This is to certify that the

thesis entitled

Ascospore Discharge and Perithecium Development in Gibberella zeae.

presented by

Corrie L. Andries

has been accepted towards fulfillment of the requirements for

Masters degree in Science

Frances Trail

Major professor

Date 5/11/00

ASCOSPORE DISCHARGE AND PERITHECIUM DEVELOPMENT IN *GIBBERELLA ZEAE*

By

Corrie L. Andries

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

2000

ABSTRACT

Fusarium Head Blight (FHB), caused by *Gibberella zeae*, has incurred billions of dollars in yield losses to wheat and barley growers from 1993 to 1999. The fungus overwinters in crop debris and undergoes sexual reproduction to form perithecia that contain ascospores. These ascospores are forcibly discharged and deposited on the developing grain head during flowering. I conducted two studies: a field study to pinpoint the timing of perithecium formation in the field on crop debris, and a random mutagenesis project to understand the mechanism of forcible discharge of ascospores.

A total of 2186 samples of infected corn and wheat stubble were collected monthly from 1997 to 2000. Of these, 1532 samples were corn debris and 654 samples were wheat debris. A total of 241 corn debris samples (15%) and 31 wheat debris samples (3%) had perithecia of *G. zeae*. The number of perithecia present was highest in May, June, and July. Average temperatures at 4 days and at two-weeks prior to sample collection were correlated with the presence of perithecia.

In a separate study, 5005 transformants were generated with random insertional mutagenesis of a plasmid harboring the hygromycin resistance gene. Transformants were screened for a loss of the ability to forcibly discharge ascospores. Southern blot analysis indicated the insertion of the vector was random and remained stable through meiosis and mitosis. Four mutants were found that were unable to forcibly discharge their ascospores. One of these mutants (designated 729G-112) appeared morphologically normal and the other three had mutations associated with the development of mature perithecia. Isolate 729G-112 also exuded irregularly shaped cirrhi.

ACKNOWLEDGEMENTS

I would like to thank the following people for their guidance and assistance while completing my masters project and thesis: Dr. Frances Trail, Iffa Gaffoor, Dr. John Linz, Dr. L. Patrick Hart, Dr. Andy Jarosz, David Johnson, Luis Velasquez, Cheryl Calhoun, Rachel Loranger, Dr. Haixin Xu, Dr. Seanna Annis, Dr. John Halloin, Jennifer Schaupp, Dr. Gerry Adams, Dr. John Scott-Craig, and Kerry Pedley.

I would also like to thank my husband, Erik Andries and my parents, Kathy and John Jacomino, my nana, Kathryn Kiser, and my sister, Kimberly Jean Platt for their support and encouragement throughout my education.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

CA: carrot agar
CMC: carboxymethylcellulose medium
Dis$^-$: discharge minus
Dis$^+$: wild type for discharge
DON: deoxynivalenol
FHB: Fusarium Head Blight
Hyg^R: hygromycin resistant
Hyg^S: hygromycin sensitive
Kb: kilobase
MMTS: minimal medium with Tergitol Type NP-10
Nit$^-$: nitrate non-utilizing, auxotrophic
Nit$^+$: nitrate utilizing
PEG: polyethylene glycol
REMI: restriction enzyme mediated integration
RH: relative humidity
RIM: random insertional mutagenesis
RM: regeneration medium
RT: room temperature
Tx: transformants
VM: V8 juice medium
WA: water with 2% agar
YEPD: yeast extract / peptone / dextrose broth
YES: yeast extract/ sucrose broth

INTRODUCTION

Disease overview. Fusarium Head Blight (FHB) or scab of wheat (*Triticum aestivum* L.) has reached epidemic proportions in the United States over the last ten years. The causal agent, *Gibberella zeae* (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe), also causes seedling blight, ear rot of maize and affects other small grain cereals such as barley and rye. The U.S. Wheat and Barley Scab Initiative reports that FHB has incurred over 4 billion dollars in yield losses to wheat and barley growers between 1993 and 1999 (McMullen, 1999). Yield losses in wheat occur primarily as a result of sterile florets and diminished, low weight kernels (McMullen, 1999). Infested

wheat kernels that are harvested, may produce a poor flour quality with a lowered protein content (Parry *et al.*, 1995). Barley infected with *G. zeae* can result in uncontrolled "gushing" during beer brewing which has led the brewing companies to establish "zero tolerance" levels for FHB (Parry *et al.*, 1995). *Gibberella zeae* also produces mycotoxins including deoxynivalenol (DON) and zearalenone. DON or vomitoxin, is a trichothecene produced in diseased kernels that causes vomiting and feed refusal in livestock. The Food and Drug Administration (FDA) has limited levels of DON in grains to 1, 5, and 10 ppm for human, swine, and cattle consumption, respectively (McMullen, 1999). Zearalenone, an estrogenic compound, has been shown to cause reproductive disorders when fed to young female pigs (Long *et al.*, 1982). There is also evidence it can accumulate in milk from dairy cattle (Kuper-Goodman, 1987). The National Veterinary Services Laboratories at United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA, APHIS) has advised that detectable levels of zearalenone in grains remain below 1 and 10 ppm, for swine and cattle consumption, respectively (Stanton, 1995).

The disease cycle of FHB (Figure 1) has been well described. *Gibberella zeae* reportedly overwinters as saprophytic mycelia on crop debris of maize, wheat, and barley. The fungus undergoes sexual reproduction and produces ascospores in a fruiting body known as a perithecium. Ascospores are forcibly discharged from perithecia and become airborne. The spores are deposited on the wheat head sometime during flowering (Paulitz, 1996; Sutton and Procter, 1982) when susceptibility is highest (Fernando *et al.*, 1997). Ascospores serve as the primary source of inoculum, although the asexual macroconidia may also produce some localized infection due to wind and splash dispersal

(Fernando *et al.*, 1997; Parry *et al.*, 1995; Sutton, 1982). The hyphae then penetrate the glume stomata (Pritsch, 2000), and spread throughout the wheat head resulting in chlorosis of infected kernels (Parry *et al.*, 1995; Sutton, 1982). Favorable conditions for disease outbreaks appear to coincide with extended periods of high relative humidity (RH) and warm temperatures (24 - 29°C). (McMullen *et al.*, 1997a). If warm, wet conditions are prolonged, secondary infection can occur from production of ascospores and macroconidia on infested wheat plants (McMullen *et al.*, 1997b).

Control of FHB has been problematic. Currently, there are no known wheat or barley cultivars entirely resistant to FHB and most are highly susceptible (Parry *et al.*, 1995). Durum wheat and barley are the most susceptible, but all classes of wheat, including spring and winter varieties, are affected (McMullen *et al.*, 1997a). Four types of resistance have been described: inhibition of initial infection (Type I), limiting the spread of infection throughout the host (Type II) (Schroeder and Christensen, 1963), ability to degrade DON (Type III), and tolerance of high DON concentrations (Type IV) (Parry *et al.*, 1995).

Chemical seed treatments can inhibit seedling blight, but do not prevent FHB (McMullen, 1999). Currently, an effective fungicide or biocontrol agent that can consistently control FHB outbreaks has not been established. Delivery of fungicides is also difficult because the disease develops inside the grain head (Parry *et al.*, 1995) and food safety and economic considerations limit the frequency of application (McMullen *et al.*, 1999). Even when fungicides achieve some reduction of disease severity, high levels of DON may still be present (Moss and Frank, 1985). A disease forecasting system is

under development but its efficacy in predicting disease outbreaks has not been established (McMullen *et al.*, 1997a).

Crop rotation and tillage practices have an impact on the severity of FHB. Incidence of FHB increases when wheat is planted immediately after maize or cereals (wheat, barley, oats) as opposed to soybean or other non-host crops (McMullen, 1999; Teich and Hamilton, 1985; Teich and Nelson, 1984; Tusa *et al.*, 1981). Tillage practices that bury crop debris have been shown to be effective in reducing levels of primary inoculum (Teich, 1989). However, the effectiveness of these cultural controls is variable and is it not always possible due to the practice of soil conservation tillage and grower commitments to the USDA to produce specific crops (Bai and Shaner, 1994; McMullen *et al.*, 1997a; Miller *et al.*, 1998; Parry *et al.*, 1995). We believe study of the causal organism, specifically, the mechanism of forcible discharge of ascospores and the timing of perithecium formation in field conditions, may lead to a better understanding of the spread of this disease and lead to novel methods for control.

Timing of perithecium formation in the field. Fungi in the Phylum Ascomycota are characterized in *Ainsworth and Bisby's Dictionary of the Fungi* (Hawksworth *et al.*, 1996) by the production of ascospores following meiosis. Ascospores are contained within sac-like cells known as asci. Certain ascomycetes bear asci in flask-shaped fruiting bodies called perithecia. *Gibberella zeae* is a member of Hypocreaceae De. Not., a family whose members are characterized by brightly colored perithecia (Alexopoulos *et al.*, 1996). Members of Hypocreaceae, along with those of Nectriaceae and Clavicipitaceae are all able to forcibly discharge their ascospores when mature. The morphological development of perithecia of *G. zeae* has been described in detail (Trail

and Common, 2000). First, the vegetative hyphae start to enlarge and develop into a perithecium body. Inside the perithecia, sterile hyphae, termed paraphyses begin to grow from the top and later attach to the base. Another type of sterile hyphae, called periphyses, form inside the developing ostiole. Once these periphyses and paraphyses mature, they will collapse, creating open space in the perithecia. The collapse of the periphyses creates the opening in the ostiole and the shrunken paraphyses leave an area for the maturing asci. The nucleus within the developing ascus undergoes meiosis, followed by one sequence of mitosis, which creates eight nuclei. These nuclei then become delimited by the formation of ascospore walls (Trail and Common, 2000). The mature perithecia are bright blue-black, globose in shape, with asci that contain eight, one to three septate ascospores in a biseriate arrangement.

Gibberella zeae is subdivided into two groups, Group 1, which does not form perithecia in culture and seldom forms them in the field, and Group 2, which easily forms perithecia under both conditions (Burgess *et al.*, 1975; Francis and Burgess, 1977). Khonga and Sutton have found that *G. zeae* can form perithecia and ascospores for up to three years in the field on infected wheat and corn debris; two years longer than macroconidia formation. This may be related to the nutrient content (high C:N ratio) at later stages of decay being more conducive to perithecium than macroconidium production (Khonga and Sutton, 1988). Formation of perithecia appear to be primarily limited by exhaustion of the host substrate and competition with other organisms (Pereyra *et al.*, 1999). Although these long-term studies have helped to understand the longevity of perithecium formation, little is known about what triggers perithecial formation in the field.

Perithecium formation in *G. zeae* and other fungi appear to favor surfaces that are exposed to direct light (El-Gholl *et al.*, 1979; Halama and Lacoste, 1992; Khonga and Sutton, 1988; Reis, 1990b). It has also been suggested that near ultra-violet (UV) light and RH may be a significant factor in triggering perithecium development in *G. zeae* and other fungi (Halama and Lacoste, 1992; Paulitz, 1996). Under laboratory conditions, a perithecium typically forms and discharges its ascospores within seven days of induction (Trail and Common, 2000). As part of this study, we collected wheat and corn stubble over the course of year, examined it for the presence of mature perithecia, and compared it to local weather data to determine if any correlation exists between perithecium formation and environmental parameters.

Forcible discharge of ascospores. Ingold has described the physiological events of ascospore discharge in detail. First, the mature ascus extends its tip through the ostiole and then rapidly ejects the ascospores. Once an ascus has been emptied, it collapses back into the perithecium body and the next ascus moves into place and fires its spores. This is repeated until all asci have been emptied (Ingold, 1971). Three mechanisms have been proposed to explain how an ascus builds up the necessary force to eject its ascospores: force induced by explosion of a gas-bubble inside the ascus, a spring mechanism involving microtubules, and the bursting of turgid ascus which forces ascospores through its narrow opening.

It was first proposed that a gas-filled bubble might build up enough pressure to forcibly discharge ascospores (Olive, 1964). However, it was later found that ascospore discharge in a species of *Sordaria* was not influenced by changes in air pressure but wase primarily affected by moisture (Ingold and Dann, 1968).

In a transmission electron microscopy study, Czymmek and Klomparens found an extensive microtubule network in the epiplasm of mature ascospores of *Thelebolus crustaceus*. Since microtubules have been implicated in active movement of cells and their organelles, they suggested these microtubules could contract, drawing the ascospores together, and eject the ascospores through a spring mechanism (Czymmek and Klomparens, 1992). It has also been found that benomyl, which inhibits microtubule formation, reduces the discharge of ascospores in *Venturia inaequalis* (Miller, 1970).

