To the Graduate Council:

I am submitting herewith a dissertation written by Rodney J. Scott entitled "New approaches to the study of antheridiogen sensitivity in Ceratopteris richardii." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.

Leslie G. Hickok, Major Professor

We have read this dissertation
and recommend its acceptance:

Otto J. Schwarz

James W. Hughes

W. S. Riggs

Accepted for the Council:

Vice Provost
and Dean of the Graduate School

NEW APPROACHES TO THE STUDY OF ANTHERIDIOGEN SENSITIVITY IN *CERATOPTERIS RICHARDII*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Rodney J. Scott

August 1989

DEDICATION

I dedicate this work to my wife and to my mother. Both of these women have greatly influenced and inspired me and both have loved me unconditionally.

I also dedicate it to my Lord and Savior Jesus Christ. He has given me life and has shown me how to live it abundantly.

ACKNOWLEDGMENT

I would like to express my appreciation to the following individuals who have inspired, encouraged and supported me as I've worked on this project.

To those in the Department of Botany at the University of Tennessee, Knoxville, who provided me with the opportunity to study there.

To Dr. Leslie G. Hickok, my major professor. I thank him for providing the initial inspiration for this project. I also thank him for providing me with many resources which enabled me to carry out this research. Above all, I thank him for sharing his time, wisdom and friendship with me and for serving as a model of an excellent researcher and teacher.

To Drs. K.W. Hughes, W.S. Riggsby and O.J. Schwarz who served on my committee. I thank them for their helpful insights and guidance.

To Dr. M.I. Cousens who was my undergraduate advisor. I thank him for his continued friendship and professional support.

To all those who have worked alongside me in the lab. To Dr. Thomas Warne whose friendship and professional interactions have inspired me. To the other graduate students, Shawn, Paulette, and Meeta and to the technicians and undergraduate researchers. I am thankful for having known and worked with each one.

To all of the graduate students in the Botany Department. I

thank them for their friendship and professional interactions.

To my dear brothers and sisters at Christ Chapel. I thank them for their friendship and for their support in deeds and prayers.

To my wife Donna whose friendship, love, support and patience have been invaluable to me. She has stood alongside me during the difficult times and her presence has made the good times better.

And to my Lord Jesus Christ. I thank him for all things, but now I thank him especially for his many blessings as I prepared my dissertation. I truly believe that I could not have finished when I did without his guidance and provision.

ABSTRACT

The pheromone antheridiogen promotes differentiation of males in some fern gametophyte. Percentages of males in multispore cultures reflect antheridiogen sensitivity in homozygous strains of Ceratopteris richardii. Two homozygous strains (176D and H) with different sensitivities to antheridiogen were genetically characterized by Scott and Hickok (1987). They demonstrated that the difference in sensitivity was associated with either one or two linked genes. The present study was based on this work and had three goals: 1) improve the antheridiogen source by concentrating and partially purifying a crude aqueous filtrate (CAF); 2) develop better techniques for assessing antheridiogen sensitivity; and 3) distinguish between the two models proposed earlier or suggest others.

Extraction techniques used for gibberellic acid were used to partially purify CAF. Concentrated antheridiogen purified by extraction (CAPE) was from 112 to 170 times as active as CAF when autoclaved. If not autoclaved, CAPE exhibited reduced activity. Assays of CAPE effects on gametophytic growth indicated that it did not affect germination timing or reduce overall growth. Spectrophotometric analysis indicated that the purification procedure reduced the heterogeneity of this substance. Strains 176D and H were characterized on CAPE supplemented media with higher antheridiogen concentrations than previously possible.

A new technique was described for assessing antheridiogen sensitivities of individual gametophytes. This involved growing gametophytes in darkness on CAPE supplemented medium and counting the antheridia they produced (dark antheridiogen response). Strains with different antheridiogen sensitivities exhibited characteristic dark antheridiogen responses. This technique when applied to hybrid-derived spore collections, allowed determination of segregation patterns.

F2s from a cross between 176D and H were characterized on CAPE supplemented media in the light and in darkness. Results indicated that at least four response classes were present. Dark antheridiogen responses determined for several hybrids indicated that 176D and H differ in their antheridiogen response by two linked genes separated by as much as 36 map units.

Characterization and genetic analysis of a mutant which exhibited a high degree of dark germination (>95%) was also described. Dark-germination is associated with a single nuclear gene which functions at the gametophyte level.

TABLE OF CONTENTS

CHAPTER PAGE

CHAPTER PAGE

VI. (Continued)

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

FIGURE PAGE

CHAPTER I

GENERAL COMMENTS

Introduction

General characteristics of antheridiogen.

Antheridiogens are pheromones which induce antheridia differentiation on gametophytes of sensitive fern species (Dopp, 1950; Naf, 1979). Dopp first demonstrated that undifferentiated gametophytes of Pteridium aquilinum could be induced to form antheridia precociously if they were grown on medium from mature cultures (Dopp, 1950). Subsequently, species in several genera have been shown to have antheridiogen systems (Naf, 1979). Of these, the antheridiogens of P. aquilinum (Dopp, 1950), Anemia phyllitidis (Naf, 1959), Lygodium japonicum (Naf, 1960) and Ceratopteris thalictroides (Schedlbauer and Klekowski, 1972) have been most fully studied. Each antheridiogen (A_{Pt}, A_{An}, A_{Ly}, and A_{Ce} respectively) has been characterized chromatographically and by its biological activity, and each has been found to be unique (Naf et al., 1975; Schedlbauer, 1974; 1976a).

The antheridiogen of Pteridium has been studied extensively and has been shown to be active in many other polypodiaceous ferns. However, A_{Pt} is completely inactive in the schizaeaceous ferns Anemia phyllitidis and Lygodium japonicum and in Ceratopteris thalictroides (Parkeriaceae). There is some cross-reactivity between the two

schizaeaceous ferns in that L. japonicum responds to A_{An}. However, A. phyllitidis is unresponsive to A_{Ly}. Thus far, A_{Ce} is known to be active only within the genus Ceratopteris (Schedlbauer, 1974).

In addition to their ability to induce antheridia formation, some antheridiogens are also capable of substituting for the light requirement for spore germination in some fern species. A_{Pt} acts in this manner for some members of the Polypodiaceae (sensu Bowers) and A_{An} and A_{Ly} promote dark germination in spores of their own source species and show cross-reactivity similar to that for antheridia induction (Naf et al., 1975). The antheridiogen from Ceratopteris lacks the ability to promote dark germination in the spores of its own source species, or in those of any other tested ferns (Schedlbauer, 1976a). However, several authors have reported that dark germination of Ceratopteris spores may occur under certain conditions, even without applied A_{Ce} (see Miller, 1968). A recent study using C. richardii provided results which conflict with these observations and suggested that earlier observations of dark germination in this genus may be attributed to the use of freshly collected spores (Cooke et al., 1987). It has been reported for several species that fresh spore collections exhibit limited dark germination, while older collections do not (see Miller, 1968 for review).

Gametophyte development and sequence of antheridia development.

During the earliest stages of development, gametophytes are sexually undifferentiated and do not possess an organized meristem

region. Antheridia are inducible at an early stage and frequently can be produced during all subsequent stages of growth. However, archegonia (female sex organs) can only be formed following the development of an organized meristem region, which leads to the formation of a heart shaped, or cordate gametophyte (Miller, 1968).

In multispore cultures of fern gametophytes, two or three types of differentiated gametophytes can generally be distinguished. Some gametophytes possess an organized meristem region and considerable vegetative tissue. These are either hermaphrodites or females which are capable of ultimately producing both sex organs. The other type of gametophyte possesses little vegetative tissue and no organized meristem region and, under the prevailing conditions of the culture, produces only antheridia (Miller, 1968).

To investigate the timing of antheridiogen-mediated development, Naf and coworkers (1975) observed multispore cultures of Pteridium aquilinum and conducted bioassays of media from cultures of various developmental stages. They concluded that gametophytes of P. aquilinum do not begin to produce antheridiogen in effective quantities until they themselves are insensitive to it. They surmised that this insensitive phase begins shortly after the formation of an organized meristem that leads to the attainment of the heart shape. This proposed sequence accounted for the typical range in morphologies observed in cultures. The most rapidly growing individuals reached the archegonial stage without ever producing antheridia; this was attributed to a total lack of antheridiogen in the medium. The

majority of the remaining gametophytes formed a few antheridia before producing archegonia. The few remaining individuals, whose development was continually being influenced by a high concentration of antheridiogen, became ameristic, exclusively antheridiate gametophytes. Although this pattern of antheridiogen production and gametophyte development has been generally accepted for other genera, studies by Hickok and Kiriluk (1984) demonstrated that gametophytes of Ceratopteris thalictroides produce small amounts of A_{Ce} long before they actually become insensitive to it. However, in its basic form, the same general scheme of antheridiogen action as that established for Pteridium has been demonstrated for gametophytes of Anemia, Lygodium (Naf, 1960; 1967) and Ceratopteris (Schedlbauer, 1976b).

Mode of action of antheridiogen.

Based on his own experiments and the observations of others, Naf (1961) proposed a general theory for the mode of action of antheridiogens. In the ferns for which antheridiogen systems have been studied most completely, it has been shown that gametophytes begin to produce antheridiogen and become insensitive to it at nearly the same stage (Naf, 1959; 1960; 1975; Schedlbauer, 1976b). Naf (1961) demonstrated that both removal of the meristem tissue of insensitive individuals and temporary plasmolysis of insensitive tissues can restore antheridiogen sensitivity. He further demonstrated that excision of the meristem tissue in young gametophytes can lead to spontaneous induction of antheridia without the intervention of

antheridiogen. These observations prompted Naf (1961) to suggest that antheridiogens exercise their effects simply by overcoming a block to antheridia formation which is maintained by some substance produced in the meristem. It is, he suggests, the accumulation of this substance in maturing gametophytes which ultimately renders them insensitive to antheridiogen. Hickok (1983) and Schraudolf (1987a) have demonstrated that antheridia induction in Ceratopteris richardii and Anemia phyllitidis respectively, can be blocked by application of abscisic acid (ABA). Along with studies by Warne and Hickok (unpublished) and Cheng and Schraudolf (1974), which show that spores of these species contain ABA, these finding may indicate ABA as a possible candidate for the natural antheridia blocking agent. However, these studies were unable to demonstrate a role for ABA in spores.

Factors affecting antheridiogen responses.

Various ratios of males:females:hermaphrodites are typically observed in multispore cultures when grown under different conditions. Many studies have demonstrated a correlation between conditions favoring slow vegetative growth and the tendency for development of unisexual, male gametophytes. Alternately, unisexual, female, or hermaphroditic gametophytes develop under conditions favoring rapid growth (Miller, 1968). Schedlbauer (1976b) studied three factors associated with gametophytic growth and development and related these to antheridiogen sensitivity in Ceratopteris thalictroides. The factors studied were spore size, germination time and growth rate.

Spore size provided the best predictor of ultimate sexual morphology. Individuals destined to develop first and become cordates could be predicted with 74% accuracy based upon spore size. Schedlbauer suggested that individuals developing from larger spores have an increased storage capacity for developmental substances (i.e. enzymes, ribosomes, etc.), and therefore develop more quickly, escaping the effects of antheridiogen.

Some gibberellic acids have antheridiogen-like activity.

Some naturally occurring gibberellic acids (GAs) can substitute for antheridiogen, both in antheridia induction and in promoting dark germination for some schizaeaceous ferns (Voeller, 1964a; Schraudolf, 1964; Takeno and Furuya, 1975; Weinberg and Voeller, 1969). The functional relationship between GAs and the antheridiogens of schizaeaceous ferns appears to have a basis in structural similarities. The structure of the antheridiogens of several species of Anemia (Nakanishi et al., 1971; Corey and Meyers, 1985; 1986; Zanno et al., 1972; Yamane et al. 1987; Nester et al., 1987) and one from Lygodium japonicum (Yamane et al., 1979) have been elucidated and shown to be similar to the general structure of GAs (see following section for further details). Furthermore, the synthetic conversion of GA7 to the structure assigned to A_{An} has been successfully accomplished (Furber and Mander, 1987).

To date, no tested GAs substitute for the antheridiogen activity of the polypodiaceous ferns or Ceratopteris (Naf et al. 1975;

Schedlbauer, 1974). Recent work by Warne and Hickok (1989) using blockers of GA biosynthesis indicates that production of Ceratopteris antheridiogen involves some of the same, or similar reactions as those of GA biosynthesis. However, no direct evidence is presently available to indicate a relationship between the GAs and either A_{Ce} or A_{Pt}.

Chemical characterization of antheridiogen.

Work done by a number of researchers (Naf, 1959, 1960; Nakanishi et al., 1971; Pringle, 1961; Pringle et al., 1960; Schedlbauer, 1976a) on the four main antheridiogens has indicated that all share a number of basic chemical characteristics. They all behave as weak acids with a pKa around five, all appear to have a carboxyl group which is necessary for their activity, and all have low molecular weights (<2,000 as determined using dialysis tubing) (Schedlbauer, 1976a).

Nakanishi and coworkers (1971) carried out the first complete chemical characterization of an antheridiogen. They determined that the chemical formula of A_{An} is $C_{19}H_{22}O_6$, and its structure is similar to that of natural GAs. They proposed that a compound with this structure could easily be produced from natural GAs by way of a suggested intermediate. Corey and Meyers (1985) synthesized this antheridiogen and demonstrated a slightly different stereochemical conformation. Final confirmation of the structure came with direct comparison of the native antheridiogen to the synthetic form (Corey et al., 1986). With this confirmation, the molecule was given the name antheridic acid.

The antheridiogen molecule of Anemia hirsuta (Zanno et al., 1972) and those of A. rotundifolia and A. flexuosa (Yamane et al., 1987) are structurally identical to antheridic acid. However, based upon the partial characterization of A. mexicana (Nester et al., 1987), it appears that although this antheridiogen is a GA-like molecule, it does not have the same structure as antheridic acid. This result demonstrates that structural diversity of antheridiogen molecules extends to the species levels.

Although most structural characterizations of antheridiogen molecules have been accomplished for the genus Anemia, some insight is available concerning the structure of one such molecule in another genus of the Schizaeacae. Yamane et al. (1979) have demonstrated an antheridiogen effect for the compound GA_9 methyl ester which was isolated from medium supporting Lygodium japonicum gametophytes. They demonstrated that this compound can both induce antheridia formation and inhibit archegonial development in Lygodium.

Antheridiogen bioassays.

Since antheridiogen is not commercially available and must be obtained directly from the experimental organisms, the development of accurate bioassays has been an important subject of research. Naf (1958) assayed the antheridiogen activity of medium from variously aged cultures of Pteridium aquilinum to determine the time course of the secretion of the pheromone. He tested the medium by creating a dilution series of conditioned medium mixed with new medium and

determined the point at which 50% activity was obtained. For these experiments, he used Onoclea sensibilis as the assay organism. Gametophytes of this species do not form antheridia spontaneously under the prevailing conditions of culture, but do so readily when exposed to Pteridium antheridiogen. This phenomenon allowed Naf to easily determine what dilution of conditioned medium caused 50% response.

Voeller (1964b) carried out similar experiments with Pteridium aquilinum to investigate factors which increase antheridiogen production. He showed that Pteridium produced more (or more active) antheridiogen when the pH of the medium was low, when light intensity was between 600 and 800 foot candles and continuous, when temperature was maintained around 20 C, and when coconut milk was added to the basic nutrient media.

Takeno and Furuya (1975) have commented on the assays of Naf and Voeller and concluded that both allow for the existence of too many variable factors to provide clear insight into the amount of antheridiogen actually present. These authors developed a novel assay, in which the activity of the antheridiogen of Lygodium japonicum was assayed against protonemata of this species. Protonemata are filamentous gametophytes which have been kept from undergoing normal differentiation by being cultured in red light or darkness. In their assay, germinated spores were grown in darkness for three days and then transplanted onto media supplemented with various dilutions of antheridiogen. The cultures were then maintained for a week of additional dark growth. Protonemata were then scored for the presence

or absence of a single antheridium. The authors stated that their assay was more accurate than previous ones because the protonemata were more sensitive to antheridia induction and they were all at a uniform growth stage when the antheridiogen was applied. In addition, the entire assay was performed in darkness, eliminating photosynthetic and photomorphogenic influences on growth and development.

Genetic studies of antheridiogen responses.

Considerable variation exists with regard to antheridiogen responses within species. Several studies have demonstrated that different strains of the same species often show different sex ratios when gametophytes are grown in multispore cultures (Schedlbauer, 1974; Lloyd and Warne, 1978; Cousens, 1979; Warne and Lloyd, 1981). For instance, Schedlbauer (1974) tested the intraspecific activity of antheridiogen from two strains of Ceratopteris thalictroides and observed markedly different responses.

Another striking example of intraspecific variation in Ceratopteris has been examined by Scott and Hickok (1987). Two completely homozygous strains of C. richardii were used in this study. The two strains, H and 176D, exhibit markedly different responses to antheridiogen which result in gametophyte populations with either high or low frequencies of males. Scott and Hickok (1987) analyzed F1 hybrids and an F2 generation produced from these strains and concluded that the differences in response are due either to a single nuclear gene or to two linked, nuclear genes. This study provided the first

experimental evidence supporting the inheritance of antheridiogen sensitivity by one or more nuclear genes.

In another study using C. richardii, Warne and coworkers (1988) utilized mutants which exhibited altered sensitivities to antheridiogen. Three mutant strains were studied, two of these exhibited total insensitivity, while one was much less sensitive than the wild-type. The three mutant strains were used to generate various crosses with different wild-type strains and the responses of F1 gametophyte populations were analyzed. The results of these analyses were most easily explained by assuming that each of the mutants differ by one nuclear gene from the wild-type.

Objectives of This Project

The investigation described in this report was initiated to improve techniques for analyzing responses to antheridiogen in Ceratopteris richardii and to clarify the genetic model proposed by Scott and Hickok (1987). Each of the remaining five chapters deals with a specific aspect of this project.

Chapter two deals with the purification of Ceratopteris antheridiogen. In this chapter, a procedure for concentration and partial purification of antheridiogen is described. Descriptions of bioassays to characterize the resultant antheridiogen substance are also given and the effect of highly concentrated antheridiogen on two previously characterized strains of Ceratopteris is discussed.

Chapters three and four deal with the antheridiogen response of

individual gametophytes. In previous studies with Ceratopteris, the response to antheridiogen has been characterized as the percentage of male gametophytes in a genetically homogeneous population (Scott and Hickok, 1987; Warne et al., 1988). The obvious limitation of this approach is that it does not allow meaningful characterizations of genetically heterogeneous populations. This limitation is severe for studies involving genetic characterization, because hybrid progeny cannot be directly analyzed.

In chapter three, a study which demonstrates that genetically heterogeneous populations can be analyzed in such a way will be discussed. This study investigates the relationship between the sexual morphologies of gametophytes growing in multispore cultures and the genes for antheridiogen response which they carry.

Chapter four describes a novel procedure for determining antheridiogen responses of individual gametophytes in genetically heterogeneous populations. Examples of the general utility of this procedure will be provided.

The fifth chapter describes experiments which were performed to clarify the genetic model proposed by Scott and Hickok (1987). These experiments utilized purified antheridiogen and employed techniques for characterizing genetically heterogeneous gametophyte populations such as those described in chapter four.

The final chapter details the characterization of a mutant strain of Ceratopteris which germinates in the absence of light. This mutant

was selected in an attempt to isolate a strain which responds to antheridiogen by dark germination.

Materials and Methods

In this section, materials and methods which have been used routinely throughout the project will be described. When unique materials and methods are used in subsequent chapters, they will be described there.

Spore source.

All spore collections were taken from completely homozygous sporophytes or sporophytes generated by specific hybridizations (see below for selfing and crossing techniques). The term "strain" will be used to refer to all sporophytes of the same, completely homozygous genotype (including clones and individuals produced by further selfing) and spores derived from them. The term "hybrid" will be used to refer to individuals produced from specific crosses.

Two strains derived from field collected specimens were used. These were the H strain and the 176D strain. The H strain was derived from spores of a specimen collected in Cuba (Killip 44595, GH) and the 176D strain was derived from spores collected by R.M. Lloyd in Guyana (Lloyd 4815, BHO).

Eleven additional strains were derived from the F2 generation produced by Scott and Hickok (1987). These strains are designated as H1761-12, H1761-52, H1761-58, H1761-100, H1761-118, H1761-166, H1761-173, H1761-177, H1761-179, H1761-196, and H1761-198.

Three mutant strains derived from the H strain were also used. These strains are the antheridiogen-insensitive mutant HaC23 (Warne et al., 1988), a strain designated 57.45 which carries a gene for antheridiogen-insensitivity (from HaC57, described by Warne et al., 1988) and a separate unlinked, recessive gene which confers tolerance to the herbicide paraquat in both gametophytes and sporophytes, and the strain originally isolated for paraquat tolerance, strain HaPQ45 (Hickok and Schwarz, 1986).

Gametophyte selfing and hybridization.

Completely homozygous sporophytes were obtained by isolating gametophytes in sterile cultures prior to sexual maturity and watering frequently to induce self fertilization. Isolated gametophytes were monitored for the presence of young sporophytes. If no sporophytes were produced, these gametophytes were discarded.

Hybridizations were achieved by isolating young cordate gametophytes from the intended female strain and flooding the isolate cultures with a spermatozoid solution derived from a gametophyte culture of the intended male strain. The solution bearing spermatozoids was obtained by watering a culture with gametophytes having abundant antheridia, viewing the culture with a dissecting microscope for the presence of swimming spermatozoids and transferring the spermatozoid bearing solution to the isolates, by way of a sterile pipette.

Attempted crosses were examined under a dissecting microscope

three and four days after inoculation and screened for the presence of an embryo. Gametophytes which failed to produce an embryo were discarded and the rest were retained as putative hybrids. True hybrids were selected from among these on the basis of the expression of intermediate phenotypes in the F1 gametophyte generations, or by specific mutant marker traits. Hybrids are referred to with the egg parent listed first.

Sporophyte maintenance.

Young sporophytes were transplanted from agar-solidified medium into styrofoam cups filled with soil and and having small holes in the bottom. These were maintained in a greenhouse in trays of standing water which were flushed periodically. Larger sporophytes were grown in plastic pots with drip tubes providing water.

Medium preparation.

All cultures were grown in 60 mm X 20 mm petri plates on agar-solidified (1%) Parker/Thompson inorganic nutrient medium which was modified from Klekowski (1969b). The pH of this basal medium was adjusted to 6.0 prior to autoclaving.

Preparation of antheridiogen supplemented media.

Antheridiogen was added to media in two forms. In some experiments, a crude aqueous filtrate (CAF) derived from medium which supported mature gametophyte cultures of the H strain was used (see below). In other experiments, antheridiogen was supplied in a the

form of a concentrated antheridiogen purified by extraction (CAPE) derived from CAF. A detailed description of the production of CAPE will be provided in chapter two.

Antheridiogen supplemented medium was prepared by adding various quantities of CAF or CAPE, volume for volume, in place of distilled water in the basal medium. These antheridiogen supplements were added to medium prior to autoclaving. Preliminary experiments showed that autoclaving had no measurable effect on the antheridiogen activity of CAF. The effects of autoclaving CAPE will be discussed in chapter two.

Antheridiogen source.

Gametophytes of the H strain were grown in liquid medium in wide bottom, 2.8 liter flasks, as described by Warne and coworkers (1988) with slight changes in temperature and light. In each flask, 20 mg of spores was grown in one liter of basal medium without agar, under cool white fluorescent light (90 $\pm$ 7 umol s^{-1} m^{-m} photon flux density) at 27 $\pm$ 2 C. on a culture shaker at 50 rpm for 20 days.

The liquid medium containing antheridiogen activity was harvested by removing gametophytes with suction filtration. This procedure yielded slightly less than 1,000 ml of liquid medium for each culture. The medium was made up to one 1,000 ml by adding sterile distilled water. This conditioned, liquid medium is referred to as crude aqueous filtrate (CAF). Each liter of CAF was frozen for later use.

Spore sterilization and sowing.

Spores were sterilized as described by Warne and coworkers (1986). They were soaked in water in a conical centrifuge tube overnight and treated with a 1:5 solution of commercial bleach (5.25% Na hypochlorite by weight) and water for three minutes. Spores were washed in sterile distilled water. This water was removed and the spores were resuspended in sterile distilled water and sown by depositing drops of suspension on the medium and spreading with a wire loop. Removal of the water and sterilizing solution in each of the above procedures was accomplished by placing the tip of a Pasteur pipette firmly on the bottom of the conical centrifuge tube and withdrawing the liquid by aspiration, leaving the spores in the tube.

Spore density.

A constant spore density was utilized in all experiments. Five drops of a standard density spore suspension were sown onto each culture plate. The standard suspension contained ca. 0.36 mg spores/0.1459 ml water (ca. 0.1459 ml/5 drops) and medium in each culture plate had a surface area of 22.5 cm^2, giving a spore density of ca. 20 spores/cm^2.

Culture conditions.

Cultures were maintained under a constant thermo-photoperiod of 27 $\pm$ 2 C and 90 $\pm$ 7 umol s^{-1} m^{-2} photon flux density.

Germination counts.

Culture plates were viewed with a dissecting microscope at a magnification of 120X. A grid with 6 mm X 6 mm squares was placed beneath the cultures so that it could be seen through the medium. The squares were used to mark off individual fields of view so that spores could be counted. Squares were sampled successively until 100 spores had been scored for germination (based on the presence or absence of an observable primary rhizoid).

Sex ratio determination.

Three characteristic morphologies were identified in multispore cultures: 1) exclusively male gametophytes (males) which bore only antheridia and possessed no organized meristem region, 2) gametophytes which did possess an organized meristem region (cordates) and had either produced or were capable of producing both kinds of gametangia, and 3) gametophytes which had neither an organized meristem or gametangia of either kind (neuters).

To score the ratio of males to cordates to neuters, a permanent slide was made for each culture. This was done at 8 days following soaking (DFS) of spores in water. Slides were made by removing all gametophytes from a randomly selected section of the culture and mounting them on a slide with a 4:1 mixture of Hoyer's (Beeks, 1955) solution and 0.5% acetocarmine with iron.

When less than 200 gametophytes were present on a slide, all

gametophytes were counted. When more than 200 were present, the sex ratio was determined by scoring 200 individuals at random.

CHAPTER II

CONCENTRATION AND PURIFICATION OF ANTHERIDIOGEN

Introduction

The antheridiogens of several schizaeaceous ferns have been characterized and are similar in structure to known gibberellic acids (GAs; see chapter one, introduction). Certain GAs can also substitute for the natural antheridiogens of these species (see chapter one, introduction). However, in general, there are no commercially available compounds which act as antheridiogens for other fern species and the native compounds must be obtained from gametophytes.

The effects of naturally-derived antheridiogens on fern gametophytes have been studied in two main ways. The pheromone has either been added to growth medium as part of an aqueous filtrate from medium which previously supported gametophyte growth (i.e. Dopp, 1950; Naf, 1959; 1960; Schedlbauer, 1976a), or it has been partially purified prior to addition (i.e. Naf, 1968; Endo et al. 1972; Gemmrich, 1986; Nester and Coolbaugh, 1986).

The use of an aqueous filtrate as the source of antheridiogen was first employed by Dopp (1950) in early experiments with Pteridium. This method has been used widely because of the simplicity of the technique. In most applications, gametophytes were first grown to maturity on agar-solidified medium. They were then removed and the

medium was frozen and thawed. Freezing and thawing agar-solidified medium produces a semi-solid phase and an aqueous phase which may be separated by filtration. The aqueous phase contains antheridiogen activity and may be used as a source of the pheromone in experimental applications. A variation of this method which has been successfully employed with Ceratopteris simplifies the procedure by growing antheridiogen production cultures in liquid medium (Warne et al., 1988).

Although aqueous filtrates have been used effectively, they have the following limitations. Because only a small fraction of such filtrates are biologically active, they are bulky and difficult to store. Because they are crude filtrates, they are undefined with regard to other compounds which may be present besides antheridiogen. And finally, since they are aqueous and not concentrated, the activity of media supplemented with them are bounded by upper limits which may fall short of inducing maximal responses in some strains (for example, see Scott and Hickok, 1987).

Techniques involving concentration and partial purification of antheridiogen (see Endo et al., 1972; Gemmrich, 1986; Naf, 1968; Nester and Coolbaugh, 1986; Nester, Veysey and Coolbaugh, 1987; Pringle et al., 1960; Takeno and Furuya, 1980; Yamane et al., 1979) have made use of the structural similarities between antheridiogens and known GAs by employing standard purification techniques used for GAs (i.e. Crozier and Durely, 1983). For large scale purifications, the CAF is first concentrated in a rotary evaporator. Subsequent

purification involves partitioning and extracting the filtrate with organic solvents to isolate the antheridiogen activity.

Past experiments using GA3, GA4 and GA7 have indicated that Ceratopteris gametophytes are not sensitive to this class of compounds (Schedlbauer, 1974; Warne and Hickok, pers. comm.). Such results might suggest that GA purification protocols would be unsuccessful for purifying Ceratopteris antheridiogen. However, it should be noted that while three GAs have been tested for antheridiogen activity in Ceratopteris, at least 53 natural GAs are known (Goodwin and Mercer, 1983). Additionally, work by Warne and Hickok (1989) with GA biosynthesis inhibitors indicates that the antheridiogen of this taxon is produced by a pathway which shares some steps in common with gibberellin biosynthesis, or at least involves similar reactions.

This chapter describes the successful use of techniques such as those described above, to partially purify the antheridiogen of Ceratopteris. The activity of the partially purified substance is compared to the activity of a crude aqueous filtrate (CAF) of medium which previously supported gametophytic growth. The effects of the purified substance on two strains of Ceratopteris, which were previously characterized on CAF supplemented medium (Scott and Hickok, 1987), are described.

Materials and Methods

Concentration and purification of CAF.

A total of 13.5 liters of CAF was concentrated and partially purified. This volume was concentrated in individual aliquots ranging from 380 ml to 600 ml. Each aliquot was first titrated to pH 8.0. Preliminary experiments indicated that recovery of antheridiogen activity was more complete near this pH. The CAF was dried in a rotary evaporator at temperatures between 35 and 40 C. The dried residue was resuspended in a 0.5 M potassium phosphate buffer (pH 8.0) to $1/30^{th}$ the original volume and frozen for later purification.

Partial purification was accomplished using a standard procedure for group separation of GAs described by Crozier and Durely (1983). Aliquots of the concentrate in buffer were thawed, pooled and partitioned five times against 1/2 volumes of toluene to remove nonpolar materials. The antheridiogen activity was removed from the buffer by lowering the pH to 2.5 and extracting five times with 2/5 volumes of ethyl acetate. The combined ethyl acetate fractions were dried at temperatures below 35 C, resuspended in distilled water $1/4^{th}$ the volume of the buffer solution (i.e. $1/120^{th}$ the original volume of CAF) and frozen in 1 ml aliquots for later use. This aqueous antheridiogen solution is referred to as concentrated antheridiogen purified by extraction (CAPE).

