigma) were added per approximately 100 ml of inoculum as a surfactant. Seedlings were sprayed until run-off, using a devilbis-type atomizer and an air pressure of approximately 1.0 to 1.4 kg/cm^2. Inoculated seedlings were then allowed to air dry and

subsequently placed in a misting chamber for 72 hours (Fig. 3). An ultrasonic humidifier provided continuous leaf wetness. Four isolates were used in each inoculation run with three replications for each isolate. Isolate 96-36 was used as a control in each trial and barriers were used to separate each set of differential wheat lines, inoculated with the same isolate, to prevent cross-contamination of isolates in the misting chamber. Plants were then allowed to dry, removed from the misting chamber, and placed on a growth chamber bench with the same temperature and light conditions prior to inoculation. Plants were fertilized weekly with a 20-20-20 soluble fertilizer (Plant Pro) solution (5g/1L).

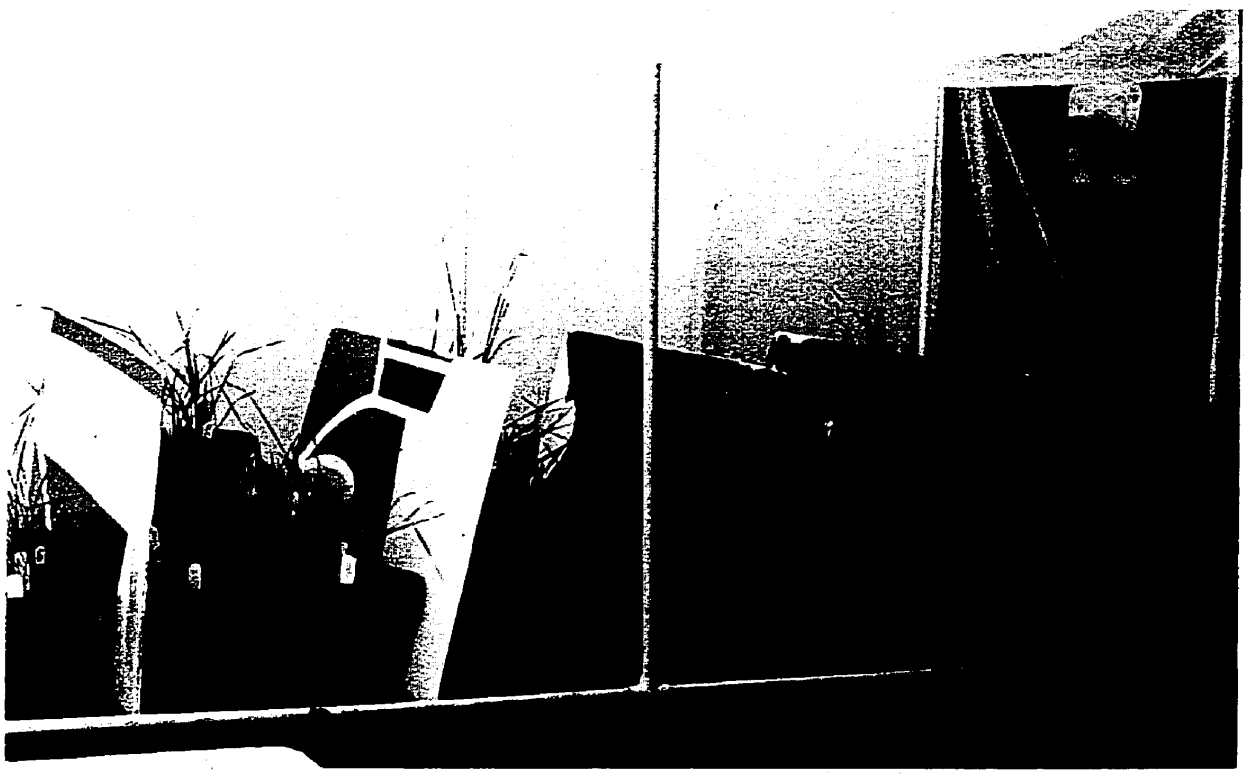
Adult Plant Inoculation

The six host differentials were also inoculated as adult plants. However, only isolates 96-36 and 96-W, representing the two major virulence patterns in this study, were used in adult plant inoculations. The plants were inoculated at the same time period to ensure that inoculum and growth conditions were identical. All varieties were at the flag leaf stage, except for Salamouni, which was at the penultimate leaf stage when the inoculation was performed. Plants were incubated for 72 hours, as described above for the seedlings, and subsequently transferred to a greenhouse bench until rated two and three weeks later.

Rating System

Disease assessment was conducted twice for each trial, 14 and 21 days after inoculation, to ensure that reaction type for each wheat line was consistent between the

Figure 3. Misting chamber containing the differential set of six wheat cultivars inoculated with four isolates of *Mycosphaerella graminicola*.



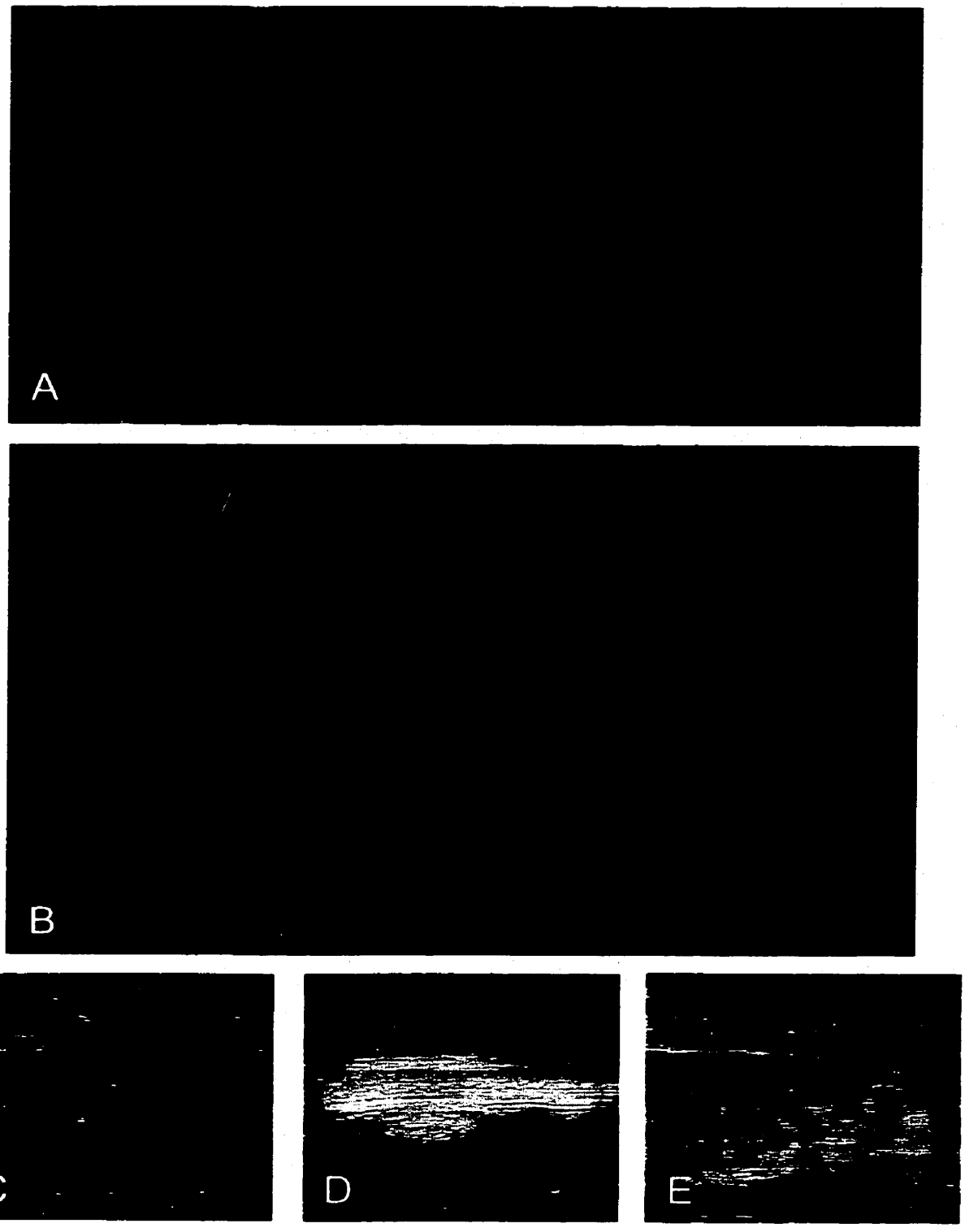
two rating dates. A qualitative method of disease scoring was adapted from Rosielle (1972). Disease ratings as described by Rosielle (1972) are as follows: 0 = (Imm) immune with no pycnidial formation, no visible symptoms or occasional hypersensitive fleck; 1 = (HR) highly resistant with no, or only an occasional isolated pycnidium formed, particularly in older leaf tissue, hypersensitive flecking in younger leaf tissue; 2 = (R) resistant with very light pycnidial formation, some lesions coalesced, mainly towards the leaf tip and in older leaf tissue; 3 = (I) intermediate characterized by light pycnidial formation, coalescence of lesions normally evident towards the leaf tips and elsewhere on the leaf blade; 4 = (S) susceptible with moderate pycnidial formation, lesions much coalesced; 5 = (VS) very susceptible characterized by large, abundant pycnidia, lesions extensively coalesced. An X following the reaction type was also used for those reactions which showed extensive leaf necrosis but light pycnidial formation (Rosielle, 1972). Only the second and third leaves were rated to ensure that premature senescence of the primary leave did not interfere with accurate symptom evaluation in the present study. These leaves were the two oldest leaves at the time of seedling inoculation.

4.0.3 Results

Two pathogen races could be distinguished among the 74 isolates of *M. graminocola* tested, as was determine by lesion type (Table 1; Fig. 4A and B). No isolate was found to be either virulent or avirulent on all wheat lines in the host differential set. Salamouni (*T. aestivum*) and Coulter (*T. durum*) were resistant to all isolates tested and each cultivar exhibited the same reaction to all isolates. Salamouni developed an immune to near-immune reaction that was characterized by the development of very small chlorotic and sometimes necrotic flecks (Fig. 4A, B and C). The small chlorotic flecks became visible four to five days post-inoculation and did not enlarge with increasing post-inoculation period. The flecking response of Salamouni was occasionally entirely absent depending on the isolate used. This hexaploid cultivar did not develop any visible pycnidia to any of the isolates tested when observed 21 days after inoculation. The tetraploid line, Coulter, exhibited a highly resistant response, as larger chlorotic flecks (1–2 mm) were visible on the inoculated leaf as early as three to four days post-inoculation. As the post-inoculation period increased, the early chlorotic fleck response developed into a necrotic fleck, without pycnidial formation at any post-inoculation period (Fig. 4A, B and D).

Conversely, the additional tetraploid line used in the differential set, 4B 1149, was susceptible to all isolates tested and produced abundant pycnidia and extensive necrosis, developing two to three weeks following inoculation (Fig. 4A and B). The bread wheat lines, Erik (Fig. 4A, B and E) and Amazon (Fig. 4A and B), were also susceptible to all isolates tested. The susceptible reaction of Erik, Amazon, ST 6 and 4B1149, varied in intensity depending on the isolate used.

Figure 4. A) Adult plant host differential set infected with isolate 96-W of *Mycosphaerella graminicola*. Wheat cultivars from L-R: ST 6, Amazon, Salamouni, Erik, Coulter, and 4B1149. B) The host differential set at the seedling stage inoculated with isolate 96-36 of *Mycosphaerella graminicola*. Wheat cultivars from L-R; ST 6, Amazon, Salamouni, Erik, Coulter, and 4B1149. C) Hypersensitive flecking present on the cultivar Salamouni at the seedling stage, following inoculation with the isolate 96-36. Cirrhi of pycnidiospores are being produced from pycnidia. D) Hypersensitive flecking observed on the resistant line, Coulter, after inoculation with the isolate 96-36 at the seedling stage. E) The cultivar Erik inoculated at the seedling stage with isolate 96-36.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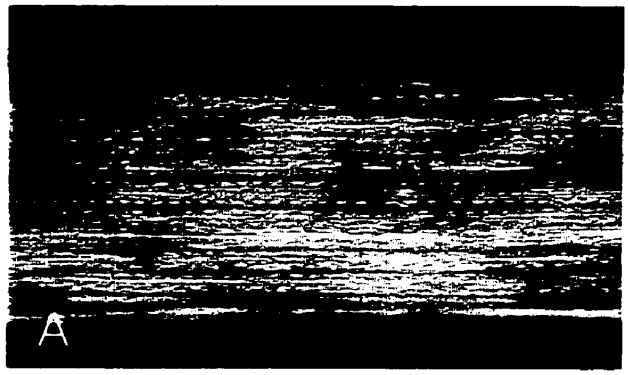
Line ST 6 was the only line that exhibited a differential reaction to the isolates used in this study. This line was susceptible to pathogen isolates from race I and resistant to those from race II. The susceptibility of this cultivar, to isolates from race I, was expressed as extensive necrosis and large dark pycnidia, which formed after two to three weeks of incubation (Fig. 4B). Chlorotic blotches characterized the resistance of ST 6, to isolates of race II, occurring at two and often three weeks post-inoculation. Patches of green tissue surrounded by chlorosis and small necrotic patches occasionally harboring a few pycnidia (Fig. 4A, 5A, 5B and 5C), were seen in ST 6 in response to infection by isolates from race II. Symptom development occurred slowly, compared to the other resistant cultivars, as no indication of a resistance response was seen until two to three weeks post-inoculation. Necrotic patches sometimes developed in the location of the chlorotic blotches and occasionally there were small patches of both necrosis and pycnidia on some of the leaf tissue three weeks post-inoculation. However, the lesions did not coalesce and there was no evidence of hypersensitive flecking.