The idea that turgor pressure is behind forcible discharge was proposed by C.T. Ingold (1968). He observed a stretching of the ascus wall in *Loramyces sp.* as it built up osmotic pressure under moist conditions. This stretching eventually caused the ascus to burst and subsequently contract, which forced the ascospores out of a narrow slit at the top of each ascus (Ingold, 1968). In a field study of *Venturia inaequalis*, a relationship was found between leaf surface wetness and the mean distance ascospores were fired. A correlation between increased ascospore discharge and high RH or rainfall has been observed in several fungi *Venturia inaequalis* (Gadoury *et al.*, 1998) (Stensvand *et al.*, 1998), *Anisogramma anomala* (Pinkerton *et al.*, 1998), and *Pleospora allii* (Prados Ligero *et al.*, 1998). Some early studies showed a similar correlation in *G. zeae* (Chen and Yuan, 1984; Reis, 1990a). However, a later study by Paulitz (1996) did not find a definitive correlation between ascospore discharge by the perithecia of *G. zeae* and moisture in the field. During periods of heavy rainfall, ascospores were not forcibly discharged, but instead oozed out of the ostiole in a mucilaginous spore-tendril, or cirrus. However, since microclimates on the surface of the debris were difficult to evaluate, he concluded that high moisture levels were still important for perithecium and ascospore

formation, and RH may play a role in discharge in addition to cirrhi production. (Paulitz, 1996). Under laboratory conditions, it has also been observed that although discharge occurred at low RH levels, the maximum number of spores were released in 100% RH conditions (Trail *et al.*, 1998).

Since many fungi are known to actively discharge their spores, determining the mechanism behind this phenomenon would lead to an increased understanding of the physiology of spore dissemination. The role of environmental parameters is difficult to determine in fungi, due in part, to microclimates that exist around a perithecium on its host. Physiological study of the thin-walled, delicate asci are also problematic. Instead, we have chosen to study this mechanism by disabling ascospore discharge through a random gene disruption process, and study phenotypic changes *in vitro*. Based on current studies, some mutant phenotypes that might affect discharge would be the following: inability to build up osmotic pressure in the ascus, limited microtubule formation, changes in composition of the epiplasm of ascospores, and morphological mutations of the paraphyses, ostiole, or asci.

Molecular study of ascospore discharge. Random insertional mutagenesis can be useful for a large-scale search for genes involved in a specific mechanism. A large number of tagged transformants can be generated and then screened for a change in the phenotype of interest. In this study, a screening method was developed that quickly distinguished isolates that had lost their ability to forcibly eject their spores. Mutants showing a change in this phenotype could then be studied in further detail.

Random mutagenesis had been traditionally performed by exposure of the organism to UV light or a mutagenic chemical. Ultra-violet (UV) mutagenesis has been used for

G. zeae as an effective method of generating large numbers of mutants (Leslie, 1983). However, UV and chemical mutagenesis do not leave any molecular tag indicating the location of the affected gene(s). Recombinant DNA technology has currently become a powerful tool in molecular research. This technology allows for integration of foreign DNA into the host genome, leaving a molecular marker at a specific gene. Mechanical methods of fungal cell wall disruption such as the gene gun or biolistics (Armaleo *et al.*, 1990; Lorito *et al.*, 1993) (Durand *et al.*, 1997) (Yu Jieh and Cole Garry, 1998), and electroporation (Chakraborty *et al.*, 1991; Redman and Rodriguez, 1994), have been successfully used to allow incorporation of foreign DNA. Polyethylene-glycol (PEG) mediated transformation of protoplasts has also been shown to achieve an increased rate of transformation, particularly when coupled with restriction enzyme mediated integration (REMI) in some fungi (Brown *et al.*, 1998; Redman and Rodriguez, 1994). Dissolution of the cell wall occurs during enzymatic digestion followed by the uptake of foreign DNA in a PEG solution (transformation). Foreign DNA is linearized with restriction enzymes prior to transformation. REMI transformation, originally developed for *Dictyostelium discoidium* (Kuspa and Loomis, 1992), uses additional restriction enzymes to nick the host DNA. This is thought to facilitate insertion of the linearized plasmid. The REMI system has been successfully used to isolate desirable mutants in many fungi including *Saccharomyces cerevisiae* (Schiestl and Petes, 1991), *Ustilago maydis* (Bolker et al, 1995), *Cochliobolus heterostrophus* (Lu *et al.*, 1994), *Magnaporthe grisea* (Shi *et al.*, 1995; Sweigard *et al.*, 1998), *Aspergillus nidulans* (Sanchez *et al.*, 1998), and *Penicillium paxilli* (Itoh and Scott, 1997).

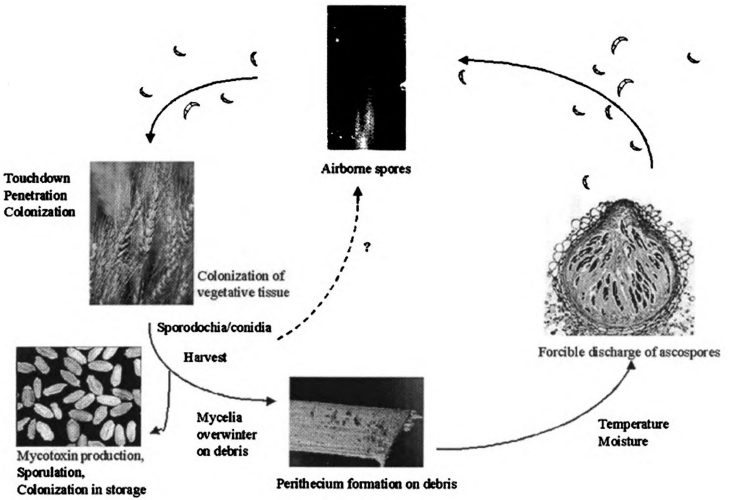

Figure 1: Disease cycle of *Gibberella zeae* (*Fusarium graminearum*)

CHAPTER 1

PERITHECIA SURVEY OF *G. ZEAE*

INTRODUCTION

Fusarium Head Blight (FHB) or scab of wheat (*Triticum aestivum* L.), caused by *Gibberella zeae* (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe), has reached epidemic proportions in the United States over the past decade. Fusarium Head Blight has incurred over 4 billion dollars in yield losses to wheat and barley growers in the U.S. between 1993 and 1999 (McMullen, 1999). Yield losses in wheat occur primarily as a result of sterile florets and diminished, low weight kernels (McMullen, 1999). *Gibberella zeae* also produces the mycotoxins deoxynivalenol (DON) and zearalenone that cause vomiting, feed refusal, and reproductive disorders when contaminated grain is fed to livestock (McMullen, 1999; Stanton, 1995).

The species is subdivided into two groups: Group 1, seldom forms perithecia in the field and does not form them in culture; Group 2, easily forms perithecia under both conditions (Burgess *et al.*, 1975; Francis and Burgess, 1977). The disease cycle of FHB has been well described (Parry *et al.*, 1995; Sutton, 1982). *Gibberella zeae* overwinters as saprophytic mycelia on the crop debris of maize, wheat, and barley. The fungus undergoes sexual reproduction and produces ascospores in a fruiting body known as a perithecium. Ascospores are forcibly discharged from perithecia and become airborne. The spores are deposited on the wheat head sometime during flowering (Paulitz, 1996; Sutton and Procter, 1982) when susceptibility is highest (Fernando *et al.*, 1997). Ascospores serve as the primary source of inoculum, although the asexual macroconidia may also produce some localized infection due to wind and splash dispersal (Fernando *et*

al., 1997; Parry *et al.*, 1995; Sutton, 1982). The hyphae then penetrate the glume stomata (Pritsch, 2000), and spread throughout the wheat head resulting in chlorosis of infected kernels (Parry *et al.*, 1995; Sutton, 1982). Favorable conditions for disease outbreaks appear to coincide with extended periods of high relative humidity (RH) and warm temperatures (24 - 29°C). (McMullen *et al.*, 1997a).

An effective and consistent method to control FHB has not yet been established (Parry *et al.*, 1995). Crop rotation and tillage, when employable, can reduce the severity of FHB. Incidence of FHB increases when wheat is planted immediately after maize or cereals (wheat, barley, oats) (McMullen, 1999; Teich and Hamilton, 1985; Teich and Nelson, 1984; Tusa *et al.*, 1981) as compared to a non-host crop. Deep tillage that can bury crop debris has been shown to be effective in reducing levels of primary inoculum (Teich, 1989).

Gibberella zeae can form perithecia and ascospores for up to three years in the field on infected wheat and corn debris; two years longer than macroconidia formation (Khonga and Sutton, 1988). Formation of perithecia appear to be primarily limited by exhaustion of the host substrate and competition with other organisms (Pereyra *et al.*, 1999). Although these long-term studies have helped to understand the longevity of perithecium formation, little is known about what environmental triggers may induce formation. Perithecium formation in *G. zeae* and other fungi appears to favor the light-exposed surfaces of plant debris (El-Gholl *et al.*, 1979; Halama and Lacoste, 1992; Khonga and Sutton, 1988; Reis, 1990b). It has also been suggested that day length, light intensity, wavelength and relative humidity (RH) may all be significant factors in triggering perithecium development in *G. zeae* and other fungi (Halama and Lacoste,

1992; Paulitz, 1996). Under laboratory conditions, perithecia typically form and discharge ascospores within 7-14 days of induction (Trail and Common, 2000).

Knowledge of the timing of formation of perithecia in the field with respect to flowering of wheat would be important in designing novel control methods. To evaluate the timing of perithecium formation in the field, we collected wheat and corn stubble from commercial fields throughout the year from 1997 to 2000. Additionally, we analyzed the timing of perithecium formation to determine if it positively correlated to local temperature and rainfall. Based on previous research, our hypothesis was that the timing of perithecium formation coincides with the cool, wet weather conditions that that typically occur near the time of wheat flowering.

MATERIALS AND METHODS

Field collection. Between June 1997 to March 2000, thirteen commercial fields were surveyed that had experienced previous outbreaks of Fusarium Head Blight (FHB) in Ingham County, Michigan. The traditional crop rotation scheme is corn, soybean, and wheat, however rotation patterns often varied. Fields containing wheat or corn stubble from the previous year's harvest were sampled (Table 2). A decreasing number of fields were sampled in 1999 and 2000 due to progressive replacement of wheat by soybean in the crop rotation scheme.

Debris samples that had visible perithecia were collected monthly unless impeded by snow cover. During the period of wheat flowering, sampling was increased to two-week intervals. Eight locations were sampled along transects in each field (A1-D2) in a diamond pattern (Figure 2). The length of transects were scaled to the acreage of each field. Two to four samples exhibiting symptoms of infestation were collected from each

location. Samples were stored in plastic bags at –20° C. Daily weather data, from the nearest National Weather Service (NWS) station at the Lansing Capitol Airport, was obtained from the National Oceanic and Atmospheric Administration National Climatic Data Center (NOAA-NCDC) database (NOAA-NNDC, 2000). All fields were within a 60-mile radius of the NWS station.

Identification. Corn and wheat stubble were microscopically examined at 70X magnification for the presence or absence of perithecia. Species identification was confirmed by perithecium wall structure, color and ascospore morphology at 400X magnification as described by Nelson *et al.* (1983). Samples harboring one or more developing perithecia of *G. zeae* were scored as positive.

Data Analysis. Data was expressed as a proportion of samples with perithecia per total samples collected. The proportion of samples with perithecia was compared to calendar days. Wheat stubble samples were not included in analyses due to the low number of mature perithecia.

The distribution of proportion data was normalized with the arcsine square root transformation. All analyses were performed using proc REG of SAS version 7.0 (SAS Institute, Cary, N.C.). Single factor regression analysis was used to evaluate the effect of rainfall and temperature. A stepwise regression analysis for all variables was used to determine if combined variables increased correlation. Variables used are defined in Table 1.

Table 1. Variables for regression analysis.