Sterilization of CAPE and post-autoclave addition.

To sterilize CAPE so that it could be added to medium after autoclaving, the substance was passed through a 0.2 um filter under pressure from a 3 cc syringe. Autoclaved, basal medium was allowed to cool for 10 minutes prior to the addition of sterile CAPE.

Size determinations of gametophytes.

Spores were soaked and then sown at standard densities on test medium (i.e. plain, 0.2% CAPE supplemented or 24% CAF supplemented) and allowed to grow for eight days following soaking. Individuals of uniform size exhibiting early meristem development were transferred to fresh plates of test medium at densities of 15 gametophytes per 60 x 15 mm culture plate. At nine days following transfer, slides were made. The surface areas of mounted gametophytes were determined using an IBM (TM) personal computer with Bioquant (TM) software and a Hipad digitizing pad.

Spectrophotometric analysis.

Samples of CAF and CAPE were analyzed on a Shimadzu UV-160, UV/Visible spectrophotometer. The samples were analyzed in the scanning mode at wavelengths between 200 and 800 nm.

Statistical analyses.

The averages of size measurements were compared for significant differences using Duncan's multiple range test and Tukey's Studentized

range test. These tests were run using a SAS (TM) software program on an IBM (TM) personal computer.

Results

CAPE activity.

The process of autoclaving affected the activity of CAPE. Figure 2-1 shows the results of a dose response experiment which employed media supplemented with CAPE added both before and after autoclaving the media. Both the H strain and the 176D strain exhibited greater responses on media to which CAPE was added before autoclaving. However, this difference was most pronounced in the H strain.

The activity of CAPE was between 112 and 170 times that of CAF (see figure 2-2). The CAF, here produced by liquid culture techniques, yielded an activity similar to that of the CAF derived from agar-solidified cultures such as that used by Scott and Hickok (1987). The percentages of male gametophytes on media supplemented with 0.025, 0.05 and 0.10% CAPE were equal to those which would be predicted for cultures grown on 4.25, 5.6 and 12% CAF supplemented media respectively (see figure 2-2).

Effect of CAPE and CAF on growth and development.

The addition of CAPE to the basal growth medium had no affect on the timing of germination. A comparison of germination data for strains H and 176D on plain and 0.2% CAPE supplemented medium is shown in figure 2-3. Although the two strains differed slightly, such that

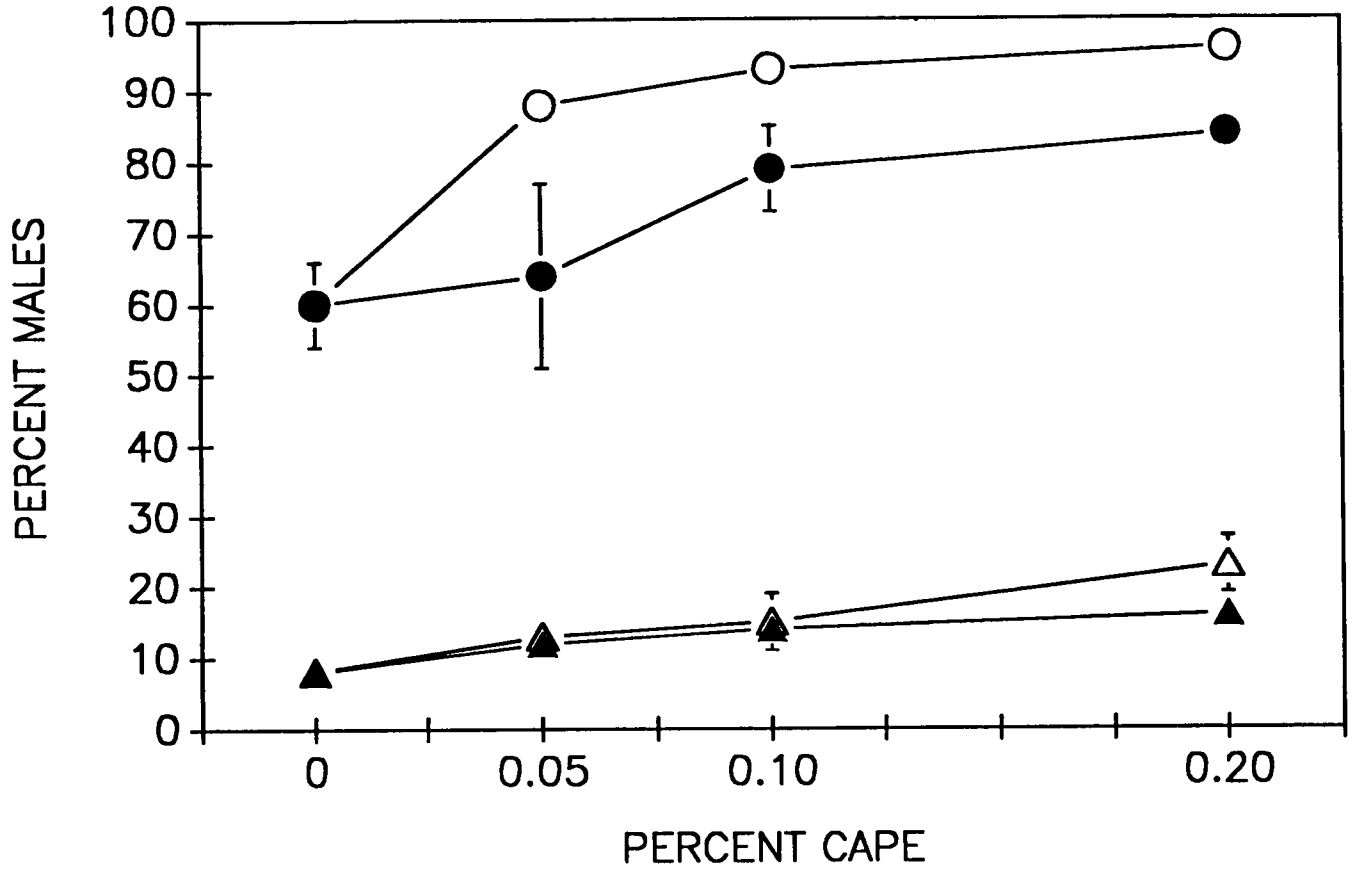

Figure 2-1. The effects of CAPE added to media before and after autoclaving the media for strains H and 176D. The H strain is symbolized by circles and the 176D strain is symbolized by triangles. Open symbols represent conditions for which CAPE was added prior to sterilization. Filled symbols represent conditions for which CAPE was added after sterilization. Each point represents the average of three replicates ± the standard deviation of the sample.

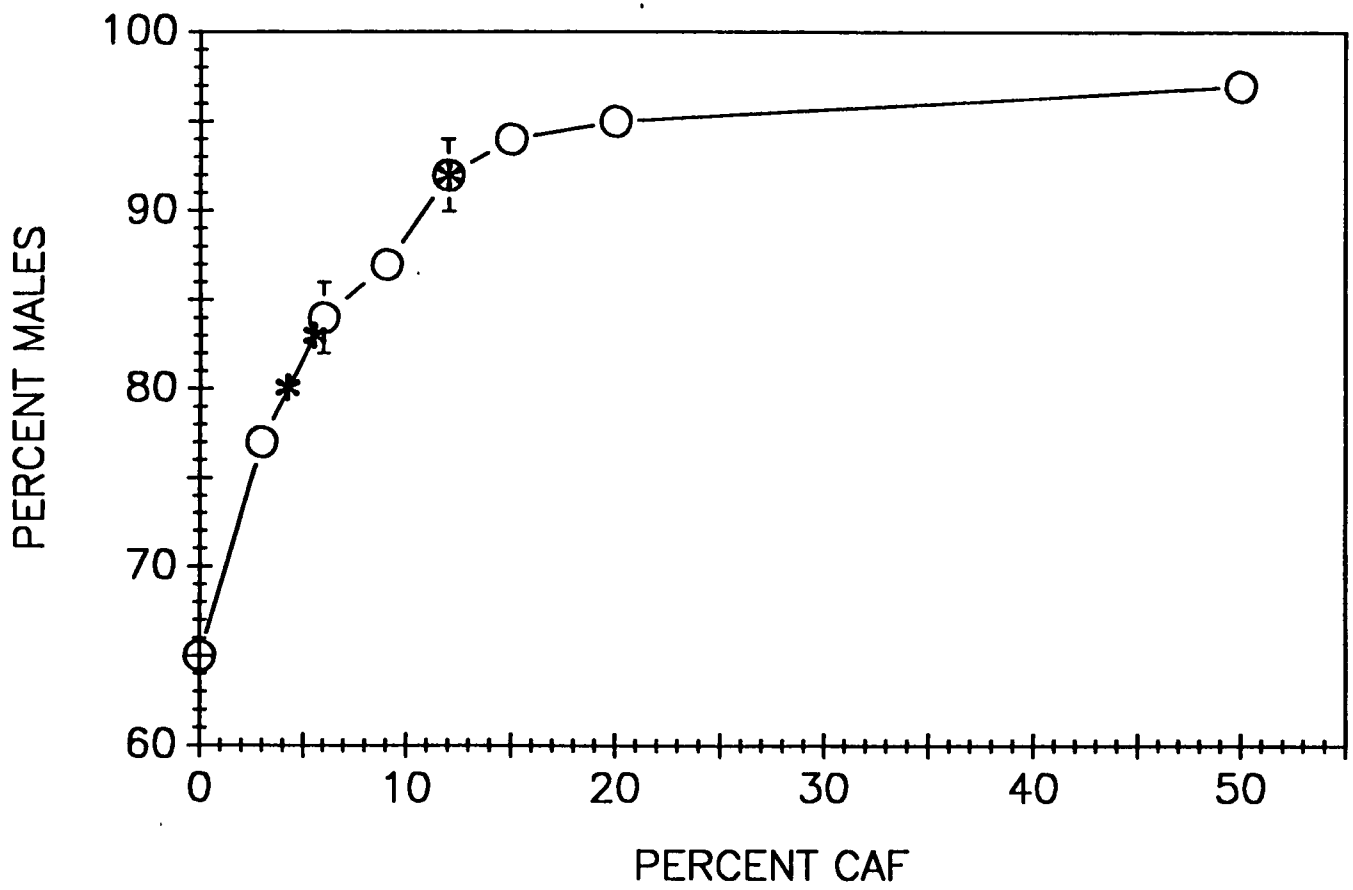

Figure 2-2. A comparison of the activities of CAF and CAPE assayed against the H strain. The three points representing the responses to 0.025% CAPE, 0.05% CAPE, and 0.1% CAPE are shown as asterics in that order from left to right. Each point represents the average of three replicates ± the standard deviation of the sample.

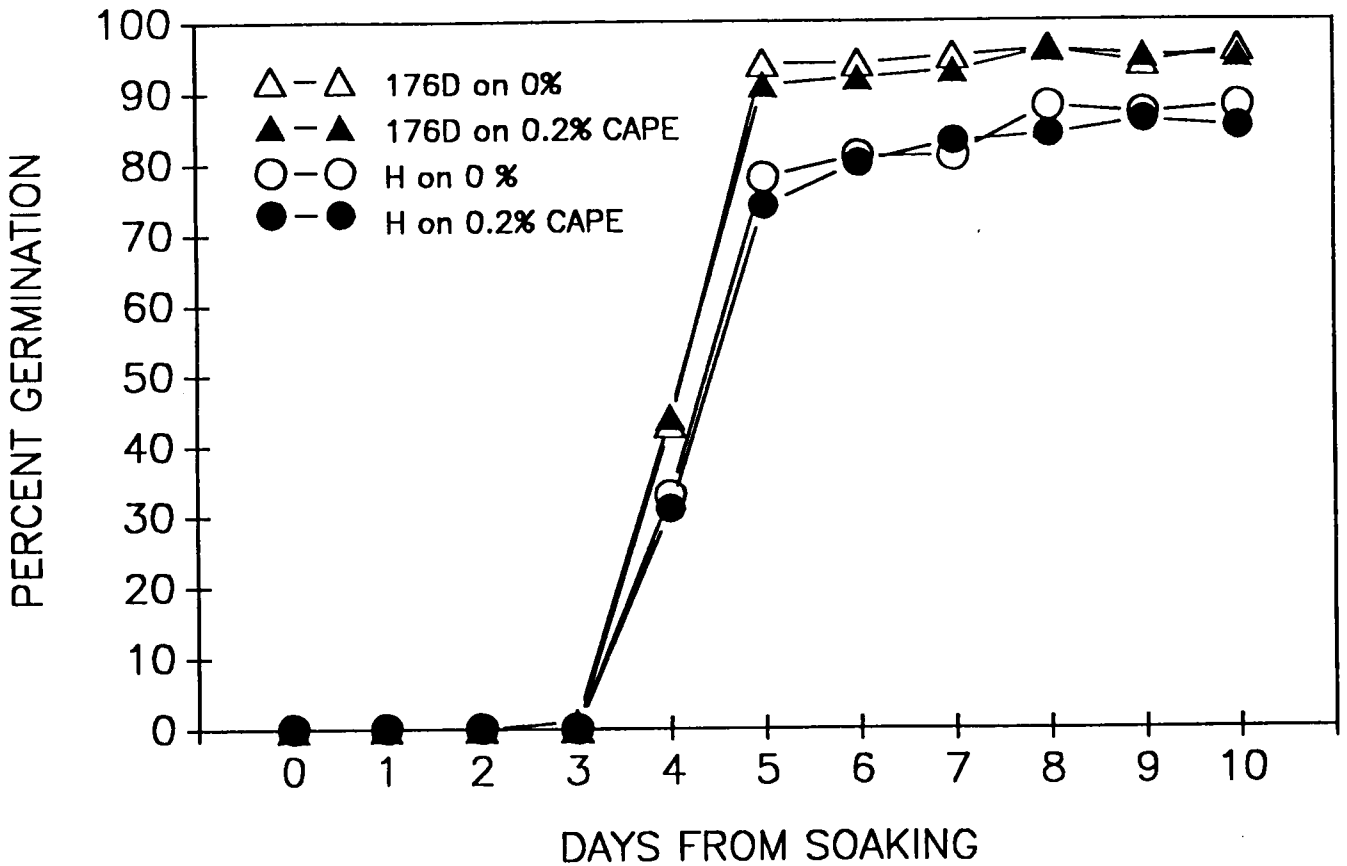

Figure 2-3. A comparison of the germination timing of strains H and 176D on plain and 0.2% CAPE supplemented medium. Each point represents the average of three replicates. The greatest standard deviation was ± 9% of the average.

176D exhibited a high degree of germination earlier than H and a greater overall level of germination, no consistent difference was observed between treatments within strains.

The trends in gametophyte sizes on the three types of media (plain, 0.2% CAPE supplemented and 24% CAF supplemented) varied among the three strains tested (see table 2-1). Gametophytes of the H strain were smallest on plain medium while the average sizes of individuals on 0.2% CAPE supplemented medium and those on 24% CAF supplemented medium were the same. Two statistical tests confirmed that the average size on plain medium was significantly different from those on the other two types of media (see table 2-1). The average sizes of 176D gametophytes varied less than did those of the H strain. The largest of these gametophytes grew on 0.2% CAPE supplemented medium, while those which grew on plain medium were somewhat smaller and those on 24% CAF supplemented medium were the smallest. Statistical analyses indicated that the average size of gametophytes on plain medium was not significantly different from those of gametophytes growing on either type of antheridiogen supplemented medium. However, the average sizes of gametophytes on the two types of supplemented media were significantly different. The average sizes of HaC23 gametophytes, a CAF insensitive strain (Warne et al., 1988), were nearly identical on all three types of media.

Table 2-1. The effect of Antheridiogen on Gametophyte[a] Sizes[b] compared by statistical analyses[c].

	Treatments		
Strain	Plain	0.2% CAPE	24% CAF
H	3.7 ± 0.5 A	4.3 ± 0.7 B	4.3 ± 0.6 B
176D	5.7 ± 0.8 A B	5.8 ± 0.7 A	5.4 ± 0.7 B
HaC23	3.3 ± 0.4 A	3.2 ± 0.4 A	3.3 ± 0.4 A

[a]Gametophytes were grown for 17 days prior to size determination.

[a]Sizes indicates the mean size in mm^2 of 35 gametophytes ± the standard deviation of the sample.

[c]Duncan's multiple range test and Tukey's Studentized range test were used to compare the average sizes among treatments for each strain. Both tests gave the same results. For each strain, average sizes which were not significantly different are shown with the same letters indicated to the right of the values.

Spectral characterization of CAF and CAPE.

The UV/visible spectra (200 - 800 nm) for CAF (undiluted) and CAPE ($1/120^{th}$ dilution to give approximate equivalence) are shown in figure 2-4. In general, CAF exhibited much greater absorbance in this range, with a maximum of 2.5. The main absorbance occurred between 200 and 350 nm with a peak at 212. However, the absorbance spectrum of CAF appeared to extend to wavelengths below 200 nm as well. The maximum absorbance for CAPE (both autoclaved and non-autoclaved) occurred between 200 and 350 nm. However, the maximum degree of absorbance in this range was considerably less than that of CAF. For the nonautoclaved CAPE, a maximum absorbance of 0.045 was observed, for the autoclaved CAPE, this value was 0.05. No peak was observed for either form of CAPE in this range, indicating that CAPE absorbs additional energy below 200 nm.

Characterization of strains 176D and H on CAPE supplemented media.

Two strains of C. richardii which were previously characterized on CAF supplemented media (Scott and Hickok, 1987), showed similar patterns of antheridiogen response when grown on a range of CAPE concentrations (Figure 2-5). However, the maximum percentage of males (93%) in cultures of the H strain was slightly lower than it was in Scott and Hickok's study using CAF (98%). The two strains exhibited markedly different responses over the range of CAPE concentrations tested. The magnitude of the responses indicates a similar degree of

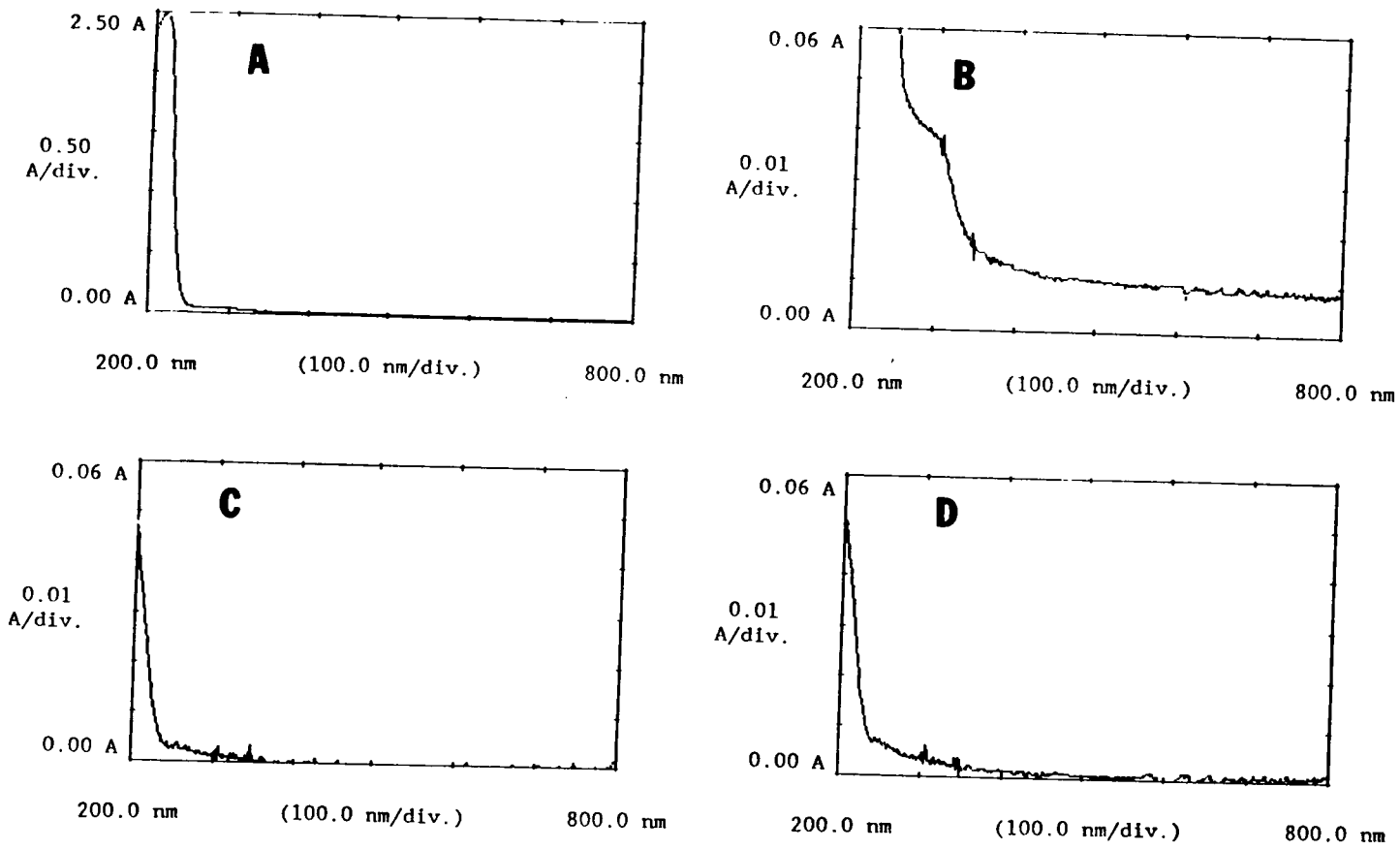

Figure 2-4. A comparison of the UV/visible spectra of CAF and CAPE. A) The spectrum of full strength CAF. B) The spectrum of full strength CAF with the scale adjusted to equal that of c. and d. C) The spectrum of 1/120 diluted CAPE which has not been autoclaved. D) The spectrum of 1/120 diluted CAPE which has been autoclaved.

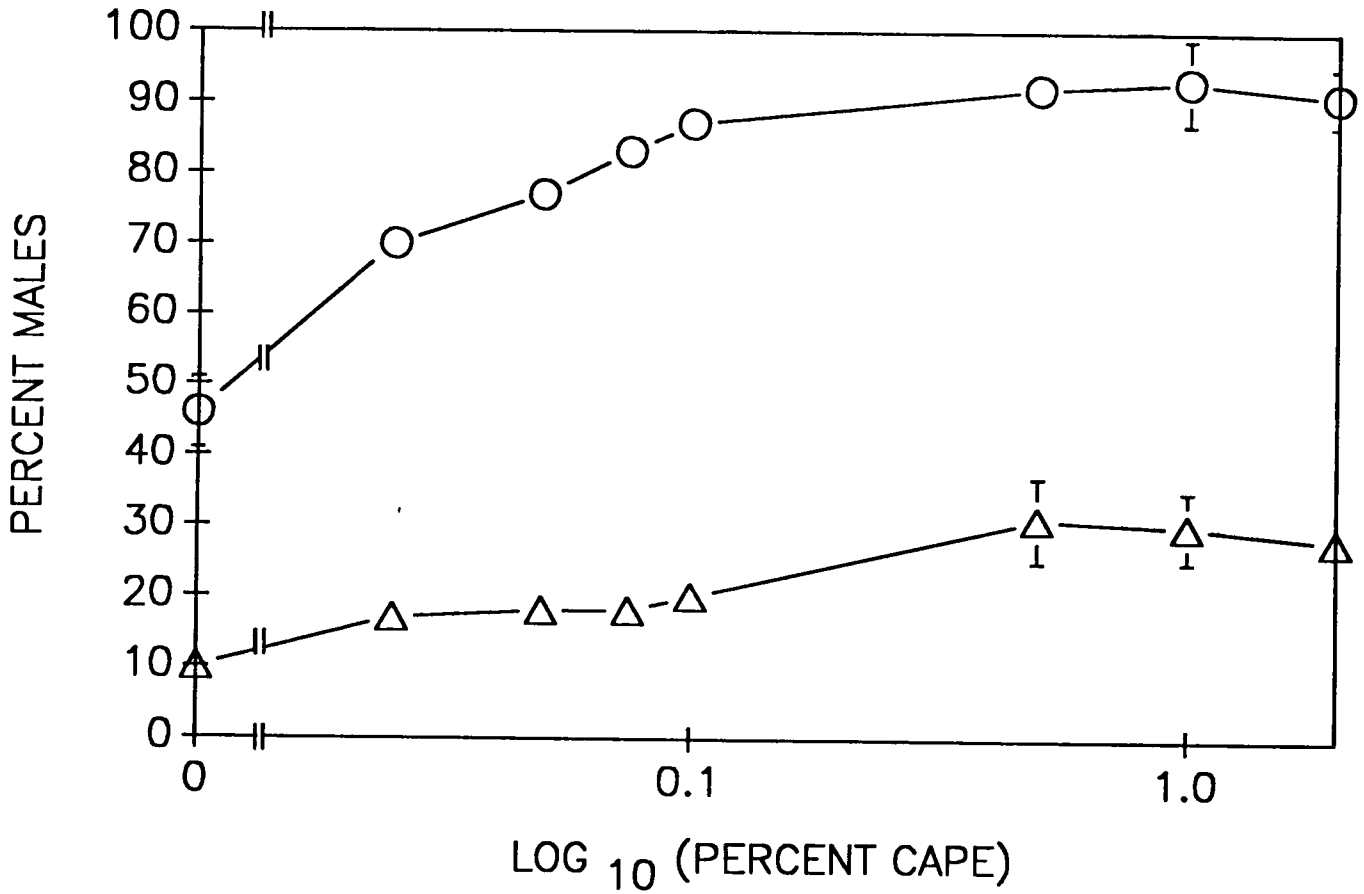

Figure 2-5. The responses of gametophytes of the H strain (circles) and the 176D strain (triangles) to a range of CAPE concentrations. Each point represents the average of three replicates ± the standard deviation of the sample.

antheridiogen concentration as determined from the experiment illustrated in figure 2-2.

Both strains exhibited a general trend of increasing response with increasing antheridiogen concentrations. However, at higher concentrations, the response of each strain reached an apparent maximum and became level. For the 176D strain, this maximum was around 30% males, and for the H strain, it was around 93%.

Discussion

CAPE activity.

The observation that the activity of CAPE is enhanced when added to medium before autoclaving the medium was somewhat unexpected since many biologically active compounds loose activity when heated. However, this result is not entirely without precedent in the study of antheridiogens. A similar observation was made by Naf (1965) in a study involving the antheridiogen of *Onoclea sensibilis*. Naf observed that the crude aqueous filtrate of *Onoclea* medium lacked the ability to induce antheridia unless it was first heated. Two alternative hypotheses were suggested by these results: 1) heating the filtrate inactivated an antheridiogen inhibitor, or 2) heating converted an inactive form of antheridiogen to an active form. Although Naf was unable to disprove either hypotheses, his results favored the latter.

The results of this investigation are different from those reported by Naf for *Onoclea* antheridiogen in several ways. Firstly, Naf worked with a compound which exhibited no activity unless it was

heated. In this study, the substance showed activity both when added to medium before and after autoclaving; the activity being greater when the substance was autoclaved with the medium. Secondly, Naf observed this phenomenon in an aqueous filtrate of Onoclea medium, whereas the results reported here pertain to a partially purified form of Ceratopteris antheridiogen. Hickok (1983) reported that autoclaving Ceratopteris CAF had no effect on its antheridiogen activity. Additionally, Warne et al. (1988) used autoclaved CAF and in their experiments the H strain responded with high frequencies of males on media supplemented with as little as 10% CAF.

It is likely that some step in the purification process used in this study reduced the antheridiogen activity of CAPE. This reduction in activity was then apparently reversed by exposure to high temperatures. A possible explanation is that the antheridiogen molecules crystallized during evaporation and some portion remained insoluble until heated. Another alternative is that during the purification procedure, some of the antheridiogen molecules became conjugated with other types of organic molecules and subsequent heating eliminated this association. A final possibility is that two or more antheridiogens are present in CAPE and one or more of these is heat activated.

The possibility that some chemical alteration occurred when the CAPE was autoclaved with the medium is supported to some degree by comparison of the absorption spectra for nonautoclaved versus autoclaved CAPE (figure 2-4 C and D). Although these two spectra are

nearly identical, the spectrum for autoclaved CAPE shows a slightly higher degree of absorbance over the entire range. However, these observations do not provide sufficient basis for further speculation regarding the chemical nature of this phenomenon.

That the activity of autoclaved CAPE appears to be from 112 to 170 times that of CAF suggests that concentration and purification procedures were successful. Since the total volume of CAPE was $1/120^{th}$ the volume of the CAF used to produce it (see Materials and Methods), it might be expected that a very efficient protocol would yield activity increased by as much as 120 times. Additionally, it is possible that partial purification might have removed some compound with inhibitory effects, allowing isolation of a substance with even higher activity. Hickok (1983) reported that abscisic acid inhibits the action of Ceratopteris antheridiogen. Although it is not known if this substance is present in cultures of C. richardii, Hickok and Warne (pers. comm.) have found that it does occur in spores of this species.

Comparison of the spectra illustrated in figure 2-4 also suggests that the concentration and partial purification procedure was successful. This figure shows that these processes reduced the overall heterogeneity of CAPE as compared to CAF. Although the CAPE was diluted to give a similar antheridiogen concentration (based on activity) to that of the CAF, the overall absorbance was greatly reduced for CAPE. This suggests that the purification protocol selectively retained antheridiogen and removed other substances. Whether these substances

included ones inhibitory to antheridiogen effects can not be determined from the data presented here.

Gametophyte growth and development on CAPE supplemented media.

That antheridiogen supplied as CAPE did not affect the timing of germination (Figure 2-3, page 28) agrees with an earlier report by Scott and Hickok (1987) in which antheridiogen was supplied as CAF. In addition, it demonstrates that the concentration and partial purification protocol did not concentrate allelopathic substances inhibitory to germination, or contaminate CAPE with chemicals which could inhibit this process.

The trends in growth for the strains H, 176D and HaC23 on three different kinds of media (Table 2-1) were variable, and at least for the H strain, unexpected. This was the only strain which exhibited consistent, significant differences in the size of gametophytes grown on different media. The largest individuals were those which grew on 0.2% CAPE and 24% CAF supplemented media. This result contradicts the expected results because antheridiogen, in its role of promoting antheridia production, leads to reductions in vegetative growth in males. Therefore, the expected results were either a reduction in growth or no effect at all on gametophytes of the cordate morphology. It seems unlikely that the pheromone actually promotes growth in gametophytes which are otherwise unaffected by it.