The majority of isolates (59 out of 74) tested exhibited the virulence pattern of race II (Table 1). All 13 isolates from the 1999-cropping season were of race II. During 1996, 1997 and 1998 both races were found and in most cases only a single isolate was tested from each field sampled. Isolates collected from the same field all exhibited the same virulence patterns. Although isolates selected from the same leaf exhibited the same virulent patterns, some isolates were occasionally less aggressive on the susceptible cultivars when compared to the susceptible reaction caused by 96-36, the control isolate (Appendix 3).

Figure 5. The resistant reaction of ST 6 to various *Mycosphaerella graminicola* isolates collected from Manitoba and Saskatchewan. A) The differential line ST 6 inoculated with the *Mycosphaerella graminicola* isolate 96-W. Macroscopically chlorosis was the only evidence of any host tissue disruption. B) ST 6 inoculated with the isolate 98MG37-6. Note the chlorotic rings of tissue surrounding patches of green host tissue. C) Large chlorotic rings on the cultivar ST 6 inoculated with isolate SC37-4.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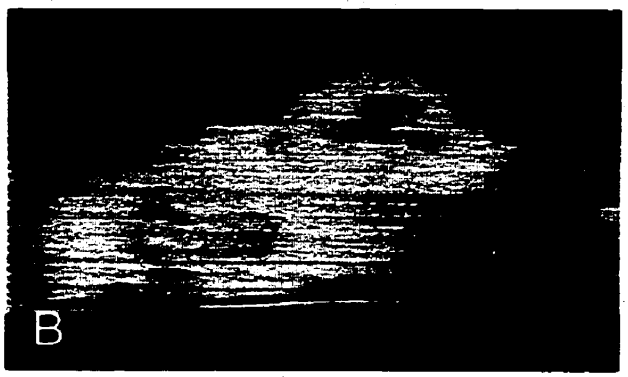
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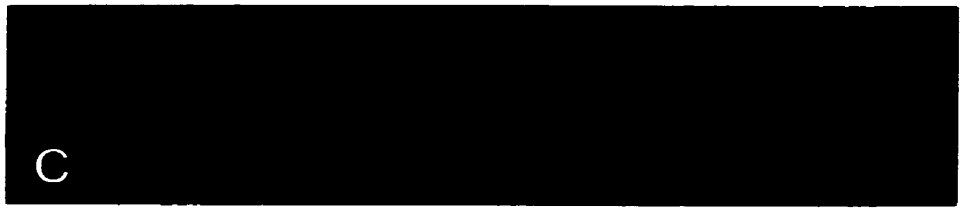

C

Table. 1 Evaluation of 74 isolates of *Mycosphaerella graminicola* for virulence on six wheat differential lines/cultivars.

Pathogen Race	Wheat cultivars						
	Erik	Amazon	ST 6	Salamouni	4B 1149†	Coulter†	Total No.of Isolates
I	4 - 5	4 - 5	5	0	4 - 5	1	15
II	4 - 5	4 - 5	2	0	4 - 5	1	59
							74

†Indicates durum wheat genotypes
0 = Immune; 1 = Highly resistant; 2 = Resistant; 3 = Intermediate; 4 = Susceptible and 5 = Very susceptible

Adult plant inoculations, with one isolate from each of the races identified in this study, indicated that symptom development at maturity corresponded with that seen at the seedling stage (Fig. 4 A and B). Parallel, necrotic lesions containing pycnidia were formed on flag leaves and the penultimate leaf in susceptible lines (Fig. 4A). Time from inoculation to symptom development increased, as rating was conducted 25 days post-inoculation. No symptom development was evident on any cultivar, apart from chlorotic flecking on the resistant cultivars, 14 days after inoculation in contrast to seedling inoculations. Lesion formation on leaves of adult plants was also much more distinct as parallel lesions containing dark pycnidia were formed in the susceptible differential lines (Fig. 4A). Lesions produced on seedlings coalesced much more frequently than those of adult plants (Fig. 4B).

4.0.4 Discussion

A clear understanding of host-pathogen interactions within a pathosystem is required to effectively identify and incorporate resistance into agronomically desirable cultivars. Host resistance to *M. graminicola* isolates from Manitoba and Saskatchewan was identified in both *T. aestivum* and *T. durum* cultivars. Two races of *M. graminicola* were identified in the pathogen population of the Canadian prairie region based on the differential response of a single wheat line (ST6) in the differential set used in the present study. ST 6 was susceptible to isolates of race I and resistant to those from race II.

The existence of physiologic races in *M. graminicola* is still, to a certain extent, a matter of controversy. Evidence for the existence of races has been reported in some studies (Eyal et al., 1973; Eyal & Levi 1987; Eyal et al., 1985; Kema et al. 1996a; Kema et al. 2000) but not in others (Nelson and Marshall 1990; Van Ginkel & Scharen 1988). The quantitative methods used in assessing host response may have played a confounding role in the identification of true differential interactions between isolates of the pathogen and host lines (Kema et al 1996d). To circumvent the complexities inherent to quantitative evaluation techniques, we used a set of differential lines that provided us with clear resistant (Salamouni, Coulter) and susceptible (Erik, 4B1149, Amazon) reactions, amenable to evaluation on a purely qualitative basis. The advantage of using a qualitative rating scale, such as the one described by Rosielle (1972), is self-evident when attempting to identify differential responses of host genotypes to individual isolates of *M. graminicola*.

In addition to the differential response of line ST6, which separated the isolates into two distinct races, quantitative variation between isolates was also observed.

However, this variation was based on the amount of symptoms caused on susceptible genotypes and was equated with aggressiveness. Qualitative and quantitative variation, as revealed in this and other studies, need not be mutually exclusive. Differences in aggressiveness between isolates are expected to occur in any pathogen population and can easily be measured on a susceptible host genotype. By contrast, qualitative variation in virulence requires the use of effective differential lines, capable of expressing resistance and susceptibility. A situation similar to that in the wheat-*M. graminicola* system existed in tan spot of wheat, until appropriate host differential lines and evaluation techniques were used to identify races. This was the beginning of an understanding as to the basis of specificity in the wheat-*Pyrenophora tritici-repentis* system (Lamari & Gilbert 1998).

These immune and highly resistant reactions of Salamouni and Coulter, involving a hypersensitive-like response, appear to be unique to the present study. Others assessing host-pathogen interactions within the wheat-*M. graminicola* pathosystem reported that the response of resistant genotypes was often characterized by limited necrosis (1-5 %) and pycnidial development (Eyal et al., 1973; Kema et al, 1996a; Saadaoui, 1987). The hypersensitive-like responses in the resistant lines observed in this study made it possible to differentiate between pathogen races based solely on lesion type. Previous studies investigating the pathogen have often lacked evidence of any complete resistance (Van Ginkel and Scharen, 1988) leading to the use of statistical procedures to distinguish between pathotypes, on a quantitative basis. Eyal and co-workers (1973) evaluated host response to *M. graminicola* by measuring pycnidial densities, which inherently leads to a quantitative method of disease assessment. Saadaoui (1987) determined virulence

patterns of 19 *M. graminicola* isolates from Morocco based on percent infection. This method incorporated both disease severity and pycnidial density, but did not consider lesion type. Likewise, Kema and co-workers (1996a) measured disease severity based on percent necrosis and percent pycnidia. Kema et al. (1996b) suggested that the numerous methodologies used to assess host reaction to *M. graminicola* may be the reason for the lack of consensus regarding physiological specialization within the pathosystem.

The use of qualitative disease assessment scales was critical in the study of other pathosystems, such as tomato-*Cladosporium fulvum* (Van den Ackerveken et al., 1992), wheat-*Puccinia graminis* (Roelfs, 1998) and wheat-*Pyrenophora tritici-repentis* (Lamari & Gilbert 1998) as it led to the distinction of pathotypes and/or races in the pathogen populations. Pathogen races were discovered in the wheat-*P. tritici-repentis* system when reactions were evaluated according to lesion types (Lamari and Bernier, 1989a). Prior to such assessment, isolates were found to quantitatively differ in virulence, lesion size and the % infected leaf area (da Luz and Hosford, 1980; Krupinsky 1992).

The limited number of races found among the isolates tested in the present study suggests that variability within the pathogen population of Canada may not be as diverse as was found in other wheat producing countries. In a number of areas, the presence of the teleomorphic state was shown to produce ascospores which initially infect crops, later followed by conidial infections (Garcia and Marshall, 1992; Hunter et al., 1999; Scott et al., 1988; Shaw and Royle, 1989). The hypothesis that genetically diverse founding populations could result from ascospore infections and subsequently localized colonal populations could result from asexual reproduction, was supported by the considerable genetic variation found in localized population as determined by the use of restriction

fragment length polymorphism (RFLP) markers (McDonald and Martinez 1990b). Recently, the pathogen was shown to be capable of ascospore infection throughout the growing season (Hunter et al., 1999; Zhan et al., 1998) and to be capable of completing several sexual cycles per year (Kema et al., 1996d). This could possibly lead to extensive genetic variation and the development of new founder populations of the pathogen throughout the cropping season (Kema et al., 1996d). When measuring immigration and sexual reproduction in field experiments, Zhan et al. (1998) concluded that the majority of isolates originated through asexual reproduction. However, 10% of isolates were found to be immigrants and 24% were due to sexual recombination, as determine by RFLPs and DNA fingerprinting, suggesting that migration and sexual recombination do play a critical role in the genetic variation associated with certain pathogen populations.

The occurrence of the teleomorphic state of the pathogen has not been conclusively demonstrated in the Canadian prairies as in other countries (Gilbert et al., 1998). The lack of evidence of the teleomorphic state may simply be due to the limited number of studies concerned with the identification of this state, as Septoria leaf blotch is a disease of very recent history in our region. The limited amount of variation in virulence could also result from limited sexual recombination. Unlike the situation in temperate regions (Hunter et al., 1999; Kema et al., 1996d; Shaw and Royle, 1989), it is not likely that the fungus undergoes multiple sexual reproduction events within a single season under western Canadian conditions. The prolonged (up to six months) winter sub-zero temperatures and the short spring and summer seasons (< 100 days) would likely be major limiting factors for the pathogen. In more temperate locations such as the United Kingdom, pseudothecia development predominantly occurred during December and

January and pseudothecia were depleted by February (Hunter et al., 1999). As well, ascospore infection of winter wheat crops was also show to occur primarily in autumn (Shaw and Royle, 1989). By analogy, *P. tritici-repentis,* which shares the same ecological niche as *M. graminicola*, forms sexual structures on two year-old stubble under western Canadian conditions as opposed to a single-year cycle in eastern Canada (Sutton and Vyn, 1990) and elsewhere (Summerell and Burgess, 1988). Therefore, the absence or the limited occurrence of the sexual state may have had a stabilizing effect on the pathogen population examined in the current investigations thus, the small number of pathogenic races found in the area. An appropriate study determining the genetic variability and sexual recombination would provide insight into the diversity of the *M. graminicola* population in the prairies.

Another possible hypothesis for the small number of pathogenic races found in the current study, may be due to the recent prevalence of the pathogen on the Canadian prairies (Gilbert et al., 1995). Commercial cultivars currently grown in western Canada are all susceptible to the pathogen, consequently imposing little or no selection pressure on the pathogen to produce new virulent races. The resistance of Salamouni to both races identified in this study and of St 6 to race I is not of any significance to the evolution of virulence in the pathogen in western Canada, as these lines were never grown in the region.

The effectiveness of the differential host set may also be an obstacle when assessing variation in the *M. graminicola* population. The pathogen has been suggested to follow the gene-for-gene model (Eyal et al., 1985; Kema et al., 1996a and Kema et al., 2000). Therefore, races of the pathogen, which may be present locally, could go

undetected due to a lack of host resistance genes to match avirulence genes in the pathogen population.