Label	Time period included
T4	Average daily temperature 1 – 7 days preceding sampling
T7	Average daily temperature 4 – 10 days preceding sampling
T14	Average daily temperature 11 – 17 days preceding sampling
R4	Average daily rainfall 1 – 7 days preceding sampling
R7	Average daily rainfall 4 – 10 days preceding sampling
R14	Average daily rainfall 11 – 17 days preceding sampling

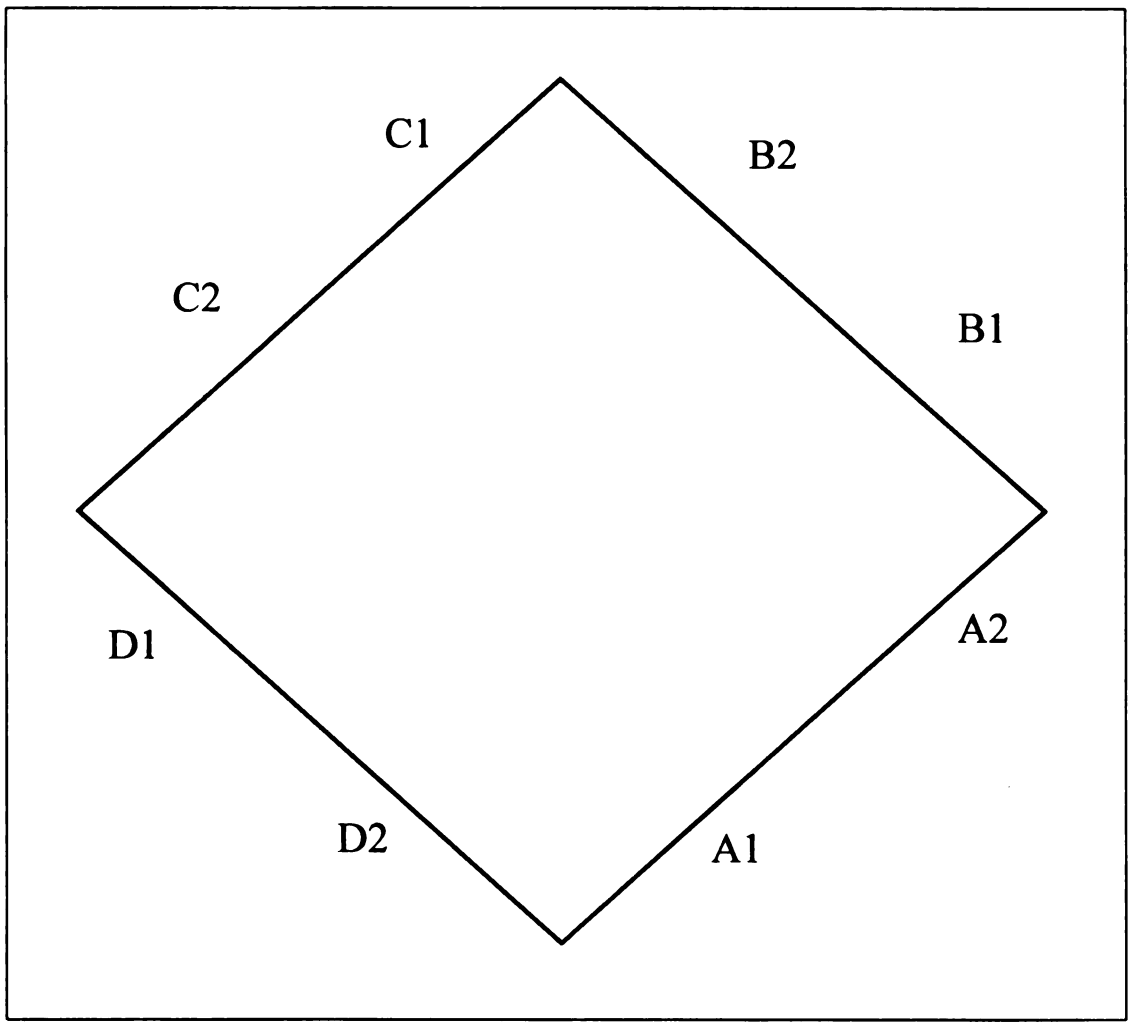

Figure 2. Sampling pattern for crop debris collection. Outer square indicates field perimeter, diamond indicates sampling transect. Letter and number notations locate sampling locations along the transect.

RESULTS

Of the 2186 samples collected, 272 were found to have perithecia of *G. zeae* (Table 1). Approximately 83% of the samples with perithecia were found on corn debris [(241 of the 1532 corn debris samples or 15%) (31 of the 654 wheat debris samples or 3% had perithecia)]. Over the three years sampled, the highest proportion of samples with perithecia were found during the summer months, with the highest number in May, 1997 and 1998 and July, 1999 (Figure 3).

Table 2. Field data summary.

Sampling year	Average No. of fields	No. Corn debris fields	No. Wheat debris fields	Total No. corn samples	Total No. wheat samples	Corn samples w/ perithecia	Wheat samples w/ perithecia
Spring 1997 – Fall 1997	10	8	2	320	80	31	1
Fall 1997-Fall 1998	11	7	4	368	312	40	24
Fall 1998-Fall 1999	9	7	2	684	200	161	6
Fall 1999-Winter 2000	8	5	2	160	62	9	0
TOTAL	**38**	**27**	**10**	**1532**	**654**	**241**	**31**

In single variable regression, variables T4 and T14 were found to have the highest correlation with the proportion of corn debris samples with perithecia (Table 2). Temperatures below 9° C appear to inhibit perithecium formation (Figure 4). If data points of T14 below 9° C are removed from the analysis, the R^2 of T14 increases to 0.75.

Table 3. Effect of temperature on development of perithecia.

Condition at days prior to collection	F value	$P^a > F$	R^2	Adjusted R^2
T4	12.290	0.0017	0.2949	0.2949
T14	13.168	0.0012	0.3107	0.3107
T4 and T14 days	7.42	0.0030	0.3725	-
T14 using 9° C threshold	50.866	0.0001	0.7495	0.7348

[a] Regressions were significant at $P < 0.05$

Stepwise regression analysis showed that inclusion of all other variables (T7, R4, R7, R14) in the T4 and T14 model improved the R^2 (including data below the 9° C threshold) to 0.4811, but no individual variable had a large effect on the predictivity of the model. Combination of T4 and T14 with a stepwise regression analysis showed that R^2 was only slightly improved, indicating that the variables may covary.

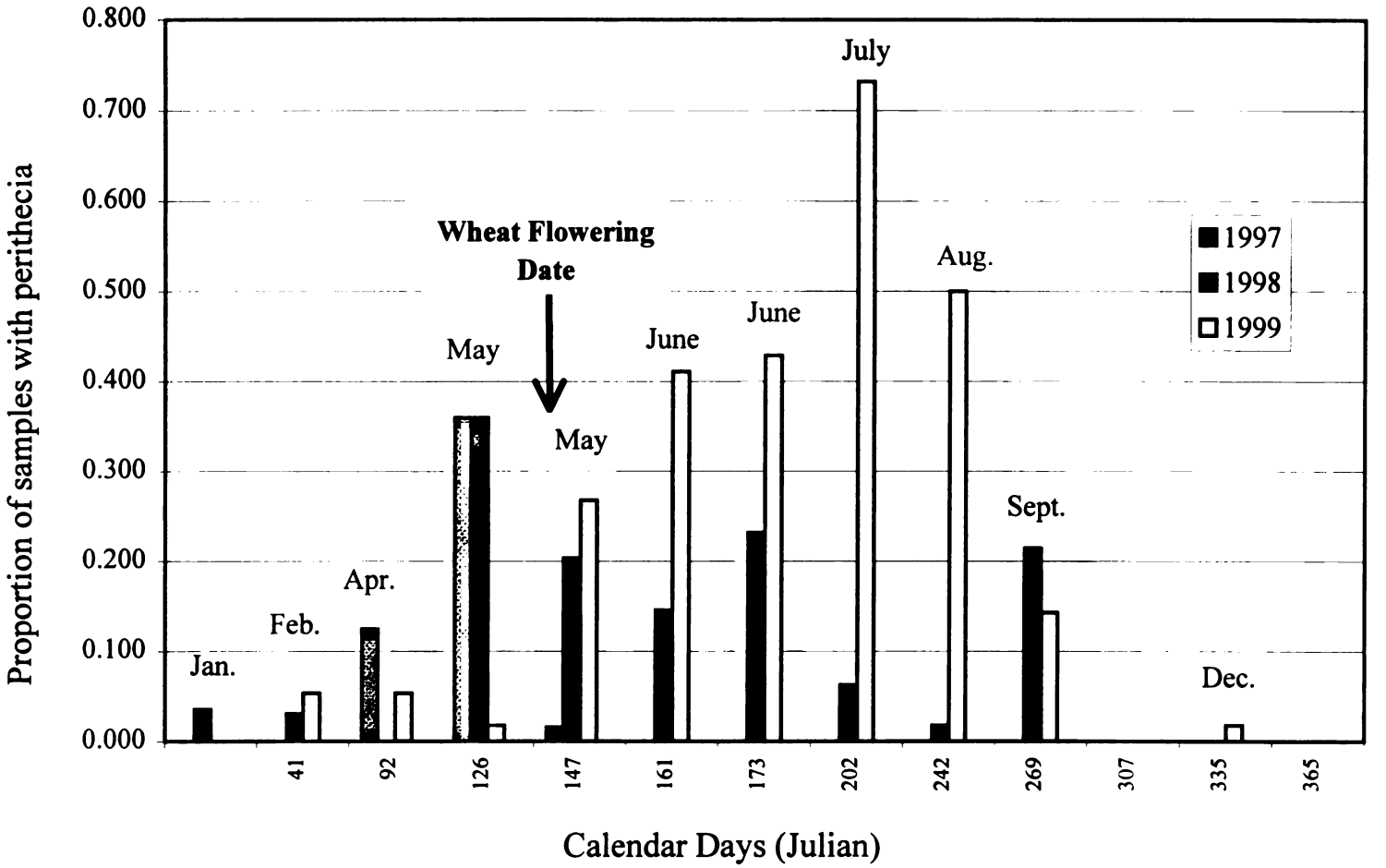

Figure 3: Timing of perithecium production on corn debris samples 1997 - 1999

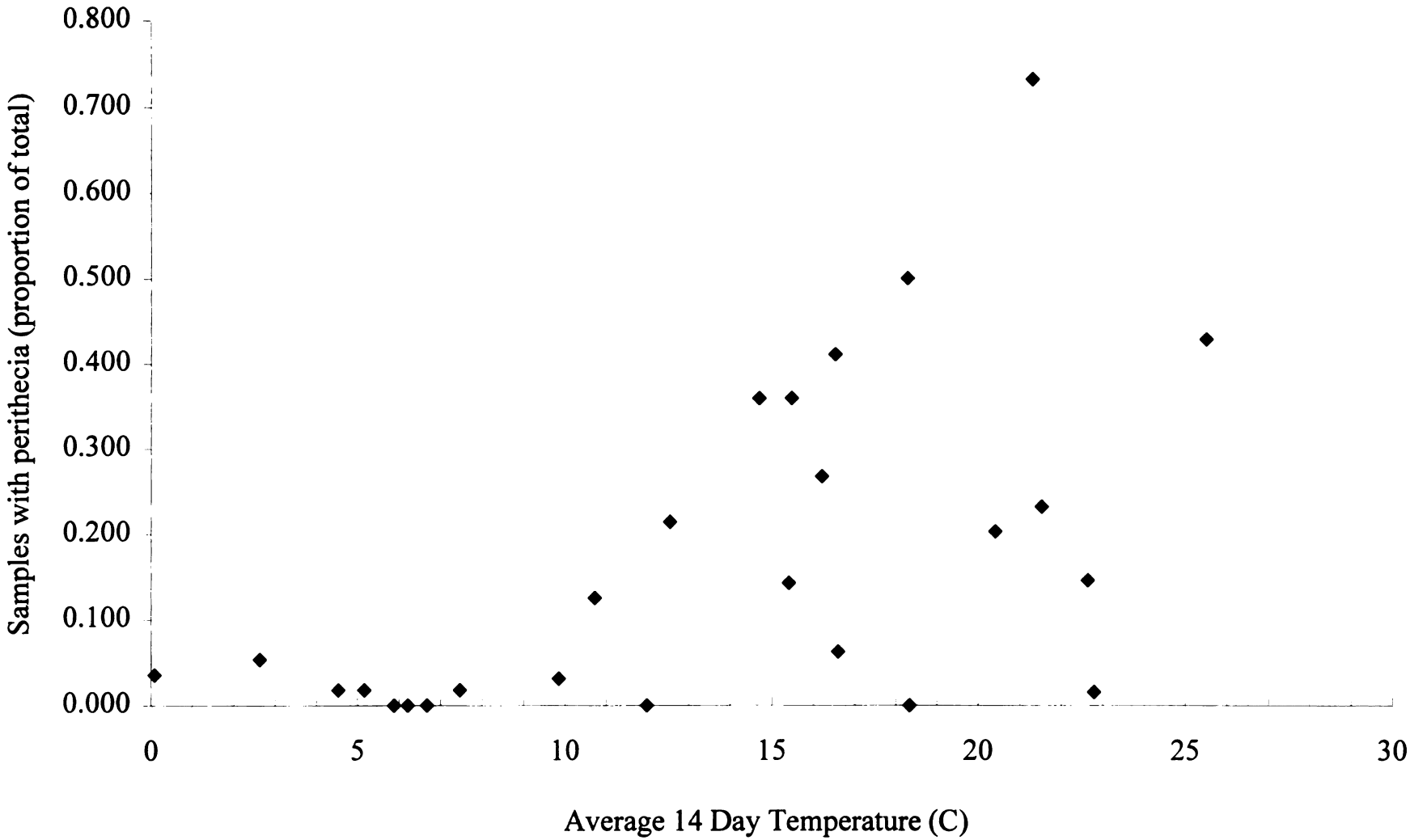

Figure 4. Proportion of perithecia in relation to average 14 day temperature

DISCUSSION

In the study area, corn stubble was the predominant substrate for perithecium formation of *G. zeae* (83% of total debris samples with perithecia). However, the disease outbreak of FHB in wheat fields was minimal in 1998 and 1999 due to hot, dry conditions during the spring (Hart, 1998; 1999). Weather conditions that decreased colonization of the wheat plants would have resulted in lower numbers of perithecia on wheat stubble.