A likely explanation of these results is that they do not illustrate any effect of antheridiogen, but instead are due to

unintentional selection of gametophytes with different growth potentials. The protocol used here was adapted from a standard procedure used in the laboratory of L.G. Hickok to study the effects of herbicides and other chemical growth inhibitors on gametophytes. In this protocol, gametophytes are grown on the test medium over the entire duration of the experiment to insure that the test substance is present when the gametophytes are sensitive to it. After a sufficient number of gametophytes reach the early meristematic stage (precordate stage), these are transferred to low density cultures where they continue to grow under the influence of the test compound.

When this method was used to investigate the effects of antheridiogen, the transfer of individuals at the precordate stage may have biased the results for the H strain gametophytes. When transfers were made for this strain from the cultures on plain medium, a small number of gametophytes were randomly chosen from among many precordate individuals. However, when such individuals were transferred from media supplemented with either 0.2% CAPE or 24% CAF, only a small number of precordates were present in each culture, the rest having become males. It seems likely that transferring these individuals resulted in the unintentional selection of gametophytes which were able to escape the effects of antheridiogen. This interpretation suggests an increased capacity for rapid growth was the characteristic which allowed development of cordates.

This interpretation does not reveal whether antheridiogen directly affects growth in gametophytes of the H strain. However, it

does provide support for an established model pertaining to the relationship between the developmental sequence of gametophytes and antheridiogen sensitivity. This model seeks to explain how two or more sexual morphologies could occur in a genetically homogeneous gametophyte population (i.e. produced from spores of a completely homozygous sporophyte). Naf et al. (1975) suggested that epigenetic factors affecting growth and development could account for these differences in multispore cultures on plain medium. They proposed that the most rapidly growing gametophytes developed as cordates because there was little or no antheridiogen present in the medium. However, these individuals produced antheridiogen as they grew, causing gametophytes which developed later to differentiate as males. The interpretation discussed above suggests that the H gametophytes which were selected as precordates on antheridiogen supplemented media were representative of these more rapidly growing individuals.

The average areas of 176D strain gametophytes differed significantly only for those growing on antheridiogen (CAPE or CAF) supplemented media. Based on the small degree of variation and the inconsistency of the results, it seems likely that differences in size for this strain were the result of random variation rather than the effects of the treatments. Since this strain is relatively less sensitive to antheridiogen, it is not surprising that the pheromone exhibited no effect on growth.

Gametophytes of the HaC23 strain exhibited no significant variation between the three treatments. Again, since this strain is

insensitive to antheridiogen, no effect on growth would have been predicted.

A final observation which can be made from these data is that in no instance did the presence of CAPE or CAF significantly reduce gametophyte growth. This serves as further confirmation that these substances do not contain any compounds which are inhibitory to the growth of gametophytes.

Responses of strains 176D and H to CAPE.

Since CAPE is a concentrated, partially purified form of CAF, it is not surprising that the responses of 176D and H to lower concentrations of CAPE (see figure 2-5, page 33) were similar to their responses on previously tested CAF supplemented media (Scott and Hickok, 1987). However, in this experiment, 176D and H were also grown on media with much higher antheridiogen concentrations than previously tested. In this experiment, the highest CAPE concentration (2.0%) was equivalent to 224 to 340% CAF (based on a 112 to 170 concentration factor). That gametophytes of both strains exhibited a maximum response on medium supplemented with as little as 0.5% CAPE was somewhat unexpected.

Not only did both strains exhibit a maximum response on relatively lower CAPE concentrations than expected, but the gametophytes of the H strain failed to respond as highly to any concentration of CAPE as they did on CAF in the experiments of Scott and Hickok (1987). This observation can be accounted for by differences in the pH of the

growth medium utilized in these experiments. In the present report, the medium was titrated to a pH of 6.0, whereas the medium used by Scott and Hickok was titrated to a pH of 5.4. This change in methods was implemented because preliminary experiments (unpublished) using CAF showed that a pH of 6.0 provided optimum growth conditions. This allowed gametophytes to mature earlier and the duration of experiments could be reduced from 10 to 8 days. A side effect of this alteration was that the antheridiogen response was reduced (presumably because of enhanced growth). In the preliminary experiments, the response to CAF at pH 6.0 was reduced to a similar extent as described above for the response to CAPE.

Even though the response to high concentrations of CAPE were reduced by the effects of pH, they still provide new insights into the effects of antheridiogen in multispore cultures. The observation that both strains exhibited a stable maximum response over a wide range of concentrations indicates that antheridiogen was not the limiting factor in preventing further response. It appears instead that a certain proportion of each population (a small proportion in H cultures and a larger one in 176D cultures) were insensitive to the effects of the pheromone regardless of the concentration. This insensitivity is not based on a genetic difference since both spore collections are genetically homogeneous.

A scheme proposed by Naf (1958) to account for variations in antheridiogen responses in genetically homogeneous gametophyte populations has been generally accepted (see chapter one,

introduction). In this scheme, asynchrony in growth and development combined with antheridiogen accumulation were the only factors identified as being associated with different responses. Although these factors appear to play a role in determining antheridiogen response on medium with low antheridiogen concentrations (see above), they cannot account for the responses of gametophytes at high concentrations.

A simple modification Naf's original model would make it compatible with these observations. Naf suggested that only gametophytes which grew fast enough to differentiate before antheridiogen accumulated, became cordates. However, it seems more likely that rapidly growing gametophytes may escape the effects of the pheromone even if it is present in high concentrations. This would be possible if antheridiogen induction occurred only during a sensitive phase as previously indicated (Naf, 1958), but a minimum length of time was required for induction to occur. If this were the case, gametophyte which were insensitive to antheridiogen would be ones which grew so rapidly that they existed in the sensitive phase for too brief a time to become induced. This model would explain why certain gametophytes are never induced by antheridiogen, regardless of the concentration and would also explain why altering growth conditions (i.e. by changing the pH of the medium) changes the maximum percentage of male gametophyte produced.

Summary.

The activity of the partially purified form of antheridiogen (CAPE) was altered by the process of autoclaving. As previously reported for the antheridiogen of Onoclea (Naf, 1965) this process increased the activity of this substance. Autoclaved CAPE exhibited an activity which indicated a high degree of efficiency for the partial purification process. Furthermore, the absorption spectra of CAPE and CAF indicated that the overall heterogeneity of CAPE was greatly reduced.

The presence of CAPE in growth medium did not affect the timing of germination in either strain H or 176D. Neither the presence of CAPE nor CAF reduced the average sizes of gametophytes of strains H, 176D or HaC23. Although growth increases were observed for H strain gametophytes on both types of antheridiogen supplemented media, it appeared that these results were due to an artifact in the experimental methods. However, these results do support the theory that gametophytes which escape the effects of antheridiogen are those which grow most rapidly.

The responses of strains H and 176D to a range of CAPE supplemented media were similar to those reported for these strains on CAF supplemented media (Scott and Hickok, 1987). However growth on media with higher antheridiogen concentrations revealed that both strains reached a maximum level of response on submaximal concentrations of CAPE. This suggested a new theory for the action of antheridiogen. It is proposed that when antheridiogen is present in sufficient con-

centration, it is the duration of the sensitive phase of gametophyte growth which determines whether or not induction will occur.

CHAPTER III

ANTHERIDIOGEN RESPONSE OF INDIVIDUAL GAMETOPHYTES

Introduction

Multispore cultures of fern gametophytes typically exhibit varying ratios of males:females:hermaphroditic individuals (Miller, 1968). This is the case even when spores are derived from completely homozygous sporophytes and are therefore genetically homogenous (Schedlbauer, 1976b; Scott and Hickok, 1987). This observation suggests that various factors which are not associated with the gametophytic genotype influence antheridiogen response. Such factors will be described as non-genetic factors in this report. This term requires further definition because its usage may not be self-evident in reference to gametophytes.

Fern gametophytes represent an unusual situation because many traits which may affect development (i.e., spore size, maternal RNAs etc.) are determined at the level of the sporophyte during sporogenesis. While such traits may be genetically as well as environmentally controlled, they are not necessarily related to the genotype of the gametophyte. Therefore, such factors are non-genetic with regard to this generation. The extent to which antheridiogen response is influenced by non-genetic factors versus genetic factors is the subject of this chapter.

Based on experiments with Pteridium aquilinum, Naf (1958) suggested that the variety of sexual morphologies in multispore cultures was due to accumulation of antheridiogen together with the effects of asynchronous growth and development. He found that gametophytes did not begin to secrete antheridiogen in effective quantities until they themselves had become insensitive to it. Based upon this observation, he suggested that the most rapidly growing individuals reached the archegonial stage without being influenced by antheridiogen. However, gametophytes which germinated later or developed more slowly did so under high concentrations of antheridiogen produced by the mature individuals, and became males. Similar observations have also been made on cultures of Lygodium japonicum (Naf, 1960), Anemia phyllitidis (Naf, 1959) and Ceratopteris thalictroides (Schedlbauer, 1976b) and this scheme for the action of antheridiogen has been generally accepted.

Subsequent investigations of this phenomenon have focused primarily on factors which may affect rates of growth and development in gametophytes. For instance, Schedlbauer (1976b) considered spore size, germination time and growth rate to determine which of these factors most influenced whether gametophytes of Ceratopteris thalictroides could escape the effects of antheridiogen. He concluded that spore size provided the best indicator. This was presumably because larger spores had increased storage capacity for substances important to developmental processes (i.e., enzymes, ribosomes etc.), and therefore developed more rapidly.

The effect that specific genes may have on antheridiogen response has only recently been investigated. Scott and Hickok (1987) demonstrated that differences in antheridiogen response between two strains of Ceratopteris richardii were associated with one or more nuclear genes. They proposed two possible models to account for segregation patterns observed in an F2 generation. One model suggested that the difference in antheridiogen response was due to the effects of a single gene, while the second model accounted for this difference by the action of two linked genes. Warne and associates (1988) approached the problem of genetic characterization by selecting and characterizing mutants with altered antheridiogen responses. Three mutants were characterized and genetically analyzed. Inheritance patterns indicated that all three mutant phenotypes were associated with the activity of single nuclear genes.

Although the importance of both non-genetic and genetic factors in determining antheridiogen response has been demonstrated, the relative importance of these factors remains unknown. This chapter, addresses this question. Gametophytes were selected on the basis of their sexual morphologies from an F1 hybrid between two strains which exhibited different antheridiogen responses. An F2 generation was produced by selfing these gametophytes and the F2s were tested for their responses to antheridiogen. The F2 responses were considered to determine whether they could have been predicted from the response of the F1 gametophytes (i.e., sexual morphology) under the relatively uncontrolled conditions used here (cf. chapter four).

The hybrid used to produce the F2 generation was obtained by crossing two strains which differed greatly in their sensitivity to antheridiogen (see figure 3-1 for an outline of the production of the F2s). These strains were derived from the two most extremely responsive individuals of the F2 generation described by Scott and Hickok (1987). To distinguish the F1 hybrid and F2 generation utilized in the present study from those described by Scott and Hickok, those of the present study will be termed the F1′ and the F2′. Cultures of strains H1761-177, one of the strains used to generate the F1′, contain less than 5% males at most tested antheridiogen concentration. Cultures of the other, H1761-198, contain more than 95% males at many of the same concentrations. The hybrid between these strains yields spores which produce gametophyte populations which exhibit about 50% males on antheridiogen supplemented medium.

Based on the genetic analyses of Scott and Hickok (1987), the two parental strains (H1761-177 and H1761-198) are likely to differ by either one gene or two linked genes. According to their model, the extreme levels of response exhibited by these strains (i.e., as compared to 176D and H) are due to other independently assorting, minor genes which indirectly affect antheridiogen sensitivity. In either case (one or two linked genes), the F1′ gametophyte population should be composed of two main groups in equal proportions, having parental type genotypes. Since the F1′ gametophytes exhibit an approximate 1:1 sex ratio on antheridiogen supplemented medium, it should be possible to directly test whether the sexual morphologies of gametophytes can

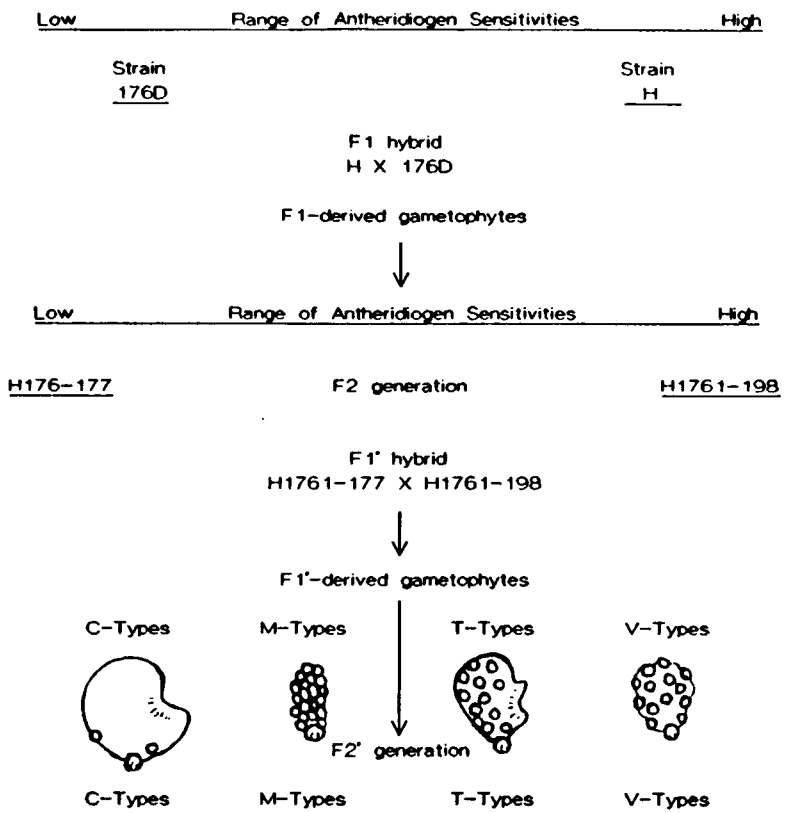

Figure 3-1. A schematic outline showing the production of an F2 (F2') generation from selected gametophytes. The lines labeled "Relative Antheridiogen Sensitivity" allow comparison of the antheridiogen sensitivities of the original parental strains (176D and H) and the two F2s (H1761-177 and H1761-198) which were used to generate the F1' sporophyte. The illustrations of the four types of F1'-derived gametophytes show the criteria used in selecting individuals for production of the F2' generation.

be used to predict the response of sporophytes produced from them by selfing. If this is the case, F1' gametophytes selected for their sexual morphologies should produce F2's with predictable antheridiogen sensitivities.

Materials and Methods

Gametophytes were selected from the F1'-derived gametophyte population based on their individual antheridiogen responses. The F1' gametophytes were grown on 6% CAF supplemented medium for selection.

Gametophytes were isolated from the F1' population to produce F2' sporophytes if they exhibited one of four distinct morphologies: 1) Gametophytes which possessed a well defined meristem region (i.e., a meristematic notch) and three or fewer antheridia were termed cordate-types (C-types); 2) Smaller, ameristic gametophytes which were almost entirely covered with antheridia were termed male-types (M-types); 3) Gametophytes which had 6 or more antheridia and a small, axial meristem (which appeared to have formed after considerable antheridia production) were termed transitional-types (T-types); 4) Gametophytes which possessed no organized meristem region and had many antheridia, but were not entirely covered with antheridia (i.e. these were larger than M-types and had exposed vegetative tissue) were termed vegetative-types (V-types). These morphologies are illustrated in figure 3-1.

These F1' gametophytes were isolated prior to fertilization and allowed to grow and differentiate. In isolate cultures, all types

eventually produced both antheridia and archegonia. After gametophytes produced both types of sex organs, they were watered to induce fertilization. Resulting sporophytes were grown as described in chapter one and spores were collected and tested for antheridiogen response as described in chapter one. Antheridiogen response was determined on 0.1% CAPE supplemented medium.

Results

Within each of the four types of F2's, most individuals responded similarly (see figures 3-2, 3-3, 3-4 and 3-5). In each group, most of the individuals exhibited either high or low percentages of male gametophytes. These responses may be compared to those of the parents, H1761-177 and H1761-198, which exhibited 4 and 99% males respectively, and to those of the 176D and the H strains, which exhibited 23 and 84% males respectively. The M-types exhibited the most variation. This group of 14 F2's exhibited a combined average of 85% males, but this included two individuals with means of 8 and 36%. The main group of M-type responses ranged from 76 to 100% males and exhibited a slightly discontinuous distribution. The C-type F2's also responded in a fairly uniform fashion with only a small discontinuity in the main distribution. The main distribution of responses ranged from 0 to 29%. However, this F2' group also included an individual which which exhibited a response unlike the others. While the combined average percentage of males among the 14 C-type F2's was 17%, this individual exhibited an average of 83% males. The T-type and V-type F2's (with a

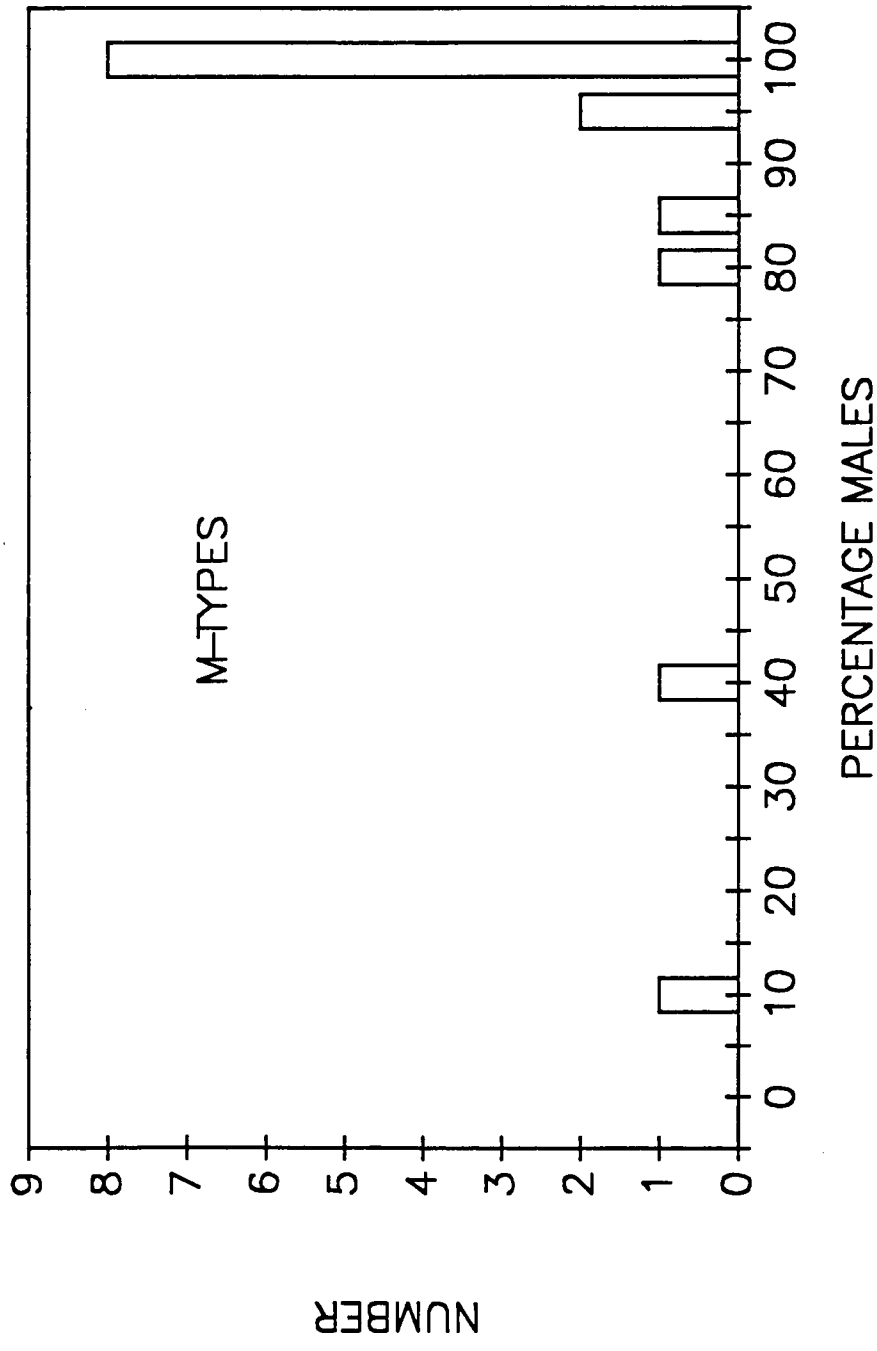

Figure 3-2. Distribution of antheridiogen responses, based on average percentages of male gametophytes, from cultures of M-type F2's. Cultures were grown on 0.1% CAPE supplemented medium. Data represent the average of three replicates for each spore collection.

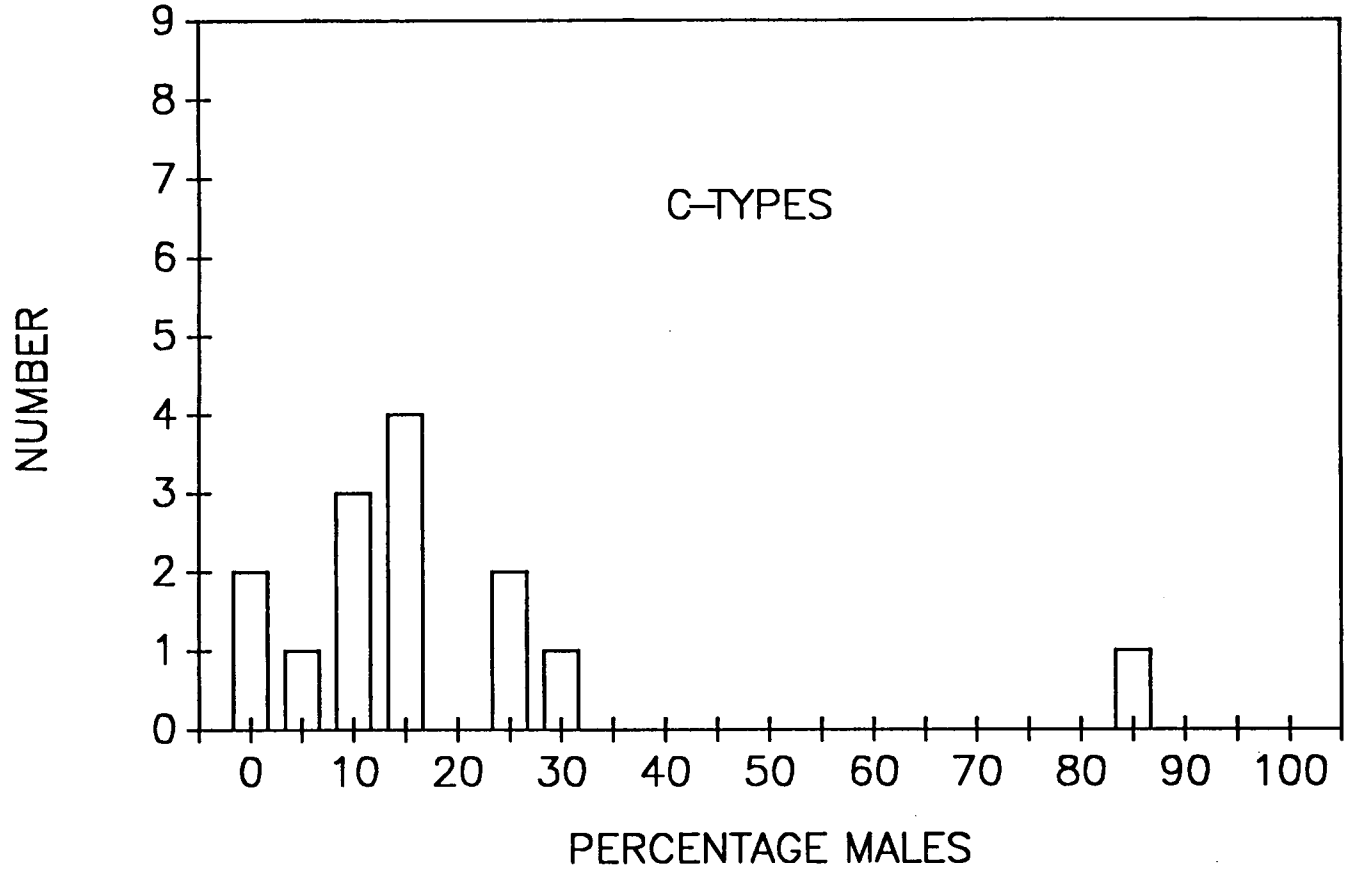

Figure 3-3. Distribution of antheridiogen responses, based on average percentages of male gametophytes, from cultures of C-type F2's. Cultures were grown on 0.1% CAPE supplemented medium. Data represent the average of three replicates for each spore collection.

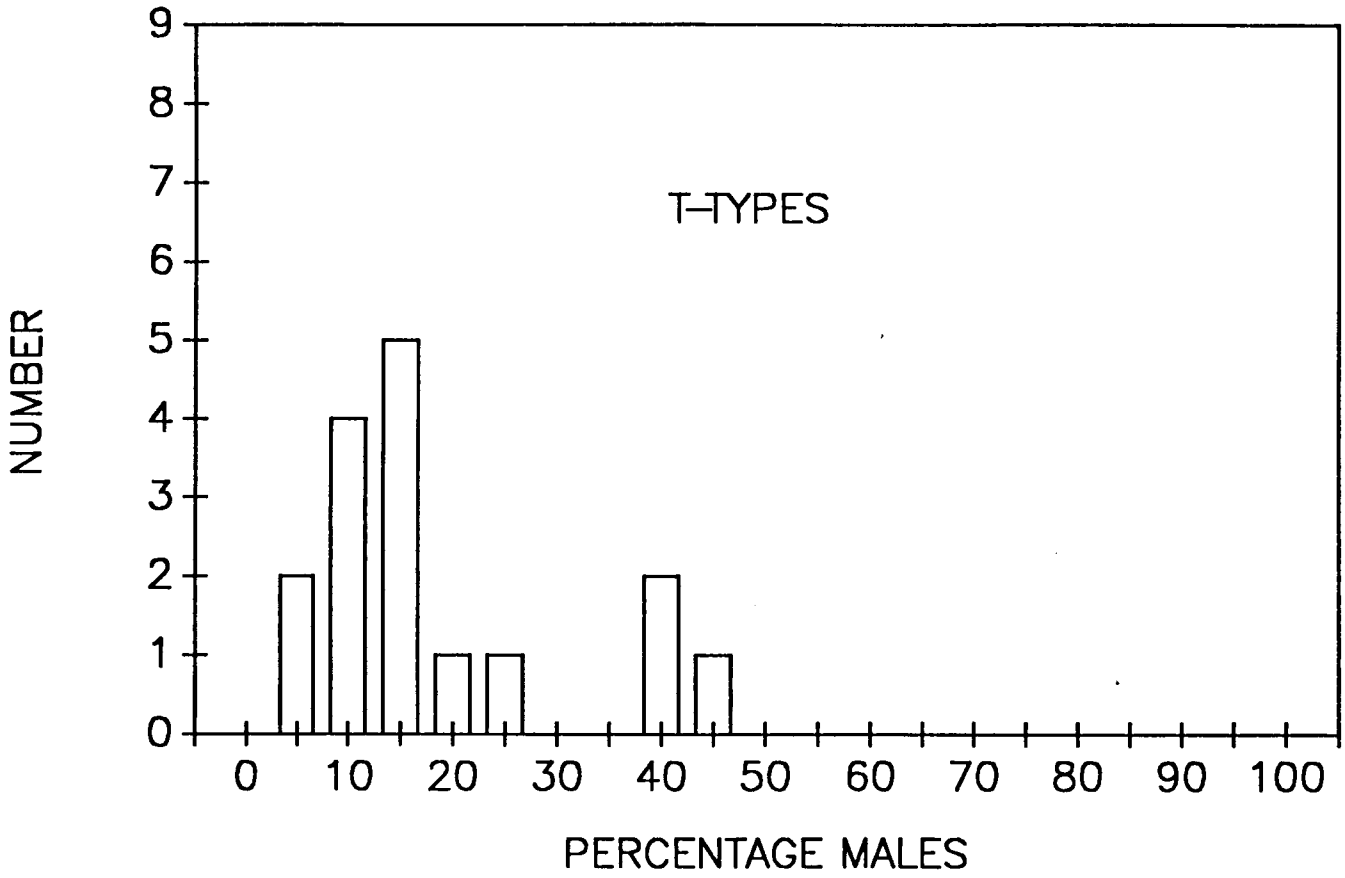

Figure 3-4. Distribution of antheridiogen responses, based on average percentages of male gametophytes, from cultures of T-type F2's. Cultures were grown on 0.1% CAPE supplemented medium. Data represent the average of three replicates for each spore collection.