In this study, host cultivar-specialization, rather than host species-specialization, seems to characterize the interaction between wheat and *M. graminicola* contrary to what was previously suggested (Kema et al., 1996a; Van Ginkel and Scharen, 1988). All bread wheat isolates tested in the present study exhibited, equally, virulence and avirulence to both hexaploid and tetraploid wheat lines/cultivars. The isolates of the pathogen from western Canada appear to be similar to those from eastern Algeria, which are believed to be virulent on both hexaploid and tetraploid wheats, whereas those from neighboring Tunisia appear to be virulent on durum wheats only (Sayoud, personal communication). However, no durum wheat isolates were used in this study, as there is only limited durum wheat production locally. It would be of benefit to challenge the resistance of the durum wheat cultivar, Coulter, with isolates from durum wheats grown in western Canada to determine if this cultivar would succumb to the disease. Preliminary screening of durum wheat isolates from Tunisia showed that Coulter is susceptible to some isolates (Lamari, unpublished).

In this study, adult plant reactions were in agreement with those observed at the seedling stage. Others have also found a good correlation between adult and seedling plant reactions (Eyal et al., 1973) and disease severity was shown not to be affected by the growth stage of the host (Wainshilbaum and Lipps, 1991). Conversely, a greater susceptibility was generally seen in adult plants when compared to seedlings, after being inoculated under field conditions (Kema and van Silfhout, 1997). Correlations between adult and seedling plant reactions were significant, for particular isolates only (Kema and

van Silfhout, 1997). Likewise, Brokenshire (1976) determined that certain cultivars resistant at the seedling stage exhibited susceptibility as adult plants, confirming the importance of adult plant evaluation.

Symptom development on adult plants was delayed in comparison with seedling inoculations. Environmental conditions may have been of consequence, as these plants were incubated under greenhouse conditions rather than in the growth chamber. However, in a comparative study of seedling and adult reactions, Kema and van Silfhout (1997) also observed an increase in the number of days to symptom development when adult plants were evaluated. The extended latent period may be partly due to plant growth stage, as differences in latent period are known to occur between the flag leaf and the lower leaves (Shaw, 1990). Latent period was also seen to increase with high average temperatures (Shaw, 1990), which may have occurred in the greenhouse setting, where the temperature cannot be accurately controlled.

Obvious differences in virulence patterns strongly suggest that physiological specialization does occur in *M. graminicola*. The results of this study also suggested that races of the pathogen do exist in the pathogen population of the Canadian prairies. An improvement of the host differential set or testing *M. graminicola* isolates from populations collected elsewhere may provide better insight into the nature of host-specific interactions in the wheat-*M. graminicola* pathosystem.

4.1 Cytological studies of host-pathogen interactions in the wheat-*Mycosphaerella graminicola* pathosystem

4.1.0 Abstract

Mycosphaerella graminicola (anamorph *Septoria tritici*) the causal fungus of Septoria leaf blotch of wheat, induces chlorosis, necrosis and pycnidia in susceptible hosts. A resistant reaction is characterized by immune, chlorotic/necrotic flecking or minimal symptom production on the host. Four wheat lines were inoculated with a single spore culture of the pathogen collected from Manitoba and designated as race I from a previous study. Two durum varieties, 4B1149 and Coulter, and two bread wheat varieties, Erik and Salamouni, were chosen based on the susceptibility and resistance within each species, respectively. An aniline blue clearing-staining technique and a fluorescence technique were used to study the infection and colonization processes and the host cell response to the pathogen. Stomatal penetration was similar in both susceptible and resistant lines. However, host tissue colonization differed. Mesophyll cell disruption was evident in the resistant lines 3 to 4 days post-inoculation and host tissue colonization remained limited. Intercellular hyphal growth of the fungus could be seen in the undisrupted mesophyll tissue of the susceptible wheat lines 8 days post-inoculation and by 10 days pycnidial initials were evident in the substomal chamber of infected stomates. Host cell disruption was seen in the mesophyll tissue beginning 8 days after inoculation, at which time chlorosis and necrosis were macroscopically visible in the susceptible reaction. Subsequently, sporulating pycnidia were evident in the compatible reaction 12 to 14 days post-inoculation. No pycnidia or pycnidial initials were seen in the resistant line, Salamouni. However, Coulter harbored very low levels of pycnidial initials 21 days post-inoculation. Mesophyll cell collapse and densely staining

mesophyll cells directly surrounding the substomatal chamber of infected stomates were typical of the resistance response, suggesting that a hypersensitive-like reaction occurred. There was no evidence of mesophyll cell penetration in either the compatible or incompatible response suggesting that the resistance mechanism is possibly active in the apoplast of the mesophyll tissue.

4.1.1 Introduction

The pathogen, *Mycosphaerella graminicola* (Fückel) Schröeter (anamorph: *Septoria tritici* Rob.ex Desm.), the causal agent of Septoria leaf blotch inflicts wheat crops worldwide (Eyal, 1981; Leath et al., 1993). The disease, which can severely decrease the grain filling capacity of the plant due to leaf tissue damage (Cornish et al., 1990), has been the focus of study by many wheat breeders and pathologists. Resistance to the disease occurs in both *Triticum aestivum* and *T. durum* (Eyal et al., 1973; Rosielle, 1972).

The durability of presently resistant wheat cultivars has been questioned due to previous studies examining plant-pathogen interactions (Ahmed et al., 1996; Van Ginkel and Scharen, 1998). Wheat cultivars shown to be resistant in some countries were found to be susceptible when grown in Israel (Eyal et al., 1973). Subsequent studies on physiological specialization within the pathosystem indicated the existence of pathogenic races (Eyal et al., 1973; Saadaoui, 1987). Eyal and colleagues (1973) stated that bread wheat varieties, once mildly afflicted by the disease, became increasingly diseased as the acreage of bread wheat increased, replacing durum varieties. Selection for an increase in both virulence and aggressiveness was found to occur in the pathogen population of Oregon, again threatening the durability of resistant cultivars (Ahmed et al., 1996). The presence of the teleomorphic state of the pathogen, found in many wheat producing countries (Brown, et al., 1978; Garcia and Marshall, 1992; Hunter et al., 1999; Scott et. al., 1988), further complicates the situation by providing a rapidly changing pathogen population (Mcdonald et al., 1995) in a relatively static population of wheat genotypes.

Understanding the genetic basis of this resistance and incorporating resistance genes into agronomically viable cultivars is crucial for disease management practices. Knowledge of the physiological and molecular basis of resistance may provide even greater insight for developing more effective control of the pathogen. Histological investigations of the pathosystem have not confirmed possible resistance mechanisms and only a few studies have examined the interaction between host and pathogen at the cytological level. Cohen and Eyal (1993) suggested that the resistance response be due to the arrestment of immature pycnidia in host tissue. Although traces of callose and lignin were found in the host tissue, these two compounds could not be confirmed as the cause of host resistance. Kema et al. (1996c) found no evidence of lignification or polyphenolic compounds related to cell wall strengthening, compartmentalization or a hypersensitive response when the same wheat lines were studied.

Symptoms of chlorosis, necrosis and production of pycnidia characterize the reaction of susceptible wheat cultivars. Resistant reactions have been associated with minimal pycnidial production and were frequently based on quantitative measurements since complete resistance was not often observed (Van Ginkel and Scharen, 1988). A qualitative assessment of symptom production, however, provides an unambiguous distinction between classes. Rosielle (1972) classified an immune reaction as the absence of pycnidial production with no visible symptoms other than minimal hypersensitive flecking. A highly resistant response was described as infrequent pycnidial formation in older leaf tissue and hypersensitive flecking in younger leaves, and a resistance response classified by very light pycnidial formation and some coalescence of lesions in older leaf tissue. Differential host lines screened with isolates from Manitoba indicated that races

of the pathogen existed, and that resistant cultivars could be clearly distinguished using a similar qualitative method of symptom assessment (Grieger et al., unpublished).

The primary objective of this study was to microscopically examine the unique resistance response of the wheat cultivars, Salamouni (*T. aestivum*) and Coulter (*T. durum*), which exhibit an immune and a near-immune to highly resistant response, respectively. The extent of host response and tissue colonization was investigated in resistant and susceptible hexaploid and tetraploid wheats.

4.1.2 Methods and Materials

Host Material and Inoculation

Two susceptible wheat lines Erik (*T. aestivum*) and 4B1149 (*T. durum*), and two resistant lines Salamouni (*T. aestivum*) and Coulter (*T. durum*) were selected for this study. To ensure even germination, seeds were pre-germinated on moist filter paper in petri dishes and place in the dark for 48 hours at 4 °C, followed by 48 hours at 22 °C. Eight pre-germinated seeds of a single line were sown in 10-cm-diameter clay pots containing a mixture of soil: sand: peat (2:1:1 v/v), amended with 11-53-00 granular fertilizer.

The wheat lines were inoculated with a single conidial isolate (96MG36) collected from southern Manitoba. Inoculum was harvested from five day old cultures grown on Yeast Malt Agar (Appendix 1) under continuous light at 24 °C. The spore suspension was adjusted to 1 x 10^7 pycnidiospores/ml and amended with 2 drops of Tween 20 (polyoxyethylene-sorbitan monolaurate /Sigma)/100ml. A control of water + Tween 20 was used.

Seedlings at the 2.5 to 3- leaf stage were inoculated with the pycnidiospores suspension using a spray mist atomizer. The plants sprayed until runoff and allowed to dry for 10 minutes, proceeding an incubation period of 72 hours in a misting chamber. Pots were then moved to a growth room bench for 18 days at 22/18 °C (day/night) and a 16-hour photoperiod under fluorescent light at 181 $\mu E.m^{-2}.s^{-1}$. Plants were then rated 14 and 21 days after inoculation. Relative humidity following inoculation fluctuated between 75-90%. Post-inoculation conditions, excluding relative humidity, were similar in both the misting chamber and the growth room.

Sampling and Staining Procedure

Infected leaf tissue samples were taken from all four wheat lines at 6, 12, 24, and 48 hours after inoculation as well as 4, 6, 8, 10, 12, 14, and 16 days after inoculation. Tissue from the uninoculated control plants was sampled only at 6 and 48 hours and at 8 and 16 days after inoculation. Five leaves were sampled from each line at each post-inoculation date. Through out this study, the second leaf of each plant was sampled for observation.

The first cytological study was conducted using a clearing/staining technique described by Bruzzese and Hasan (1983). This technique, which combines a one-step staining and clearing for whole mounts, was used to study host-specificity of rust fungi. Lamari and Bernier (1989a) adapted this procedure to observe the infection process of *Pyrenophora tritici-repentis* in wheat. Clearing and staining was achieved by immersing 1-1.5 cm pieces of fresh leaf tissue in the clearing/staining solution consisting of 95% ethanol (300 ml), chloroform (150 ml), lactophenol (660 ml) (Note: 660 ml of lactophenol was substituted in place of lactic acid and phenol), chloral hydrate (450 g) and aniline blue (0.6 g) (CI #42755 Fisher Scientific) (Appendix 2) for a minimum of 72-96 hours. The destaining procedure as stated by Bruzzese and Hasan (1983) and Lamari and Bernier (1989a) was omitted due to rapid destaining of the fungal tissue in addition to the leaf tissue. Leaf tissue was rinsed or stored in lactophenol until mounted in 0.01% aniline blue in lactophenol and observed with the Zeiss compound microscope.

Fluorescence Microscopy

The aniline blue/lactophenol clearing/staining technique was not always effective in staining the fungal hyphae within the wheat host tissue. To circumvent this problem, we used the KOH-aniline blue fluorescence technique developed by Hood and Shew (1996). This method produced a high degree of contrast between the host tissue and the fungal tissue for fungi of the Deuteromycotina, Ascomycotina, Basidiomycotina and the Mastigomycotina (Hood and Shew, 1996). Plants were inoculated and incubated as previously stated and the same hexaploid and tetraploid wheat lines were sampled at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14 and 21 days after inoculation. Uninoculated control plants were sampled only at 1, 3, 4, 6, 8, 14 and 21 days. Five secondary leaves from each wheat line were sampled at each post-inoculation period. Segments of fresh leaf tissue were placed in 50 ml of 1 M KOH and allowed to stand at room temperature for 24 hours before autoclaving at 121°C for 15 min. Specimens were subsequently rinsed three times in deionized water and mounted in a staining solution consisting of 0.05% aniline blue (CI #42755 Fisher Scientific) in 0.067M K_2HPO_4 at pH 9.0 (Fig. 6). Whole mounts were examined with a Zeiss microscope fitted with an ultraviolet excitation filter (G365) used in combination with a dichromatic beam splitter (FT395) and barrier filter (LP420) transmitting above 420nm.

Figure 6. Flasks containing KOH and diseased leaf tissue infected with *Mycosphaerella graminicola*. The flask on the left contains a clear solution and has not been autoclaved, the other has been autoclaved (right) to clear the leaf tissue. Whole mounts of diseased leaf tissue were then mounted in a solution of aniline blue and 0.067M K_2HPO_4.