Average daily temperatures below 9° C appeared to limit perithecium formation at T14, and temperatures above 9° C at T4 and T14 were found to have a linear correlation to the proportion of perithecia present. Combining these two factors in a stepwise regression only increased the R^2 slightly, which indicates a covariance. The covariance of these two factors indicates they may both be necessary for perithecium formation. Mature perithecia of *G. zeae* form within 7-14 days following induction under laboratory conditions (Trail *et al.*, 1998). The data did not show a limiting high temperature for perithecium formation.

There was a peak in the proportion of samples with perithecia in 1997 and 1998 just prior to wheat flowering. In 1999, the peak occurred in July after heavy rainfall in late June and early July (approximately 7 in). Rainfall in May (near the time of wheat flowering) was minimal (approximately 1.7 in.) and may have delayed perithecium formation until conditions became more favorable in June and July. Although it is likely moisture also plays a role (Paulitz, 1996), the rainfall data used in this study was collected 25 – 60 miles away from the field sites and may not adequately represent field conditions.

Perithecium formation may also be linked to day length and solarization (El-Gholl *et al.*, 1979; Halama and Lacoste, 1992). Although microclimates on the surface of the debris were not monitored they would be a major factor in perithecium development (Paulitz, 1996). Further studies that monitor on-site weather conditions and controls for factors such as plant variety, pesticide and fertilizer applications, and initial inoculum are needed to construct a predictive model for *G. zeae* perithecium development.

CHAPTER 2

USE OF RANDOM INSERTIONAL MUTAGENESIS TO STUDY THE MECHANISM OF ASCOSPORE DISCHARGE IN *GIBBERELLA ZEAE*

INTRODUCTION

Fusarium Head Blight (FHB) or scab of wheat (*Triticum aestivum* L.), caused by *Gibberella zeae* (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe), has reached epidemic proportions in the United States over the last ten years. Fusarium Head Blight has incurred over 4 billion dollars in yield losses to wheat and barley growers in the U.S. between 1993 and 1999 (McMullen, 1999). Yield losses in wheat occur primarily as a result of sterile florets and diminished, low weight kernels (McMullen, 1999). *G. zeae* also produces the mycotoxins deoxynivalenol (DON) and zearalenone that cause vomiting, feed refusal, and reproductive disorders when contaminated grain is fed to livestock (McMullen, 1999; Stanton, 1995).

The disease cycle of FHB has been well described (Parry *et al.*, 1995; Sutton, 1982). *Gibberella zeae* overwinters as saprophytic mycelia on crop debris of maize, wheat, and barley. In the spring, the fungus undergoes sexual reproduction and produces ascospores in a fruiting body known as a perithecium. The forcible discharge of ascospores, possibly along with wind and splash dispersal of the asexual macroconidia, serves to distribute the primary inoculum. Although both spore types may be deposited on the wheat head sometime during flowering, (Paulitz, 1996; Sutton and Procter, 1982) the forcible release of ascospores likely plays a major role in spreading the disease over large distances (Fernando *et al.*, 1997).

The process of ascospore discharge has been described by Ingold (1971). A mature ascus swells and then rapidly ejects its ascospores through the ostiole. Once an ascus has been emptied, it collapses back into the perithecium body and the next ascus moves into place and fires its spores. This is repeated until all asci have been emptied. The mechanism for pressure build-up inside the ascus is not well understood in any fungus.

The idea that turgor pressure is behind forcible discharge was proposed by C.T. Ingold (1968). He observed a stretching of the ascus wall in *Loramyces sp.* as it built up osmotic pressure under moist conditions. This stretching eventually caused the ascus to burst and subsequently contract, which forced the ascospores out of a narrow slit at the top of each ascus. The role of moisture in ascospore discharge has been described in field studies of several fungi, *G. zeae* (Chen and Yuan, 1984; Reis, 1990a), *Venturia inaequalis* (Gadoury *et al.*, 1998) (Stensvand *et al.*, 1998), *Anisogramma anomala* (Pinkerton *et al.*, 1998), and *Pleospora allii* (Prados Ligero *et al.*, 1998). Under laboratory conditions, it has also been observed *G. zeae* discharged the maximum number of spores in 100% RH conditions (Trail *et al.*, 1998).

In addition, a spring mechanism has been proposed based on the presence of an extensive microtubule network in the epiplasm of mature asci of *Thelebolus crustaceus* (Czymmek and Klomparens, 1992). It has also been found that benomyl, which inhibits microtubule formation, reduces the discharge of ascospores in *Venturia inaequalis* (Miller, 1970).

Random insertional mutagenesis can be useful for a large-scale search for genes involved in a specific mechanism. A large number of tagged transformants can be generated and then screened for a change in the phenotype of interest. In this study, a

screening method was developed that quickly distinguished isolates that had lost their ability to forcibly eject their spores. Mutants showing a change in this phenotype could then be studied in further detail.

Polyethylene-glycol (PEG) mediated transformation of protoplasts has also been shown to achieve an increased rate of transformation, particularly when coupled with restriction enzyme mediated integration (REMI) in some fungi (Brown *et al.*, 1998; Redman and Rodriguez, 1994). REMI transformation, originally developed for *Dictyostelium discoidium* (Kuspa and Loomis, 1992), uses additional restriction enzymes to nick the host DNA which is thought to facilitate insertion of the linearized plasmid. The REMI system has been successfully used to isolate desirable mutants in many fungi *Saccharomyces cerevisiae* (Schiestl and Petes, 1991), *Ustilago maydis* (Bolker et al, 1995), *Cochliobolus heterostrophus* (Lu *et al.*, 1994), *Magnaporthe grisea* (Shi *et al.*, 1995; Sweigard *et al.*, 1998), *Aspergillus nidulans* (Sanchez *et al.*, 1998), and *G. pulicaris* (Salch and Beremand, 1993).

We have chosen to elucidate the mechanism of discharge in *G. zeae* through a random gene disruption strategy. We believed random disruption of a large number of isolates would produce a mutant(s) that was unable to discharge their spores. Subsequent characterization of the disrupted genes may lead to a better understanding of the processes involved in forcible ascospore discharge. Since many fungi are known to actively discharge their spores, determining the mechanism behind this phenomenon would lead to an increased understanding of the physiology of spore dissemination and novel methods for control of these fungi.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Two strains of *G. zeae* (*Fusarium graminearum*) were used for transformation. Strain W-8 (U-5373) (Adams *et al.*, 1987) was provided by G. Adams (Michigan State University). Strain PH-1 is a field isolate from Michigan provided by L.P. Hart (Michigan State University). Nitrate-nonutilizing mutants 4232 and 4233, provided by R. Bowden (Kansas State University) were used for meiotic stability tests.

Two plasmids were used for insertional mutagenesis, both harboring the coding region on *E. coli* for the hygromycin B phosphotransferase gene (*hph*) (Appendix A). Plasmid pHA1.3 has the *hph* coding region linked to the *Aspergillus parasiticus trpC* promoter and terminator sequences (Redman and Rodriguez, 1994). Plasmid pUCH2-8 (Hohn, 1997) has the *hph* coding region fused to promoter 1 from *Cochliobolus heterostrophus* (Turgeon *et al.*, 1987).

Spore suspensions of fungal strains were prepared by flooding 4 day old cultures on carrot agar (CA) medium (Bowden and Leslie, 1999) with approximately 0.01% Tween 20. All strains were maintained as soil stocks at –20°C (Nelson *et al.*, 1983).

Transformation. Transformations were performed on germinated conidia using a previously published protocol (Hohn, 1997) with the following modifications. Approximately 0.3 g soil stock was used to inoculate carboxymethylcellulose (CMC) medium and incubated for 72 hr at 25°C at 250 rpm (Cappellini and Peterson, 1965).. Conidia were harvested by centrifugation and germinated in YEPD (0.3% yeast extract, 1% bactopeptone, and 2% D-glucose) broth for 12 - 14 hours at room temperature (RT) at 175 rpm. Isolation of protoplasts occurred in 25 mg/ml driselase, 0.05 mg/ml chitinase

(Sigma Chemical Co., St. Louis), and either 0.5 mg/ml mureinase (USB-Amersham Pharmacia Biotech Inc., Piscataway, NJ) or 5 mg/ml lysing enzyme (Sigma Chemical Co., St. Louis) in a 1.2 M KCl buffer. Protoplasts were collected by filtration through a 30 μm Nitex nylon membrane (Tetko Inc., Kansas City, MO) and washed three times in STC buffer (1.2 M sorbitol; 10 mM Tris-HCl, 50 mM $CaCl_2$, pH 8.0). Transformation took place in the presence of 30% polyethylene glycol solution (10 mM Tris-HCl, 50 mM $CaCl_2$, pH 8.0), STC buffer and linearized plasmid. Restriction enzyme mediated integration (REMI) transformations included an additional 10, 50, or 100 units of *NdeI* or *HindIII*. Protoplasts recovered on Regeneration Medium (RM): 0.1% yeast extract, 0.1% casein enzyme hydrosylate, 0.8 M sucrose , 1% agarose, for 15 hr and then were overlaid with 10 ml of RM amended with 150 μg/ml hygromycin B (HygB) (Calbiochem-Novabiochem Corp., San Diego, CA). Putative transformants were selected within 4 - 7 days and selected again on V8 juice (VM) medium amended with HygB. Unless otherwise noted, hygromycin transformants were screened on 450 μg/ml for strain PH-1 and 300 μg/ml for strain W-8. Hygromycin resistant (Hyg^R) colonies were transferred to a 2% water agar (WA) medium and hyphal-tipped to obtain genetically pure isolates. Detailed protocols for small and large-scale transformations are presented in Appendix B and C.

Screening for mutants. To induce perithecium formation, transformants were placed on CA (Klittich and Leslie, 1988) in 24 cell well microplates [17 mm well diameters (Evergreen Inc., Los Angeles, CA)] or 60 mm Petri plates. Cultures were maintained under continuous 15-watt cool white and black light fluorescent lighting. After 2 - 4 days of growth, perithecia were induced from vegetative hyphae with 200 μl (microplates) or 1

ml (Petri plates) of 2.5% aqueous Tween 60 solution (Bowden and Leslie, 1999). Approximately 7 - 14 days after perithecium induction, the plate lid was examined under a dissecting microscope at 50X magnification for discharged ascospores. Cultures with plate lids that had no visible accumulation of ascospores were screened more vigorously as follows. Agar blocks containing approximately 20 - 40 perithecia were removed from mature cultures and placed on a glass slide so that spores would be discharge in a trajectory that ran parallel to the slide. Blocks were placed in a high humidity chamber. The perithecia were kept in the chamber for 1 - 2 days away from direct light and re-examined microscopically. A detailed screening protocol is presented in Appendix D. Discharge mutants were embedded and sectioned as described in Trail and Common (2000) and examined microscopically.

Screening for auxotrophic mutants. Transformants were tested for nitrate utilization by growing on Minimal Medium (Corell, 1987) amended with 0.05% Tergitol Type NP-10 (Sigma Chemical Co., St. Louis, MO) and 2% sorbose (MMTS) (Bowden and Leslie, 1999). Nitrate non-utilizing (Nit^-) and nitrate utilizing (Nit^+) phenotypes have distinct morphologies on MMTS and are easily distinguished. Transformants unable to grow on MMTS were then rescreened on Czapeks medium (Difco Laboratories, Detroit, MI) which would not support the growth of Nit^- mutants.

Molecular analyses of transformants and progeny. Transformants were grown in a 2% yeast extract, 6% sucrose (YES) broth for 4 - 6 days. Mycelium was collected by filtration and stored at -80° C and subsequently ground to a fine powder using liquid nitrogen. DNA was isolated either according to the manufacturer's instructions using

DNeasy Plant Maxi Kit (Qiagen Inc., Valencia, CA) or by the Autogen DNA isolation system.

Plasmid DNA isolation and Southern hybridization analyses were performed using published procedures (Ausubel *et al.*, 1987). Digested genomic DNA was separated on an 0.8% agarose gel in TBA buffer (0.44 M Tris-HCl, 0.01 M EDTA, 0.44 M boric acid, pH 8.0) and subsequently transferred to a nylon Hybond-N+ membrane (USB-Amersham Pharmacia Biotech Inc., Piscataway, NJ). Restriction enzymes were used as instructed by supplier (Gibco BRL, Grand Island, NY, or Boehringer Mannheim Biochemicals, Indianapolis, IN). Linearized probe DNA (pHA1.3 or pUCH2-8) was labeled with [α-^{32}P]dCTP using Gibco Random Primers DNA Labeling System (Gibco BRL, Grand Island, NY).

Mitotic stability test. Twelve transformants that were Hyg^R (six containing pHA1.3 or six containing pUCH2-8) were placed on VM amended with HygB, and incubated at 25° C for 7 days. Mycelia were transferred to VM without HygB and incubated under the same conditions. This procedure was repeated three times.