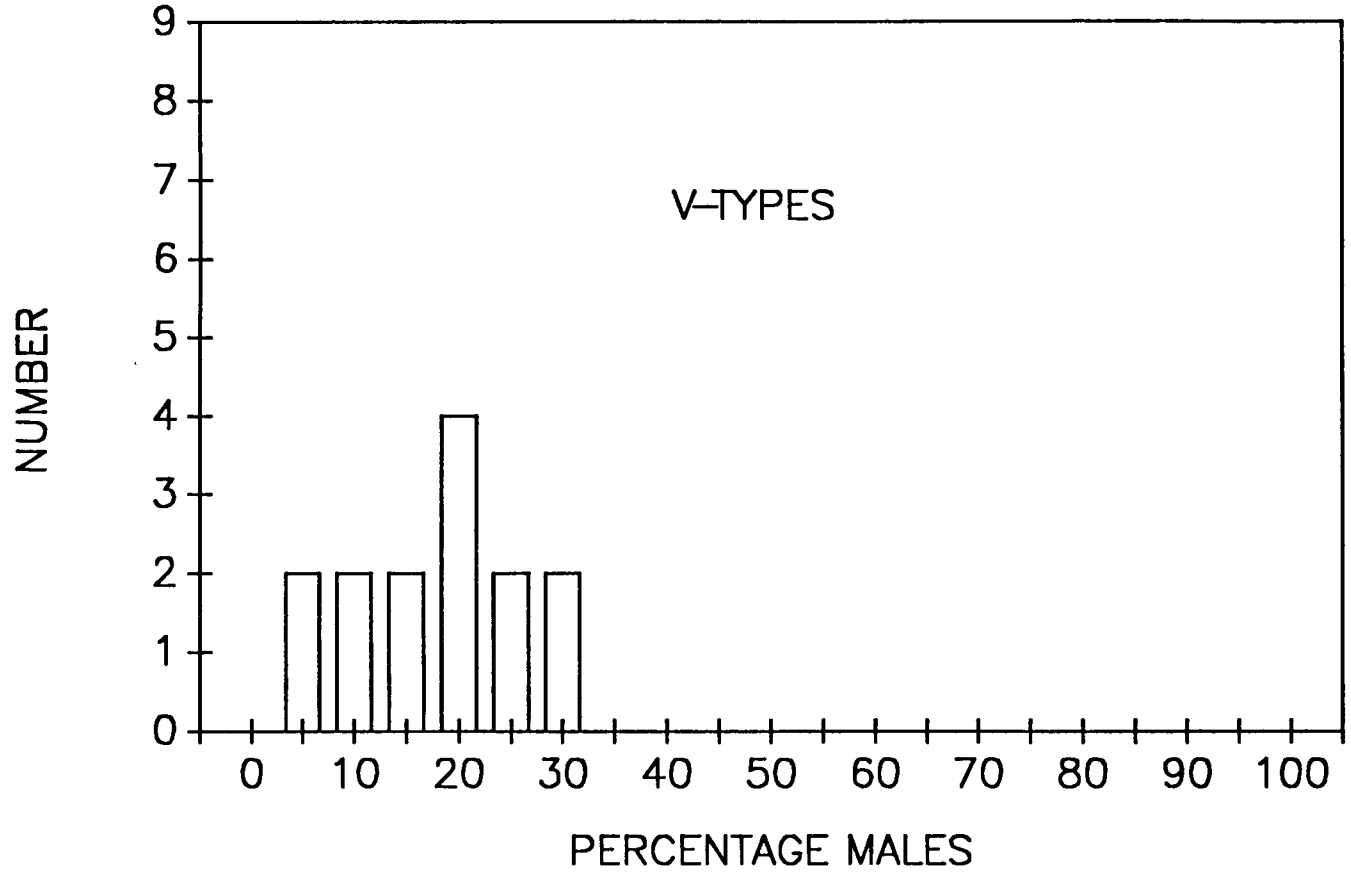

Figure 3-5. Distribution of antheridiogen responses, based on average percentages of male gametophytes, from cultures of V-type F2's. Cultures were grown on 0.1% CAPE supplemented medium. Data represent the average of three replicates for each spore collection.

total of 16 and 14 individuals respectively) contained no collections which exhibited clearly distinct responses. However, the range of responses for T-types was somewhat broad, ranging from 3 to 44% males, and discontinuous. The range of responses among the V-type F2's was narrower, from 1 to 29% males, and continuous. The average percentages of males for these F2' groups were both 16%.

The characteristic responses of the F2' categories (i.e. either generally high or generally low percentages of males) were similar to the degrees of response exhibited by the F1' gametophytes from which they were derived in three out of the four cases. The only exception was the general response of the V-types. The C-types and the T-types were produced from gametophytes which exhibited the potential to become hermaphroditic in multispore cultures. Both of these groups exhibited generally low percentages of male gametophytes. Alternatively, the M-types, which were produced from highly induced male gametophytes, generally exhibited high percentages of male gametophytes in their cultures. The V-types however, which were also selected as males, exhibited responses which were similar to those of the C-types and T-types, but not to those of M-types.

The frequency of each gametophyte type (i.e. M-type, C-type, T-type and V-type) in the F1' population from which the F2's were derived, was not readily determined. Some gametophytes had morphologies which did not correspond to the selection criteria (for instance, cordate gametophytes which had more than 3 antheridia but did not possess an axial meristem were not selected). Sex ratios were

determined, using only the categories "males" (i.e., exhibiting no meristem region and possessing antheridia) and "cordates" (i.e., exhibiting a meristem region and therefore having the potential to become hermaphroditic) and these revealed that the hybrid exhibited 58% males on selection medium (6% CAF).

Because of the unexpected response of the V-type F2's, the frequency of this type of gametophyte in the F1' population was determined. It was found that 5% of the F1' gametophytes were V-type males.

Discussion

The importance of genetic versus non-genetic factors in determining gametophyte response.

In general, the relationship between F1' gametophyte response and F2's response indicates that the influence of genotype is more significant than the influence of non-genetic factors. In most cases, the F2's of a particular group shared similar responses. And for all but one of the four groups, the general F2' response was similar to the responses of the F1' gametophytes from which they were derived.

Among M-type F2's, 86% of the spore collections (12 out of 14) exhibited similar, high degrees of response to antheridiogen. Since the M-type F1' gametophytes exhibited highly induced male morphologies, this general F2' response was expected if the gametophyte phenotype actually reflects its genotype for antheridiogen response. The two M-type F2's which exhibited low percentages of male

gametophytes in their cultures illustrate the effects of non-genetic factors. Presumably, these F2's were derived from gametophytes which carried the gene(s) for low sensitivity to antheridiogen, but were influenced by non-genetic factors to differentiate as males in the F1' gametophyte population.

Among cultures of the C-type F2's, 93% (13 out of 14) exhibited similar, relatively less sensitive responses. Again, these are the expected results if the gametophyte genotype is more important than the influence of non-genetic factors. The majority of these F2's exhibited responses similar to those of the F1' gametophytes from which they were derived. The single C-type F2' which exhibited a high sensitivity to antheridiogen shows the effects of non-genetic factors. The gametophyte from which this F2' was derived apparently carried a gene(s) which should have caused it to be sensitive to antheridiogen, however, other factors allowed it to differentiate as a cordate.

That fewer C-types were strongly influenced by non-genetic factors than M-types, may or may not be significant. Since the sample sizes were relatively small, this observation may be due to chance alone. However, if this observation represents an actual trend, it suggests that non-genetic factors are more likely to increase sensitivity to antheridiogen than they are to decrease sensitivity. This concept is consistent with previous models which suggested that factors which cause slower growth and development increase the influence of antheridiogen. However, since these cultures were grown on medium supplemented with a relatively high concentration of

antheridiogen, these observations may suggest a different mechanism than simply the interaction between slow growth and the accumulation of the pheromone.

The range of T-type F2' responses was wider and more discontinuous than were the ranges among the main groups of responses for the C-types and M-types. However, all of these F2's produced cultures with relatively low frequencies of males (less than 50%). Therefore, all of the T-types could be considered to have exhibited similar responses. Alternatively, if the T-type distribution is considered to be composed of two types of responses (see figure 3-4), then 81% of these F2's exhibited similar responses. In either case, the influence of the gametophyte genotype was apparently stronger than that of non-genetic factors.

The relatively wide range of responses of the T-type F2's may be related to the intermediate character of the sexual morphologies of the T-type F1' gametophytes. These gametophytes were among the general category typically classed as "cordates" because of the presence of an organized meristem region. However, they differed from C-type cordates because they possessed larger numbers of antheridia and appeared to have secondary meristems. The F2's produced from them, exhibited relatively less sensitive responses, somewhat similar to the responses of the C-type F2's. However, though less sensitive to antheridiogen than the M-type F2's, several exhibited responses greater than that of all but one of the C-types (whose response was considered atypical).

If T-types are assumed to have the same major gene or genes for antheridiogen response as the C-types (a theory consistent with the two possible models proposed by Scott and Hickok, 1987) then the wide range of variability exhibited by these individuals may be due to the influence of other minor genes which indirectly influence antheridiogen response. Alternatively, some or all of the T-types might represent individuals with different major genes for antheridiogen sensitivity than the C-types. If this is the case, these individuals could be accounted for by assuming that antheridiogen sensitivity is inherited by two linked genes (Scott and Hickok, 1987) and that they represent recombinant types. Alternatively, they may represent individuals with genotypes not predicted by either the one or two linked genes theory.

The V-type F2's exhibited a relatively narrow range of responses and all were similar. However, the relatively less sensitive response of this F2's group was unexpected based on the male morphology of the V-type F1' gametophytes. This incongruity suggests two possible interpretations. One alternative is that these individuals simply represent a group which was more influenced by non-genetic factors than by the gametophyte genotype. If this is the case, these results do not strongly contradict the concept that genotype is more important than non-genetic factors, since only 5% of the F1' gametophytes were V-types.

Another possible interpretation is that these gametophytes simply represent an earlier growth phase of the T-types. It is possible that

these individuals go on to elaborate secondary meristems and eventually become hermaphrodites. If this is the case, then the ultimate sexual morphology of a V-type gametophyte does correspond to the F2' response, and the gametophyte genotype is ultimately more important than non-genetic factors.

Summary and insights on the mode of inheritance of antheridiogen sensitivity.

The results of this experiment were in general agreement with the models proposed by Scott and Hickok (1987) for the inheritance of antheridiogen sensitivity. However, they did not provide new insights which would allow discrimination between these two models, nor did they specifically substantiate these models.

Both models predict that the F1' gametophyte population used here, should be composed of primarily two types of individuals present in equal proportions. One main group should carry a gene or genes associated with relatively low sensitivity to antheridiogen, while the other should carry a gene or genes associated with high sensitivity. In this experiment, the M-types, which represented 53% of the gametophyte population generally exhibited high sensitivity to antheridiogen, while the other three groups all exhibited low sensitivity to the pheromone.

The difference between the two models is that the single gene model predicts that only individuals with high and low antheridiogen sensitivity should occur, while the two linked gene model predicts

that recombinant types with intermediate sensitivities should also be present. The techniques used in this study could not distinguish between parental types and possible recombinant types.

The finding that gametophytes do respond to antheridiogen primarily on the basis of genes which they carry, suggests that it is theoretically possible to determine the antheridiogen sensitivities of individual gametophytes. This would allow determination of segregation patterns directly from gametophyte populations. However, it is also evident from this study that such characterization is not possible if based solely upon variations in the normal sexual morphology of the gametophytes. To distinguish groups with different antheridiogen sensitivities from gametophyte populations, a quantifiable response is needed. Such a response, unlike those presently known, must be observable at the gametophyte level. The identification of such a trait is the subject of the following chapter.

CHAPTER IV

ASSESSMENT OF GAMETOPHYTE ANTHERIDIOGEN RESPONSE

Introduction

The response of gametophytes to antheridiogen varies under differing culture conditions. For example, a relationship between conditions which favor slow vegetative growth and an increased proportion of males is well known for various species (Miller, 1968). A specific modification which changes the antheridiogen response of gametophytes of the fern Lygodium japonicum is growth in darkness. Takeno and Furuya (1975) germinated spores of this species in light and placed them in darkness on media with various concentrations of antheridiogen. The spores produced filamentous outgrowths known as protonemata which either did or did not produce a single antheridium in response to the pheromone.

In the genus Ceratopteris, under standard culture conditions, antheridia are produced on two morphologically distinct types of gametophytes. Cordate-shaped gametophytes with an organized meristem region produce both types of sex organs, while smaller, ameristic gametophytes produce only antheridia. Although antheridia production on both types of gametophytes is presumed to be induced by antheridiogen (Schedlbauer and Klekowski, 1972), the most obvious effect of antheridiogen in this genus is an increase in the frequency of male

gametophytes in multispore cultures (Schedlbauer and Klekowski, 1972). This effect has been used as a quantitative trait for measuring antheridiogen response in Ceratopteris in several studies (Schedlbauer and Klekowski, 1972; Schedlbauer, 1974; Hickok, 1983; Scott and Hickok, 1987; Warne et al., 1988).

In Ceratopteris, it is possible to generate completely homozygous sporophytes which produce spores that are genetically homogeneous (homogeneous). In multispore cultures, although all spores are genetically identical, various non-genetic factors (see chapter three) result in populations containing both male and hermaphroditic gametophytes. For a given genotype, the ratio of males to hermaphrodites under standard conditions is relatively constant and can be viewed as the phenotypic expression of the antheridiogen response. In other words, the "population response" of genetically identical gametophytes can be used to predict the antheridiogen genotype of the individual gametophytes. Although this method has been useful for gross characterization of antheridiogen responses, it is limited by the fact that it can only be used to characterize homogeneous gametophyte populations, and not individual gametophytes. The ability to characterize the antheridiogen phenotypes of individual gametophytes would be valuable for studying genetically heterogeneous (heterogeneous) populations. Such analyses would be especially advantageous for genetic characterizations utilizing hybrid gametophyte populations.

For many traits in Ceratopteris, genetic characterizations using hybrid gametophyte populations have been successfully accomplished.

The expression of many genes can be directly observed in and segregation patterns can be determined from gametophyte populations (Hickok et al. 1987). Thus, genetic analyses (i.e. segregation analyses) require only that the phenotypes of randomly sampled, F1-derived gametophytes be determined and it is unnecessary to extend these analyses to include F2 generations.

In the genetic analysis of antheridiogen response described by Scott and Hickok (1987), it was not possible to assess segregation patterns from F1-derived gametophytes, and the analysis was extended to an F2 generation. This was necessary because the antheridiogen response (phenotype) could only be measured at the population level (i.e. based on the percentage of male gametophytes in homogeneous populations) and homozygous F2s had to be generated from individual, F1-derived gametophytes. The additional step of analyzing an F2 generation adds at least 4 months to such an analysis and limits the sample size to the number of individual F2s which can be generated and maintained.

This chapter describes a procedure developed to enable the identification of individual antheridiogen response phenotypes in gametophytes of heterogeneous populations. The technique employed involved growing gametophytes in darkness on CAPE supplemented medium and determining their responses by counting antheridia. This technique will be referred to as the dark assay for antheridiogen response. A description of it and tests of several homogeneous spore collections and one hybrid collection are presented.

Materials and Methods

Analysis of antheridiogen responses of dark-grown gametophytes.

Spores were soaked in darkness to synchronize germination. Preliminary experiments (unpublished) and a published account (Cooke et al., 1987) indicate that such treatment optimizes germination in *Ceratopteris*. After one day of dark soaking, spores were removed from darkness and sterilized as described in chapter one. Such brief exposures to light did not induce germination. Cultures were soaked for an additional four days giving a total of five days of dark soaking. Spores were removed from darkness and the water in which they were soaking was replaced with a filter sterilized, 0.2% CAPE solution. The suspension was sown on 0.2% CAPE supplemented medium and culture plates were placed individually into small plastic bags. These cultures were exposed to light to induce germination. Typically, cultures were exposed to light from 32 to 35 hours. However, this treatment failed to induce high levels of germination in some spore collections. Therefore, the procedure was repeated with these spore stocks, using a 40 hour light induction period. Following light treatment, culture plates were wrapped in three sheets of aluminum foil to exclude light, and were maintained under standard culture conditions for an additional six to seven days.

Slides of dark-grown gametophytes were made as described in chapter one. The number of antheridia on each gametophyte was determined by viewing slides at 500X with a dissecting microscope. A

Bioquant II (TM) software program in conjunction with a Hipad digitizer and an IBM (TM) personal computer was used to tally antheridia number and to determine gametophyte length for each gametophyte counted. The average number of antheridia per gametophyte in these experiments will hereafter be referred to as the dark response to antheridiogen.

Results

General characteristics of the dark-grown gametophytes.

The general results described in this section apply the experiments described both in this chapter and in chapter five. The 33 to 40 hour light exposure given to spores to induce germination had very little immediate effect on germination. At the end of the light exposure period, cultures exhibited from 0 to 29% germination. However, after maintaining light-induced cultures in darkness for six to seven days, they generally exhibited greater than 80% germination. For several strains and one hybrid which were initially given 32 to 35 hour light treatments, considerably lower percentages of germinated spores were observed after the dark growth period. In cases in which the light controls gave higher percentages of germination, the experiments were repeated, giving the cultures 40 hours of light. For all collections given additional light, except for one strain (H1761-100), this modification elevated the percentage of germinated spores to 80% or above. In these cases the basic results of the experiments were not altered (i.e. the average numbers of antheridia were approximately

the same as when percent germination had been low). Therefore, the original results are given for these collections. In cases in which germination was low under light conditions as well as dark, this was considered the result of using spores with low viability (i.e., these spores were from old collections) rather than experimental conditions. Detailed germination data for these experiments are given in table A-1 in the appendix.

Dark grown gametophytes exhibited an etiolated growth form. The different strains ranged in average length from 800 (± 129) to 1831 (± 158) um (see table A-1 in the appendix). The elongated thalli were two or more cells in width at the base of the gametophyte and gradually increased in number toward the tip. Basal cells were long and narrow, while distal cells had an appearance more similar to those of light-grown gametophytes (i.e. squarish to round depending on their position in the gametophyte). In larger, highly vegetative gametophytes (i.e., bearing fewer antheridia), a distal area with the appearance of an early meristem region could be identified. At least some individuals of each strain produced antheridia. The frequency of these sex organs depended on the genotype of the gametophytes in culture (see below).

Characterization of the parental strains based on antheridia number.

Gametophytes of the H and 176D strains exhibited characteristic dark responses to antheridiogen. The differences between these

strains are illustrated in figure 4-1. Although the responses of a few individuals of each strain exhibited overlap, in general, they were discrete. For the 176D strain, the number of antheridia per gametophyte ranged from 0 to 5, with an average of 2.6 (±1.2). While in the H strain, the values for this trait ranged from 2 to 23 with an average of 15.4 (±3.6).

Comparison of the standard antheridiogen response to the dark response using a well defined hybrid.

Two mutant strains of C. richardii, 57.45 and HaC23, both of which were derived from the H strain, are known to exhibit complete insensitivity to antheridiogen when grown in light (Warne et al., 1988), while the H strain is highly responsive (see chapter 2). These responses are again documented in this report (Table 4-1). In addition, new data are presented for the response of the hybrid between the two mutants (table 4-1). The gametophytes of this hybrid developed as both cordates (76%) and males (24%) on CAPE supplemented medium in the light.

Characterization of these three strains and the hybrid by their dark response to antheridiogen, revealed a similar pattern (figures 4-2 and 4-3). Although the responses of the two mutants were identical when grown in the light, they exhibited a slight difference in sensitivity when grown in darkness. HaC23 gametophytes produced an average of 0.4 (±0.7) antheridia per individual, while 57.45 gametophytes produced an average of 2.2 (±0.9). The H strain gametophytes

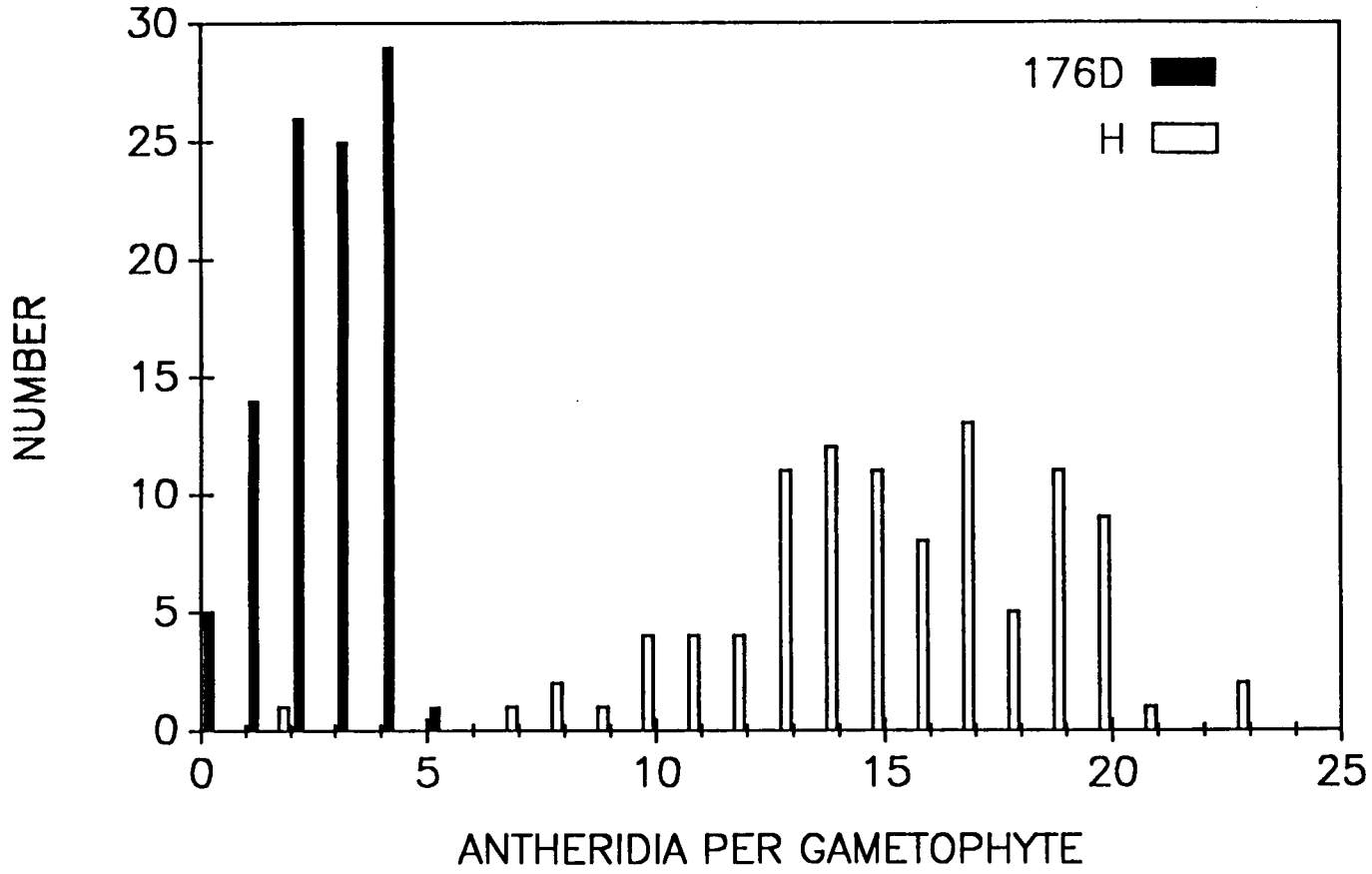

Figure 4-1. Distribution of antheridiogen responses, based on numbers of antheridia per dark-grown gametophyte, for strains 176D and H. Cultures were grown on 0.2% CAPE supplemented medium. (N = 100 for each strain).

Table 4-1. Antheridiogen[a] Responses of Strain H, two Antheridiogen-Insensitive Mutants and a Hybrid between the Mutants.

Spore Collection	M:C:N[b]	%M[c]
H	79:3:1 102:14:2 140:5:1	92±6
57.45	0:96:3 0:87:1 0:123:1	0±0
HaC23	0:173:0 0:156:1 0:137:1	0±0
F1(57.45 x HaC23)1	25:81:0 25:97:0 34:88:0	24±4

[a]Antheridiogen was supplied as 0.2% CAPE.

[b]M:C:N indicates numbers of male, cordate and neuter gametophytes.

[c]%M indicates percentage of male gametophytes ± the standard deviation of the sample (n=3).

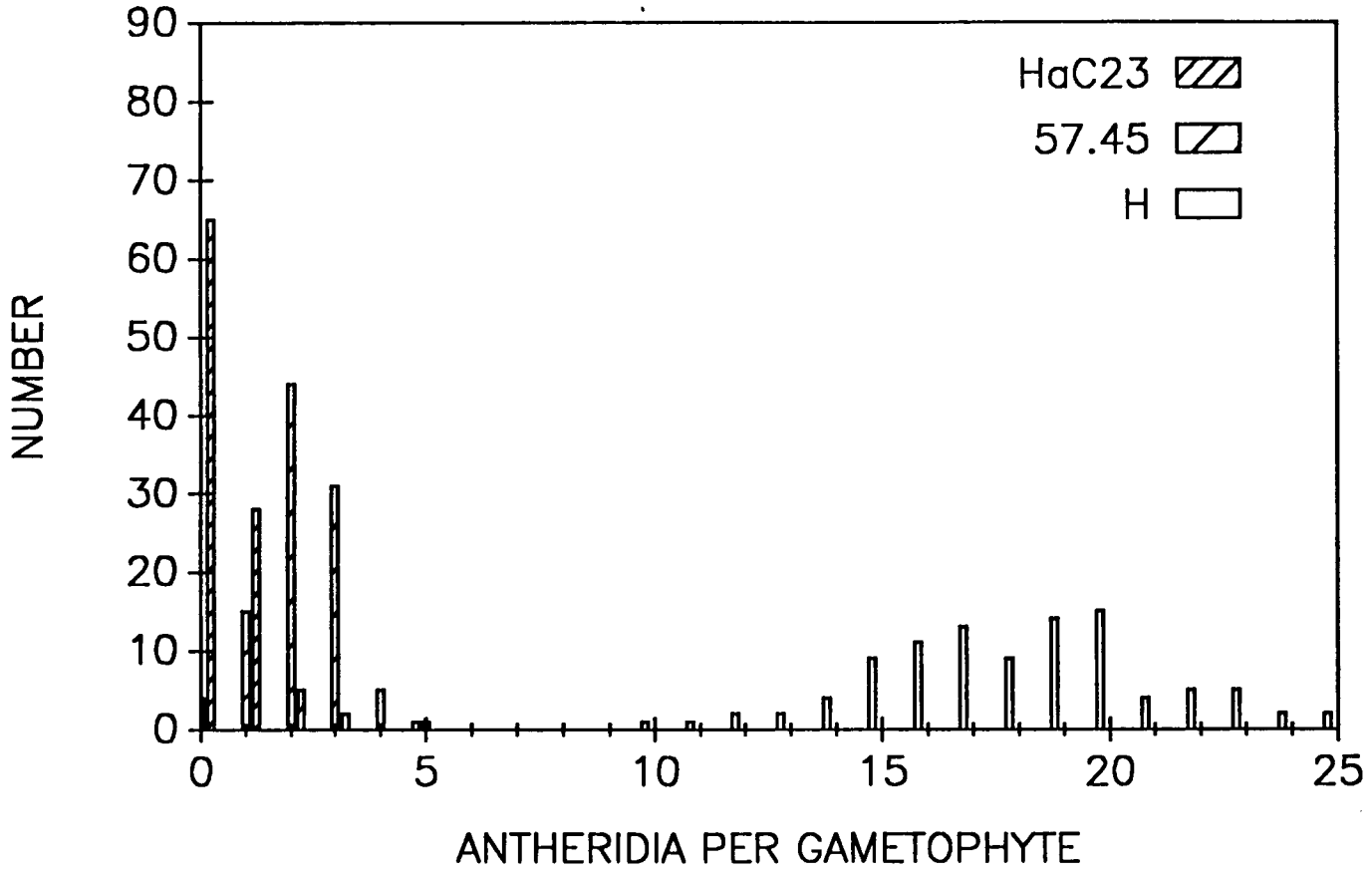

Figure 4-2. Distribution of antheridiogen responses, based on numbers of antheridia per dark-grown gametophyte, for strains 57.45, HaC23 and H. Cultures were grown on 0.2% CAPE supplemented medium. (N = 100 for each strain)

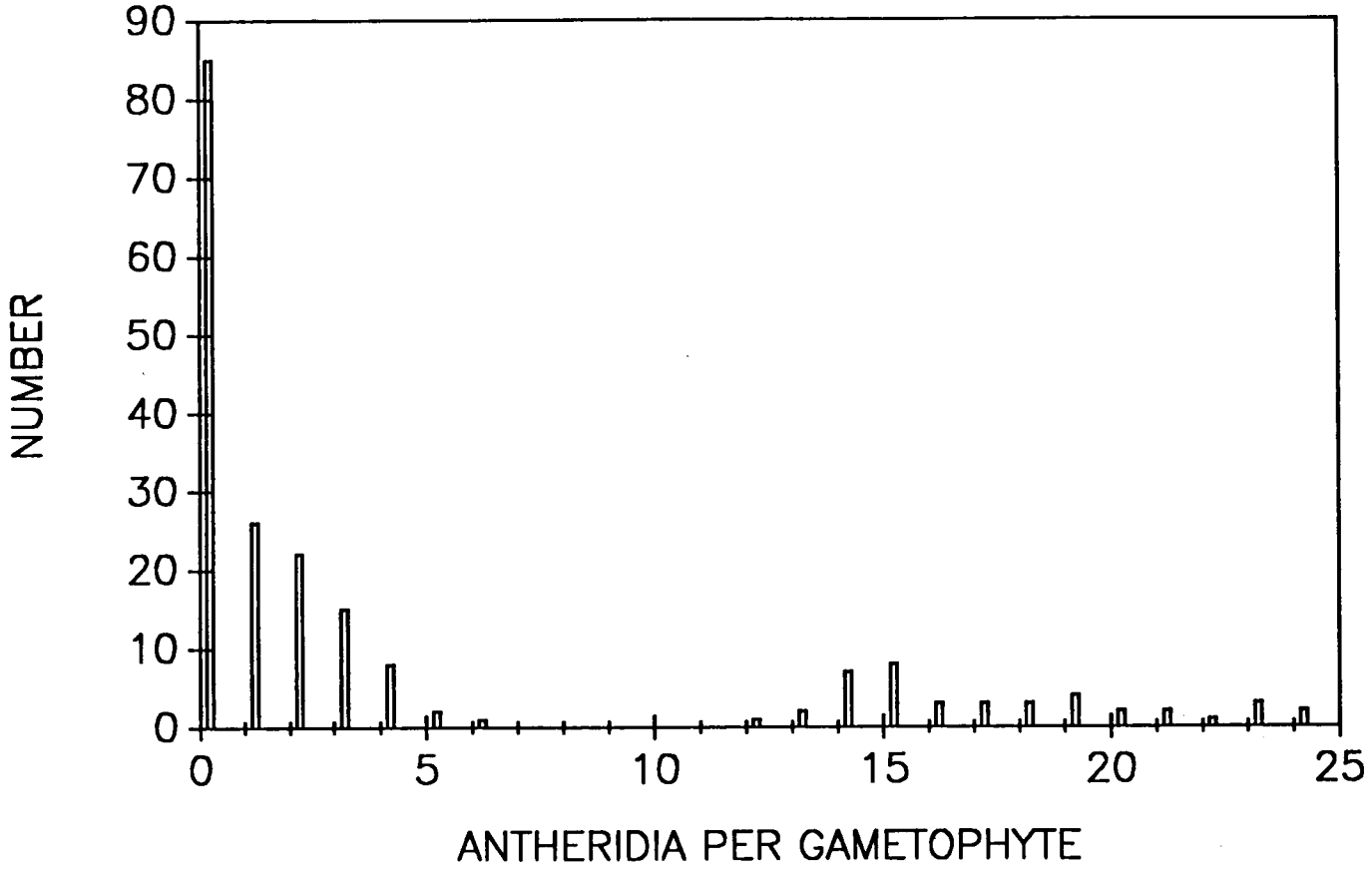

Figure 4-3. Distribution of antheridiogen responses, based on numbers of antheridia per dark-grown gametophyte, for the F1 hybrid, F1(57.45 x HaC23). Cultures were grown on 0.2% CAPE supplemented medium. (N = 200).

exhibited phenotypes which were generally distinct from the mutants. Although one H gametophyte exhibited a response which was contiguous with that of the most responsive 57.45 gametophyte, in general, the H response was much higher. The average number of antheridia for the H strain in this experiment was 18.0 (±3.3).