4.1.3 Results

Host Penetration

Germ tubes could be seen extending from the terminal ends of the 2 to 7 celled pycnidiospores on the plant surface 6 hours post-inoculation (Fig. 7A), irrespective of the wheat line used. Growth and elongation of the germ tubes continued until 24 - 48 hours post-inoculation, at which time the fungal hyphae could be seen penetrating through the stomatal opening and into the substomatal chamber, in both the resistant and susceptible lines. Multiple penetrations were often seen to occur through the same stomatal aperture (Fig. 7B). Penetration appeared to occur randomly as hyphae often grew in various directions and across the stomatal aperture without penetrating. The post-inoculation time of penetration also seemed to vary on the same wheat lines due the random and indirect mode of penetration through the stomatal opening. However, penetration generally occurred within 1 to 2 days after inoculation in both the susceptible and resistant lines/cultivars.

Appressorium-like structures were frequently seen to form over the stomatal aperture and occasionally at the junction of epidermal cells (Fig. 7C). These structures were not associated with penetration, as penetration was not observed in the presence of these appressorium-like structures at the junction of the epidermal cells. As well, penetration over the stomatal aperture often occurred without the formation of these structures. Hyphal branching was seen to occur, as this appressoria-like structure entered the stomatal aperture to begin colonization of the intercellular mesophyll tissue (Fig. 7D).

Host Colonization

The staining/clearing technique for conventional light microscopy was sufficient to visualize fungal penetration of the host tissue but was limited when determining host colonization. The cytological structure and damage sustained by the host tissue, such as mesophyll cell disruption and cell collapse, were well visualized using this technique, as densely stained cells were often apparent once damage had occurred in the resistant lines. Fluorescence microscopy provided a clear visualization of the fungal tissue and the colonization process associated with host tissue. The fungal hyphae were well detected both on the leaf surface and within the wheat host tissue. Although host tissue did fluoresce slightly in both inoculated and control treatments, this often aided in the interpretation of the infection and colonization processes of the fungi, which fluoresced much more brightly than the host tissue.

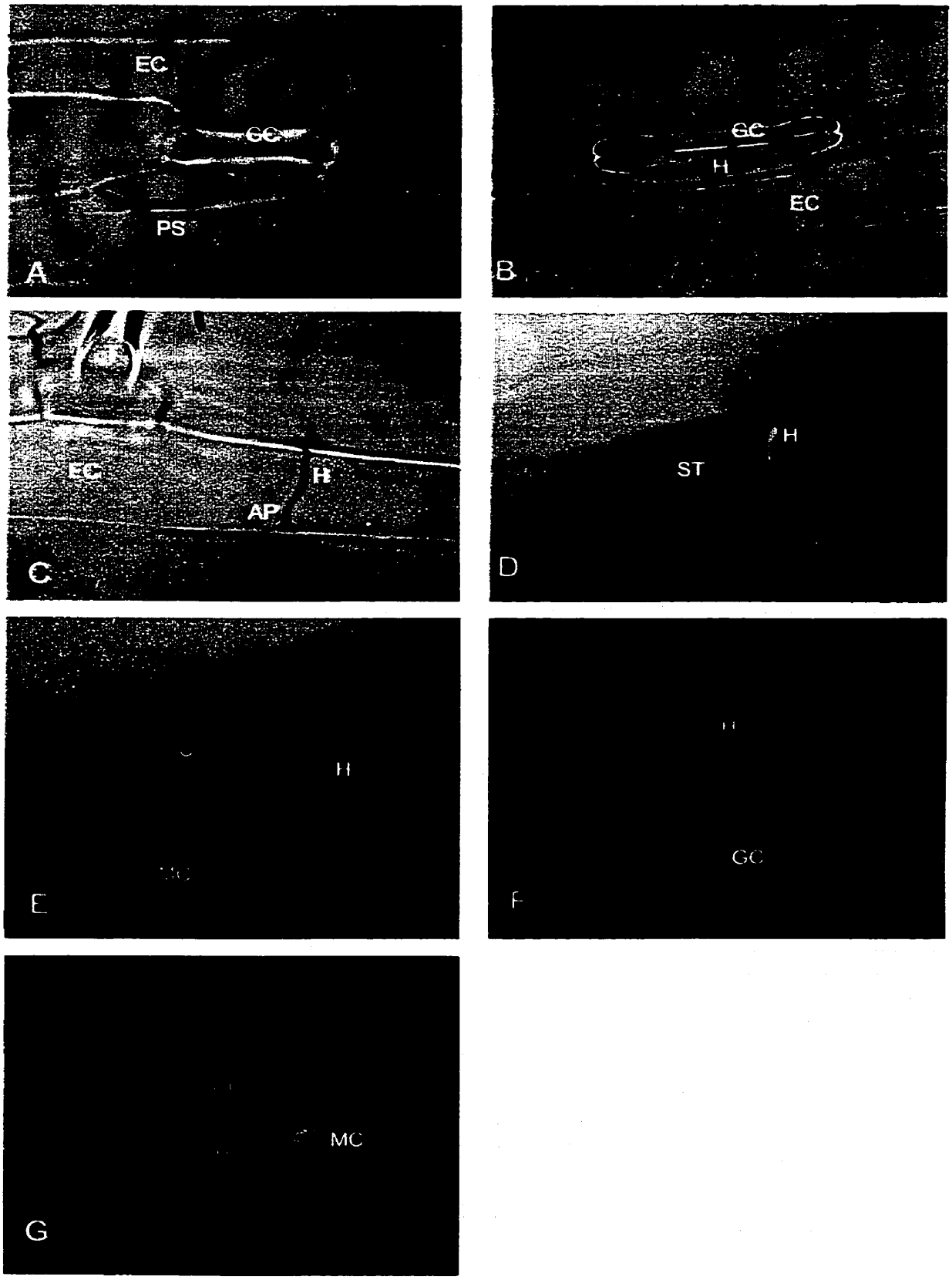
Initial colonization of the host tissue appeared to be similar in both the resistant and susceptible wheat lines 1 to 2 days post-inoculation. Following stomatal penetration on both abaxial and adaxial leaf surfaces, hyphae grew through the stomatal chamber and approached the mesophyll cells closest to the infection site within the first two days after inoculation. However, all mesophyll cells remained turgid and no cell disruption was seen to occur in any of the wheat lines (Fig. 7E).

Compatible Interactions

A differentiation in host colonization between the susceptible and the resistant lines was evident 3 days after inoculation. Colonization progressed in susceptible lines with no or little host tissue disruption (Fig. 7F). Erik and 4B1149, the susceptible lines,

Figure 7. Spore germination, host penetration and initial colonization processes of *Mycosphaerella graminicola*. A) Germinating pycnidiospore seen 6 hours post-inoculation on the near-immune cultivar Salamouni. (x500). B) Multiple stomatal penetrations, 48 hours after inoculation on the susceptible durum cultivar, 4B1149. (x500). C) Appressorial-like structure seen at the junction of two epidermal cells visible on the susceptible cultivar Erik 14 days post inoculation, no penetration occurred at this site. (x 750). D) Differentiation of hyphae seen entering the stomate upon host penetration, visible on the resistant cultivar Salamouni 8 days after inoculation. (x500). E) Hyphae seen in the substomatal chamber of the resistant cultivar, Salamouni, 2 days after inoculation. (x500). F) Intercellular hyphal growth in the susceptible line, Erik, 3 days after inoculation. There are no disrupted or fluorescing mesophyll cells present. (x500). G) Penetrated stomate and hyphae leading to fluorescing mesophyll cells in the resistant cultivar, Coulter, 3 days post inoculation. (x500).

Abbreviations: AP = appressorial-like structure, EC = epidermal cell, GC = guard cell, H = hyphae, MC = mesophyll cell, PS = pycnidiospore, ST = stomate, T = trichome


EC
GC
PS
A
GC
H
EC
B
EC
H
AP
C
H
ST
D
H
E
GC
F
MC
G

harbored low levels of fungal colonization, as hyphal growth extended intercellularly throughout the mesophyll tissue from the substomatal chamber of infected stomates. The mesophyll cells appear to be turgid and undisrupted by the presence of fungal hyphae.

Intercellular hyphae in the susceptible line, Erik, extended from the penetration site to the substomatal chambers of satellite stomates above and below the initial infected stomate 4 days post-inoculation. Intercellular hyphal growth in 4B1149 was seen to extend 5-15 mesophyll cells in length at the same post-inoculation time. Host colonization continued intercellularly without visible damage or disruption to the host tissue, as mesophyll cells remained turgid until 8 to 10 days post-inoculation. Fluorescing mesophyll cells were not present in the susceptible lines at any time during colonization of the host tissue.

Incompatible Interactions

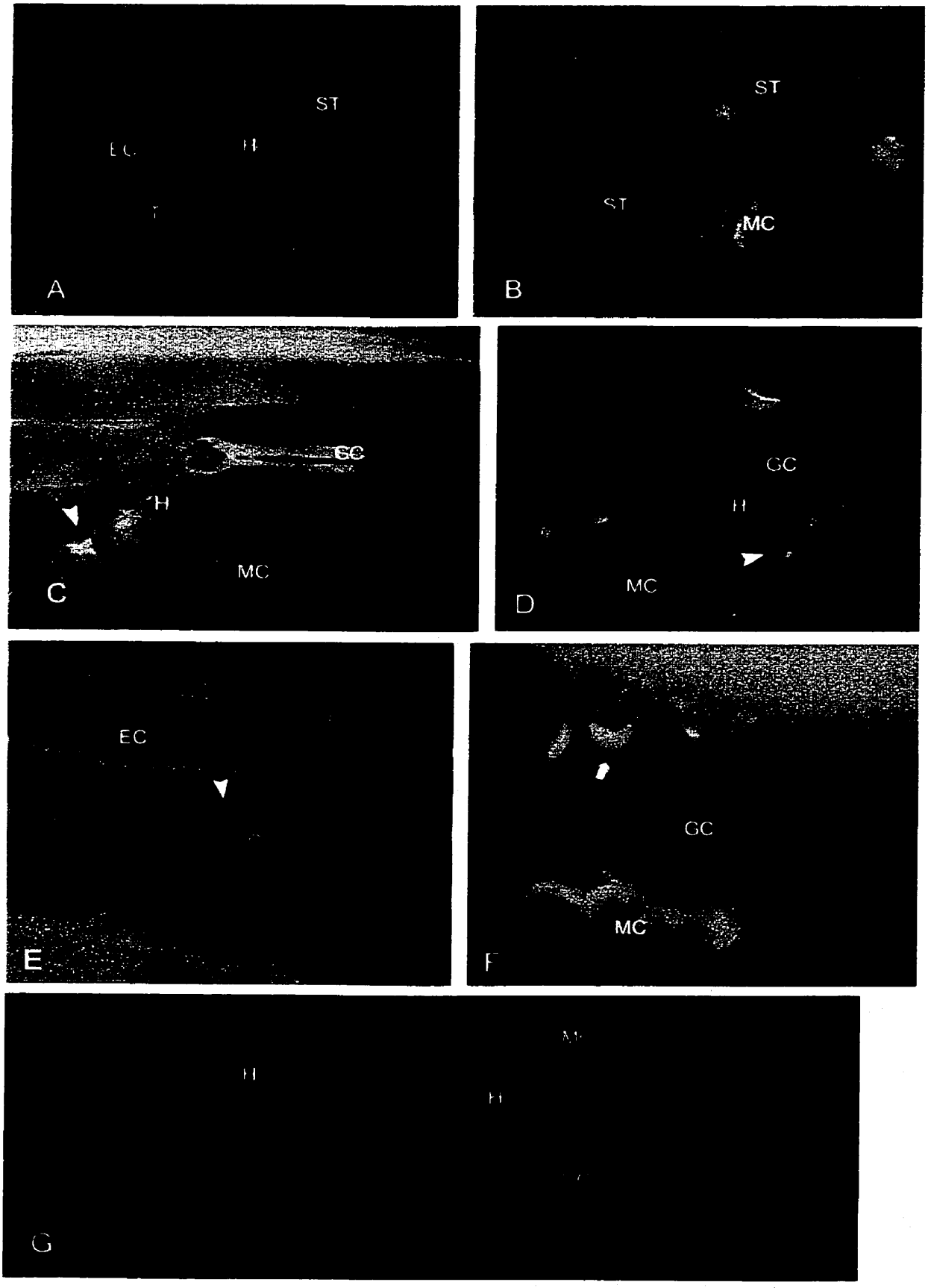
Visible disruption of the host tissue was evident in the resistant lines, Salamouni and Coulter, 3 days post-inoculation (Fig. 7G). In Salamouni, intercellular hyphal growth extended from the substomatal chamber of an infected stomate to mesophyll cells immediately surrounding the substomatal chamber. Brightly fluorescing mesophyll cells were evident at many, but not all of these infection sites and frequently intercellular hyphal growth extended 1 to 4 mesophyll cells into the host tissue (Fig. 8A and B). Macroscopically, a hypersensitive or near-immune reaction was visible, as chlorotic flecking was visible in Salamouni 4 to 5 days post-inoculation.