Meiotic stability test. Transformants showing deviations in perithecium formation or ascospore discharge were selfed and rescreened for phenotype. Transformants were also mated with nitrate-non-utilizing (Nit^-) hygromycin sensitive (Hyg^S) mutants 4232 and 4233. Ascospores were selected from eight perithecia and germinated on MMTS medium. Colonies were then placed on VM amended with HygB. Inheritance of mutations was compared to expected Mendelian ratios.

RESULTS

Two fungal strains of *G. zeae* were used to generate and screen 5005 transformants (139 with W-8 and 4866 with PH-1). Of these, four mutants (all PH-1) were identified that failed to forcibly discharge ascospores in our assays. Three of the isolates were developmental mutants that did not form mature asci and ascospores. One transformant, designated 729G-112, appeared to develop normal perithecia and ascospores and was studied in further detail.

Transformation. Transformation of protoplasts of *G. zeae* was conducted by incubation of protoplasts of linearized plasmid DNA, with and without the presence of restriction enzymes (10, 50, or 100 units of *NdeI* or *HindIII*). Rates of transformation were higher without REMI (0.2 - 2.37 transformants (Tx)/μg plasmid DNA using linearized pHA1.3 only and 0.08 Tx/μg plasmid DNA from pHA1.3 using REMI). The transformation rate of protoplasts incubated with linearized pUCH2-8 was 0.2 - 1.2 Tx/μg plasmid DNA. No transformants were recovered from control transformations without vector DNA. Strain PH-1 was weakly resistant to low concentrations of HygB (100 – 400 μg/ml) so only transformants able to grow vigorously on VM containing 450 μg/ml HygB were selected. Strain W-8 was more sensitive to HygB and was selected at 300 μg/ml.

Molecular analysis of transformants. Examination of 20 putative transformants (10 from pHA1.3 and 10 from pUCH2-8) by Southern analysis indicated that both plasmids had successfully integrated into the genome by single or multiple-copy insertion (Figure 5).

Analysis of pUCH2-8 transformants digested with *ClaI*, which cuts outside, indicated single, multiple, and possibly partial copy insertions. Lanes 2 and 7 – 10 are considered to be single-copy insertions with the additional bands attributed to partial digestion of high concentrations of genomic DNA. The presence of three or more bands in lanes 1, 3, and 4, indicates multiple insertion of pUCH2-8. Lanes 1 and 6 may contain partial copy insertions as each contain a band(s) approximately 1 – 2 Kb smaller than the size the whole plasmid.

Analysis of pHA1.3 transformants digested with *ClaI*, which cuts once within the plasmid, revealed the majority of transformants had a single-copy insertion (lanes 11 - 18, 20). One isolate, shown in lane 19, has four bands, which indicates multiple insertions of pHA1.3. This isolate was transformed with pHA1.3 DNA that had been cut by *NdeI*, which cuts twice within the plasmid. This may have resulted in multiple insertions of truncated copies of pHA1.3.

Isolates in lanes 15 - 18 and 20 were further analyzed by digestion with *XhoI* which does not cut within the plasmid. The presence of a single band in each sample, ranging from 4 – 6 kilobases (Kb) in size (lanes 15a – 18a, and 20a), suggests these isolates do not result from a tandem repeat insertion. A tandem repeat would be indicated by the presence of a fragment at least twice the size of the plasmid (>12 Kb) when digested with a restriction enzyme that cuts outside the plasmid.

Undigested samples separated on 0.8% agarose gel did not show migration of low molecular weight DNA that would indicate an autonomous plasmid (data not shown). Southern analysis did not reveal any hybridization to bands that were similar in size to an

autonomous plasmid (data not shown). Both DNA probes, linearized labeled pHA1.3 and pUCH2-8, did not hybridize to untransformed PH-1 (data not shown).

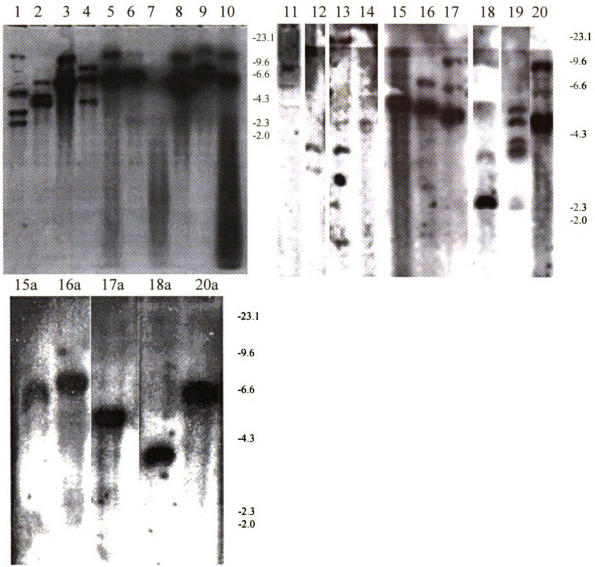

Figure 5. Southern-hybridization analysis *G. zeae* transformants. DNA from *G. zeae* isolates transformed with pUCH2-8 and digested with restriction enzyme *ClaI* (recognizes no restriction sites in the vector) (lanes 1 – 10). DNA from *G. zeae* isolates transformed with pHA1.3 and digested with restriction enzyme *ClaI* (recognizes one restriction site in the vector) (lanes 11 – 20) and *XhoI* (recognizes no restriction site in the vector) (lanes 15a-18a and 20a). Lane 11, discharge minus mutant. Probed with ^{32}P labeled with *HindIII* cut pUCH2-8 (lanes 1-10) or pHA1.3 (lanes 11 – 20).

Mitotic stability and insertional pattern. Twelve isolates (six transformed with each vector) were subjected to a mitotic stability test by transferring three successive times on and off VM amended with HygB. Southern hybridization indicated the integrated plasmid typically remained stable throughout these transfers (Figures 6 and 7). However, a distinct heavy band appeared in DNA at week 7 of the isolate shown in lanes 2a and b in Figure 6. Differences in the size of large fragments in lanes 3a and b, 4a and b, and 5a and b are attributed to partial digests of genomic DNA.

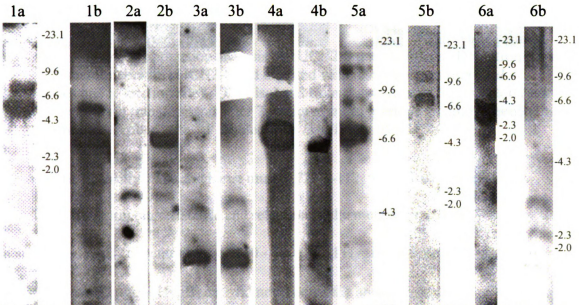

Figure 6. Southern hybridization analysis of pHA1.3 transformants. DNA from *G. zeae* isolates transformed with pHA1.3 and digested with restriction enzyme *ClaI* (recognizes one restriction site). DNA isolated from transformants from week 0 (lanes a) DNA isolated from transformants after 7 weeks of transfer on/off medium amended with HygB (lanes b). Probed with ^{32}P labeled with *HindIII* cut pHA1.3.

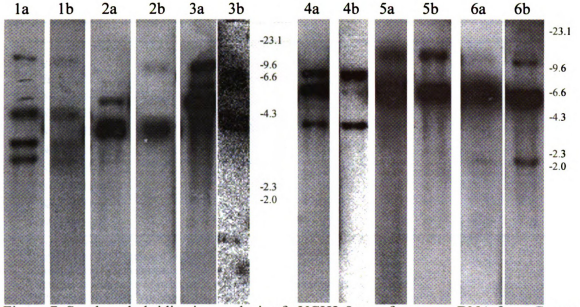

Figure 7. Southern hybridization analysis of pUCH2-8 transformants. DNA from *G. zeae* isolates transformed with pUCH2-8 and digested with restriction enzyme *ClaI* (recognizes one restriction site). DNA isolated from transformants from week 0 (lanes a) DNA isolated from transformants after 7 weeks of transfer on/off medium amended with HygB (lanes b). Probed with ^{32}P labeled with *HindIII* cut pUCH2-8.

Meiotic Stability. The discharge mutant 729G-112, was selfed and tested for stability of hygromycin resistance and retention of the mutant phenotype. Progeny were resistant to HygB concentrations of 150 - 300 μg/ml HygB (compared to parental resistance at 450 μg/ml HygB) and did not recover the ability to discharge ascospores.

The genetic inheritance of mutations was tested by mating selected mutants with isolates unable to utilize nitrate (Bowden and Leslie, 1999). Progeny were tested on MMTS for nitrate utilization, screened for perithecia production and ascospore discharge on CA, and then screened on VM amended with 150 - 300 μg/ml HygB. Cosegregation of the phenotype and hygromycin resistance was observed in 729G-112 and 729R-57 for the majority of progeny. Transformants 720R-2 and 610A-173 produced few viable ascospores after mating with Nit$^-$ isolates (Table 4). The Nit$^-$ phenotype segregated independently.

Table 4. Segregation analyses of mutations.

	Mutants			
	729G-112[a]	720R-2[b]	729R-57[b]	610A-173[b]
Vector[c]	PHA1.3	PUCH2-8	PUCH2-8	PHA1.3
Enzyme[d]	*HindIII*	*HindIII*	*HindIII*	*HindIII*
$Nit^{+[e]}Dis^{+[f]}Hyg^{R[g]}$	1	0	0	0
$Nit^{+}Dis^{-}Hyg^{S}$	1	0	0	1
$Nit^{+}Dis^{-}Hyg^{R}$	15	0	3	0
$Nit^{+}Dis^{+}Hyg^{S}$	17	0	4	1
$Nit^{-}Dis^{-}Hyg^{R}$	9	0	3	0
$Nit^{-}Dis^{+}Hyg^{S}$	14	3	4	3
$Nit^{-}Dis^{+}Hyg^{R}$	0	0	0	0
$Nit^{-}Dis^{-}Hyg^{S}$	2	0	0	0

[a] Discharge minus mutant
[b] Perithecium developmental mutant
[c] Plasmid used in transformation
[d] Restriction enzyme used to linearize plasmid prior to transformation
[e] Nitrate utilizing (Nit^{+}) or nitrate non-utilizing (Nit^{-})
[f] Ability to forcibly discharge ascospores
[g] Hygromycin resistant (R) or Hygromycin sensitive (S)

Specific mutants. Of the 5005 transformants generated, four appeared to have lost the ability to forcibly discharge ascospores. After microscopic examination, it was confirmed three of the mutants were arrested during perithecium formation. Cross-sections of perithecia are presented in Figure 8. Perithecia of 729G-112 (Figure 8A) have mature ascospores, and wild type morphology of paraphyses, periphyses, and ostiole. This mutant also produced amorphous cirri instead of the distinct tube formation of the wild type cirrhi (Figure 9). Mutant 720R-2 (Figure 8B) lacked mature asci and was arrested at an early stage of perithecial development. Mutants 729R-57 and 610A-173 were also arrested in early stages of development (data not shown).

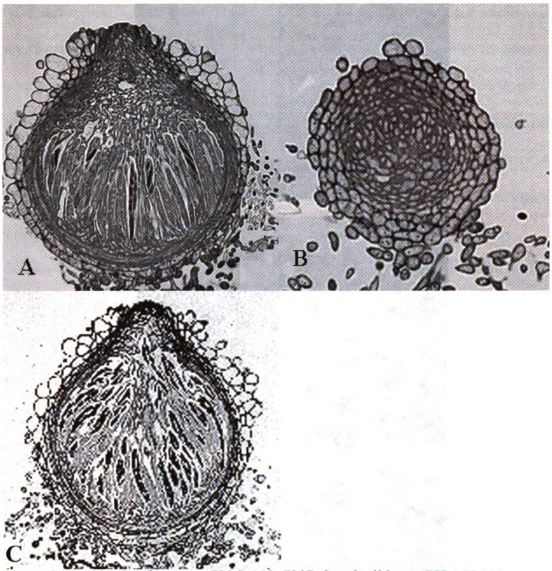

Figure 8. Perithecia of mutants 729G-112, 720R-2 and wild type PH-1 X 400.
A) Discharge minus mutant 729G-112 at earlier stage maturity B) Developmental mutant 720R-2 C) Wild type perithecia at full maturity.

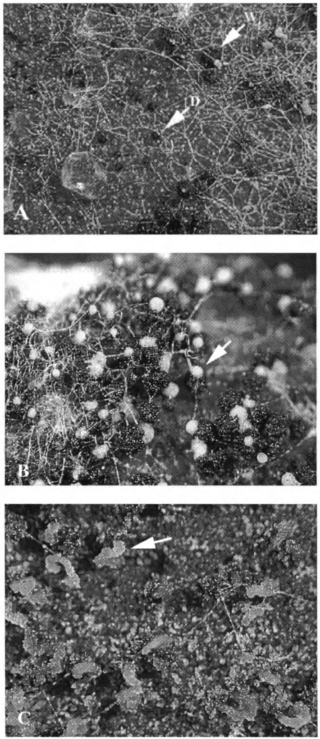

Figure 9. Perithecia and cirrhi of 720R-2, 729G-112 and wild type PH-1. **A)** Perithecium at 50X of: **D.** developmental mutant 720R-2 **W.** wild type. **B)** Ascospore discharge mutant 729G-112 at 70X (**arrow**) cirrus **C)** Wild type perithecia at 70X (**arrow)** cirrus.