The gametophytes from the hybrid between the two mutants segregated into two distinct groups, one with low numbers of antheridia (0 to 6) and one with high numbers of antheridia (12 to 24) (figure 4-3). The range of the group with low numbers of antheridia was very similar to the combined ranges of the mutants, while the range of the group with high numbers of antheridia was similar to that of H. There were 159 individuals with low numbers of antheridia and 41 with high numbers of antheridia.

Discussion

Antheridia number on dark-grown gametophytes as an indicator of antheridiogen sensitivity.

Counts of antheridia number on dark-grown gametophytes provided clear distinction between some strains which exhibit different frequencies of males under standard conditions. Although a small number of H gametophytes exhibited uncharacteristically low numbers of antheridia (see figures 4-1 and 4-2) the majority of these gametophytes exhibited responses clearly distinct from the other strains described here.

The responses of strains 176D, 57.45 and HaC23 were overlapping.

However, slight differences in the average numbers of antheridia (2.6, 2.2 and 0.4 respectively) suggested subtle differences in sensitivity such that 176D could be ranked as the most sensitive of the three, with 57.45 next and HaC23 ranked as the least sensitive. This ranking is consistent with previous observations inasmuch as 176D is known to be more sensitive to antheridiogen than 57.45 and HaC23, both of which carry a mutant gene for antheridiogen insensitivity (Warne et al., 1988). However, differences between the antheridiogen sensitivity of 57.45 and HaC23 have not previously been detected.

In the initial characterization of these mutants, it was not possible to determine whether they represented distinct mutations (Warne et al., 1988). The results reported here suggest that the two mutations are in fact different, even though their phenotypes are indistinguishable in gametophytes grown in the light. That the techniques used in this study should provide more precise insights regarding the phenotypes of these two mutants is not unexpected. The methods employed were devised to reduce variability in several ways. Spores were soaked in darkness for five days prior to light induction to increase germination synchrony. Since asynchrony of germination, together with antheridiogen accumulation has has often been cited as a likely cause of sexual dimorphism in multispore cultures (Miller, 1968), reducing the effects of this factor should reduce variability. Gametophytes were grown entirely in darkness after a light induction period. This step eliminated variation due to effects of photosynthesis and photomorphogenesis. Finally, high concentrations

of antheridiogen in the form of CAPE were used to induce antheridia production. This reduced the likelihood that variation in antheridiogen production within gametophyte populations would affect the results.

Antheridiogen response of the hybrid between the two mutants.

The ratio of cordate to male gametophytes from the hybrid between the two mutants was very close to 3:1 for light grown cultures. That male gametophytes occurred, indicates that the two mutants are genetically distinct and that the males were produced by genetic reassortment. This segregation pattern can be accounted for by a simple model which is illustrated in figure 4-4. This model assumes that the mutations carried by 57.45 and HaC23 are two distinct mutations on separate chromosomes. An alternative model (not shown) which would yield the same results is one in which the two genes are linked, but so distantly that they segregate independently. Based on either model, the F1 hybrid would be heterozygous for the two genes and would produce four types of gametophytes in equal proportions. Individuals of one class would possess both genes for antheridiogen insensitivity, while those of two other classes would have a single mutant gene. Since one mutant gene is sufficient to render gametophytes insensitive to antheridiogen, individuals in all three classes (75% of the F1) would be insensitive. The remaining class (25%) would possess wild-type (H-type) genes for this trait and most would become males on CAPE supplemented medium.

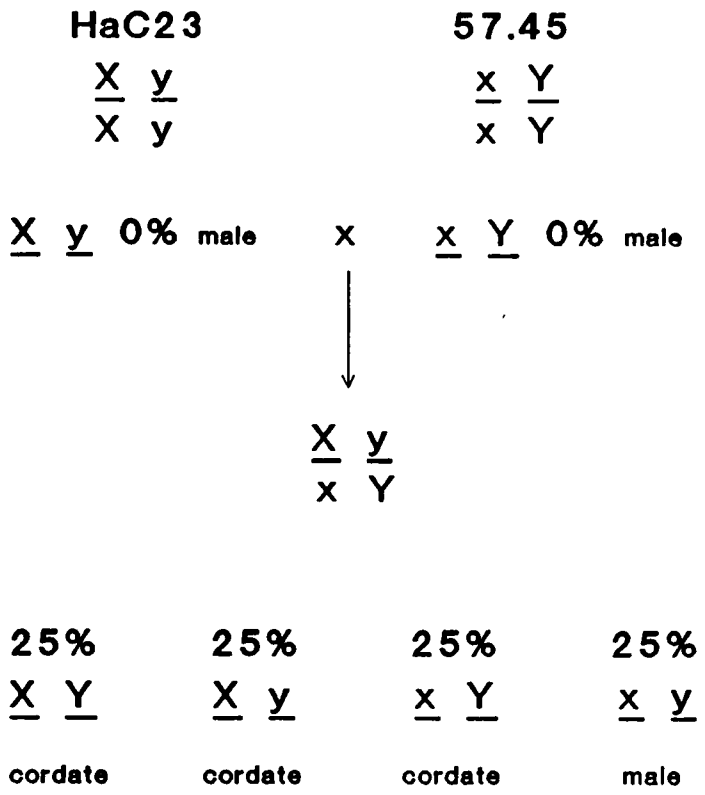

Figure 4-4. Model for the inheritance of antheridiogen insensitivity in the mutant strains 57.45 and HaC23. The model illustrates responses of different gametophyte types on antheridiogen supplemented medium. The model assumes an absolute response for H-type gametophytes.

The model described above assumes that all H-type gametophytes segregating from the hybrid become males on antheridiogen supplemented medium. Table 4-1 shows that the response of the H strain to 0.2% CAPE is not complete. In this experiment, H responded by exhibiting only 92% males. While this observation suggests that only 23% rather than 25% of the F1 gametophytes should be males it does not greatly alter the expected results from those based on a 3:1 ratio. If the model is altered to accommodate the observed H strain response, the frequencies of sexual types among the F1 gametophytes still approximates the expected frequencies ($x^2 = 0.197$, $p > 0.5$).

Determination of segregation patterns for antheridiogen responses is not generally as simple as for the cross described above. The parents exhibited an unmistakable phenotype with their total insensitivity to antheridiogen. When a novel phenotype occurred (i.e., males), it could immediately be identified. Furthermore the new response class was comprised of gametophytes which nearly all shared the same response. In most other crosses, the responses within strains would be far less uniform, making it impossible to resolve discrete classes from among the F1 progeny. For these reasons, the F1 described above represented the ideal subject for testing the usefulness of the dark response to antheridiogen for determination of segregation patterns from heterogeneous populations.

Comparison of the results in table 4-1 with those in figure 4-3 shows that comparable results were obtained. Again, a novel class with a similar response to that of the H strain segregated from the

hybrid. Based on the model described above and the responses of the parents and the H strain, a ratio close to 3:1 is expected for the two general groups in this distribution. Although the distribution is slightly skewed, with more individuals exhibiting a less sensitive response than expected, the results are still well within the expected range ($x^2 = 2.160$, $p>0.1$). These observations indicate that using the dark response of gametophytes to antheridiogen may provide a way to estimate segregation patterns among heterogeneous gametophyte populations making F2 analysis for segregation of this trait unnecessary.

Summary.

The four strains (176D, H, HaC23 and 57.45) studied here all exhibited characteristic dark antheridiogen responses. Although strains 176D, HaC23 and 57.45 exhibited overlapping responses, each was characterized by a slightly different average number of antheridia. For strains HaC23 and 57.45, this difference in response provided the first indication that the mutations responsible for their phenotypes were different mutations. Previously used techniques did not indicate differences in their phenotypes. The dark antheridiogen response of the H strain was clearly distinguishable from that of the other three strains.

The antheridiogen response of the gametophytes from the hybrid F1(57.45 X HaC23)1 were characterized by growth in both light and darkness on CAPE supplemented medium. Under both conditions, the F1-derived gametophytes segregated as less sensitive (or insensitive)

types and sensitive types in an approximate 3:1 ratio. These results indicated that the two mutant alleles are unlinked or so distantly linked that they segregate independently. Furthermore, the correspondence between the results obtained with the two assays suggested that the dark antheridiogen responses of heterogeneous gametophyte populations may be used to determine segregation patterns.

The dark assay for antheridiogen response provides a potentially valuable new method for studying this phenomenon. It has two main advantages over previously employed methods. Firstly, it was designed to reduce the variability associated with growth and development which may alter the fundamental responses of gametophytes to antheridiogen. The utility of the assay in this regard is suggested by the results which indicated different responses for the two mutant strains which were previously indistinguishable in their phenotypes. Secondly, this assay provides a new method for determining segregation patterns from hybrid sporophytes. Previous techniques required the production of a generation of F2 sporophytes and the subsequent analysis of the antheridiogen responses for each. This method was limiting both because it required a much longer period of time (up to four months) and because it limited the number of potential segregant genotypes which could be assessed to the number of F2s which could be generated and maintained. The dark assay for antheridiogen response is quicker (two to three weeks) and may be used to asses very large populations.

CHAPTER V

A RE-INVESTIGATION OF THE INHERITANCE OF ANTHERIDIOGEN SENSITIVITY

Introduction

The response of gametophytes to antheridiogen is a trait which varies at the species level. Several studies have shown that different strains of the same species often exhibit characteristic sex ratios when gametophytes are raised in multispore cultures (Schedlbauer, 1974; Cousens, 1979; Lloyd and Warne, 1978; Warne and Lloyd, 1981). However, most studies of antheridiogen responses have focused on differences between individuals within a gametophyte population (Wilkie, 1963; Naf et al., 1975; Schedlbauer, 1976a), and not differences between strains.

Naf et al. (1975) studied differences in the responses of gametophytes within populations of Pteridium aquilinum. They suggested that the only genetic basis for these variations in response lies in genetically based differences in rates of growth and development. Wilkie (1963) studied variations in antheridiogen responses within a genetically homogeneous spore stock from P. aquilinum. By hybridizing differently responding gametophytes from the homogeneous population, he showed that these differences were not transmitted by nuclear genes. Wilkie (1963) suggested that differences in response were controlled by the transmission of "heritable cytoplasmic units."

Another equally viable alternative however, is that the differences Wilkie observed were attributable to undefined epigenetic factors.

Two recent studies (Scott and Hickok, 1987; Warne et al., 1988) using the fern Ceratopteris richardii have provided the first clear evidence for nuclear inheritance of genes controlling antheridiogen response. Scott and Hickok (1987) studied natural variation in two homozygous strains which exhibited marked differences in their responses to antheridiogen. By analyzing the responses of F1 hybrids and an F2 generation, they showed that the difference between these two strains was due to the action of one or more nuclear genes. They proposed that either one or two linked nuclear genes could account for the inheritance pattern they observed.

Warne et al. (1988) used three mutants of C. richardii which exhibited altered responses to antheridiogen to study the genetic control of this trait. Two of the mutant strains exhibited complete insensitivity to antheridiogen, while one was much less sensitive than the wild-type. The three mutants and several wild-type strains were used to generate various hybrid combinations. The responses of the F1-derived gametophyte populations to antheridiogen suggested that each of the mutants differed from the wild-type by a single nuclear gene.

The project described in this chapter is based on the investigation of the natural variability in antheridiogen response conducted by Scott and Hickok (1987; see above). In the present study, recently developed techniques (described in chapters two and four) were used to

investigate the inheritance of this variation. The goal of this work was to distinguish between the two models proposed by Scott and Hickok (1987) or to suggest other models.

This investigation used spore collections from the parental strains (176D and H) and reciprocal hybrids studied by Scott and Hickok as well as collections from representative F2 strains. The F2 strains included three which were selected because their responses to antheridiogen were similar to that of strain 176D. These will be referred to as "low response F2s". Also included among the representative F2s were three selected for similar responses to the H strain. These will be referred to as "high response F2s". Furthermore, each group of three F2s consisted of low, median and high response types relative to the typical response of the parental strain (H or 176D). Five other F2s were selected because they exhibited responses intermediate to the 176D and H parental strains (Scott and Hickok, 1987). These F2s will be referred to as "intermediate F2s". Scott and Hickok's report (1987) indicated that as many as six F2s exhibited intermediate responses. However, because preliminary experiments indicated that collections from one of these F2s contained spores from two different sporophytes, this strain was eliminated from consideration.

Materials and Methods

General culture conditions were as described in chapter one. Determination of the dark antheridiogen responses of gametophytes from

various strains and hybrid combinations followed the protocol described in chapter four. Spore collections were from strains used by Scott and Hickok (1987; see introduction) and Warne et al. (1988) and also from hybrids generated by crossing these strains.

Results

Responses of representative F2s to high antheridiogen concentrations (the light assay).

The 11 F2s exhibited either relatively low or high sensitivities to all tested concentrations of CAPE (figure 5-1). The three low response F2s all exhibited relatively low degrees of sensitivity and the high response F2s all exhibited relatively high degrees of sensitivity. Of the five intermediate F2s, four exhibited low degrees of sensitivity and one exhibited a high degree of sensitivity.

The seven F2s which exhibited low degrees of sensitivity to CAPE were broadly distributed in their responses on high concentrations (figure 5-1). One strain (H1761-52) exhibited a response much like that of 176D (compare with figure 2-5). Another exhibited a higher degree of sensitivity and the remaining five exhibited lower sensitivities. One of these (H1761-177, a low response F2) never produced more than 2% male gametophytes in any of its cultures.

The four F2s which exhibited high degrees of sensitivity (figure 5-1) all shared similar responses at high CAPE concentrations. These responses were also similar to those of the H strain (compare with figure 2-5). However, one strain (H1761-198, a high response F2)

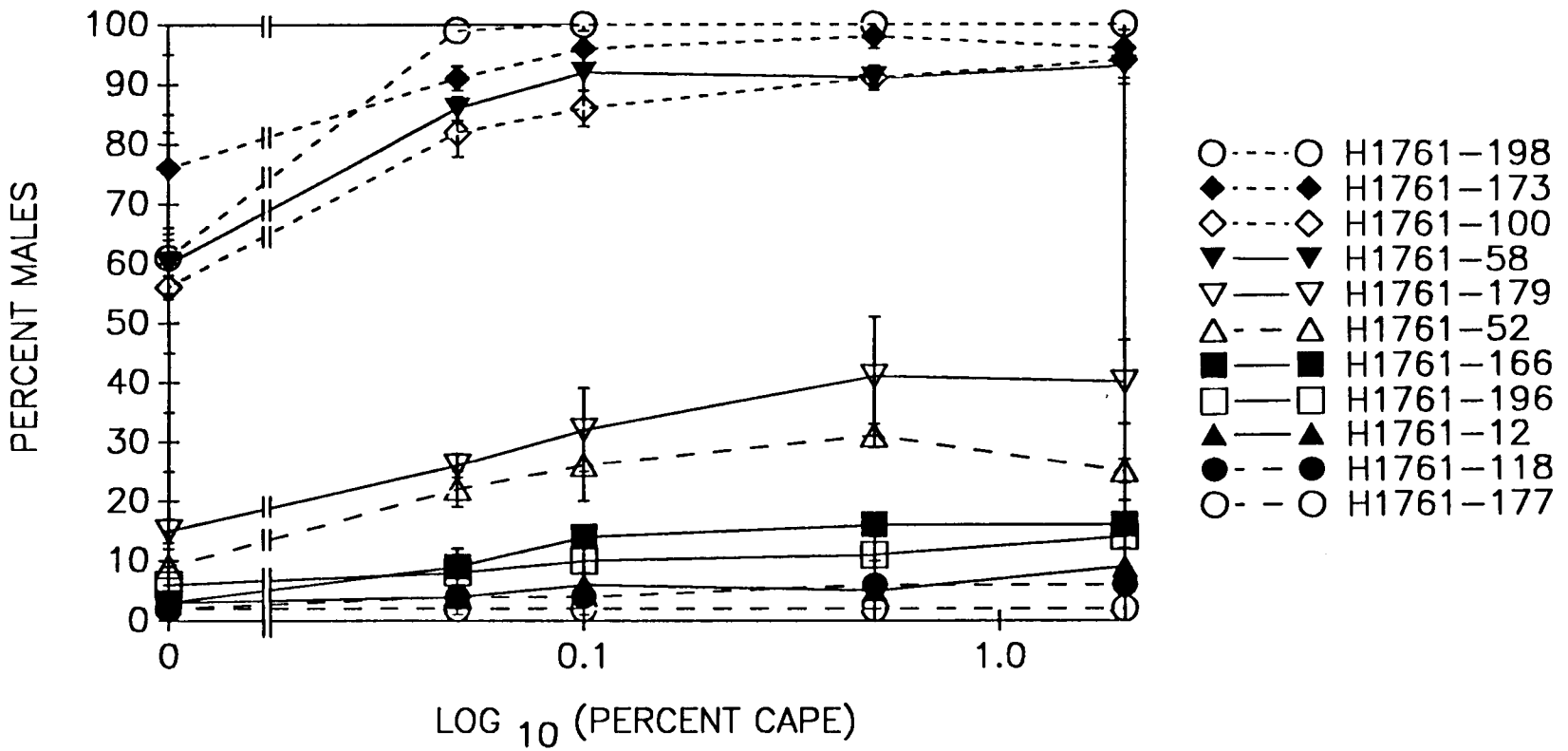

Figure 5-1. The responses of eleven representative F2 strains to various concentrations of CAPE. Symbols connected by lines with short dashes represent the responses of high response F2s, those connected by solid lines represent the responses of intermediate F2s and those connected by lines with long dashes represent the responses of low response F2s. The legend indicates which strain each symbol designates. Each point represents the average of three replicates ± the standard deviation of the sample.

exhibited a nearly maximal degree response even on lower concentrations of CAPE. This was in marked contrast to the response of the H strain (figure 2-5).

Dark antheridiogen responses of representative F2s (the dark assay).

The dark antheridiogen responses of strains 176D, H and the 11 F2s are summarized in Table 5-1. The ranges of response for these strains are illustrated in figures 5-2 through 5-4. As previously demonstrated in chapter four, strains 176D and H produced characteristically low and high numbers of antheridia respectively in darkness in response to antheridiogen (Table 5-1 and figure 5-2A).

The low response F2s were similar to each other and to 176D in the average number of antheridia produced per gametophyte (Table 5-1). The range of responses among these strains was also similar (figures 5-2B, C and D). However, the range of responses for one strain (H1761-177; figure 5-2D) was somewhat broader than that of the others.

The intermediate F2s showed variable dark antheridiogen responses (Table 5-1 and figures 5-3A, B, C, D, and 5-4A). The average number of antheridia produced per gametophyte varied from 7.9 to 17.7 (Table 5-1). All five of these strains were more sensitive to antheridiogen in this experiment than was strain 176D. Three exhibited responses considerably lower than that of H (strains H1761-12, H1761-166 and H1761-196; figures 5-3A, B, and C respectively) and two were similar in response to H (H1761-58 and H1761-179; figures 5-3D and 5-4A

Table 5-1. Dark Antheridiogen Responses[a] of Strains H, 176D and Eleven representative F2.

Parental Strains		F2 Strains					
		low response		intermediate		high response	
strain[b]	anth. number[c]	strain[b]	anth. number[c]	strain[b]	anth. number[c]	strain[b]	anth. number[c]
176D	3.0±1.3	- 52	4.1±1.5	- 12	7.9±2.9	-100	11.5±2.4
H	18.0±3.3	-118	2.6±1.0	- 58	17.7±3.6	-173	10.3±2.1
		-177	5.4±2.4	-166	10.8±3.3	-198	15.9±2.7
				-179	16.0±3.1		
				-196	12.0±2.9		

[a]Dark Antheridiogen Responses indicates the average number of antheridia formed in darkness on 0.2% CAPE supplemented medium ± the standard deviation of the sample. (N = 50).

[b]For all F2s listed, the initial portion of the code (H1761) has been omitted and only the numbers which designate each are shown.

[c]anth. = antheridia.

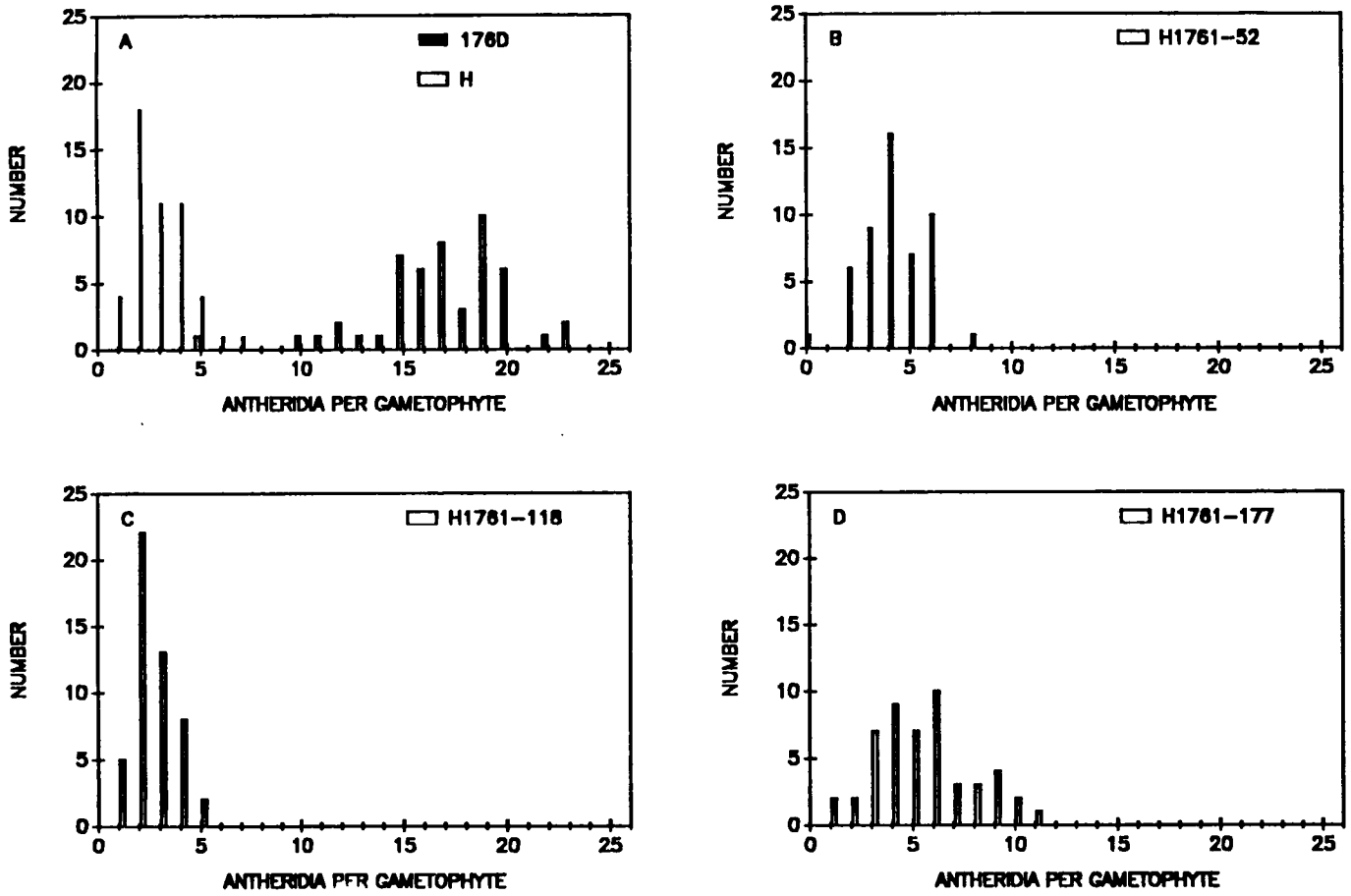

Figure 5-2. The distributions of dark antheridiogen responses among gametophytes of the parental strains and the three low response F2s. (N = 50 for each strain). A) Dark antheridiogen responses of strains H and 176D. B) The dark antheridiogen response of strain H1761-52. C) The dark antheridiogen response of strain H1761-118. D) The dark antheridiogen response of strain H1761-177.

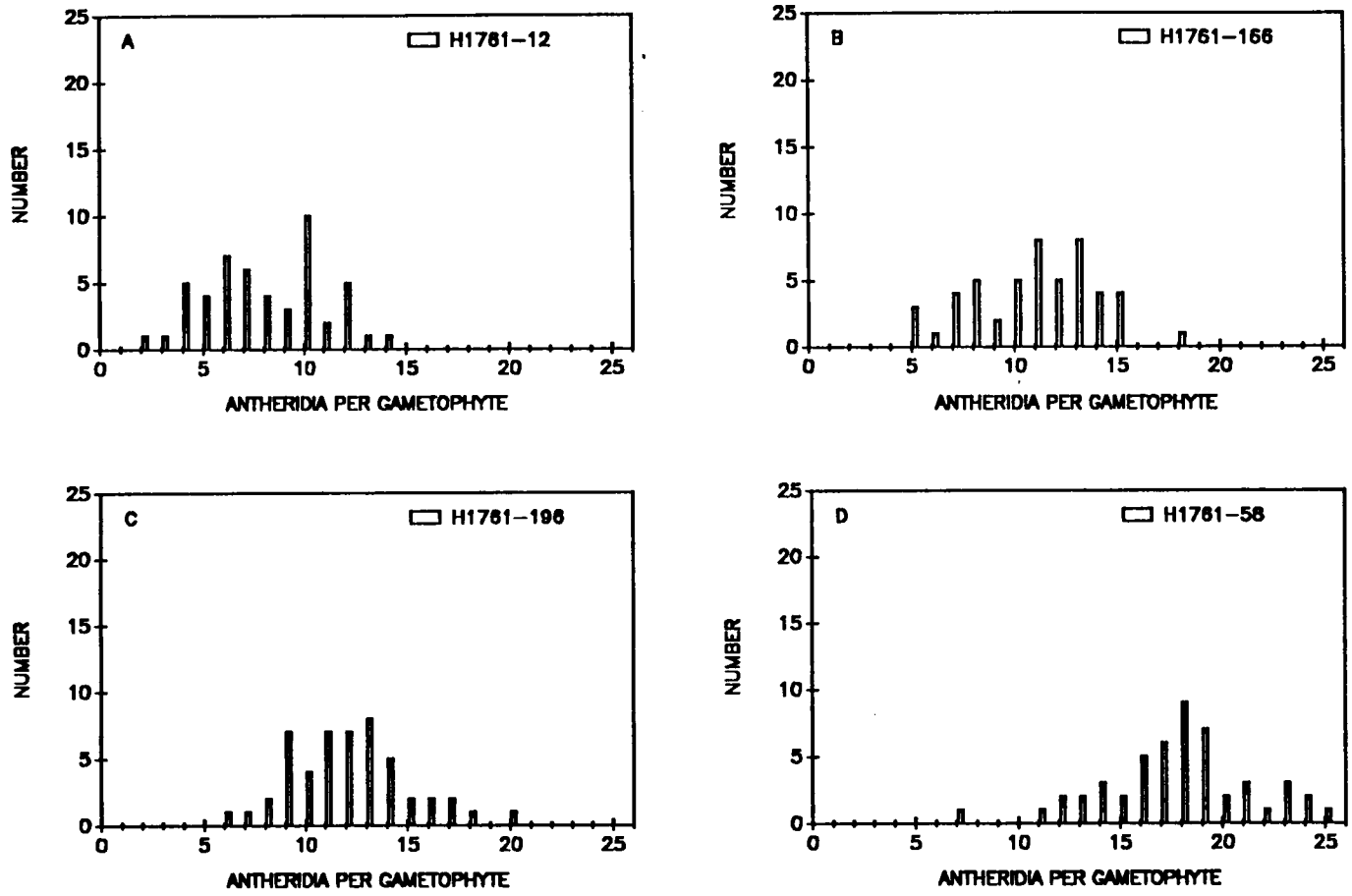

Figure 5-3. The distributions of dark antheridiogen responses of four of the intermediate F2s. (N = 50 for each strain). A) Dark antheridiogen response of strain H1761-12. B) The dark antheridiogen response of strain H1761-166. C) The dark antheridiogen response of strain H1761-196. D) The dark antheridiogen response of strain H1761-58.

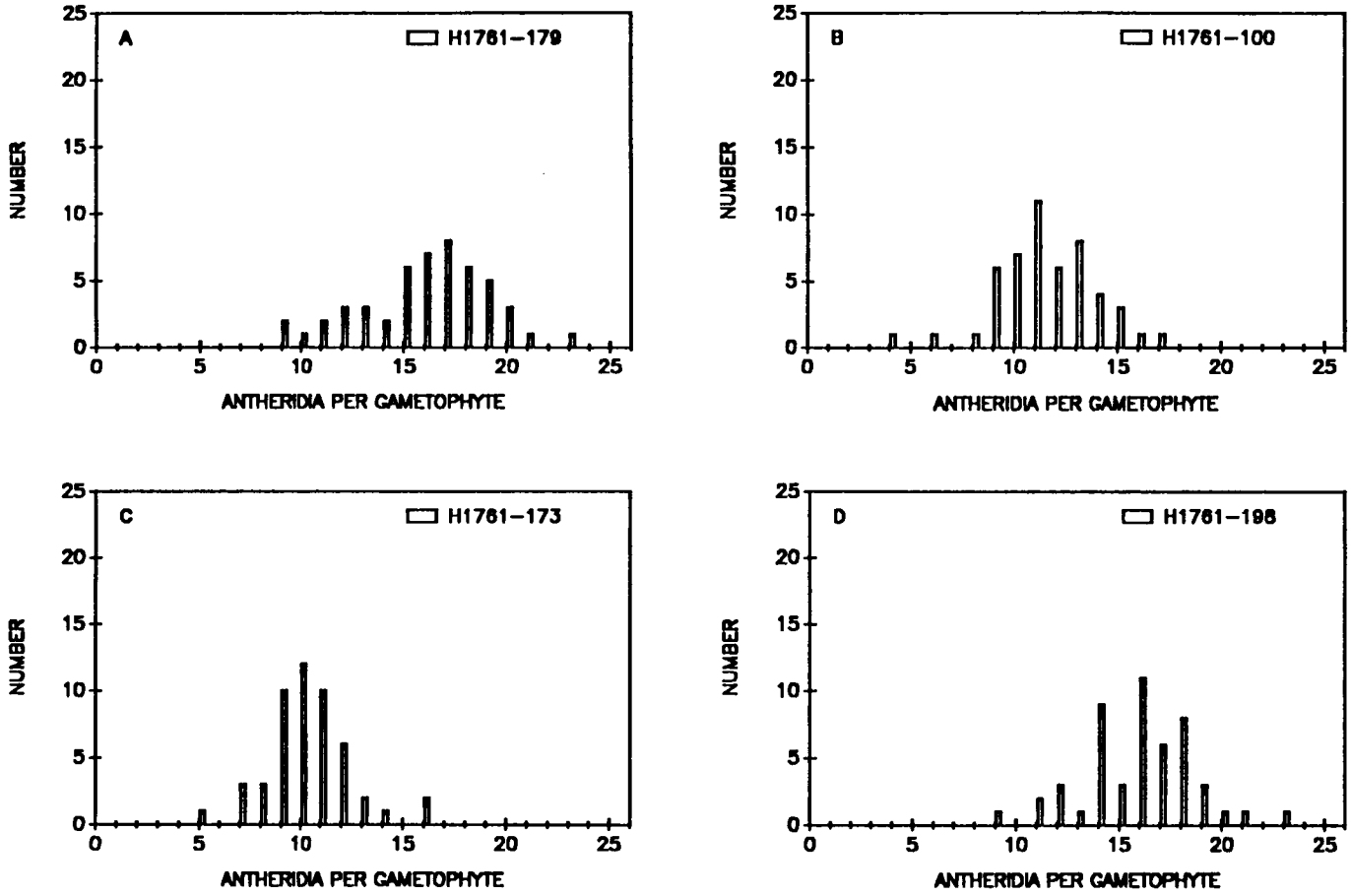

Figure 5-4. The distributions of dark antheridiogen responses of one intermediate F2 and the three high response F2s. (N = 50 for each strain). A) Dark antheridiogen response of strain H1761-179. B) The dark antheridiogen response of strain H1761-100. C) The dark antheridiogen response of strain H1761-173. D) The dark antheridiogen response of strain H1761-198.

respectively). The three with lower responses than H appeared to represent two different response types (see table 5-1 and compare figures 5-3A with figures 5-3B and 5-3C).