In Coulter, the resistant tetraploid line, intercellular hyphal growth was also seen 3 days after inoculation (Fig. 7G). At several infection sites, fungal hyphae reaching 3 to 4 mesophyll cells were present in the substomatal chamber and extended towards brightly fluorescing mesophyll cells. A single stomatal penetration could produce an area of fluorescing mesophyll cells extending to three stomates. Intercellular hyphal growth extending beyond these fluorescing areas was infrequently seen. At some infection sites, hyphal growth was seen extending through the intercellular spaces of the mesophyll tissue from one stomate to an adjacent stomate. This intercellular growth of hyphae occasionally occurred with no noticeable effect on the cellular integrity of the host tissue and no fluorescing mesophyll cells were evident. Coulter macroscopically exhibited chlorotic flecking as early as 3 to 4 days post-inoculation.

In both resistant lines, Salamouni and Coulter, fungal colonization of the host tissue remained restricted when compared to the susceptible lines, 4 to 10 days post-inoculation. Fluorescing mesophyll cells surrounding many infection sites remained visible and very few hyphae grew beyond these sites (Fig 8A, B, C, D, E and F). Most of the fluorescing mesophyll cells retained their turgidity. Although, collapsed cells closest to the substomatal chamber of infected stomates would sometimes fluoresce slightly. Periodically hyphae were seen to grow in the intercellular mesophyll tissue of the host and reach the substomatal chamber of a satellite stomate up to 5 stomates away. In this case, mesophyll cells remained undisrupted and no fluorescing mesophyll cells were seen (Fig.8G). Intercellular hyphal growth in the incompatible interaction was not as extensive as in the compatible interaction at any post-inoculation period.

Figure 8. Incompatible interaction of *Mycosphaerella graminicola* and the resistant wheat host cultivars, Salamouni and Coulter. A) Hyphal penetration through the stomatal aperture of Salamouni 4 days after inoculation. B) Fluorescing mesophyll cells surrounding the substomatal chamber of infected substomatal chamber. Note this is the same penetration site as figure 8B. C) Intercellular hyphae leading to fluorescing mesophyll cells 7 days after inoculation in the cultivar, Salamouni. D) Hyphae leading to fluorescing mesophyll cells in the resistant cultivar, Coulter, 10 days after inoculation. E) Penetration (arrow) in the resistant line, Salamouni, 10 days after inoculation. F) Focusing into the mesophyll tissue from the penetration fluorescing site in figure 8E, fluorescing mesophyll cells (arrow) are observed. Note the lack of any observable fungal tissue. G) Limited intercellular hyphae growth in Salamouni 7 days after inoculation. Note that no fluorescing mesophyll cells are evident in the region of hyphal growth. (x500 Fig. 8A-8G).

Abbreviations: EC = epidermal cell, GC = guard cell, H = hyphae, MC = mesophyll cell ST = stomate, ➢ = indicates fungal hyphae, ➡ = indicates mesophyll cell disruption


ST
EC
H
A
ST
ST
MC
B
SC
H
MC
C
GC
H
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D
EC
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GC
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F
H
H
G

Treatment with aniline blue confirmed that collapsed and densely stained mesophyll cells were found directly surrounding the substomatal chamber and in most instances a stomatal penetration was seen to occur directly above (Fig. 9A, B and C). Dark-brown or densely blue stained mesophyll cells, suggestive of major physiological change, were observed in infection sites of resistant cultivars. Mesophyll cells directly surrounding the substomatal chamber also fluoresced when observed after treatment with KOH. However, evidence of direct penetration of the mesophyll cells was not observed with either staining technique.

The hypersensitive flecking appeared to be larger when viewed with the conventional microscopy using the aniline blue staining technique. Fluorescence observations indicated that only mesophyll cells directly surrounding the infected substomatal chamber fluoresced. However, when stained with conventional aniline blue staining techniques, the hypersensitive lesions often appeared to affect not only the penetrated stomate but neighboring stomates as well (Fig. 9A and B).

The flecking observed in the host tissue of the incompatible response, as characterized by densely stained and collapsed mesophyll cells, was measured and the number of infected stomates per lesion was quantified at 14 days post-inoculation using aniline blue staining and regular light microscopy. Lesion length varied from 91.5 to 2058.7 μm and the average lesion length across four leaf sections ranged from 470.3 – 618.5 μm in Salamouni. Four whole leaf mounts of Coulter harbored necrotic flecks ranging in length from 100.7 to 1226.1 μm and the average lesion length per leaf section varied from 224.2 to 666.1 μm. Commonly 1 to 3 stomatal penetrations were seen over

Figure 9. Incompatible interaction of tetraploid and hexaploid wheat cultivars inoculated with the *Mycosphaerella graminicola* isolate 96-36. A) A lesion on the resistant line Coulter 8 days post-inoculation. (x100). B) The penetration site of the lesion seen in Figure 9a. (x500). C) Hypersensitive response of Salamouni 14 days after inoculation. (x500).

Abbreviations: MC = mesophyll cell, ST = stomate, ➢ = indicates fungal hyphae, ▬ = indicates mesophyll cell disruption.

A
B
ST
C
ST

most lesions. However, as many as 14 penetrations, through several adjacent stomates, were seen in the longest lesion measuring 2058.7 μm in the cultivar Salamouni.

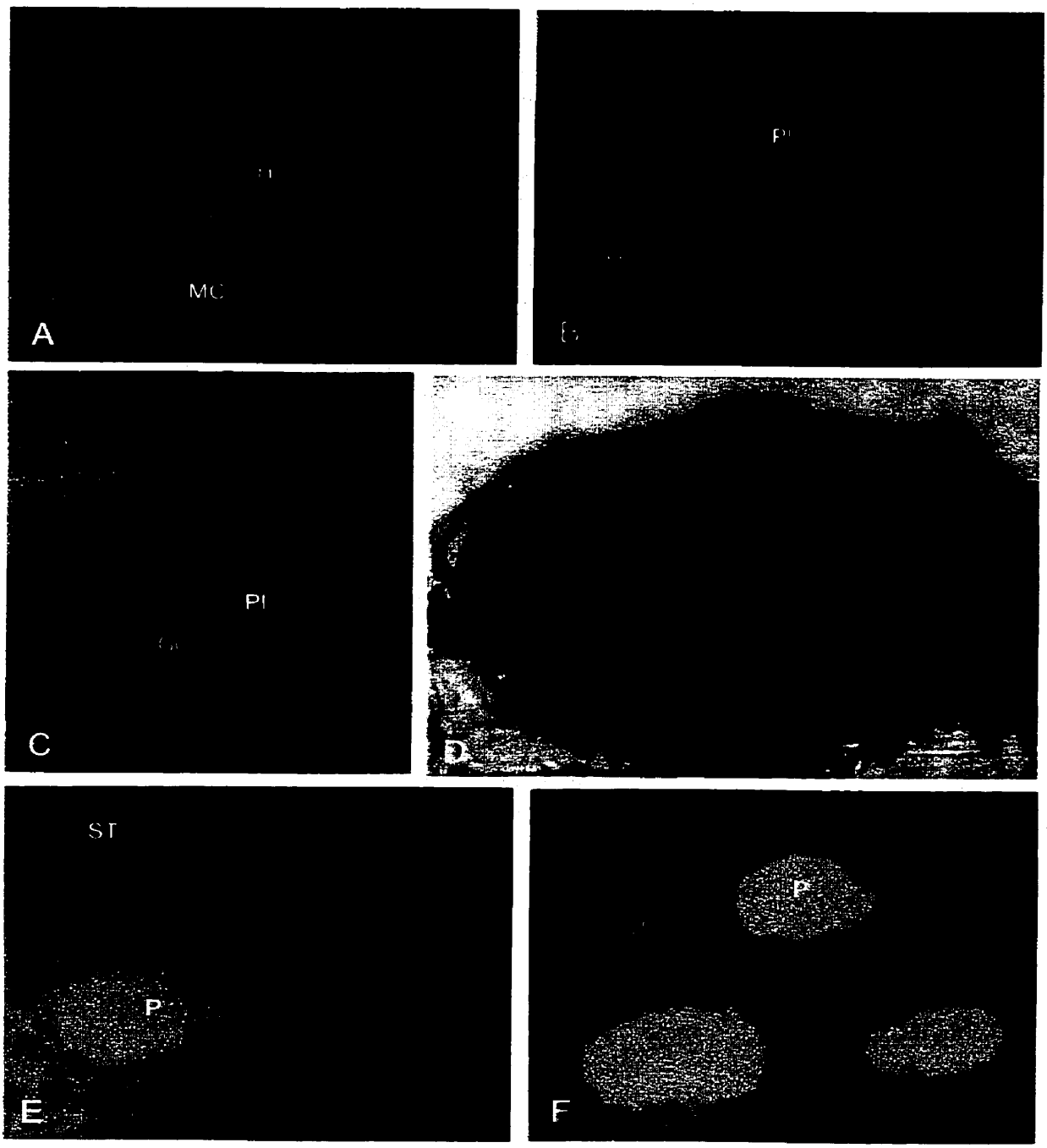
Pycnidial Formation

Intercellular hyphal growth was seen to line the interior of the substomatal chamber, 6 days post-inoculation, in the susceptible lines (Fig. 10A). Most mesophyll tissue contained intercellular hyphae at 7 days post-inoculation and by 8 to 10 days pycnidial initials began to form as hyphae aggregated in the substomatal chambers of stomates that were penetrated as well as those not penetrated (Fig. 10B and C). Mesophyll cell disruption in the susceptible lines was not evident until 8 to 10 days post-inoculation. At this time, cell disruption occurred randomly throughout the mesophyll tissue. The mesophyll cell walls did not stain densely or fluoresce, as in the resistant lines at the time of cell collapse. Mesophyll cell disruption coincided with the macroscopic appearance of lesions in the susceptible lines. Chlorotic mottling began to occur in the susceptible lines 8 days post-inoculation, and necrotic tissue and pycnidia became evident 10 to 12 days post-inoculation.

Pycnidial initials and pycnidia were present in host tissue of Erik and 4B1149, 10 to 12 days post-inoculation (Fig. 10C and D). The intercellular space of the mesophyll contained a massive hyphal network and the majority of surrounding mesophyll cells remained turgid. Mature and immature pycnidia were visible in Erik and 4B1149 between 12 and 21 days post-inoculation (Fig. 10D, E and F). Hyphal growth became irregular and no longer intercellular as extensive mesophyll cell collapse had occurred (Fig. 10F).

Figure10. Compatible response of wheat host cultivars, Erik and 4B119, to isolate 96-36 of *Mycosphaerella graminicola*. A) Hyphae circling the substomatal chamber of and infected stomate 6 days after inoculation in Erik. (x500). B) Pycnidial initials forming in the cultivar, Erik, 8 days after inoculation. (x500). C) Pycnidial initial developing in the tetraploid accession 4B1149, 10 days after inoculation. (x500). D) Sporulating pycnidia embedded in the susceptible cultivar Erik, 12 days after inoculation. (x750). E) Pycnidia and a mass of intercellular hyphal growth in Erik, 21 days after inoculation. (x300). F) Pycnidial formation initiated in the susceptible cultivar, 4B 1149, 21 days after inoculation. (x200).