DISCUSSION

Random insertional mutagenesis is an effective method to disrupt and locate a gene sequence associated with a change in phenotype. The ease of isolation of surrounding genes relies on vector insertion in a single location per genome and that the insert remains stable. Examination of 20 transformants of *G. zeae*, has shown insertions are random and remain stable in the majority of transformants. Transformants with pHA1.3 had predominantly single-copy insertions and transformants of pUCH2-8 had single and multiple-copy insertions. Multi-copy and unstable insertional events have been documented in *Gibberella pulicaris,* (Salch and Beremand, 1993), *Gibberella fujikuroi* (Leslie and Dickman, 1991), *Alternaria alternata (Tanaka et al., 1999)*, *Gibberella pulicaris* (Salch and Beremand, 1993) and *Colletotrichum sp.* (Redman and Rodriguez, 1994). We do not know the cause of the higher number of multiple copy inserts in transformants of pUCH2-8.

Although the transformation rates for *G. zeae* were low, we have modified an existing transformation protocol to make the generation of large numbers of mutants more efficient. The majority of our transformation experiments did not use REMI mutagenesis because we found it decreased the transformation rate. This was also observed in *Fusarium moniloforme* (Shim and Woloshuk, 1998). The reason for this is unknown, however it is possible that the additional restriction enzyme may have led to chromosomal breakage of the host genome and a loss of plasmid insertion. Although vector pHA1.3 has a telomeric sequence that was reported to cause chromosomal breakage and lead to a loss of plasmid integration (Redman and Rodriguez, 1994), we

observed it to yield a higher transformation rate, greater stability, and more single copy inserts than pUCH2-8.

In selfings and outcrossings with another strain, progeny of matings were more sensitive to hygromycin than the parental generation. We were not able to determine the pattern of genetic inheritance for two of the four mutants due to low ascospore viability. Ascospore abortion was also seen during a REMI mutagenesis project in *G. pulicaris* (Salch, 1993) and may be caused by the "spore-killer" gene complex similar to that found in *Neurospora crassa* (Hasunuma, 1984; Turner and Perkins, 1991).

Of the four mutants isolated during this study, the phenotypes appeared to either be normal morphologically with a loss or defect in the mechanism of ascospore discharge or, arrested early in perithecium development. The discharge mutant also has a distinct cirrhus morphology that may be related to the mechanism of forcible discharge. We have promising candidates to study the gene(s) associated with each phenotype and plan to pursue gene isolation and sequencing.

APPENDICES

APPENDIX A

Plasmid Maps: pHA1.3 and pUCH2-8

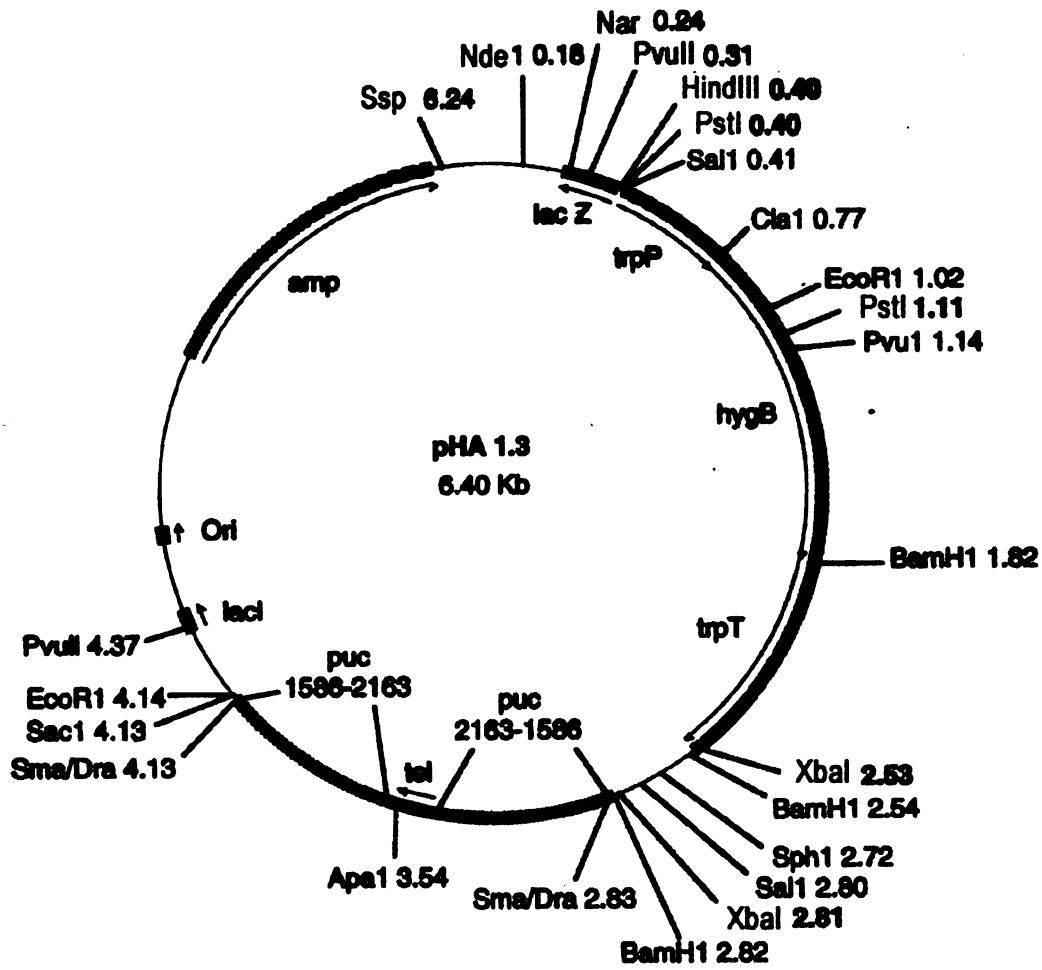

Figure 10. Map of plasmid DNA pHA1.3.

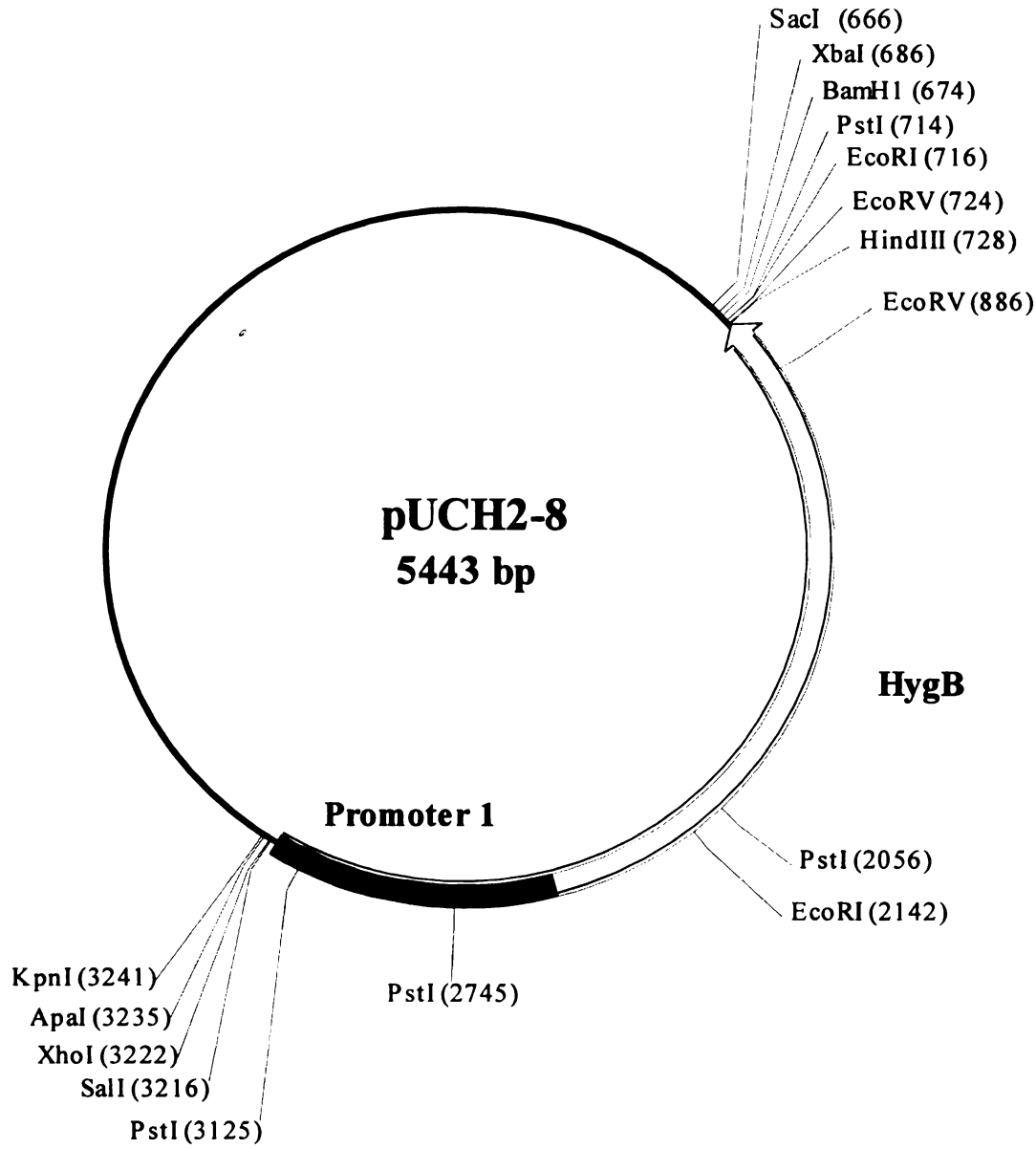

Figure 11. Map of plasmid DNA pUCH2-8.

APPENDIX B

Protocol for Random Insertional Mutagenesis

1. Inoculate 100 ml of Carboxymethylcellulose Medium (in a 250 ml Erlenmeyer flask) with 0.3 g soil suspension. Incubate for 72 hr on a rotary shaker table at 25° C at 250 rpm (Cappellini and Peterson, 1965).

2. Filter culture through sterile miracloth in a Buchner funnel under vacuum. Place into one 250 ml oakridge bottle. Spin at room temperature (RT) at 5,000 rpm (4000 g) in a GSA rotor for 10 minutes.

3. Discard all but 4–5 mls of the supernatant and resuspend solution to a concentration of 10^8 conidia/ml in the remaining solution.

4. Place conidia suspension in 100 ml of YEPD Broth (in a 250 ml Erlenmeyer flask) and grow on a rotary shaker table for 12-14 hours at 25° C at 175 rpm.

5. Filter culture through sterile miracloth in a Buchner funnel under vacuum and collect mycelial mat. Place mat in sterile 250 Erlenmeyer flask. Add Protoplasting Buffer at rate of 30 ml solution/100 ml initial YEPD culture

6. Digest for 1 hr on a rotary shaker table at 30° C at 80 rpm.

7. Filter digestion mixture through a 30 μm Nitex nylon membrane (Tetko Inc., Kansas City, MO) into a sterile beaker. Filtered solution should be turbulent due to the presence of protoplasts.

8. Distribute solution evenly in 30 ml oakridge tubes and spin at room temperature (RT) at 3,000 rpm (1500 g) for 5 minutes in a GSA rotor.

9. Discard supernatant and gently resuspend protoplasts in 10 ml of STC Buffer using wide orifice glass pipettes (Fisher Scientific, Pittsburgh, PA). Spin solution in SS-34 rotor at 5,000 rpm (4000 g).

10. Discard supernatant and gently resuspend protoplasts in 1 ml STC Buffer using wide orifice pipet tips. Transfer to a 2 ml eppendorf tube. Spin in a microcentrifuge at RT at 3,500 rpm (3500 g) for six minutes. Repeat once.

11. Resuspend protoplasts in a final volume of 300 - 600 μl (10^6 – 10^8 protoplasts /ml) and suspend in the following mixture: 100 μl - Protoplast Buffer, 100 μl -STC Buffer, 50 μl 30% PEG Solution, and 10 μl of linearized plasmid. Incubate at room temperature for 20 minutes.

12. Add 2 ml 30% PEG Solution and incubate 5 minutes

13. Add 4 ml STC Buffer and gently mix by inversion.

14. Place 600 μl of suspension in 20 ml Regeneration Medium (RM) at 47–50°C.

15. Allow protoplasts to regenerate for 15 hours and then overlay with 10 ml of RM amended with 150 μg/ml HygB (Calbiochem-Novabiochem Corp., San Diego, CA).