The three high response F2s exhibited two basic responses (Table 5-1 and figures 5-4B, C and D). Two exhibited average numbers of antheridia which were considerably lower than that of the H strain (strains H1761-100 and H1761-173; see figures 5-4B and 5-4C respectively and table 5-1), while one was very similar to H (strain H1761-198; figure 5-4D). The two with lower average numbers of antheridia were similar to each other in their ranges of response (figures 5-4B and 5-4C) and also similar to the intermediate F2s H1761-166 and H1761-196 (see above).

Dark antheridiogen responses of several hybrids (the dark assay).

Hybrids between strains 176D and H.

Strains H and 176D exhibited characteristic dark antheridiogen responses (figure 5-5) which were similar to the responses described for these strains in chapter four. For the 176D strain, the number of antheridia per gametophyte ranged from 0 to 5, while in the H strain the values for this trait ranged from 2 to 24. The ranges of response were nearly discrete (see figure 2-5), however one H strain gametophyte exhibited an unusually low degree of response (i.e., it produced only 2 antheridia).

Reciprocal hybrids between strains 176D and H were similar in their responses (figures 5-6 and 5-7). Gametophytes of both hybrid

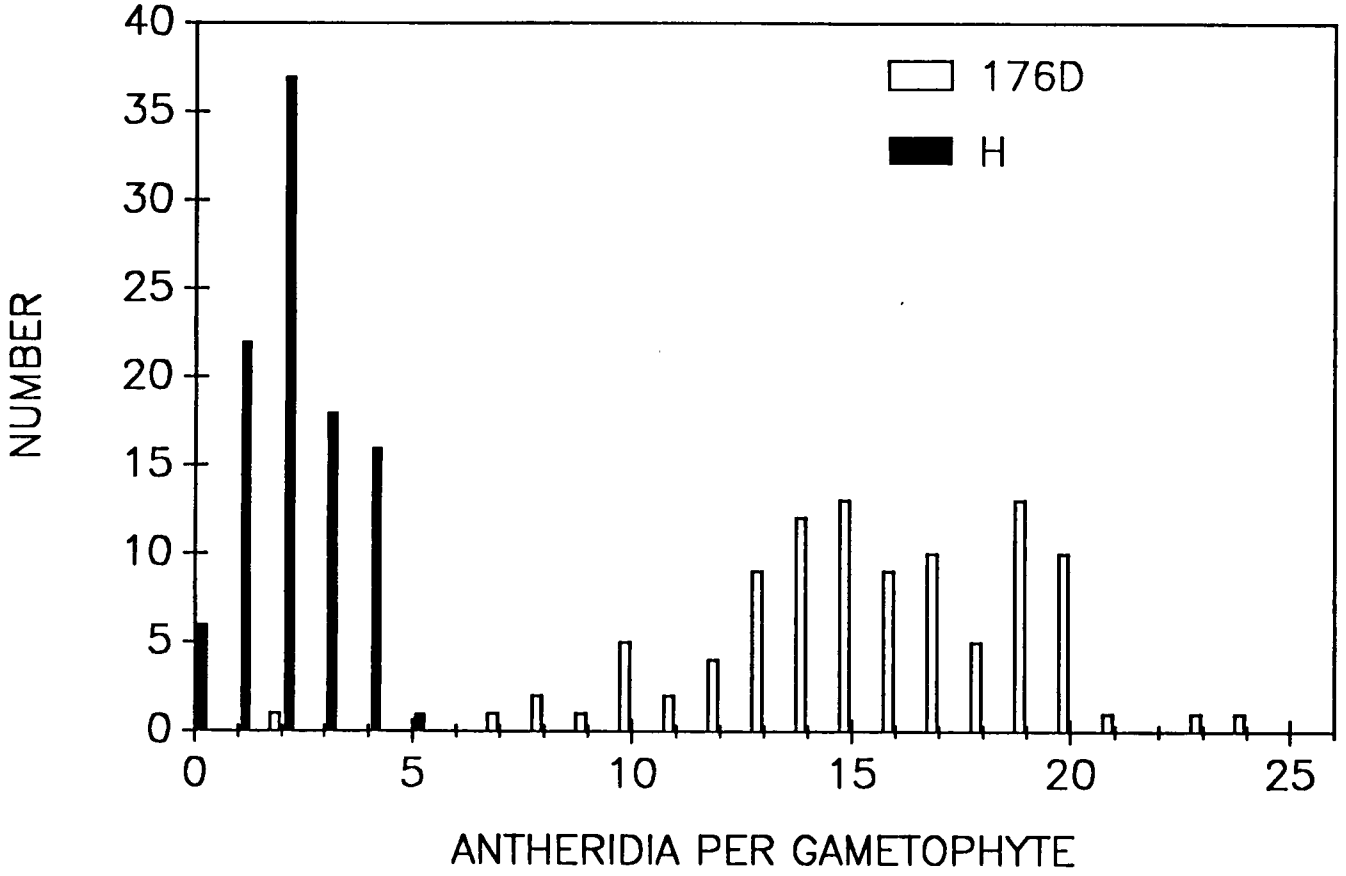

Figure 5-5. The distribution of dark antheridiogen responses among gametophytes of strains 176D and H. (N = 100 for each strain).

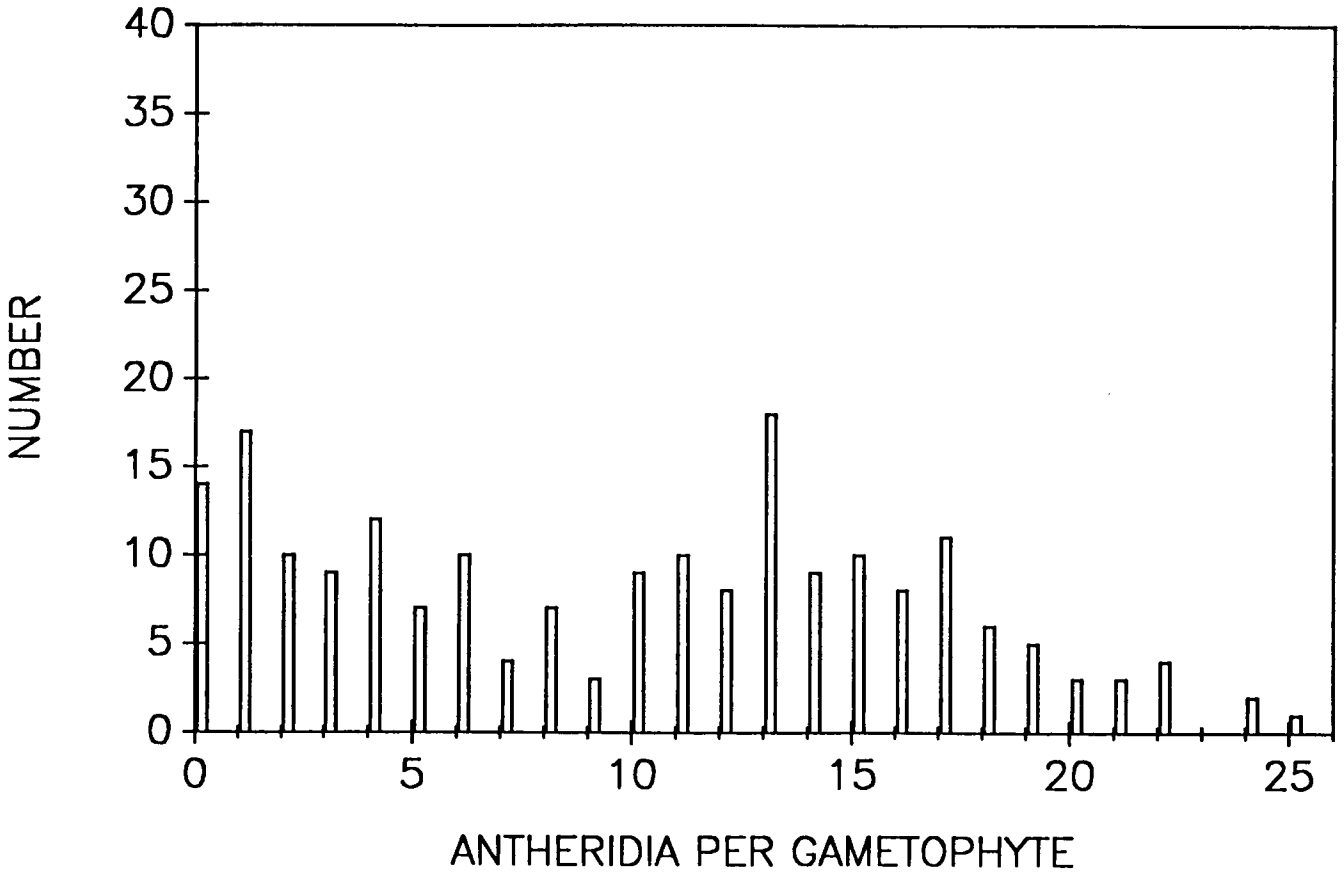

Figure 5-6. The distribution of dark antheridiogen responses among gametophytes of the Hybrid F1(176D X H)1. (N = 200).

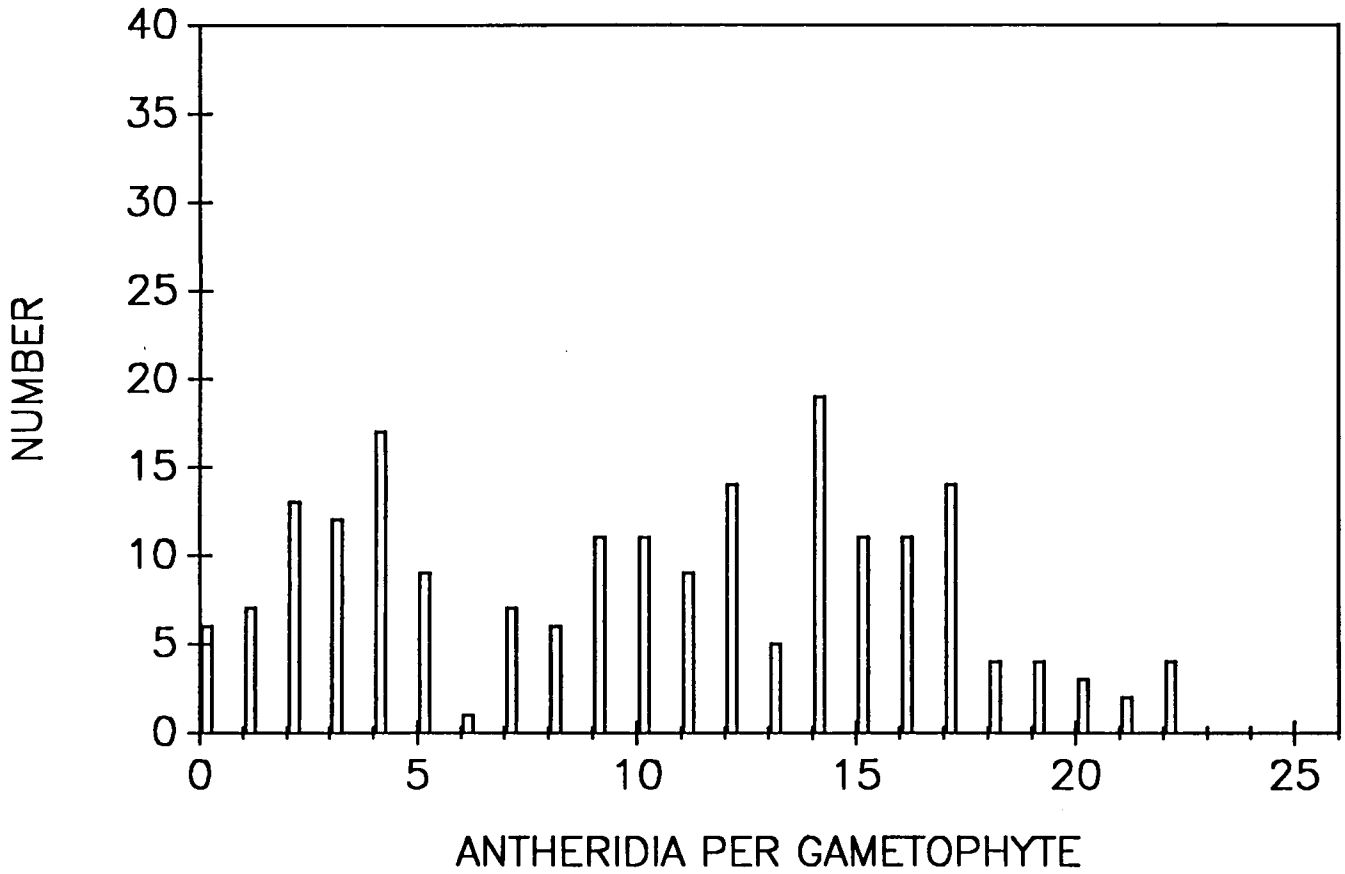

Figure 5-7. The distribution of dark antheridiogen responses among gametophytes of the hybrid F1(H X 176D)1. (N = 200).

combinations were comprised of at least two main response types. Gametophytes of one group in each hybrid population were less sensitive to antheridiogen and produced low numbers of antheridia (from 0 to ca. 5-8). The remainder in each population exhibited higher sensitivity and produced greater numbers of antheridia (up to 24 per gametophyte).

The two (or more) response types in each hybrid-derived gametophyte population were not discrete. However, the overall response of these populations resembled the combined response of the two parental strains (figure 5-5). In both hybrid populations, the number of gametophytes which exhibited responses within the range of strain 176D was much less (i.e. 32 and 34% of the entire population) than those which exhibited responses similar to strain H.

Hybrids between antheridiogen-insensitive mutants and strain 176D.

The two antheridiogen-insensitive mutants, HaC23 and 57.45 were discussed in chapter four. Their dark antheridiogen responses along with those of strains 176D and H are shown in figures 5-8 and 5-9. As in chapter four, strains HaC23, 57.45 and 176D all exhibited low degrees of sensitivity to antheridiogen in the dark assay. As before, strain HaC23 was the least sensitive, with a high proportion of gametophytes which completely lacked antheridia, strain 57.45 was slightly more sensitive and strain 176D was the most sensitive of the three.

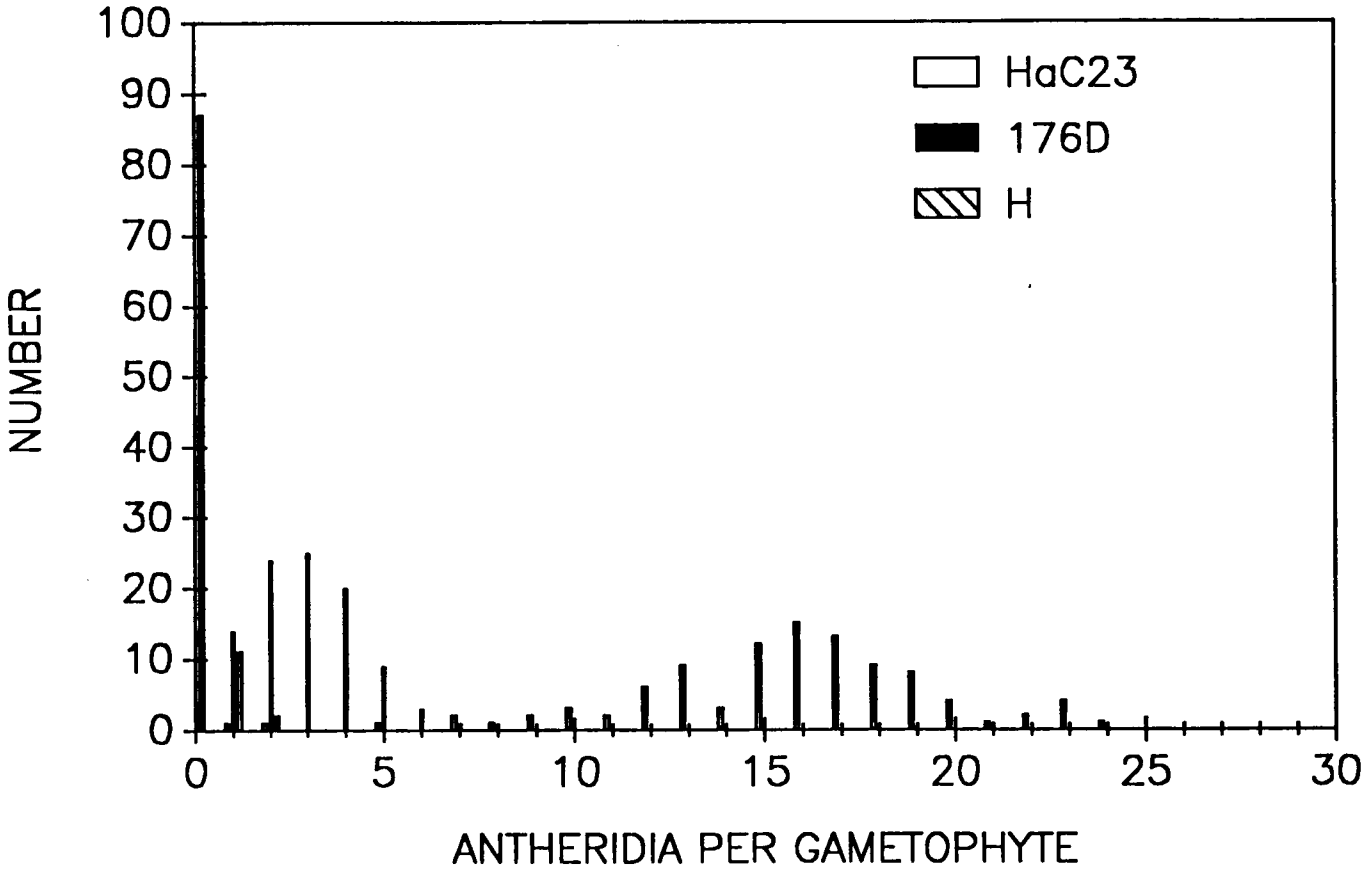

Figure 5-8. The distribution of dark antheridiogen responses among gametophytes of strains HaC23, 176D and H. (N = 100 for each strain).

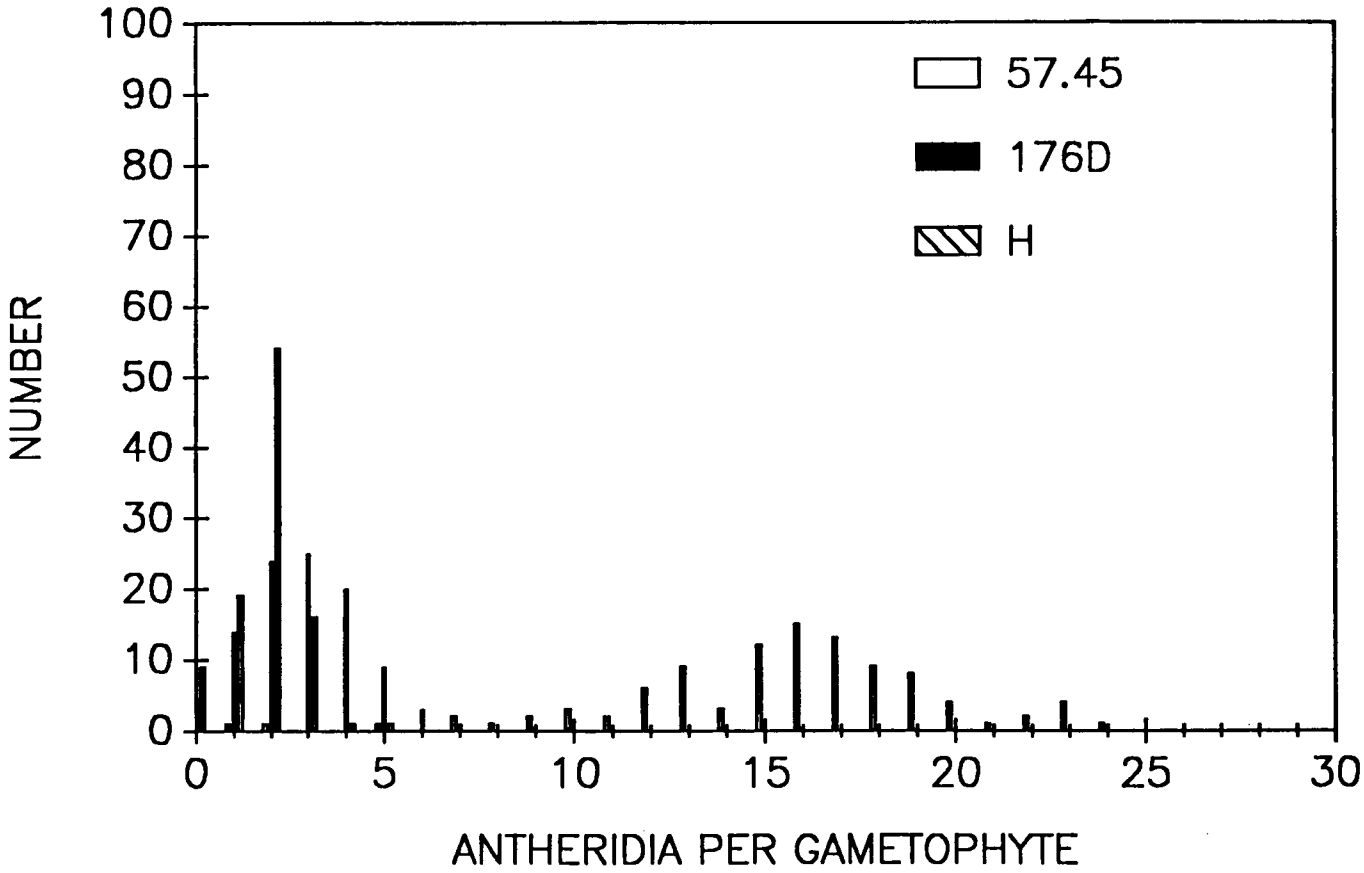

Figure 5-9. The distribution of dark antheridiogen responses among gametophytes of strains 57.45, 176D and H. (N = 100 for each strain).

The response range of strain 176D, overlapped that of each antheridiogen-insensitive mutant (figures 5-8 and 5-9). The combined ranges of strain 176D and each mutant extended from 0 to 7 antheridia per gametophyte. The range of responses for the H strain was from 1 to 24 antheridia per gametophyte. However only 3% of the H gametophytes responded by producing less than 7 antheridia. The rest (97%) ranged from 7 to 24 antheridia per gametophyte.

Figure 5-10 illustrates the dark antheridiogen response of the hybrid F1(HaC23 X 176D)1. Two main response types were observed among these hybrid-derived gametophytes. One group comprised the majority of the population and exhibited relatively fewer antheridia per gametophyte (0 to ca. 7 or 8). The other group contained fewer individuals which produced greater numbers of antheridia (ca. 8 or 9 to 26 with one individual which produced 36).

The responses of these hybrid-derived gametophytes were similar to the combined responses of strains HaC23, 176D and H (figure 5-8). In the hybrid population, 67% of the gametophytes exhibited responses as low as those of the HaC23 and 176D parental types. The rest (33%) exhibited higher responses which were similar to those of the H strain.

The response of the hybrid, F1(57.45 X 176D)1 (figure 5-11), was very similar to that of F1(HaC23 X 176D)1. Again, two main response groups could be identified, one in which the gametophytes produced fewer antheridia and another in which they produced higher numbers of antheridia. The ranges and proportions of these two groups were

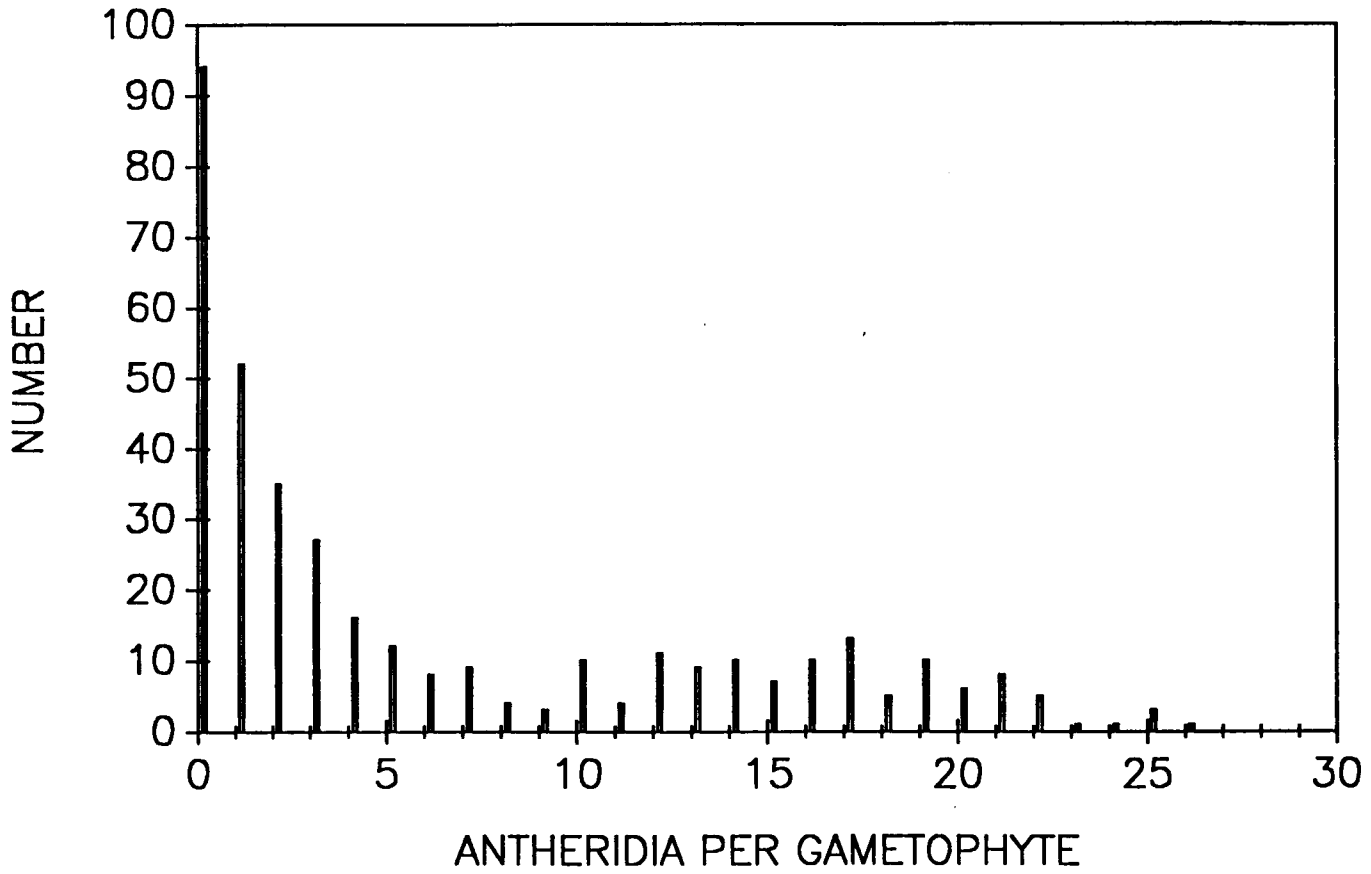

Figure 5-10. The distribution of dark antheridiogen responses among gametophytes of the hybrid F1(HaC23 X 176D)1. The response of one individual which produced 36 antheridia is not illustrated. (N = 375).