Abbreviations: GC = guard cell, H = hyphae, MC = mesophyll cell, P = pycnidium, PI = pycnidium initial, PS = pycnidiospore, ST = stomate

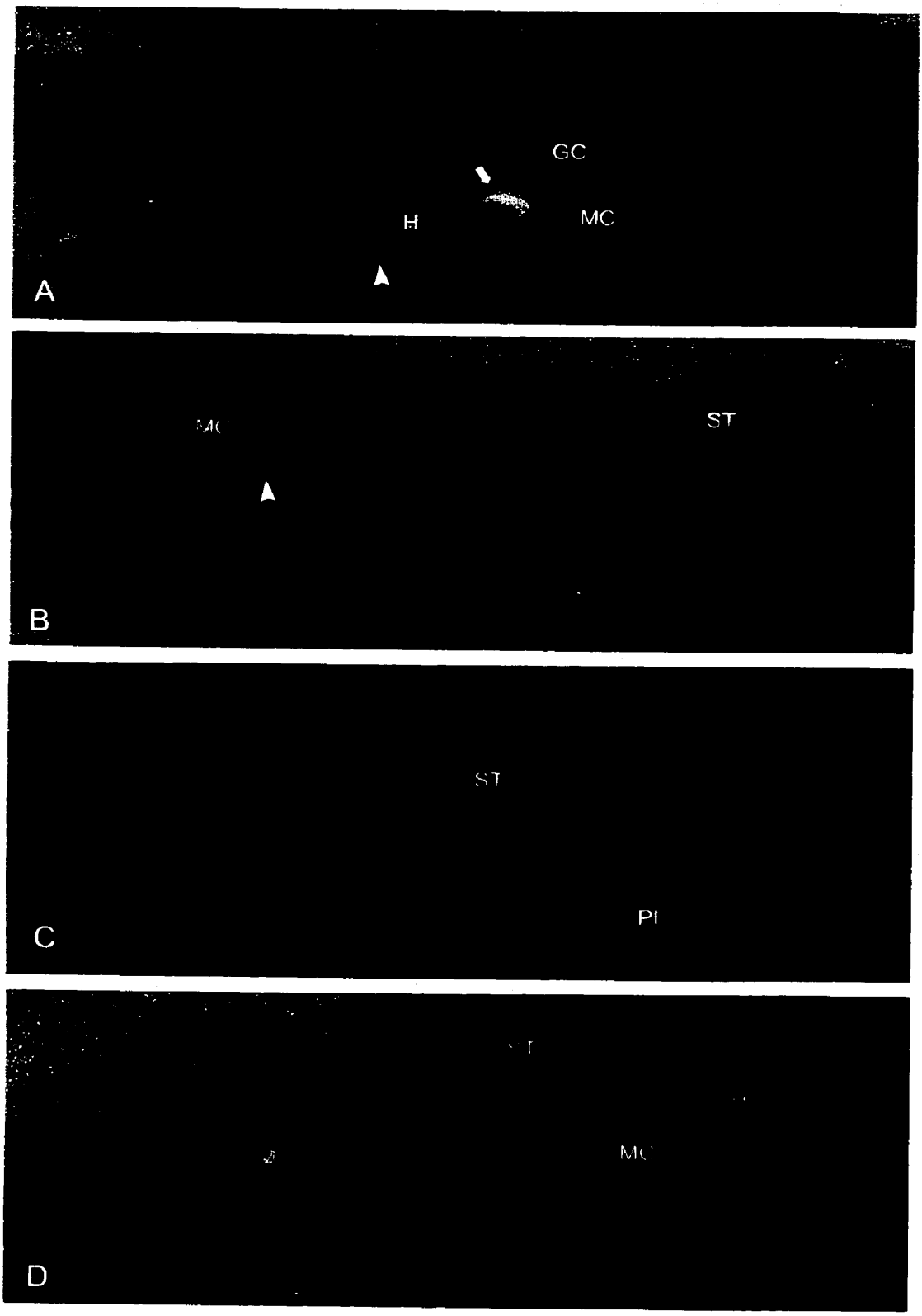

MC
A
B
PI
C
PI
D
ST
P
E
P
F

The resistant line, Salamouni, harbored no pycnidia or pycnidial initials 14 to 21 days post-inoculation, suggesting that growth of the pathogen had been severely restricted (Fig. 11A and B). Single strands of intercellular hyphae were occasionally visible in the mesophyll tissue, extending from one vascular bundle to the next. Hyphal branching was limited and the hyphae did not aggregate in the substomatal chamber (Fig.11A). A limited network of hyphae was occasionally seen along the leaf margins and at the leaf tips. Surrounding the substomatal chamber of many penetrated stomates, fluorescing mesophyll remained visible. Hyphae seen in these areas did not extend beyond the fluorescing mesophyll cells. Macroscopically, leaf tissue did not senesce and exhibited very small dark necrotic and occasionally chlorotic flecks on the green leaf tissue.

Although there were no macroscopic indications of pycnidial formation in the resistant tetraploid line, Coulter, some pycnidial initials were seen 21 days after inoculation (Fig. 11C). Pycnidial initials, as few as one and as many as 20 initials on some leaf segments, could be seen forming along the leaf margins. Fluorescencing mesophyll cells were not evident in these areas of limited intercellular growth (Fig. 11C and D). However, fluorescing mesophyll cells remained visible around many infection sites and intercellular hyphae extending from these areas occurred rarely. Hyphal proliferation was greater than in Salamouni. Macroscopically, Coulter exhibited chlorotic/necrotic flecking 21 days post-inoculation. Primary leaves and tips of secondary leaves senesced prematurely in this cultivar in contrast to the other lines used in this study.

Figure 11. Incompatible interaction of *Mycosphaerella graminicola* and the resistant cultivars Salamouni and Coulter, 14 to 21 days post inoculation. A) Limited hyphal growth in the resistant cultivar Salamouni, 14 days after inoculation. Hyphal growth is not restricted by fluorescing mesophyll cells as only one mesophyll cell contains fluorescing materials. (x500). B) Minimal intercellular hyphae (arrow) seen in the cultivar Salamouni 21 days post inoculation. (x300). C) Intercellular hyphae and a pycnidial initial seen in the tissue of Coulter 21 dpi. (x200). D) Intercellular hyphae seen in the tissue of Coulter 21 dpi. (x300).

Abbreviations: EC = epidermal cell, GC = guard cell, H = hyphae, MC = mesophyll cell, PI = pycnidial initial, ST = stomate, ➢ = indicates fungal hyphae, ▬ = indicates fluorescing mesophyll cell


GC
H
MC
A
MC
ST
B
ST
PI
C
MC
D

Sporulation

Pycnidia formed linearly, directly in the substomatal chamber of infected stomates of the susceptible wheat lines (10F). Generally one pycnidium formed in each susbstomatal chamber. Occasionally two pycnidia were found beneath the same stomate. Mature sporulating pycnidia were seen, as conidia were released through the ostiole of the pycnidium, located at the stomatal aperture, as early as 12 days post-inoculation (Fig. 10D).

4.1.4 Discussion

Spore germination, germ tube elongation and host penetration were similar in both compatible and incompatible interactions investigated in this study, suggesting that the early events of the infection process were non-specific. Germ tube elongation through the stomatal aperture occurred during the same post-inoculation time period on all four wheat lines. Multiple penetrations were often seen and hyphae frequently grew across the stomatal opening indicating that penetration of this pathogen occurred randomly. The random penetration of wheat stomates by hyphae of *M. graminicola* is in agreement with the findings of Kema et al. (1996c), which indicate that thigmotropism and chemotropism were not likely involved in the penetration process of this pathogen. The appressorium-like structures, occasionally seen at the junction of epidermal cells, on periclinal epidermal cell walls and directly over the stomatal aperture, were not associated with penetration events. The same was concluded by Rohel and co-workers (2001). These appressorium-like structures may be a differentiation of hyphae as branching begins to take place.

Salamouni, the immune to near-immune line used in the present study, exhibited small chlorotic flecks 4 to 5 days post-inoculation. No other symptoms developed other than minimal necrosis within the chlorotic flecks. The resistant to near-immune reaction of Coulter was characterized by similar flecking. However, the necrotic/chlorotic flecks appeared larger and developed 3 to 4 days post-inoculation. Rosielle (1972) suggested that these reactions were indicative of immune to highly resistant responses to *M. graminicola*.

Resistant cultivars previously used in cytological studies, investigating the wheat-*M. graminicola* pathosystem, did not produce near-immune responses as seen in Salamouni and Coulter. 'Kavakaz/K4500 l.6.a.4', a resistant line used in several studies, was reported to develop 5% necrosis and 1% pycnidial coverage, 21 days after inoculation (Kema et al., 1996c). This cultivar harbored no macroscopically visible pycnidia when tested with isolates from Israel (Cohen and Eyal, 1993). There was no reference, in the literature by either Cohen and Eyal (1993) or Kema et al. (1996c), of a hypersensitive flecking response exhibited by this cultivar.

Expression of the incompatible interaction, 3 days post-inoculation, was microscopically evident in both Salamouni (*T. aestivum*) and Coulter (*T. durum*) in response to infection by isolate 96-36 of *M. graminicola* from Manitoba. Fluorescent mesophyll cells, surrounding infected substomatal chambers, were consistently visible in leaf mounts stained with KOH-aniline blue. Hood and Shew (1996) indicated that although this technique provided a good discrimination between host tissue and the invading pathogen, substances common to host-pathogen interactions may not be observed. Callose deposition in the tissue of *Nicotiana tabacum* roots, found around penetration pegs of invading *Thielaviopsis basicola*, were often not observed following treatment with KOH-aniline blue, even though they fluoresced with conventional aniline blue stain (Hood and Shew, 1996).

The fluorescing compounds in the mesophyll cells surrounding the stomatal infection site are considered to be an indication of incompatibility in the current study. Mesophyll cells were the only affected cells, as the appearance of the epidermal cells and of base of hair cells did not differ from the controls in their fluorescence. Fluorescence

of mesophyll cells was not observed at any time in the compatible interaction in the presence of the invading hyphae. Autofluorescing materials and an increased affinity for tyrpan blue were observed by Cohen and Eyal (1993) on the anticlinal and periclinal walls of epidermal cells, in the stomates and at the base of hair cells, 48 hours after inoculation with *M. graminicola* and interpreted as an indication of incompatibility. Subsequently, the authors also found fluorescing mesophyll tissue surrounding the substomatal space.

Limited fungal colonization in the incompatible interactions was most evident at infection sites were mesophyll cells surrounding the substomatal chamber had been disrupted. When treated with aniline blue, following the procedure of Bruzzese and Hasan (1983), and observed with conventional light microscopy, mesophyll cells appeared deeply stained in blue, in the absence of any penetration by the invading fungus. Throughout this study, cell penetration was not observed and hyphal proliferation beyond the disrupted mesophyll cells was not seen. Lamari and Bernier (1989c) reported a similar finding in the wheat-*Pyrenophora tritici-repentis* and attributed symptom development to host-specific toxins (Lamari & Bernier 1989b). Recent evidence that the wheat-*M. graminicola* system was consistent with the gene-for-gene model (Kema et al.2000), suggests that the hypersensitive-like response associated with the incompatible interaction in this study may be due to the interaction of a pathogen-produced elicitor and putative resistance gene(s) product(s). The near-immune line Salamouni is known to carry two major genes for resistance to the isolate used in this study (McCartney unpublished). The issue of whether hypersensitivity occurs as the determinant or consequence of incompatibility is a point of contention, most prevalently seen in the host-

parasite specificity of the rusts (Heath, 1976). However, this question could apply to other systems as well. As hyphal growth is inhibited both in the presence and the absence of hypersensitive flecking, it is reasonable to assume that a biochemical process be involved in limiting the colonization of the host tissue by *M. graminicola*. Heath (1976) also indicated that evidence of hypersensitivity could result from a number of preceding interactions between host and pathogen. Hypersensitivity may, therefore, be morphological evidence of an earlier biochemical process occurring in the wheat-*M. graminicola* pathosystem. The lack of any host cell penetration, particularly in mesophyll cells showing evidence of disruption, also suggests that a fungitoxic, plant derived substance(s), be responsible for the incompatible interaction.

This hypothesis may also explain restricted host colonization, in the absence of rapid cell death associated with the hypersensitive response. Single strands of hyphae were seen only in the intercellular mesophyll tissue but often did not fluoresce as brightly, as in the compatible interaction. In this case, fluorescing mesophyll cells were not seen and mesophyll cell integrity remained undisrupted in these areas of hyphal growth. Evidence of restricted intercellular hyphal growth in the mesophyll tissue of the incompatible response, strengthens the probability that apoplastic movement of compounds is responsible for the success or failure of compatible reaction, as suggested by Kema et al. (1996c).

Salamouni harbored no pycnidia or pycnidial initials 21 days post-inoculation, indicating that incompatibility is not due to the arrestment of immature pycnidia, as was suggested by Cohen and Eyal (1993) as a possible resistance mechanism. As well, hyphal growth was limited even in the absence of the hypersensitive response, suggesting

that a mechanism other than that induced by the hypersensitive flecking was responsible for the limitation of hyphal growth. Coulter did harbor low numbers of pycnidial initials, visible only through microscopic examination, suggesting that this response may be similar to the one described by Cohen and Eyal (1993). However, the level of infection was extremely low and pycnidial initials were only occasionally microscopically visible along leaf margins and tips only. No pycnidia were macroscopically observed in the resistant reaction of Coulter. However, host tissue colonization was limited to a greater extent in Salamouni when compared to Coulter.

Other host-parasite systems have been studied in detail using histological methods to determine the processes associated with specific host resistance. Tomato leaf mould and cereal rusts are of particular interest because of variability within the system and notably among the incompatible interactions. Heath (1976) indicated that an approach which inclusively groups all incompatible interactions as being equivalent rather than studying each from an individual perspective was detrimental to the full understanding of each incompatible interaction. Rohringer and Heitefuss (1984) agree in stating that processes involved in one incompatible interaction within a system might not typify those of another incompatible interaction within the same system. In support of this view, incompatible interactions within the tomato-*Cladosporium fulvum* system are distinctly variable at the ultrastructural level. The immune reaction was characterized by rapid cell death and callose deposition 6 days after infection (Lazarovits and Higgins, 1976). However, in the resistant reaction there was limited callose deposition occurring 8 days after inoculation (Lazarovits and Higgins, 1976).