16. When transformants emerge (generally within 4 - 7 days), screen putative transformants on V8 Medium containing 450 μg/ml HygB (strain PH-1) or 300 μg/ml HygB (strain W-8).

CMC Medium
15 g carboxymethylcellulose (low viscosity) Sigma
1.0 g NH_4NO_3
1.0 g KH_2PO_4
0.5 g $MgSO_4$-$7H_2O$
1.0 g Yeast Extract
in 1 L H_2O
*note dissolve CMC in warm H_2O before adding remaining ingredients
dispense 100 mls into ten 250 ml Erlenmeyer flasks

YEPD Broth
3.0 g Yeast Extract (Difco Laboratories, Detroit, MI)
10.0 g Bacto™peptone ((Difco Laboratories, Detroit, MI)
20.0 g Dextrose (D-glucose)
dispense 100 mls into ten 250 ml Erlenmeyer flasks

Protoplasting Buffer
20 ml - 1.2 M KCl
500 mg - driselase (Sigma Chemical Co., St. Louis)
1 mg - chitinase (Sigma Chemical Co., St. Louis)
either:
10 mg - mureinase (USB-Amersham Pharmacia Biotech Inc., Piscataway, NJ)
or:
100 mg - lysing enzyme (Sigma Chemical Co., St. Louis)

STC Buffer
For 100 mls
60 ml - 1.2 M sorbitol
1 ml - 1M Tris - HCl pH 8.0
5 ml - 1 M $CaCl_2$
autoclave 20 minutes

30% PEG Solution
For 10 mls
7.5 ml - 40% PEG 8000
0.1 ml - 1 M Tris-HCl, pH 8.0
0.5 ml - 1 M $CaCl_2$,
1.9 ml - H_2O
Filter sterilize in a 0.45 μm Millex-HA filter (Millipore, Bedford, MA).

Regeneration Medium
For 1 L (500 ml each solution)
2X solution
1.0 g - yeast extract
1.0 g - casein enzyme hydrosylate
10 g - agarose
bring to 500 mls
Sucrose solution
547 g – sucrose
bring to 500 mls
autoclave each solution 20 minutes and then combine

V8 Medium
163 ml V8 Juice
g $CaCO_3$
15 g agar
in 1 L H_2O

APPENDIX C

Protocol for Large Scale Random Insertional Mutagenesis

1. Inoculate thirty-two flasks of 100 ml CMC Media (in 250 ml Erlenmeyer flasks) with 9.6 g soil suspension (0.3 g each flask). Incubate for 72 hours on a rotary shaker table at 25°C at 250 rpm (Cappellini and Peterson, 1965).

2. Filter cultures through sterile miracloth in a Buchner funnel under vacuum. Divide into sixteen 250 ml oakridge bottles. Spin at room temperature (RT) at 5,000 rpm (4000 *g*) in a GSA rotor for 10 minutes.

3. Discard all but 10 mls of the supernatant and resuspend solution to a concentration of 10^8 conidia/ml in the remaining solution in each bottle.

4. Distribute conidia suspension (from the sixteen 250 ml oakridge bottles) evenly into thirty-two 100 ml cultures of YEPD Broth and grow for 12 - 14 hours on a rotary shaker table at 25°C at 175 rpm.

5. Filter cultures through sterile miracloth in a Buchner funnel under vacuum. Collect mycelial mats and evenly divide into eight sterile 250 Erlenmeyer flasks. Add Protoplasting Buffer at rate of 30 ml solution/100 ml initial YEPD culture

6. Digest for 1 hour at on a rotary shaker table 30° C at 80 rpm.

7. Filter digestion mixture through a 30 µm Nitex nylon membrane (Tetko Inc., Kansas City, MO) into a sterile beaker. Filtered solution should be turbulent due to the presence of protoplasts.

8. Distribute solution evenly in four 250 ml oakridge bottles and spin at room temperature (RT) at 3,000 rpm (1500 *g*) for 5 minutes in a GSA rotor.

9. Discard supernatant and gently resuspend protoplasts in 10 ml of STC Buffer using wide orifice glass pipettes (Fisher Scientific, Pittsburgh, PA). Transfer to four 30 ml oakridge bottles.

10. Discard supernatant and gently resuspend protoplasts in 1 ml STC Buffer. Transfer to sixteen 2 ml eppendorf tubes. Spin in a microcentrifuge at RT at (3500 g) for six minutes. Repeat once.

11. Resuspend protoplasts in 2 ml tubes a final volume of 600 µl ($1 \times 10^6 - 1 \times 10^8$ protoplasts /ml). Combine two tubes for a total of eight tubes. Prepare eight 50 ml sterile conical polypropylene centrifuge tubes with the following mixture: 600 µl - protoplast solution, 600 µl -STC Buffer, 300 µl 30% PEG Solution, and 60 µl of linearized plasmid. Incubate at room temperature for 20 minutes.

12. Add 12 ml 30% PEG Solution and incubate 5 minutes

13. Add 24 ml STC Buffer and gently mix by inversion.

14. Gently pour each conical tube into 120 ml Regeneration Medium (RM) at 47–50°C. Aliquot 20 mls into each 60 x 15-mm Petri plate dish.

15. Allow protoplasts to regenerate for 15 hours and then overlay with 10 ml of RM amended with 150 μg/ml HygB (Calbiochem-Novabiochem Corp., San Diego, CA).

16. When transformants emerge (generally within 4 - 7 days), screen putative transformants on V8 Medium containing 450 μg/ml HygB (strain PH-1) or 300 μg/ml HygB (strain W-8).

CMC Medium
15 g - carboxymethylcellulose (low viscosity) Sigma
1.0 g - NH_4NO_3
1.0 g - KH_2PO_4
0.5 g - $MgSO_4$-$7H_2O$
1.0 g - yeast extract (Difco Laboratories, Detroit, MI)
in 1 L H_2O
*note dissolve CMC in warm H_2O before adding remaining ingredients
dispense 100 mls into ten 250 ml Erlenmeyer flasks

YEPD Broth
3.0 g - yeast extract (Difco Laboratories, Detroit, MI)
10.0 g - Bacto™peptone ((Difco Laboratories, Detroit, MI)
20.0 g - dextrose (D-glucose)
dispense 100 mls into ten 250 ml Erlenmeyer flasks

Protoplasting Buffer
960 ml - 1.2 M KCl
24 g - driselase (Sigma Chemical Co., St. Louis)
48 mg - chitinase (Sigma Chemical Co., St. Louis)
480 mg - mureinase (USB-Amersham Pharmacia Biotech Inc., Piscataway, NJ)
stir for 30 minutes and filter sterilized in a 0.45 μm Millex-HA filter (Millipore, Bedford, MA).

STC Buffer
For 100 mls
60 ml - 1.2 M sorbitol
1 ml - 1M Tris-HCl, pH 8.0
5 ml - 1 M $CaCl_2$
autoclave 20 minutes

30% PEG Solution
For 10 mls
7.5 ml - 40% PEG 8000
0.1 ml - 1 M Tris-HCl, pH 8.0
0.5 ml - 1 M $CaCl_2$,
1.9 ml - H_2O
Filter sterilize in a 0.45 μm Millex-HA filter (Millipore, Bedford, MA).

Regeneration Medium
For 1 L (500 ml each solution)
2X solution
1.0 g - yeast extract
1.0 g - casein enzyme hydrosylate
10 g - agarose
bring to 500 mls
Sucrose solution
547 g – sucrose
bring to 500 mls
autoclave each solution 20 minutes and then combine

V8 Medium
163 ml V8 Juice
1.0 g $CaCO_3$
15 g agar
in 1 L H_2O

APPENDIX D

Protocol for Screening of Discharge Minus / Perithecia Mutants

Transformants were placed on carrot agar (CA) medium (35% carrots, 2% agar) (Klittich and Leslie, 1988) in 24 cell well microplates with 17 mm well diameters (Evergreen, Los Angeles, CA) or 60 mm Petri plates. Plates were maintained under continuous 15-watt cool white and black light fluorescent lighting. Atmospheric moisture was intensified by covering incubation area with plastic sheeting. During dry periods (winter months) a standard room humidifier was used to further supplement air moisture. Prior to induction, conidia were harvested in sterile water containing a few drops of Tween 20. After 2 - 4 days of growth, vegetative hyphae was covered with 200 µl or 1000 µl of 2.5% aqueous Tween 60 solution (Bowden and Leslie, 1999) in 17 mm cell well plates and 60 mm Petri plates, respectively. Hyphae was flattened using a glass rod or hockey stick in 17 mm cell well plates and 60 mm Petri plates, respectively. Approximately 5 - 7 days after perithecium formation, the cell well microplate lid was examined under a microscope at 5X magnification for discharged ascospores.

Cultures that had no visible signs of forcible discharge after two trials were rescreened more vigorously as follows. Agar blocks containing approximately 20 - 40 perithecia were removed from mature cultures and placed on a glass slide so that spores would be parallel to the slide in a high humidity chamber. The perithecia were kept in the chamber for 1 - 2 days away from direct light and re-examined microscopically.

BIBLIOGRAPHY

National Climatic Data Center National Oceanic and Atmospheric Administration. [Online] Available http://www.ncdc.noaa.gov/ol/climate/climatedata.html#DAILY, 2000.

Adams, G., Johnson, N., Leslie, J. F., and Hart, L. P., 1987. Heterokaryons of *Gibberella zeae* formed following hyphal anastomosis or protoplast fusion. *Exp. Mycol.* **11**(4): 339-353.

Alexopoulos, C. J., Mims, C. W., and Blackwell, M., 1996, *Introductory Mycology*, John Wiley & Sons, Inc., New York, pp. 869.

Armaleo, D., Ye, G. N., Klein, T. M., Shark, K. B., Sanford, J. C., and Johnston, S. A., 1990. Biolistic nuclear transformation of *Saccharomyces cerevisiae* and other fungi. *Curr. Genet.* **17**(2):97-103.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Siedman, J. G., Smith, J. A., and Struhl, K., 1987. *Current protocols in molecular biology*. John Wiley & Sons, New York.

Bai, G. H., and Shaner, G., 1994. Scab of Wheat - Prospects For Control. *Plant Dis.* **78**(8):760-766.

Boelker, M., Boehnert, H. U., Braun, K. H., Goerl, J., and Kahmann, R., 1995. Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). *Mol. Gen. Gen.* **248**(5):547-552.

Bowden, R. L., and Leslie, J., F., 1999. Sexual recombination in *Gibberella zeae*. *Phytopathology* **89**(2):182-188.

Brown, J. S., Aufauvre Brown, A., and Holden, D. W., 1998. Insertional mutagenesis of *Aspergillus fumigatus*. *Mol. Gen. Gen.* **259**(3):327-335.

Burgess, L. W., Wearing, A. H., and Toussoun, T. A., 1975. Surveys of Fusaria Associated with Crown Rot of Wheat in Eastern Australia. *Aust. J. Agric. Res.* **26**:791-799.

Cappellini, R. A., and Peterson, J. L., 1965. Macroconidium Formation in Submerged Cultures by a Non-Sporulating Strain of *Gibberella zeae*. *Mycologia* **57**:962-966.

Chakraborty, B. N., Patterson, N. A., and Kapoor, M., 1991. An electroporation-based system for high-efficiency transformation of germinated conidia of filamentous fungi. *Can. J. Microbiol.* **37**(11):858-63.

Chen, X. M., and Yuan, C., 1984, Application of microcomputer in studying wheat scan epidemiology and forecasting, *Zhejiang Agric. Sci.* **2**:55-60.

Corell, J. C., Klittich, C.J.R., Leslie, J.F, 1987. Nitrate Nonutilizing Mutants of *Fusarium oxysporum* and Their Use in Vegetative Compatibility Tests. *Phytopathology* **77**:1640-1646.

Czymmek, K. J., and Klomparens, K. L., 1992. The ultrastructure of ascosporogenesis in freeze-substituted *Thelebolus crustaceus*: enveloping membrane system and ascospore initial development. *Can. J. Bot.* **70**(8):1669-1683.

Durand, R., Rascle, C., Fischer, M., and Fevre, M., 1997. Transient expression of the beta-glucuronidase gene after biolistic transformation of the anaerobic fungus *Neocallimastix frontalis*. *Curr. Genet.* **31**(2):158-61.

El-Gholl, N. E., Schoulties, C. L., and Ridings, W. H., 1979. Factors affecting perithecial production in *Gibberella* (*Fusarium*) *tricincta*. *Can. J. Bot.* **57**:2497-2500.

Fernando, W. G. D., Paulitz, T. C., Seaman, W. L., Dutilleul, P., and Miller, J. D., 1997. Head blight gradients caused by *Gibberella zeae* from area sources of inoculum in wheat field plots. *Phytopathology* **87**(4):414-421.

Francis, R. G., and Burgess, L. W., 1977. Characteristics of *Fusarium roseum 'Graminearum'* in Eastern Australia. *Trans. Brit. Myc. Soc.* **68**:421-427.