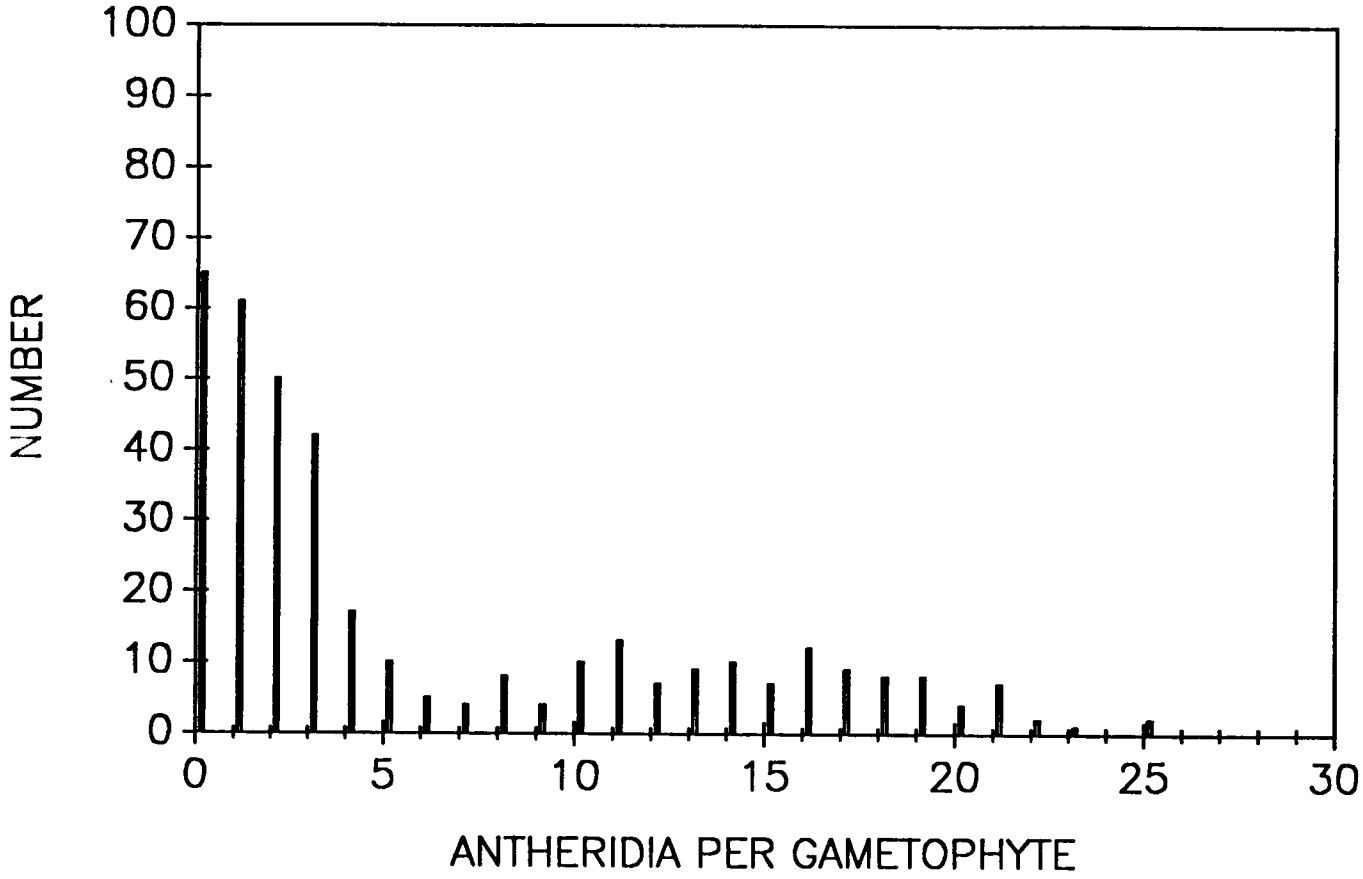

Figure 5-11. The distribution of dark antheridiogen responses among gametophytes of the hybrid F1(57.45 X 176D)1. (N = 375).

similar to those for the hybrid described above with one exception. Although the less sensitive response type for both hybrids was composed of approximately equal numbers of individuals, there were fewer gametophytes which completely lacked antheridia among those derived from hybrid F1(57.45 X 176D)1 than from F1(HaC23 X 176D)1 (compare figures 5-10 and 5-11).

The responses of the gametophytes from hybrid F1(57.45 X 176D)1 were similar to the combined responses of strains 57.45, 176D and H (figure 5-9). In the hybrid population, 68% of the gametophytes exhibited responses as low as those of the HaC23 and 176D parental strains. The rest (32%) exhibited higher responses which were similar to those of the H strain.

Discussion

Analysis of F2 responses to high antheridiogen concentrations.

All of the F2s exhibited either generally high or generally low sensitivities to a range of antheridiogen concentrations (see figure 5-1, page 86). This could be interpreted to indicate that only two response types (i.e. parental types) exist. All of the high response F2s exhibited high sensitivity, all low response F2s exhibited low sensitivity, and the intermediate F2s exhibited one or the other of these general responses. However, within these two general response categories, the different F2 strains exhibited a range of variations in sensitivity. This observation could suggest that additional response types besides parental types are also present.

Scott and Hickok (1987) suggested that the effects of genes associated with growth rate or other developmental traits could alter fundamental responses to antheridiogen. Thus, the differences demonstrated here among F2s within either response category (i.e., high or low sensitivities) could be interpreted as resulting from the activity of minor genes not directly associated with antheridiogen sensitivity. This interpretation suggests that the differences in fundamental response to antheridiogen are associated with a single gene and only parental types segregated from the F2.

Alternatively, the differences within each response category may be associated with differences in major genes which directly affect antheridiogen sensitivity. That is, genes associated with growth and development may not greatly alter the fundamental responses to antheridiogen and the observed differences may be related instead to different genotypes for antheridiogen response within each category. It is also possible that minor genes do alter the fundamental responses to antheridiogen and that this obscures differences between strains with different genotypes, both within and between response categories (i.e. between parental types and other genotypic classes).

To more clearly distinguish between the alternatives presented above, an assay for antheridiogen response which reduces the effects of factors associated with growth and development was required. The dark assay technique described in chapter four meets this requirement. With this assay gametophytes are grown on antheridiogen supplemented medium in darkness following a period of dark imbibition to

synchronize germination. Their response to the pheromone, which is termed the dark antheridiogen response, is then measured by determining the number of antheridia produced per gametophyte. This method has been applied to the representative F2s discussed in this chapter and the significance of the results (see above) are described in the following section.

The dark antheridiogen responses of representative F2s.

All of the low response F2s were similar in their dark antheridiogen responses to strain 176D (see table 5-1 and figures 5-2B, C and D, pages 86 and 87 respectively). This suggests that these strains all share the same major gene or genes associated with antheridiogen sensitivity. One strain, H1761-177 (see figure 5-2D, page 87), though similar to 176D exhibited a wider range of responses. This observation is particularly interesting because this strain exhibited the least sensitivity to antheridiogen of all the F2s when grown in the light (see figure 5-1, page 84). This suggests that the high degree of insensitivity exhibited by this strain in light-grown cultures was strongly associated with growth and developmental factors which were minimized by the conditions used here. It is possible that the dark antheridiogen response of H1761-177 indicates a different fundamental response from that of 176D. However, in comparison to other F2s, the dark response of this strain was relatively similar to that of 176D.

The remaining F2 strains, both the intermediate F2s and the high

response F2s, exhibited dark antheridiogen responses which were often dissimilar from the responses of those strains in the light (compare table 5-1 on page 86 and figures 5-3A through 5-4D on pages 88 and 89 with figure 5-1 on page 84). None of the intermediate F2s exhibited dark antheridiogen responses as low as that of strain 176D. This is in contrast to the results illustrated in figure 5-1 which indicate that several of these F2s respond like 176D. Instead, three of these F2s exhibited dark responses greater than that of 176D, but considerably less than that of H, while two were similar in their dark responses to H. The high response F2s yielded similar dark antheridiogen responses to those of the intermediate F2s. Only one exhibited a dark response similar to that of the H strain, while the other two exhibited responses greater than that of 176D, but considerably lower than that of H. These results suggest that the intermediate and high response F2s are highly subject to the effects of minor genes which indirectly affect antheridiogen response.

At least four fundamental responses to antheridiogen were observed among the F2s. Two responses were represented by those strains with dark antheridiogen responses similar to strains 176D and H. Additional responses were represented by those strains less responsive than H but more responsive than 176D. Among these strains at least two general response types were observed. These observations suggest that both parental types and recombinant types were present in the F2 generation. This supports the model proposed by Scott and Hickok (1987) for inheritance of antheridiogen sensitivity associated with two linked genes.

Other interpretations of these results could also be suggested. However, since results discussed below may influence these interpretations, further discussion of concerning the responses of the F2s will be reserved for final consideration.

Analysis of the dark antheridiogen responses of various hybrids.

Hybrids between strains 176D and H.

Gametophytes from hybrids between strains 176D and H included individuals with both parental type responses. This was an expected result. However, parental type categories were not as discrete as might be predicted from the responses of 176D and H. In addition, more individuals exhibited a high degree of response to antheridiogen than a low degree of response.

These observations suggest that one or more intermediate response types were present in the F1-derived gametophyte populations. This interpretation would account for the inability to clearly discern parental type categories from among the F1-derived gametophytes. Although the responses of strains 176D and H were fairly discrete, they were not widely separated (see figure 5-5, page 91). Therefore, individuals with intermediate responses would be expected to obscure the separation between the parental types in the F1.

This interpretation further suggests that most of the intermediate types exhibited antheridiogen sensitivities similar to less responsive gametophytes of the H strain. This would account for the higher proportion of F1-derived gametophytes which exhibited a high dark antheridiogen response (see figures 5-6 and 5-7, pages 92 and 93

respectively). The suggestion that intermediate types would generally exhibit a higher degree of dark antheridiogen response than would 176D-types is also supported by the dark antheridiogen responses of the F2 strains (discussed above).

This interpretation supports the two linked gene model for inheritance of antheridiogen sensitivity proposed by Scott and Hickok (1987). Since gametophytes which exhibited 176D-type responses were present as only 32 and 34% of the gametophyte population of the F1 hybrids, as much as 36% recombination between the two linked genes is suggested.

Hybrids between antheridiogen-insensitive mutants and strain 176D.

The dark antheridiogen responses of both hybrids between antheridiogen-insensitive mutants and strain 176D also support the two linked gene model. Previous tests (described in chapter four) have shown the two antheridiogen-insensitive mutants to be associated with separate, unlinked loci. Different possible linkage relationships between the gene or genes associated with natural variation in antheridiogen sensitivity (natural response gene(s)) and those associated with the mutant phenotypes, would be expected to produce different segregation patterns among the hybrid-derived gametophyte populations. The patterns actually exhibited and the implications of these patterns for a genetic model are described below.

Gametophytes with responses similar to those of the H strain segregated from both hybrids. This indicates that neither of the

mutant alleles are allelic with the natural response gene(s). If this were the case, only parental types would have segregated from the hybrids.

A pattern such as the ones exhibited by the hybrids could result if a mutant gene was linked to the natural response gene(s) (see figure 5-12). Since both mutants were derived from the H strain, gametophytes with responses like those of H would be expected to segregate from the hybrid as the result of recombination. If natural variation for antheridiogen response is associated with a single gene, two kinds of antheridiogen-insensitive gametophytes and 176D-types should also segregate from the hybrid (see figure 5-12A). The model would generate more complex results (see figure 5-12B) if two linked genes are associated with natural variation (i.e. intermediate-types and additional antheridiogen-insensitive types would also result from recombination). However, it is expected that differences in these segregation patterns could not be detected, since intermediate-types would probably have responses similar to less responsive H-types.

Although one of the hybrid segregation patterns could be explained using a model such as that described above, it is highly unlikely that these models could account for both hybrid patterns. Because the mutant alleles assort independently of each other (see chapter four), it is not likely that both are linked to the natural response gene(s). The only way such a model could be used to account for both hybrid responses is if the mutant alleles were both linked to the natural response gene(s) on opposite sides and so distantly separated from each other that they assorted independently. Furthermore,

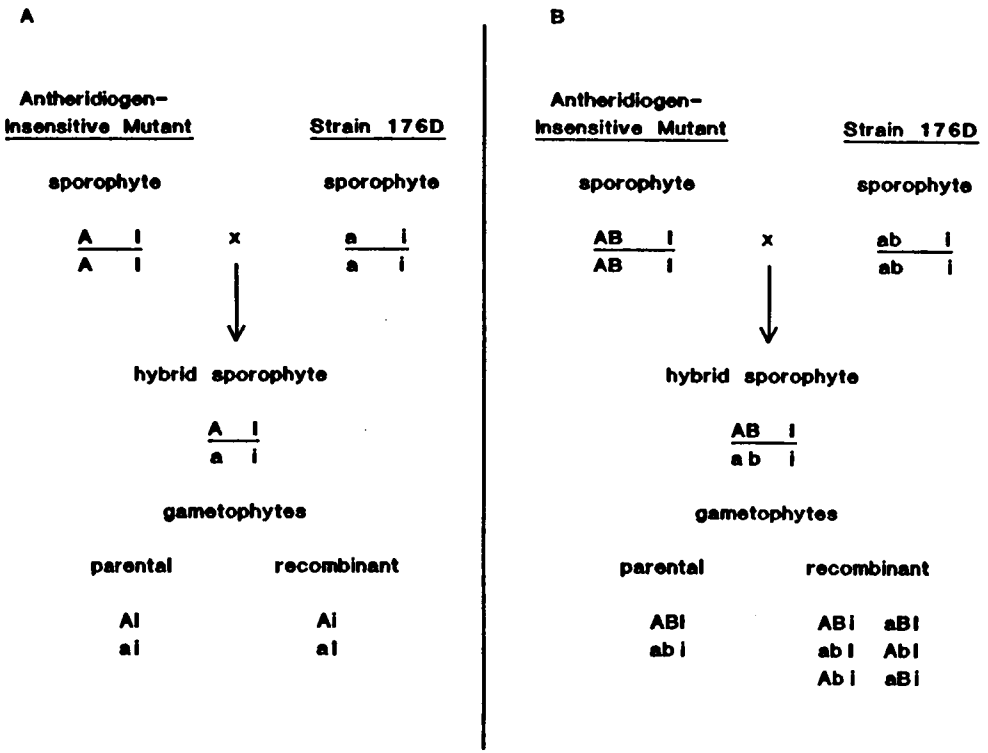

Figure 5-12. A model for the inheritance of an allele which confers antheridiogen-insensitivity in Ceratopteris richardii showing this allele linked to the natural response gene(s). The allele indicated as "I" is the allele which confers insensitivity to antheridiogen, the allele indicated as "i" is the wild-type form of this gene A. Inheritance patterns which result if the natural response is Associated with a single gene. The Allele indicated as "A" represents the natural response allele of strain H and the allele indicated as "a" represents this allele for strain 176D. B. Inheritance patterns which result if the natural response is associated with two linked genes. The alleles indicated as "A" and "B" represent the two natural response genes of strain H, the alleles indicated as "a" and "b" represent these alleles for strain 176D.

for this model to explain the nearly identical patterns exhibited by the two hybrids, it would be necessary to assume that the mutant genes are each separated from the natural response gene(s) by nearly the same genetic distance.

The patterns exhibited by both hybrids can more easily be accounted for by assuming that both mutant alleles are on separate chromosomes from the natural response gene(s) (see figure 5-13). Since the mutants were derived from the H strain, genetic reassortment in the hybrid would be expected to produce H-type gametophytes.

If a single gene is associated with natural variation, two kinds of antheridiogen-insensitive gametophytes, 176D-types, and H-types should all be present in equal proportions (see figure 5-13A). Since the responses of antheridiogen-insensitive gametophytes and 176D-types cannot be distinguished by the methods use in this study, this model would predict a 3:1 ratio of less responsive to more responsive individuals. The patterns of response exhibited by both hybrids were close to 3:1, but in both cases, more highly responsive individuals were observed than expected (for F1(HaC23 X 176D)1, $x^2 = 11.35$, $p < 0.01$; for F1(57.45 X 176D)1, $x^2 = 10.65$, $p < 0.01$). Among gametophytes from the hybrid F1(HaC23 X 176D)1, 32% exhibited responses greater than those of either parental strain, while among gametophytes from the hybrid F1(57.45 X 176D)1, this figure was 33%.

If two linked genes are associated with natural variation, this model predicts a more complex pattern of segregation from the hybrids (see figure 5-13B). In addition to the four types described in the preceding paragraph, the hybrid gametophyte population would contain

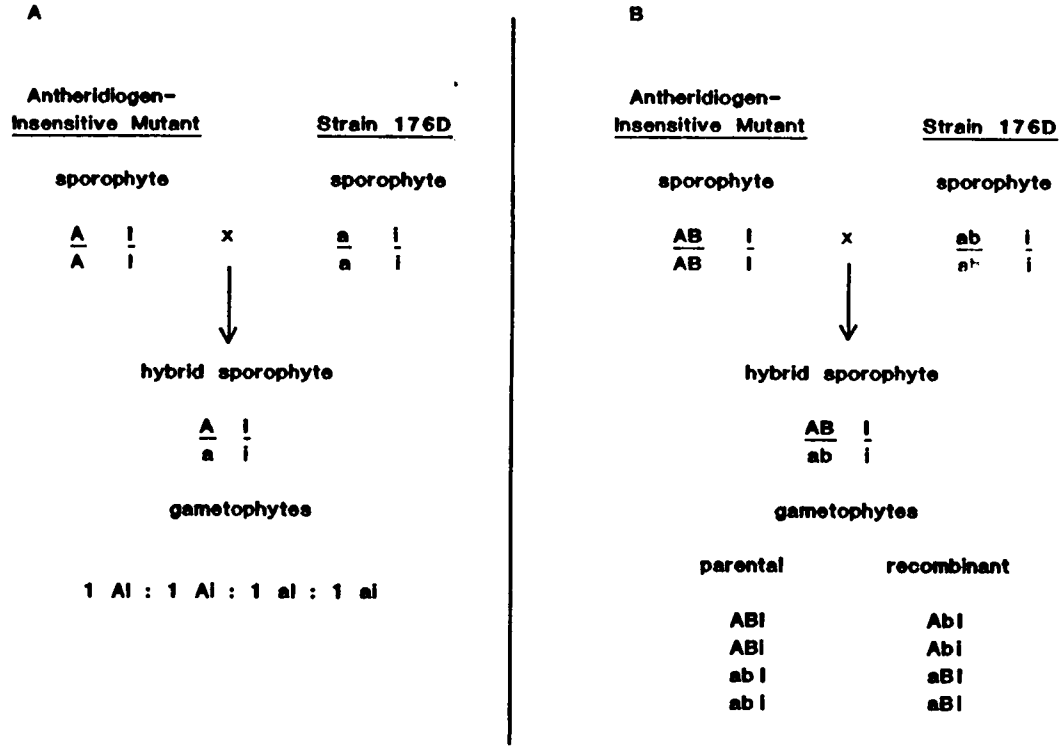

Figure 5-13. A model for the inheritance of an allele which confers antheridiogen-insensitivity in Ceratopteris richardii showing this allele on a separate chromosome from those associated with the natural response. The allele indicated as "I" is the allele which confers insensitivity to antheridiogen, the allele indicated as "i" is the wild-type form of this gene A. Inheritance patterns which result if the natural response is Associated with a single gene. The Allele indicated as "A" represents the natural response allele of strain H and the allele indicated as "a" represents this allele for strain 176D. B. Inheritance patterns which result if the natural response is associated with two linked genes. The alleles indicated as "A" and "B" represent the two natural response genes of strain H, the alleles indicated as "a" and "b" represent these alleles for strain 176D.

four additional genotypes resulting from recombination between the two linked genes. Two of these types would be insensitive to antheridiogen and could not be distinguished from other generally less sensitive types. Two would be intermediate types and would be expected to exhibit responses similar to those of H. The proportion of hybrid-derived gametophytes with relatively high responses to antheridiogen would depend on the genetic distance between the two linked genes. If the genes were tightly linked, these individuals would comprise close to 25% of the population. The greater the genetic distance between the two linked genes however, the higher this percentage would become.

Figure 5-14 illustrates the results predicted by the model described above if the two genes are separated by 36 map units as suggested in the previous section in association with the analyses of the reciprocal hybrids between strains 176D and H. This model predicts that 66% of the hybrid-derived gametophyte population should be less responsive to antheridiogen and 34% should be more responsive. These predicted results correspond well to the observed values (68 and 32% and 67 and 33%) for the two hybrids between the mutants and strain 176D. Furthermore, this model could account for the responses of both hybrid populations since the two mutant alleles appear to be unlinked. This model predicts identical segregation patterns for hybrids with any mutant allele conferring antheridiogen insensitivity, as long as such genes assort independently from the natural response genes.

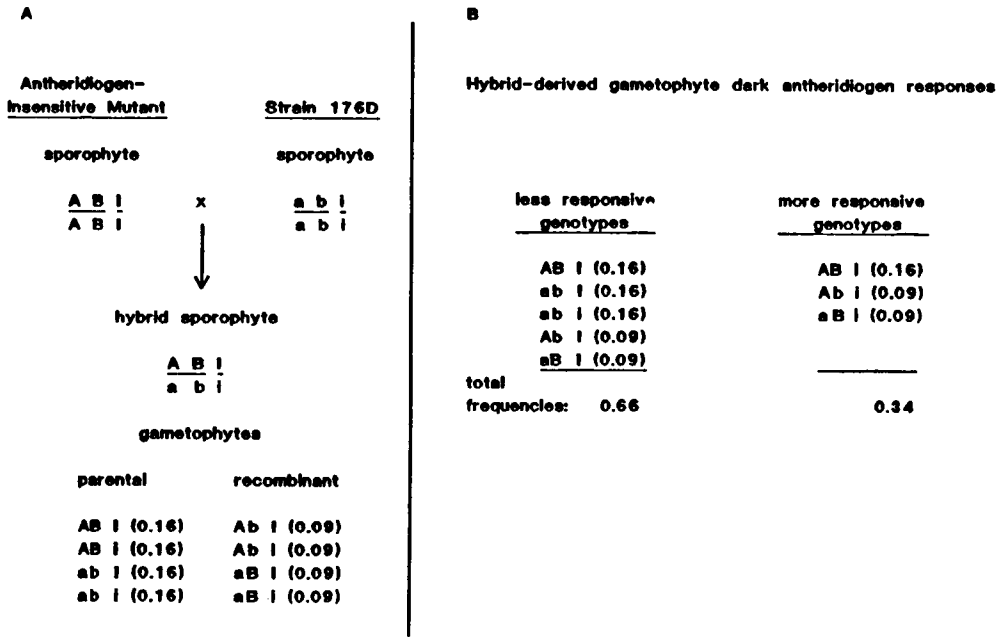

Figure 5-14. Specific segregation patterns predicted for a model of inheritance of genes associated with antheridiogen sensitivity in Ceratopteris richardii showing the allele associated with antheridiogen-insensitivity on a separate chromosome from those associated with the natural response and assuming that 36 map units separate the two linked genes associated with natural response. The allele indicated as "I" is the allele which confers insensitivity to antheridiogen, the allele indicated as "i" is the wild-type form of this gene A. Segregation of specific genotypes from a hybrid between an antheridiogen-insensitive mutant and strain 176D. The alleles indicated as "A" and "B" represent the two natural response genes of strain H, the alleles indicated as "a" and "b" represent these alleles for strain 176D. The numbers in parentheses indicate the frequencies of the specified genotypes expected if 36 map units separate the two linked genes. B. The dark antheridiogen responses which would be predicted for each genotype. Numbers in parentheses indicate the expected frequencies of each genotype.

Summary and conclusions.

The inheritance of natural variation of antheridiogen sensitivity in two strains of Ceratopteris richardii was investigated using several experimental approaches. Eleven F2 strains which were representative of the various F2s studied by Scott and Hickok (1987) exhibited either generally high or generally low sensitivity to a range of antheridiogen concentrations. However, within these two general response groups, a large degree of variation was observed. No conclusive genetic interpretation could be made from these observations.

The dark antheridiogen responses of these same eleven F2s suggested that four response types were present. The low response F2s all exhibited similar low sensitivities to antheridiogen. However, the intermediate F2s and the high response F2s exhibited variable responses. While none exhibited responses as low as those of strain 176D, some from both groups exhibited responses considerably lower than that of the H strain. Among these, two response types were observed. Other intermediate F2s and high response F2s responded similarly to the H strain. These results are interpreted to indicate that both parental types as well as two recombinant types were present in the F2 generation as would be predicted by a model of inheritance involving two linked genes.

These results also indicate that intermediate types and H-types are more likely to be influenced by minor genes which indirectly influence antheridiogen response than are 176D types. These minor genes may either enhance the response to antheridiogen (as was the case for intermediate-types which exhibited H like responses in the

light) or they may decrease the response (as was the case for H-type individuals which exhibited intermediate responses in the light).

The dark antheridiogen responses of several hybrids also supported the two gene model. Reciprocal hybrids between strains 176D and H produced gametophytes which exhibited both low and high sensitivity to antheridiogen. The patterns of response indicated that both parental types and intermediate types were present and suggested a recombination frequency of up to 36%.

Two hybrids between antheridiogen-insensitive mutants and strain 176D also exhibited patterns of dark antheridiogen response which were consistent with the two linked gene model. These results were also consistent with a model which suggested that the alleles associated with antheridiogen-insensitivity assorted independently from those associated with natural variation for antheridiogen sensitivity. This model generated results similar to the observed results if a recombination frequency of about 36% was assumed between the two linked genes.

In general, the results of this study favor the two linked gene theory proposed by Scott and Hickok (1987) and indicate that the two genes are separated by as many as 36 map units. Although more complex models may also be consistent with these results, this study has shown that this relatively simple model is sufficient to explain all observations.

Along with results reported in chapter four, this study suggests that at least three chromosomes carry genes which affect antheridiogen sensitivity in Ceratopteris richardii. However, the significance of

possible wild-type alleles at the loci of the mutant alleles of HaC23 and 57.45 is not known at this time. Furthermore, while two linked genes appear to be responsible for the differences in sensitivity between strains 176D and H, it is unknown whether these strains share other genes associated with this trait which may be identical. It is possible that other genes involved in antheridiogen sensitivity may be clustered around the two genes which were studied in this report. Alternatively, these two genes may be isolated from other genes which effect this trait. Although such questions cannot be resolved by the results reported here, it is hoped that techniques introduced in this study may provide a means for further investigation of antheridiogen sensitivity.

CHAPTER VI

CHARACTERIZATION AND GENETIC ANALYSIS OF A DARK-GERMINATING MUTANT

Introduction

The effects of light on fern spore germination have been studied extensively. In almost all cases, photoinduction is a requirement (reviewed in Miller, 1968). Investigations on the genera Dryopteris (Mohr, 1956), Osmunda (Mohr et al., 1964), Pteris (Sugai and Furuya, 1967; Sugai et al., 1984), Onoclea (Towill and Ikuma, 1973; Huckaby et al., 1982; Wayne and Helper, 1984), Lygodium (Sugai et al, 1977; Tomizawa et al, 1982; 1983), Adiantum (Sugai and Furuya, 1985) and Ceratopteris (Cooke et al., 1987), have all revealed a red light requirement, suggesting that phytochrome is the photosensitive receptor. Furthermore, experiments with at least eight different species have demonstrated the characteristic red-far red photoreversibility typically associated with phytochrome (reviewed in Howland and Edwards, 1979).

In addition to the far red light inhibition typically associated with phytochrome mediated systems, spore germination in many ferns is also inhibited by blue light. Although blue light has some capacity to change the active form of phytochrome to the inactive form, causing inhibition, evidence from several fern species implies that a novel photoreceptor is also involved in blue light inhibition (reviewed in Howland and Edwards, 1979).

In contrast to the general light requirement for germination in fern spores, some species produce spores which exhibit dark germination under certain circumstances. Miller (1968) compiled an extensive list of references which cited species that had been examined specifically for the ability to germinate in darkness. Although spores of most species failed to exhibit dark germination in any of the reported studies, some occasionally exhibited low frequencies of germinated spores in dark cultures. In these cases, Miller offered possible explanations based on trends in the experimental methodologies or on present-day observations. In some studies, high temperatures may have induced small numbers of spores to germinate in darkness. In other instances, spore age may have affected the results. Freshly collected spores frequently exhibit limited dark germination. However, this capacity is lost as the spores age. Conditions under which sporogenesis occur may also contribute to the ability of some spores to germinate in darkness. Schraudolf (1987b) demonstrated that the frequency of dark germination in spores of Anemia phyllitidis may be increased from less than 0.1% to more than 80% by culturing sporophytes under conditions of continuous red light.

In addition to the infrequent occurrences of dark germination cited for some species (Miller, 1968), three species are notable for repeated references citing their ability to germinate in darkness on mineral nutrient media alone. These species are: Pteridium aquilinum, Ceratopteris thalictroides and Osmunda regalis. Although Miller offered no explanation for this phenomenon, a recent investigation by Cooke et al. (1987; see below) suggests that at least for

Ceratopteris, dark germination may have been due to spore age or other factors.

A remaining exception to the general requirement of light for germination occurs in several species of the family Schizeaceae. Several members of this family consistently exhibit high degrees of dark germination in the presence of gibberellic acid or antheridiogen. This phenomenon was first demonstrated for Anemia phyllitidis with gibberellic acid (Schraudolf, 1962) and later with native antheridiogen (Naf, 1966). The activity of both substances was also shown for Lygodium japonicum (Naf, 1966)

A recent study of the germination response in Ceratopteris richardii (Cooke et al., 1987) demonstrated that this species shares the common light requirements of most other ferns. Mature spores of this species completely fail to germinate in the absence of light. Red light induces germination and far red light inhibits the process in induced spores. Blue light is also inhibitory in this species. Another aspect of germination in this species is that antheridiogen does not substitute for red light induction (Scott and Hickok, unpublished).

These results are difficult to reconcile with previous reports which suggested that C. thalictroides germinates in total darkness. Cooke et al. noted the possibility that dark germination is a species specific trait of C. thalictroides not shared with the related species C. richardii. However, they suggested that a more likely explanation is that earlier studies utilized immature spores. Spores of C. richardii typically exhibit limited dark germination if tested prior

to maturity (Schedlbauer, 1976a). Another alternative is that the ability of spores to germinate in darkness is a trait associated with specific genes which varies among different strains in Ceratopteris. Schraudolf (pers. comm. to L.G. Hickok) reported that spores collected from a particular sporophyte of Ceratopteris exhibited a high degree of dark germination while others did not.

In this chapter the selection of a dark-germinating mutant strain of C. richardii is reported. This mutant, HaDG7, was selected in an attempt to generate a line which germinates in darkness in the presence of antheridiogen. However, preliminary characterization showed that although spores of this strain do germinate in darkness, there is no requirement for the pheromone. Further characterization and a genetic analysis have also been accomplished for this strain. These results and a discussion of the mutant phenotype will be provided in this chapter.

Materials and Methods

Mutagenesis and selection.

A selection scheme for isolating dark-germinating mutants was modeled after that described by Hickok (1987). Spores of the wild-type strain, strain H, were soaked in darkness then sterilized under green light (see below) and replaced in darkness in a solution containing Ceratopteris antheridiogen (i.e. a crude aqueous filtrate or CAF from medium which previously supported gametophytic growth). Spores were mutagenized by exposure to X-irradiation (400R/min., 28 min.). Nine days after mutagenesis, a total of approximately 3.125 x

10^5 mutagenized spores (0.25g) and the same number of non-mutagenized spores were screened for germination. Spores were considered to have germinated if the primary rhizoid protruded from the spore wall. Spores which germinated in darkness were isolated and allowed to mature. Mature gametophytes were watered to induce self-fertilization to produce homozygous sporophytes.

Culture conditions.

Soaking of spores, sterilization and sowing were all performed in a darkened room. The only light was indirect light from a 25 watt green light bulb (General Electric, order number 25A/TG). Spores were not soaked overnight prior to sterilization as described in chapter one. Instead, spores were soaked with distilled water just prior to sterilization. Spores were sterilized and sown as described in chapter one, except that spores were sown at 1/2 standard density. Culture plates were sealed with parafilm. Cultures to be maintained in darkness (dark cultures) were wrapped in three layers of aluminum foil. All cultures (both light and dark cultures) were grown on culture decks having standard conditions of light and temperature as described in chapter one. Cultures of both HaDG7 and the wild-type strain (strain H) were always sterilized, sown and maintained under the same conditions as a control measure.

Linkage analysis.