A similar approach should be adopted when investigating the incompatible interactions in the wheat-*M. graminicola* pathosystem, as it is likely that a number of processes are involved in host response to a pathogen. Macroscopically identifiable differences exist among incompatible interactions in wheat cultivars infected with *M. graminicola* (Rosielle, 1972). A near-immune, a highly resistant and a resistant response all occurred when three wheat lines were inoculated with a single isolate from Manitoba (Grieger et al., unpublished). Therefore it is possible that there is also variability at the untrastuctural level among incompatible reactions in the wheat-*M. graminicola* system.

Recent investigations by Kema et al. (2000) point toward a gene-for-gene system controlling avirulence in *M. graminicola* and plant host resistance in wheat. Avirulence was shown to be controlled by a single locus as determined by crosses made between virulent and avirulent isolates. Studies in other pathosystems indicated that other hemi-biotrophic fungi communicate with the host tissue via molecular compounds. The tomato-*Cladosporium fulvum* system is known to follow the gene-for-gene model, as the cloned *avr9* gene produced specific elicitors that induced the hypersensitive response in tomato cultivars carrying the resistance gene *Cf9* (Van den Ackerveken et al, 1992). The infection of *C. fulvum* appears to be similar to that of *M. graminicola*. Stomatal penetration, intercellular hyphal growth in mesophyll and lack of cell penetration are common to both pathogens (Cohen and Eyal, 1993; de Wit, 1977; Kema et al. 1996c). The majority of the colonization processes of each of these fungi, within their respective host tissues, occur in the apoplast and mesophyll cell penetration via a haustorium does not occur in either system. Isolation of specific elicitors from intercellular washing fluids of *C. fulvum* were also shown to induce chlorosis and necrosis in resistant cultivars (de

Wit and Spikeman, 1992). Studies should be conducted within the wheat-*M. graminicola* pathosystem to determine if race-specific elicitors and resistant gene(s) products are responsible for the incompatible response.

5. GENERAL DISCUSSION

The distinction of only two different pathotypes/races of *M. graminicola* in the present study may result from various environmental and epidemiological factors associated with the disease. Although the teleomorph has been reported to be an important factor in many countries and could lead to increased genetic variation within the pathogen population (Garcia and Marshall, 1992; Kema et al, 1996d; McDonald et al., 1995; Scott et al., 1988), only the anamorph has been isolated from wheat fields in Manitoba (Gilbert et al, 1998). Therefore, clonal populations, from asexual reproduction, are likely to be the most prevalent means of pathogen increase, resulting in little genetic variation for virulence within the population of the pathogen from the Canadian prairies.

However, it is possible that wind-borne spore dispersal may be partially responsible pathogen distribution, due pathogen isolation from wheat fields separated by significant distances throughout both Manitoba and Saskatchewan. The asexual state of the pathogen is splash-dispersed over short distances only (Shaner and Buechley, 1995) and could not provide inoculum to infect disease free fields. The air-borne nature of ascospore dispersal provides a method of long distance dispersal for *M. graminicola* (Eyal et al, 1987; Zhan et al., 1998). Therefore, ascospores may be responsible for the infection of wheat crops on the Canadian prairies. An investigation to identify the sexual state and to determine its contribution to the genetic variation in the *M. graminicola* population of Manitoba would be of benefit.

The growing conditions throughout the season may limit dispersal of the sexual state of the pathogen in Canada. Pseudothecia were found to develop primarily in the autumn and winter months on the leaf sheaths of stubble in countries such as the United

Kingdom (Hunter et al., 1999) and the United States (Garcia and Marshall, 1992). These countries have a more temperate winter season than that of the Canadian prairies. Therefore, the production of sexual fructifications may be regulated by moderate environmental conditions that are not present during the winter season in Manitoba. Even if the sexual state were present, the ability to complete many sexual cycles within the cropping season (Kema et al., 1996d), is unlikely in Manitoba due to the short length of the growing season.

Another limitation when assessing physiological specialization within the pathogen population of Manitoba may be the inadequacy of the host differential set, as race differentiation is dependent on the wheat genotypes used (de Wit, 1992). The differential lines used in this study may be limited in their ability to detect all pathotypes/races present in this area. Furthermore, the number of lines used was limited to six for practical reasons, including the availability of growth room and misting facilities. Saadaoui (1987) also reported similar limitations. It has been suggested that regionally important isolates should be used in studies aiming at determining host specificity, if these hosts were to be incorporated into local agronomic practices (Van Ginkel and Scharen, 1998). However, a differential set composed of only host cultivars used in the same region may be inappropriate when evaluating race specificity. It is likely that many of these cultivars share common lineages and thus, common resistant genes. Detection of multiple avirulence genes within the pathogen population may, therefore, be unrecognizable.

The lack of a standard rating scale to assess wheat reaction to *M. graminicola* further complicates the issue of physiological specialization and the existence of races

within the pathogen population worldwide. A quantitative rating scale, based on percent leaf area infected, was used when three races were determined to exist in the pathogen population of Morocco (Saadaoui, 1987). The distinction between susceptibility and resistance was then determined by a separation of disease scores, ranging from 0-9. A rating less than three, 15-20% of leaf area necrotic and few pycnidia, indicated resistance and a rating of three or greater indicated susceptibility (Saadaoui, 1987). Conversely, Eyal and colleagues (1973) used only pycnidia density on live plant tissue to determine distinct virulence patterns of five isolates. They determined that disease severity corresponded with pycnidial densities. Although pycnidial density is seen to be affected by host cultivar (Hess and Shaner, 1987; Shaner and Finney, 1982), factors such as pycnidium size (Eyal and Brown, 1976), temperature (Hess and Shaner, 1987) and environmental conditions (Gough, 1978) also affect pycnidial production. Therefore, inconsistencies may arise when using pycnidial density as the sole criterion for disease assessment.

Isolates were found to differ in their degree of virulence (Ahmed et al., 1996; King et al., 1983) or aggressiveness, which would further complicate the use of quantitative disease measurements. In highly aggressive populations of *M. graminicola*, where there is the greatest amount of variability in aggressiveness (Marshall, 1985), the use of strictly quantitative disease measurements could lead to only the classification of "aggressive races" and not true differential interactions.

Virulence is used to describe the ability of the pathogen to induce disease in a susceptible host and likewise, avirulence describes the inability of the pathogen to produce disease on a resistant host (Green, 1975). Therefore, using virulence rather than

aggressiveness to develop a classification system would remove the need for statistical quantitative evaluation in determining host specificity. A compatible interaction is specified by a virulent pathogen and a susceptible host, as opposed to an incompatible interaction, which is determined by an avirulent pathogen and a resistant host. The compatible or incompatible reactions may be expressed by various host responses based on lesion type, such as immune, highly resistant, resistant or susceptible as seen in this study and reported previously (Rosielle, 1972). Classification of pathotypes or races can, therefore, be developed using a system of phenotypic expression (Lamari and Bernier, 1989a).

The infection process of *M. graminicola* isolates from Manitoba and Saskatchewan appears to be consistent with previous findings. Pycnidospore germination and host penetration are similar on both susceptible and resistant host cultivars (Cohen and Eyal, 1993; Kema et al., 1996c; Rohel et al., 2001). Colonization of the resistant host tissue seems to be limited to the apoplast, as colonization of the host tissue in both the susceptible and resistant lines is limited to the intercellular mesophyll tissue of the host (Cohen and Eyal, 1993; Kema et al., 1996c; Rohel et al., 2001).

Host penetration was also seen to occur between epidermal cell walls in the durum wheat lines, Coulter and 4B1149 (Appendix 4, Appendix 5) in the present study. This occurred rarely but was seen, as openings between the epidermal cell walls appeared to allow entry of hyphae into the mesophyll host tissue (Appendix 4, Appendix 5). The stomatal penetration seen in all cultivars may therefore, only allow entry into the host tissue as the runner hyphae "sense" an opening in the epidermal cell layer of the host tissue large enough to allow fungal penetration. Appressorial-like structures were not

seen at these penetration sites, further emphasizing that they are not required for host penetration.

Cohen and Eyal (1993) suggested direct penetration through the epidermal cells walls to be a secondary method of host invasion. Rohel and colleagues (2001) observed penetration between epidermal cell walls in a study of the infection process using confocal microscopy and transformants containing a green fluorescent protein. However, this type of penetration was limited to certain cultivar-isolate interactions (Rohel et al., 2001). This supports the present observation that penetration between epidermal cell walls was limited to the durum cultivars infected with isolate 93-36 of *M. graminicola.* This type of penetration was not observed at any time in the bread wheat lines, as the infection process in a resistant and susceptible bread wheat line was also studied. Conversely, Kema et al. (1996c) determined that fungal penetration of the host tissue was strictly stomatal.

Although the resistance mechanism of wheat hosts to *M. graminicola* have not been determined, it is evident that incompatibility is expressed early in the infection process, 2 to 3 days post-inoculation. In the present study, incompatibility was often expressed as mesophyll cell fluorescence and mesophyll cell collapse in the resistant host tissue occurring approximately 24 hours after the hyphae reacted the intercellular spaces of the mesophyll tissue. Kema et al., (1996c) noted differences between the compatible and incompatible responses 48 hours after inoculation. In the compatible interaction, condensation of chloroplasts and release of starch granules was observed in mesophyll cells of the host tissue (Kema et al., 1996c). Elucidation as to the biotrophic nature of *M. graminicola* was provided by Rohel et al., (2001) as they determined fungal uptake of

metabolite sugars occurred in the apoplastic mesophyll tissue of the host, early in the infection process. The evidence of an interface occurring between host and pathogen suggests that substances other than nutrient carbohydrates may be passed from one identity to the other.

Race-specific elicitors were found in the intercellular fluids obtained from the compatible interaction of *C. fulvum* and tomato (de Wit and Spikeman, 1982). Intercellular growth of *C. fulvum* characterizes much of the colonization process, therefore communication between host and this biotrophic pathogen occurs in the apoplast (de Wit, 1977). Induced chlorosis, necrosis and hypersensitivity in the host tissue are resultant of the interaction between the product of the avirulence gene (elicitor) and the product of the host resistant gene (receptor) (Van den Ackerveken et al., 1992). Infection and colonization are similar in both the tomato-*C. fulvum* system and the wheat-*M. graminicola* system (Cohen and Eyal, 1993; de Wit, 1977; Kema et al., 1996c). Evidence of an apoplastic interface between host and pathogen (Rohel et al., 2001) and host cell disruption (Kema et al., 1996c) early in the infection process of *M. graminicola* suggest that elicitors may be responsible for the incompatibility in the wheat-*M. graminicola* pathosystem. Recent evidence supports the operation of the gene-for-gene model within the wheat-*M. graminicola* system, also suggesting that products of avirulence genes, such as elicitors, may be responsible for host-species and pathogen incompatibility (Kema et al, 2000).

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7. APPENCICES

Appendix 1

Yeast Malt Agar

4 g sucrose
4 g yeast malt
4 g malt extract
15 g agar
1 L distilled water
.25 g chloramphenicol added following the autoclaving treatment

Appendix 2

Whole leaf clearing and staining (Bruzzese and Hasan, 1983)

300.0 ml 95% ethanol
150.0 ml chloroform
660.0 ml lactophenol (Note: 660 ml of lactophenol was substituted in place of lactic acid and phenol)
450.0 g chloral hydrate
0.6 g aniline blue (CI #42755 Fisher Scientific).

Appendix 3

Aggressiveness of 74 isolates of *Mycosphaerella graminicola* on four tetraploid and two hexaploid wheat cultivars.