Gadoury, D. M., Stensvand, A., and Seem, R. C., 1998. Influence of light, relative humidity, and maturity of populations on discharge of ascospores of *Venturia inaequalis*. *Phytopathology* **88**(9):902-909.

Halama, P., and Lacoste, L., 1992. Determinism of sexual reproduction of *Phaeosphaeria nodorum* agent of wheat septoriosis: II. Effect of temperature and light. *Can. J. Bot.* **70**(8):1563-1569.

Hart, L. P., 1998. NCR-184 Regional committee scab of wheat progress report 1998, in: *The 1998 National Fusarium Head Blight Forum* (L. P. Hart, Ward, R., Bafus, R., Bedford, K., ed.). University Printing, East Lansing, MI, Michigan State University, East Lansing Michigan, pp. 111-113.

Hart, L. P., 1999. 1999 NCR-184 State report management of head scab of small grains, in: *1999 National Head Blight Forum* (J. A. Wagester, R. Ward, L. P. Hart, S. P. Hazen, J. Lewis, and H. Borden, eds.). University Printing, Michigan State University, East Lansing, MI, Sioux Falls, S.D., pp. 201-203.

Hasunuma, K., 1984. Meiosis in Neurospora crassa: 2. Isolation and partial characterization of dominant meiotic mutants constituting meiotic gene cluster, V-2 or LG V. *Jap. J. Gen.* **59**(5):517-526.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C., and Pegler, D. N., 1996. Ainsworth and Bisby's dictionary of the fungi (I. M. Institute, ed.). CAB International, New York.

Hohn, T., 1997. Transformation Protocol for *Gibberella zeae.* USDA, Peoria, IL.

Ingold, C. T., 1968. Spore liberation in Loramyces. *Transaction of British Mycological Society* **51**(2):323-341.

Ingold, C. T., 1971. Fungal spores: their liberation and dispersal. Clarendon Press, Oxford, UK.

Ingold, C. T., and Dann, V., 1968. Spore discharge in fungi under very high surrounding air-pressure, and the bubble-theory of ballistospore release. *Mycologia* **60**:285-289.

Itoh, Y., and Scott, B., 1997. Effect of de-phosphorylation of linearized pAN7-1 and of addition of restriction enzyme on plasmid integration in *Penicillium paxilli. Curr. Genet.* **32**:147-151.

Leslie, J.F. and Dickman, M.B., 1991. Fate of DNA encoding hygromycin resistance after meiosis in transformed strains of *Gibberella fujikuroi* (*Fusarium monoliforme*). *Appl. Env. Microb.* **57**(5): 1423-1429.

Khonga, E. B., and Sutton, J. C., 1988. Inoculum production and survival of Gibberella zeae in maize and wheat residues. *Can J Plant Pathol Rev Can Phytopathol* **10**(3):232-239.

Klittich, C. J. R., and Leslie, J. F., 1988. Nitrate reduction mutants of *Fusarium moniliforme* (*Giberella fujikuroi*). *Genetics* **118**:417-423.

Kuper-Goodman, T., Scott, P.M., Watanabe, H., 1987. Risk assessment of the mycotoxin zearalenone. *Regulatory toxicology and pharmacology* **7**:253-306.

Kuspa, A., and Loomis, W. F., 1992. Tagging Developmental Genes in Dictyostelium By Restriction Enzyme-Mediated Integration of Plasmid DNA. *Proc. Natl. Acad. Sci. USA* **89**(18):8803-8807.

Leslie, J. F., 1983. Some genetic techniques for Gibberella zeae. *Phytopathology* **73**(7):1005-1008.

Long, G. G., Dickman, M., Tuite, J., Shannon, G. M., and Vesonder, R. F., 1982. Effects of *Fusarium roseum* corn culture containing zearalenone on early pregnancy in swine. *Am. J. Vet. Res.* **43**:1599-1603.

Lorito, M., Hayes, C. K., Dipietro, A., and Harman, G. E., 1993. Biolistic Transformation of *Trichoderma-Harzianum* and *Gliocladium-Virens* Using Plasmid and Genomic DNA. *Cur. Gen.* **24**(4):349-356.

Lu, S., Lyngholm, L., Yang, G., Bronson, C., Yoder, O. C., and Turgeon, B. G., 1994. Tagged mutations at the Tox1 locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. *Proc. Natl. Acad. Sci. U. S. A.* **91**(26):12649-53.

McMullen, M., Jones, R., and Gallenberg, D., 1997a. Scab of wheat and barley: a re-emerging disease of devasting impact. *Plant Dis.* **81**(12):1340-1348.

McMullen, M., Milus, G., and Prom, L., 1999. 1999 Uniform fungicide trials to identify products effective against *Fusarium* head blight in wheat. in: *1999 National Head Blight Forum* (J. A. Wagester, R. Ward, L. P. Hart, S. P. Hazen, J. Lewis, and H. Borden, eds.), University Printing, Michigan State University, East Lansing, MI, Sioux Falls, S.D., pp. 64-68.

McMullen, M. P., Stack, R.W., 1999. US Wheat and Barley Scab Initiative. North Dakota State University Extension Service. [Online] Available http://www.scabusa.org.

McMullen, M. P., Enz, J., Lukach, J., and Stover, R., 1997b. Environmental conditions associated with Fusarium head blight epidemics of wheat and barley in the northern Great Plains, North America. *Cer. Res. Com.* **25**(3 Part 2):777-778.

Miller, J. D., Culley, J., Fraser, K., Hubbard, S., Meloche, F., Ouellet, T., Seaman, W. L., Seifert Keith, A., Turkington, K., and Voldeng, H., 1998. Effect of tillage practice on fusarium head blight of wheat. *Can J Plant Pathol. March* **20**(1):95-103.

Miller, P. M., 1970. Reducing discharge of ascospores of *Venturia inaequalis* with a spring application of benomyl, thiabendazole, or urea. *Plant Dis.* **54**(1):27.

Moss, M. O., and Frank, J. M., 1985. Influence of the fungicide tridemorph on T-2 toxin production by Fusarium sporotrichoides. *Trans.Brit. Myc. Soc.* **84**:585-590.

Nelson, P. E., Toussoun, T. A., and Marasas, W. F. O., 1983. Fusarium species : an illustrated manual for identification. Pennsylvania State University Press, University Park.

Olive, L. S., 1964. Spore discharge mechanism in Basidiomycetes. *Science* **146**:542.

Parry, D. W., Jenkinson, P., and McLeod, L., 1995. Fusarium ear blight (scab) in small grain cereals-a review. *Plant-Pathology (United Kingdom)* **v. 44(2)**:p. 207-238.

Paulitz, T. C., 1996. Diurnal release of ascospores by Gibberella zeae in inoculated wheat plots. *Plant Dis.* **80**(6):674-678.

Pereyra, S. A., Dill-Macky, R., and Sims, A. L., 1999. Survival and Incolum potential of *Fusarium graminearum* in wheat residues. in: *1999 National Head Blight Forum* (J. A. Wagester, R. Ward, L. P. Hart, S. P. Hazen, J. Lewis, and H. Borden, eds.), University Printing, Michigan State University, East Lansing, MI, Sioux Falls, South Dakota, pp. 96-98.

Pinkerton, J. N., Johnson, K. B., Stone, J. K., and Ivors, K. L., 1998. Maturation and seasonal discharge pattern of ascospores of *Anisogramma anomala*. *Phytopathology* **88**(11):1165-1173.

Prados Ligero, A. M., Gonzalez Andujar, J. L., Melero Vara, J. M., and Basallote Ureba, M. J., 1998. Development of *Pleospora allii* on garlic debris infected by *Stemphylium vesicarium*, *Eur. J. Plant Pathol.* **104**(9):861-870.

Pritsch, C., Muehlbauer, G.J., Bushnell, W.R., Somers, D.A., and Vance, C.P., 2000. Fungal development and induction of defense response genes during early infection of wheat spikes by *Fusarium graminearum*. *Mol. Plant-Microbe Interact.* **13**(2):159-169.

Redman, R. S., and Rodriguez, R. J., 1994. Factors affecting the efficient transformation of Colletotrichum species. *Exp. Myc.* **18**(3):230-246.

Reis, E. M., 1990a. Effects of rain and relative humidity on the release of ascospores and on the infection of wheat heads by *Gibberella zeae*. *Fitopatologia-Brasileira (Brazil)* **15(4)**:p. 339-343.

Reis, E. M., 1990b. Survival of perithecia of *Gibberella zeae* on naturally infected wheat kernels under field conditions, *Fitopatologia-Brasileira (Brazil)* **15**(3):254-255.

Salch, Y. P., and Beremand, M. N., 1993. *Gibberella pulicaris* transformants: state of transforming DNA during asexual and sexual growth, *Curr. Gen.* **23**:343-350.

Sanchez, O., Navarro, R. E., and Aguirre, J., 1998. Increased transformation frequency and tagging of developmental genes in *Aspergillus nidulans* by restriction enzyme-mediated integration (REMI), *Mol. Gen. Gen.* **258**(1-2):89-94.

Schiestel, R.H., and Petes, T.D., 1991. Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae, Proc. Natl. Acad. Sci.* **88:** 7585-7589

Schroeder, H., and Christensen, J., 1963. Factors affecting the resistance of wheat scab caused by *Gibberella zeae*. *Phytopathology* **53**:831-838.

Shi, Z., Christian, D., and Leung, H., 1995. Enhanced Transformation in Magnaporthe-Grisea By Restriction Enzyme-Mediated Integration of Plasmid DNA. *Phytopathology* **85**(3):329-333.

Shim, W. B., and Woloshuk, C. P., 1998. Use of restriction enzyme mediated integration for *Fusarium moniliforme*. in: *American Phytopathology Society*, Las Vegas, NV.

Stanton, N., 1995. Mycotoxin levels in the 1995 midwest preharvest corn crop. The National Veterinary Services Laboratories [Online] Available http://www.usda.org. USDA, APHIS. Ames, Iowa 50010.

Stensvand, A., Amudsen, T., Semb, L., Gadoury, D. M., and Seem, R. C., 1998. Discharge and dissemination of ascospores by *Venturia inaequalis* during dew. *Plant Dis.* **82**(7):761-764.

Sutton, J. C., 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Can. J. Plant Path.* **4**:195-209.

Sutton, J. C., and Procter, R., 1982. Dispersion analysis of gibberella ear rot in maize *Gibberella zeae*. *Can. J. Plant. Pathol.* **4**(3):254-258.

Sweigard, J. A., Carroll, A. M., Farrall, L., Chumley, F. G., and Valent, B., 1998. *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. *Mol. Plant. Microbe. Interact.* **11**(5):404-12.

Tanaka, A., Shiotani, H., Yamamoto, M., and Tsuge, T., 1999. Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-specific AK-toxin in the Japanese pear pathotype of *Alternaria alternata*. *Mol. Plant. Microbe. Interact.* **12**(8):691-702.

Teich, A. H., 1989. Epidemiology of wheat (*Triticum aestivum* L.) scab caused by *Fusarium* spp. in: *Fusarium Mycotoxins, Taxonomy and Pathogenicity* (J. Chelkowski, ed.), Elsevier, Amsterdam, pp. 269-282.

Teich, A. H., and Hamilton, J. R., 1985. Effect of cultural practices, soil phosphorus, potassium and pH on the incidence of *Fusarium* head blight and deoxynivalenol levels in wheat (*Triticum aestivum*). *Appl. Environ. Microbiol.* **49**(6):1429-1431.

Teich, A. H., and Nelson, K., 1984. Survey of *Fusarium* head blight and possible effects of cultural practices in wheat fields in Lambton County in 1983. *Can. Plant Dis. Sur.* **64**:11-13.

Trail, F., and Common, R., 2000. Perithecial development of *Gibberella zeae*: A light microscopy study. *Mycologia* **92**(1):130-138.

Trail, F., Gadoury, D., and Loranger, R., 1998. Environmental parameters of ascospore discharge in *Gibberella zeae*, in: *The 1998 National Fusarium Head Blight Forum.* University Printing, Michigan State University, East Lansing Mi, pp. 11-13.

Turgeon, B. G., Garber, R. C., and Yoder, O. C., 1987. Development of a fungal transformation system based on selection of sequences with promoter activity. *Mol. Cell Bio.* **7**(9):3297-3305.

Turner, B. C., and Perkins, D. D., 1991. Meiotic drive in Neurospora and other fungi. *American Naturalist* **137**(3):416-429.

Tusa, C., Munteanu, I., Capetti, E., Pirvu, T., Bunescu, S., Sin, G. L., Nicolae, H., Tianu, A., Caea, D., Romascanu, I., and Stoika, V., 1981. Aspects of the *Fusarium* attacks on wheat in Romania, *Probleme de Protectia Plantelor* **9**:15-31.

Yu Jieh, J., and Cole Garry, T., 1998. Biolistic transformation of the human pathogenic fungus Coccidioides immitis. *J Microbiol. Meth. July* **33**(2):129-141.