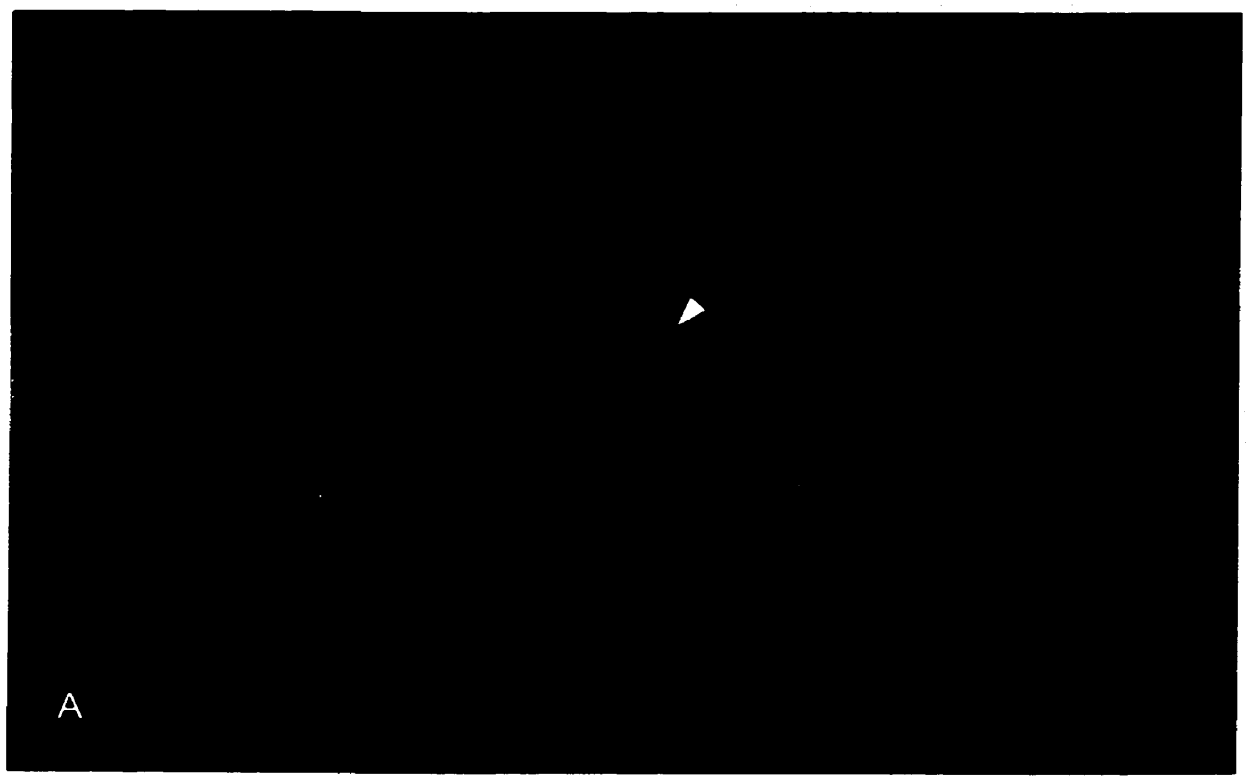
Pathogen Race	Wheat cultivars						Total No.of Isolates
	Erik	Amazon	ST 6	Salamouni	4B 1149†	Coulter†	
I	V	V	V	A	V	A	14
I	V	V	V-	A	V	A	1
II	V	V	A	A	V	A	47
II	V	V	A	A	V-	A	1
II	V	V-	A	A	V	A	2
II	V-	V-	A	A	V	A	1
II	V-	V	A	A	V-	A	5
II	V-	V-	A	A	V-	A	3
							74

†Indicates durum wheat genotypes
V = virulent, A = avirulent
+ more, or - less aggressive on the specific cultivar

Appendix 4

Penetration of *Mycosphaerella graminicola* between epidermal cell walls of the resistant durum wheat cultivar, Coulter, 3 days after inoculation. A) Hyphae penetrating through opening between epidermal cell walls (arrow). (x500). B) Hyphae from penetration seen in Fig. A, now in mesophyll tissue (arrow) of the wheat host. (x500).

Abbreviations: EC = epidermal cell; H = hyphae; MC = mesophyll cell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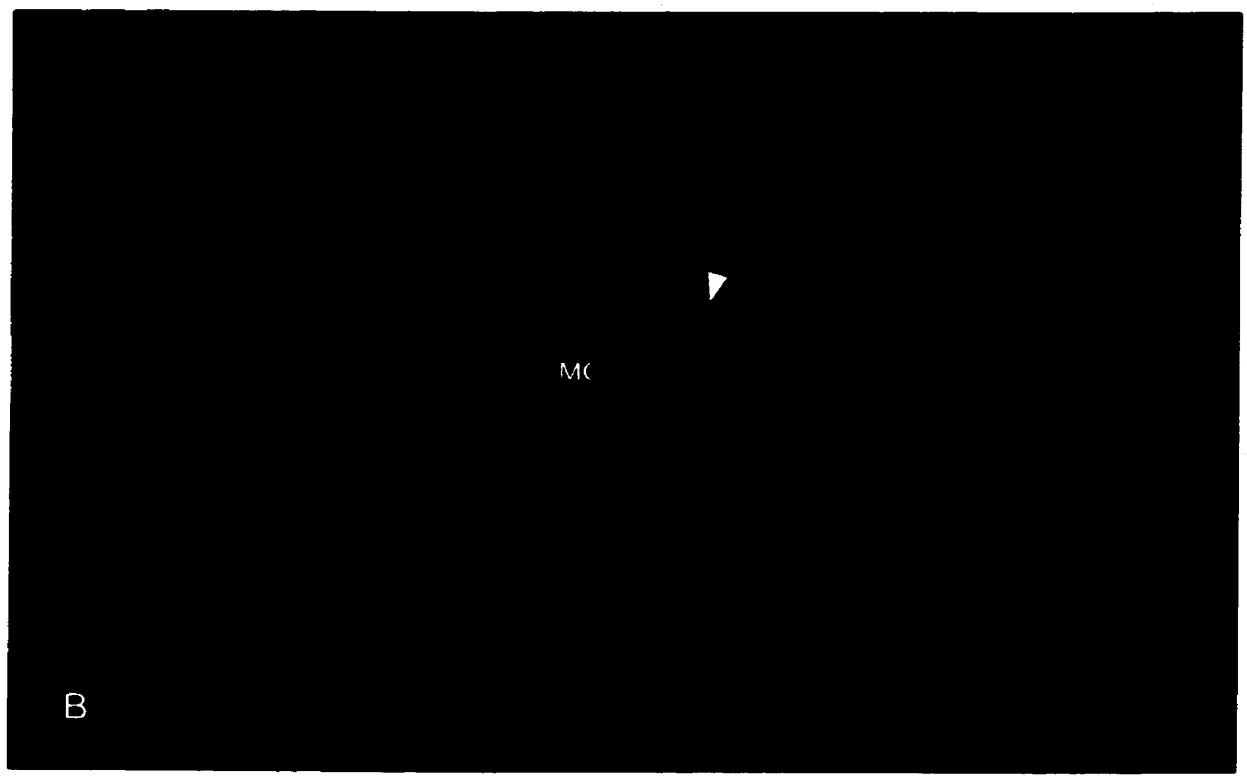
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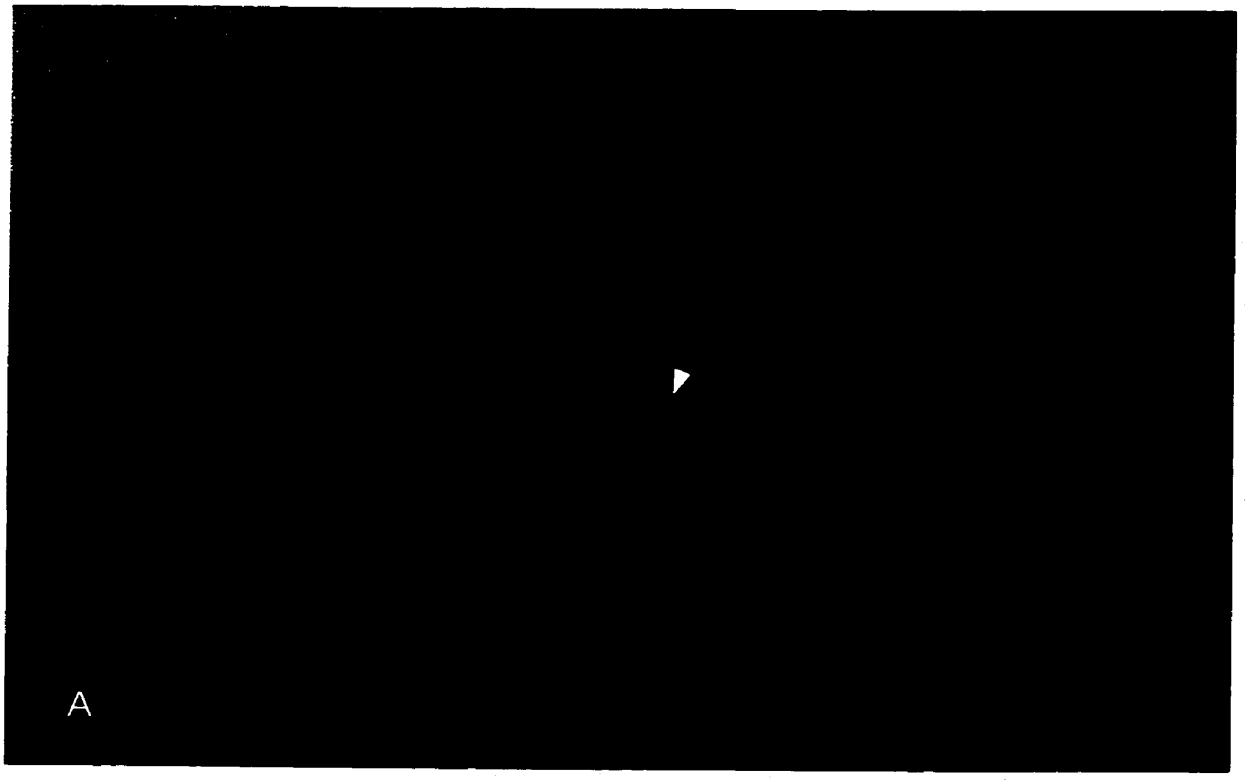
Appendix 5

Penetration of *Mycosphaerella graminicola* between epidermal cell walls of the susceptible durum wheat cultivar, 4B1149, 4 days post-inoculation. A) Hyphae penetrating (arrow) through opening between epidermal cell walls. (x500). B) Hyphae from penetration (arrow) seen in Fig. A, now extending to the mesophyll tissue of the wheat host. (x500).

Abbreviations: EC = epidermal cell; H = hyphae; MC = mesophyll cell; ST = stomate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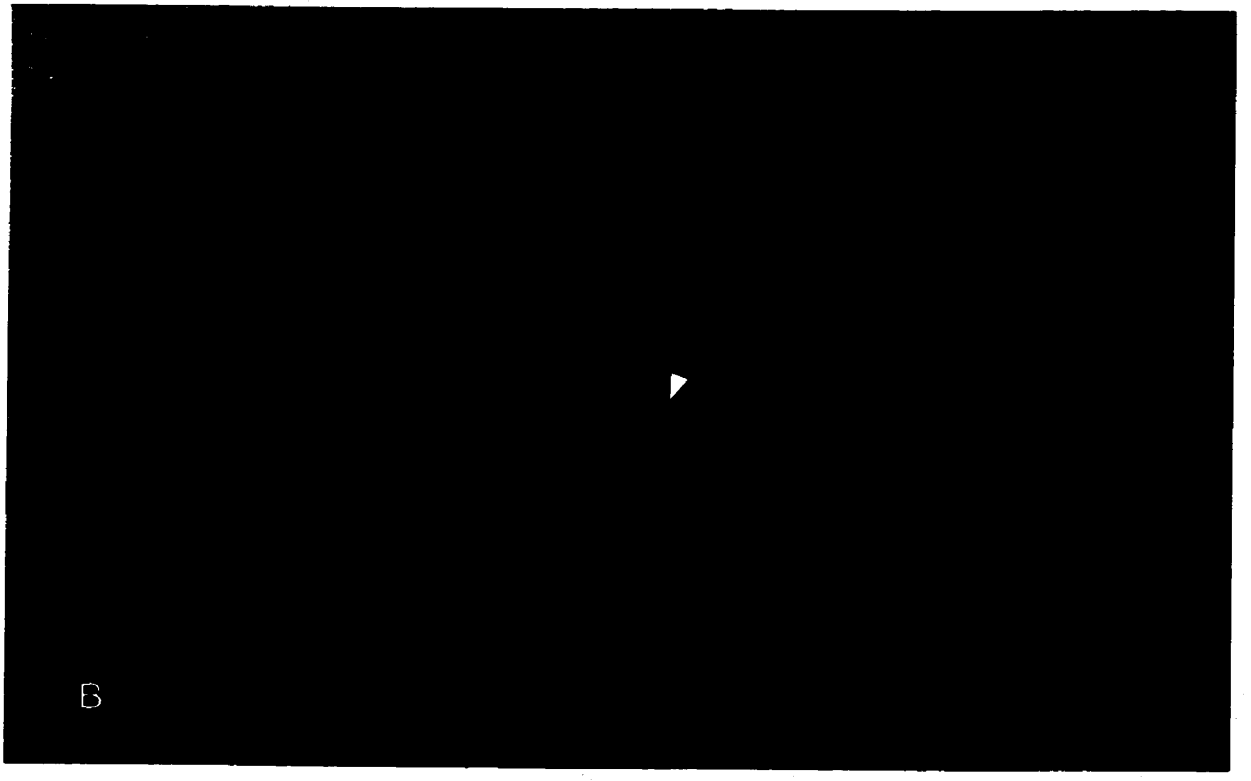
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