Spores from a hybrid between a strain which carries a gene conferring paraquat tolerance and the dark-germinating strain HaDG7 were cultured in darkness for 20 days. After 9 days of light exposure,

gametophytes which had germinated in darkness were transferred to cultures containing basal medium with 1 uM paraquat. Gametophytes of the HaDG7 were also transferred to 1 uM paraquat supplemented medium as a control. At 4 days following transfer, gametophytes were assessed to determine whether they exhibited paraquat tolerance. Gametophytes which did not exhibit tolerance were no longer green, while tolerant ones appeared normal.

Results

Selection of strain HaDG7.

Spore were observed to germinate in darkness at a frequency of 1 out of every 5500 for the mutagenized spores and 1 out of every 8000 for the non mutagenized spores. A total of 18 spore collections from strains generated from mutagenized spores and 8 from strains from non-mutagenized spores were tested for dark germination in the presence of CAF. Two collections, both from strains derived from mutagenesis, exhibited dark germination. The degree of dark germination was high in one strain, HaDG7, but low in the other, HaDG8. Only a small collection of spores was available for HaDG8 and the sporophyte grew poorly. Therefore, further characterization was accomplished only for strain HaDG7. Initial characterization for HaDG7 spores indicated that germination occurred in darkness with or without CAF in the growth medium.

Characterization of strain HaDG7.

At 10 days following soaking (DFS) of spores, strain HaDG7 exhibited nearly complete germination in darkness, but only a very slight degree of germination in light. This was in contrast to the response of strain H, which did not germinate at all in darkness, but exhibited high levels of germination in light (see table 6-1).

Spores of HaDG7 which germinated in light produced gametophytes which appeared normal. They differentiated as both cordates and males and could not be visually distinguished from gametophytes of the H strain. Gametophytes of the HaDG7 strain which developed in darkness from dark-germinated spores had the same appearance as H strain gametophytes from light-germinated spores which were subsequently cultured in darkness (see chapter three). If these gametophytes were brought into the light after dark-germination and growth, newly produced tissues developed normally, resulting in gametophytes with long etiolated sections of tissue followed by tissues which developed like normal cordate or male gametophytes.

The germination patterns of HaDG7 in darkness and H in light were virtually identical. Figure 6-1 shows the germination pattern for these strains during the period of active germination. Both strains first showed evidence of germination at four DFS. The percentage of germinated spores was nearly the same for both strains on days four, five and six. On day seven, percent germination was slightly different, however each strain exhibited nearly maximum germination (compare to table 6-1).

Spores of strain HaDG7 required between two and three days of

Table 6-1. The Percentage of Germinated Spores[a] in Cultures of Strains H and HaDG7 and a Hybrid under Various Conditions of Light and Darkness.

Spore Collection	Conditions[b]					
	10DL	10DD	20DL	20DD	10DD/10DL[c]	10DL/10DD[c]
H	91±1	0±0	92±2	0±0	----	----
HaDG7	8±2	98±1	13±2	98±1	----	----
F1(HaPQ45 x HaDG7)3	88±6	43±2	93±1	47±4	95±3	91±3

[a] The percentage of germinated spores indicates the average of three samples ± the standard deviation of the sample. For each sample, 100 spores were counted.

[b] DL indicates days of light treatment, DD indicates days of dark treatment, a / indicates that the treatment listed second directly followed the treatment listed first.

[c] These treatments were given only to F1-derived spores to determine if increased degrees of germination could be obtained.

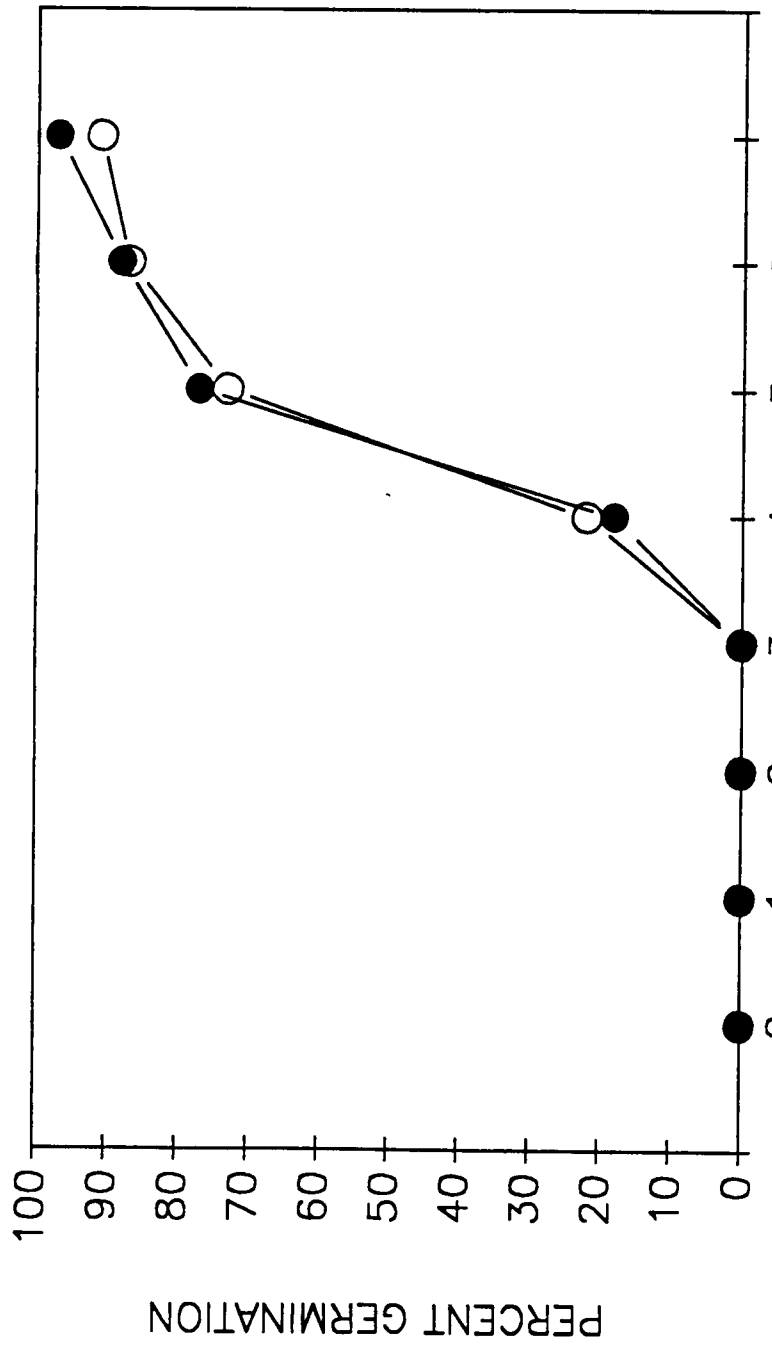

Figure 6-1. A comparison of the timing of germination for H spores in the light (open circles) and HaDG7 spores in darkness (filled circles). For HaDG7 data, cultures were exposed to light and the percentage of germinated spores was immediately determined. Each point represents the average of three replicates. The greatest standard deviation was ± 12% of the average.

darkness for maximum germination to take place. Figure 6-2 shows the effects of maintaining cultures of this strain in darkness for various lengths of time and then exposing them to light. Spores maintained in light continuously or given only 24 hours of darkness responded almost identically. These treatments stimulated no germination initially. At five DFS, the first evidence of germination was observed for these conditions. On following days the percentage of germinated spores rose gradually, until germination reached 22 and 14% respectively on day 14. Spores cultured in darkness for 48 hours and then exposed to light, exhibited much greater germination, but not all spores responded. Spores maintained under this light regime began to germinate at four days. However, the percentage of germinated spores reached a maximum between 50 and 60% and became fairly constant between 6 and 14 days. Spores allowed to remain in darkness for 72 hours or longer all responded similarly, and exhibited maximum germination.

F1 analysis.

The average percentage of germinated spores was determined under various conditions of light and darkness for cultures of the H strain, the HaDG7 strain and a hybrid between a wild type strain (HaPQ45 which carries a marker gene conferring paraquat tolerance) and HaDG7 (Table 6-1). At 10 DFS, H and HaDG7 exhibited the characteristic responses described above. If cultures were left in light or darkness for 20 days, the responses of the H strain were virtually unchanged. Cultures of HaDG7 left in darkness for 20 days showed no additional

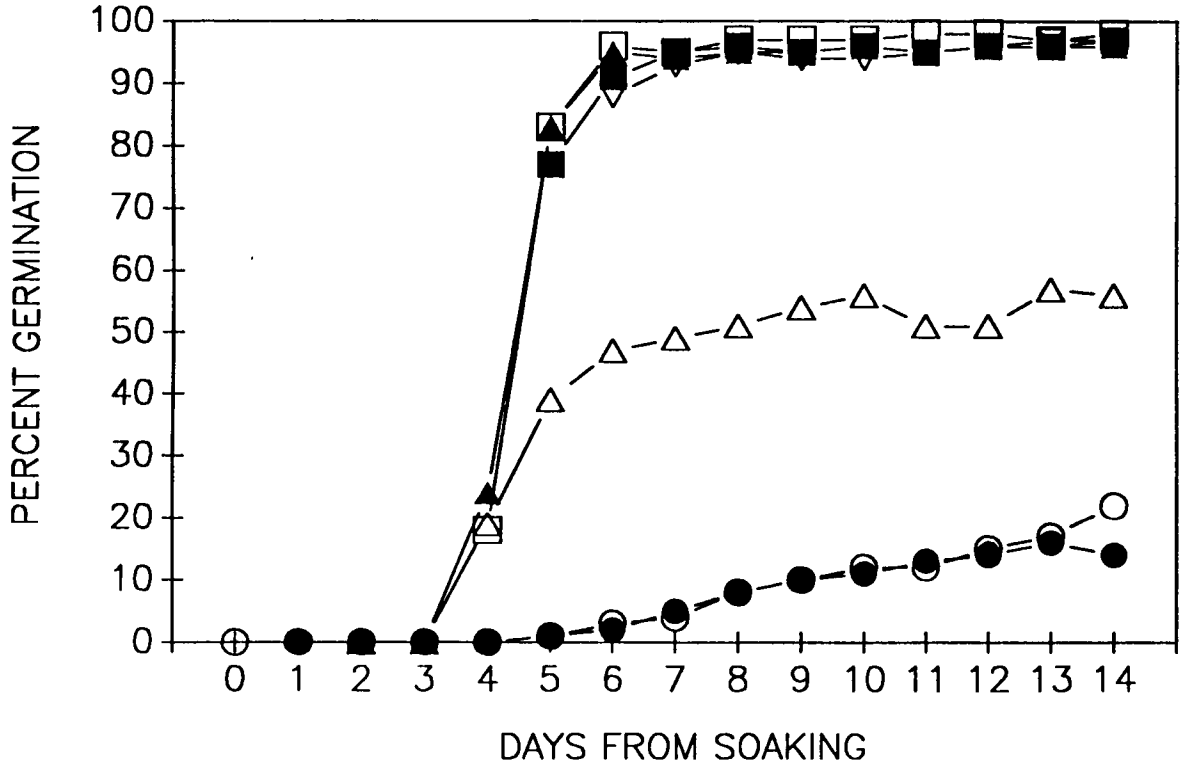

Figure 6-2. The germination responses of HaDG7 spores cultured in darkness for various lengths of time, then exposed to light. The symbols represent the various numbers of days (24 hour periods) of dark treatments as follows: open circles = 0, closed circles = 1, open upward pointing triangles = 2, filled upward pointing triangles = 3, open squares = 4, filled squares = 5 and open downward pointing triangles = 6. Cultures were also maintained in darkness for 7 days. The data for this treatment are not shown, however, the response was nearly identical to that of cultures maintained in darkness for 3 through 6 days. Each data point represents the average of three replicates. The greatest standard deviation was ± 15% of the average.

germination. However, if cultures of this strain were left in the light for 20 days, the percentage of germinated spores increased somewhat. Cultures of HaDG7 left in the light for longer periods of time continued to exhibit gradual increases in germination. For instance, after 30 days in the light, HaDG7 spores exhibited 64% germination.

Spores of the F1 hybrid exhibited a relatively high percentage of germination in the light after 10 days. However, less than 50% germinated in darkness in the same amount of time. If hybrid-derived spores were allowed to remain in the light for 20 days, the percent of germinated spores approached 100%. If they were kept in the dark for 20 days, the germination percentage increased only slightly, reaching 47%. If F1 spores were kept in the light for 10 days then placed in darkness for 10 days or given the opposite treatment, they exhibited near maximum germination. The highest percentage of germination exhibited by F1-derived spores was 95% after 10 days in darkness, followed by 10 days in light.

Linkage analysis.

Paraquat tolerance was exhibited by 55 out of 100 dark-germinated gametophytes from the hybrid between the paraquat tolerant strain HaPQ45 and strain HaDG7. The ratio of nontolerant to tolerant types among these gametophytes closely approximated 1:1 ($p > 0.3$). Gametophytes from the strain HaDG7 which were transferred to paraquat did not exhibit paraquat tolerance.

Discussion

The phenotype of HaDG7.

The spores of strain HaDG7 exhibit two unusual germination responses. The first is that they germinate in complete darkness and the second is that their ability to germinate is inhibited by light. The first of these responses, dark germination, is clearly distinguishable from the response of the wild-type (see table 6-1). The second unusual response is not as distinct. Although germination in HaDG7 spores is inhibited by light, after prolonged periods of light exposure, they exhibit increasing degrees of recovery from this inhibition. An understanding of the physiological nature of this mutation will require additional, detailed characterization. However, consideration of the results reported here will provide insights into the light regulation of germination in Ceratopteris.

That dark germination in HaDG7 followed a pattern almost identical to light germination in H (Figure 6-1) provides insights into the events of light mediated germination. Although HaDG7 spores do not require light induction, the time between soaking of spores and germination for this strain was nearly identical to that for H spores. This indicates that necessary processes which occur during this lag period may not be directly related to photoinduction.

Most investigations of germination have focused on the induction period and less is known regarding the pre-induction phase. However, Towill and Ikuma (1975) obtained results which indicated that some protein synthesis was required prior to induction in spores of

O. sensibilis. Furthermore, Tomizawa et al. (1982) measured the phytochrome content of spores of L. japonicum as they imbibed in darkness and showed that the concentration increased with time. The increase closely followed a pattern of increasing light sensitivity later established for that species (Tomizawa et al., 1983). Whether ability to germinate in wild-type or mutant Ceratopteris spores is related to protein synthesis or increasing phytochrome content is not known at this time. However further investigation into this question should supply valuable insights.

That mutant spores germinated in darkness over a period of several days, may provide insight into why germination of wild-type spores in the light is asynchronous. In multispore cultures of the H (wild-type) strain, spores germinate over a period from about three to seven days in the light (see figure 6-1). Furthermore, if H spores are kept in darkness for several days prior to light induction, germination synchrony is increased (Cooke et al., 1987; Scott and Hickok, unpublished).

Two alternative theories could explain how a uniform light stimulus causes asynchronous germination. One possibility is that some spores become competent to respond to the stimulus before others. This would involve the completion of such pre-induction processes as those described above. The alternative to this hypothesis is that some spores require less light stimulus to trigger germination than do others. Furuya (1985) discussed these two options for spores of L. japonicum, considering only increases in phytochrome content as a prerequisite to induction. Although he could not draw a definitive

conclusion from the available data, he suggested that the more likely explanation is that different spores become competent at different times.

HaDG7 spores do not require a light stimulus, but still exhibit asynchronous germination. This suggests that some HaDG7 spores become competent to germinate before others and since this strain has no light requirement for induction, spores germinate as soon as they become competent. Furthermore, the observation that germination becomes more synchronous for H spores if they are soaked in darkness prior to induction suggests that this procedure allows more spores to become competent prior to induction. Both of these observations suggest that in wild-type spores, when light is not limiting, germination asynchrony results from spores becoming competent to germinate at different times rather than from different spores having different light requirements.

The inhibition of germination by light in spores of HaDG7 (see figure 6-2) may be associated with the effects of blue light. Wild type spores of Ceratopteris exhibit inhibition of germination if exposed to monochromatic blue light (Cooke, 1987) as do the spores of other ferns (see introduction). However, it is difficult to imagine how eliminating the requirement for induction by red light would enhance the inhibitory effects of blue light. Further investigation of this phenomenon in spores of HaDG7 should increase understanding of photoinduction in fern spores.

Genetic analysis.

Dark germination in HaDG7 appears to be associated with a single gene which operates at the level of the spore. Maximum germination in spores of the F1 hybrid occurred after 10 days in darkness, followed by 10 days in light. After such treatment, F1-derived spores exhibited 95% germination, indicating that nearly all spores were viable. F1-derived spores kept in darkness for 20 days, exhibited 47% germination. If spore viability is taken into account, 49% of the viable F1-derived spores exhibited dark germination. This pattern of segregation for dark versus light-germinating spores clearly indicates single gene inheritance.

That segregation for dark germination occurred in F1-derived spores indicates that this trait is associated with a gametophytic gene (i.e. in the spore) rather than a sporophytic gene. This observation eliminates at least one possible explanation for dark germination in these spores. Schraudolf (1987b) showed that spores of Anemia phyllitidis from sporophytes grown under continuous red light exhibited high degrees of dark germination. He suggested that this phenomenon resulted from spores being preloaded with the active form of phytochrome during sporogenesis. Since only half of the F1-derived spores exhibited dark germination, it appears that preloading of spores with active phytochrome could not account for dark germination in HaDG7.

Although the mutant gene in HaDG7 acts in spores to allow dark germination, it appears that the associated phenotype of light inhibition is controlled at the level of the sporophyte. The trait is

evident in spores derived from the homozygous strain HaDG7, but not in those derived from heterozygous F1. Light inhibition in HaDG7 spores appears to result from the action of a gene which functions as a homozygous recessive in the sporophyte.

There are two alternatives for the action of this gene in the F1 hybrid. The observations that HaDG7 spores exhibit limited recovery from light inhibition and that spores of the F1 show slightly higher germination after 20 days in the light than after 10 (see Table 6-1) suggest one alternative. It is possible that the single copy of the mutant allele in the hybrid sporophyte still causes light inhibition. However, recovery from this inhibition is much quicker in the hybrid due to the action of a single copy of the normal allele. In this case, the wild-type allele could be considered to be partially dominant. A second possibility is that the slightly lower germination in the F1-derived spores at 10 days as compared to 20 days is due to an unrelated factor. If this is the case, the F1-derived spores do not exhibit light inhibition and the wild-type allele should be considered dominant.

Linkage analysis.

The approximate 1:1 segregation ratio for paraquat tolerance among the dark-germinated F1 gametophytes indicates that the genes for dark germination and paraquat tolerance segregate independently. Since Ceratopteris richardii possesses a high chromosome number (n=39), this result was not unexpected.

Summary and concluding remarks.

The mutant strain HaDG7 exhibits two unusual phenotypes, dark germination and light inhibition. The pattern of dark germination in this mutant suggests the importance of undetermined pre-induction events in the spores of Ceratopteris. The pattern further suggests that asychrony of germination in Ceratopteris spores is due to differences in the time at which spores become competent to respond to induction, rather than differences in induction requirements. Light inhibition in these spores is characterized by a gradual recovery which occurs over an extended period of time. Further investigation of this phenomenon is required.

Dark germination is associated with a single gene which operates at the level of the spore rather than the sporophyte. This gene is not linked to the gene which confers paraquat tolerance to the mutant HaPQ45. Light inhibition is controlled at the level of the sporophyte and is inherited as a recessive trait. The wild type allele of this gene behaves as either a partially or a completely dominant allele.

It is highly unlikely that two random mutations could occur in a single spore and both affect genes associated with photomorphogenesis. This suggests that the gene which controls dark germination in HaDG7 and the gene which controls light inhibition are the same. However, as indicated above, this gene is active in both the gametophytic genome and the sporophytic genome and apparently has different effects in the two generations. The designation dg_7 is given to the mutant allele which controls these effects. Further study of this mutation

should provide many additional insights on the light control of fern spore germination.

BIBLIOGRAPHY

BIBLIOGRAPHY

Beeks, R.M. 1955. Improvement in the squash technique for plant chromosomes.Aliso 3:131-133.

Cheng, C.-Y. and H. Schraudolf. 1974. Nschweis von Abscisinsaure in Sporen und Jungen Prothallien von Anemia phyllitidis L. Sw. Z Pflanzenphysiol. 71:366-369.

Cooke, T.H., R.H. Racusen, L.G. Hickok and T.R. Warne. 1987. The photocontrol of spore germination in the fern Ceratopteris richardii. Plant Cell Physiol. 28(5):753-759.

Corey, E.J. and A.G. Myers. 1985. Total synthesis of the (±)-antheridium-inducing factor (A_{An}, 2) of the fern Anemia phyllitidis. Clarification of stereochemistry. J. Amer. Chem. Soc. 107:5574-5576.

Corey, E.J., A.G. Meyers, N. Takahashi, H. Yamane and H. Schraudolf. 1986. Constitution of antheridium-incucing factor of Anemia phyllitidis. Tetrahedron Lett. 27(42):5083-5084.

Cousens, M.I. 1979. Gametophyte ontogeny, sex expression, and genetic load as measures of population divergence in Blechnum spicant. Amer. J. Bot. 66:116-132.

Crozier, A. and R.C. Durley. 1983. Modern methods of analysis of gibberellins. In. The Biochemistry and Physiology of Gibberellins. A. Crozier (Ed.) pp. 485-560. Praeger, New York.

Dopp, w. 1950. Eine die Antheridienbildung bei Farnen Fordernde Substanz in den Prothallien von Pteridium aquilinum L. Kuhn. Ber. Stsch. Bot Ges. 63:139-147.

Endo, M., K. Nakanishi, U. Naf, W. Mckeon and R. Walker. 1972. Isolation of the antheridiogen of Anemia phyllitidis. Physiol. Plant. 26:183-185.

Furber, M. and L.N. Mander. 1987. Conversion of Gibberellin A_7 into antheridic acid, the antheridium inducing factor from the fern Anemia phyllitidis: a new protocol for controlled 1,2-bond shifts. J. Am. Chem. Soc. 109:6389-6396.

Furuya, M. 1985. Photocontrol of spore germination and elementary processes of development in fern gametophytes. In Proceedings of the Royal Society of Edinburgh 86B:13-19

Gemmrich, A.R. 1986. Antheridiogenesis in the fern Pteris vittata II) hormonal control of antheridium formation. J. Plant Physiol. 125:157-166.

Goodwin and Mercer. 1983. Phytohormones and related compounds. In Introduction to plant biochemistry, 2nd ed., pp. 567-626. Pergamon Press.

Hickok, L.G. 1983. Abscisic acid blocks antheridium formation in gametophytes of the fern Ceratopteris. Can. J. Bot. 61:888-892.

_____. 1985. Genetics of gametophyte development. Proc. of the Royal Soc. of Edinburgh. 86B:21-28.

_____. 1987. Application of in vitro selection systems. Whole-plant selection using the haploid phase of Ceratopteris. In Biotechnology and Agricultural Chemistry., H.M. LeBaron, R.O. Mumma, R.C. Honeycutt and J.H. Duesing, Eds. pp. 53-65. American ChemicalSociety Symposium Series, 334, Washington, D.C.

_____. and R.M. Kiriluk. 1984. Effects of auxins on gametophyte development and sexual differentiation in the fern Ceratopteris thalictroides (L.) Brongn.. Bot. Gaz. 145:37-42.

_____. and O.J. Schwarz. 1986. An in vitro whole plant selection system: paraquat tolerant mutants in the fern Ceratopteris. Theor. Appl. Genet. 72:302-306.

_____., T.R. Warne and M.K. Slocum. 1987. Ceratopteris richardii: Applications for experimental plant biology. Amer. J. Bot. 74(8):1304-1315.

Howland G.P. and M.E. Edwards. 1979. Photomorphogenesis of fern gametophytes. In The Experimental Biology of Ferns, A.F. Dyer, Ed., pp. 394-434 London: Academic Press.

Huckaby, C.S., K. Kalantari and J.H. Miller. 1982. Inhibition of Onoclea sensibilis spore germination by far-red light and cis-4-cyclohexene-1,2-dicarboximide. Z. Phlanzenphysiol. 105:375-378.

Klekowski, E.J. 1969. Reproductive biology of the Pteridophyta. III. A study of the Blechnaceae. Bot. J. Linn. Soc. 62:361-371.

Lloyd, R.M. and T.R. Warne. 1978. The absence of genetic load in a morphologically variable sexual species, Ceratopteris thalictroides (Parkeriaceae). Sys. Bot. 3:20-36.

Miller, J.H. 1968. Fern gametophytes as experimental material. Bot. Rev. 34(4):361-440.

Mohr, H. 1956. Die Beeinflussung der keimung von Farnsporen durch licht und andere faktoren. Planta 46:534-551.

_____., Meyer and K. Hartmann. 1964. Die Beeinflussung der Farnsporenkeimung (Onoclea cinnamomea (L.) and Osmunda claytoniana (L.)) uber das phytochromsystem und die photosynthese. Planta 60:483-496.

Naf, U. 1958. On the physiology of antheridium formation in the bracken fern Pteridium aquilinum (L.) Kuhn. Physiol. Plant 11: 728-746.

_____.1959. Control of antheridium formation in the fern species Anemia Phyllitidis. Nature 184:798-800.

_____. 1960. On the control of antheridium formation in the fern species Lygodium japonicum. Proc. Soc. Exp. Biol. Med. 105:82-86.

_____. 1961. Mode of action of antheridium-inducing substances in ferns. Nature 189:900-903.

_____. 1965. On antheridial metabolism in the fern species Onoclea sensibilis L. Plant Physiol. 40:888-890.

_____. 1966. On dark-germination and antheridium formation in Anemia phyllitidis. Physiologia Plant. 19:1079-1088.

_____. 1967. On the induction of a phase inhibitory to antheridium formation in the juvenile prothallus of the fern species Anemia phyllitidis. Z. Pflanzenphysiol. 56:353-365.

_____. 1968. On separation and identity of fern antheridiogens. Plant Cell Physiol. 9:27-33.

_____. 1979. Antheridiogens and antheridial development. In The Experimental Biology of Ferns, A.F. Dryer, Ed., pp. 435-470 London: Academic Press.

_____, K. Nakanishi and M. Endo. 1975. On the physiology and chemistry of fern antheridiogens. Bot. Rev. 41(3):315-359.

Nakanishi, K., M, Endo, U. Naf and L. Johnson. 1971. Structure of the antheridium-inducing factor of the fern Anemia phyllitidis. J. Amer. Chem. Soc. 93:5579-5581.

Nester, J.E. and R.C. Coolbaugh. 1986. Factors influencing spore germination and early gametophyte development in Anemia mexicana and Anemia phyllitidis. Plant Physiol. 82:230-235.

Nester, J.E., S. Veysey and R.C. Coolbaugh. 1987. Partial characterization of an antheridiogen of Anemia mexicana: Comparison of antheridiogen of A. phyllitidis. Planta. 170:26-33.

Pringle, R.B. 1961. Chemical nature of antheridiogen-A, a specific inducer of the male sex organ in certain fern species. Sci. 133: 284.

Pringle, R.B., U. Naf and A.C. Braun. 1960. Purification of a specific inducer of the male sex organ in certain fern species. Nature 186:1066-1067.

Schedlbauer, M.D. 1974. Biological Specificity of antheridogen from Ceratopteris thalictroides (L.) Brongn. Planta 116:39-43.

_____. 1976a. Specificity of antheridogen from Ceratopteris thalictroides (L.) Brongn. Plant Physiol. 57:666-669.

_____. 1976b. Fern gametophyte development: controls of dimorphism in Ceratopteris thalictroides. Amer. J. Bot. 63(8):1080-1087.

_____. and E.J. Klekowski, Jr. 1972. Antheridogen activity in the fern Ceratopteris thalictroides (L.) Brongn. Bot. J. Linn. Soc. 65(4):399-413.

Schraudolf, H. 1962. Die wirkung von phytohormonen auf keimung und entwicklung von farnprothallien, I. Auslosung der antheridienbildung und dunkelkeimung bei schizaeceen durch gibberellinsaure. Biol. Zbl. 81:731-740.

_____. 1964. Relative activity of the gibberellins in the antheridium induction in Anemia phyllitidis. Nature 201:98-99.

_____. 1987a. Antagonistic effects of abscisic acid and ABA-analogues on hormone - induced antheridium differentiation. J. Plant Physiol. 131:433-439.

_____. 1987b. Parental predetermination of dark germination in spores of Anemia phyllitidis. Naturwissenschaften 74:138

Scott, R.J. and L.G. Hickok. 1987. Genetic analysis of antheridiogen sensitivity in Ceratopteris richardii. Amer. J. Bot. 74(12): 1872-1877

Sugai, M. and M. Furuya. 1967. Photomorphogenesis in Pteris vittata. 1. Phytochrome-mediated spore germination and blue light interaction. Plant Cell Physiol. 8:737-748.

_____. 1985. Action spectrum in ultraviolet and blue light region for the inhibition of red-light-induced spore germination in Adiantum capillus-veneris L. Plant Cell Physiol. 26:953-956.

Sugai, M., K. Takeno and M. Furuya. 1977. Divers responses of spores in the light dependent germination of Lygodium japonicum. Plant Sci. Lett. 8:333-338.

Sugai, M., K. Tomizawa, M. Watanabe and M. Furuya. 1984. Action spectrum between 250 and 800 nanometers for the photoinduced inhibition of spore germination in Pteris vittata. Plant Cell Physiol. 25:205-212.

Takeno, K. and M. Furuya. 1975. Bioassay of antheridiogen in Lygodium japonicum. Dev. Growth Differ. 17:9-18.

_____. 1980. Sexual differentiation in population of prothallia in Lygodium japonicum. Bot. Mag. Tokyo 93:67-76.

Tomizawa, K., K. Manabe and M. Sugai. 1982. Changes of phytochrome contents during imbibition in spores of the fern Lygodium japonicum. Plant Cell Physiol. 23:1305-1308.

Tomizawa, K, M. Sugai and K. Manabe. 1983. Relationship between germination and P_{fr} levelin spores of the fern Lygodium japonicum. Plant Cell Physiol. 24:1043-1048.

Towill, L.R. and H. Ikuma. 1973. Photocontrol of germination of Onoclea spores. I. Action spectrum. Plant Physiol. 51:973-978.

Voeller B.R. 1964a. Gibberellins: Their effect on antheridium formation in fern gametophytes. Sci. 143:373-375.

_____. 1964b. Antheridiogens in fenrs. In Regulateurs Naturels de la Croissance Vegetale. J.P. Nitsch, Ed., pp. 665-684.

Warne, T.R., and L.G. Hickok. 1989. Evidence for a gibberellin biosynthetic origin of Ceratopteris antheridiogen. Plant Phys. 89:535-538.

Warne, T.R., L.G. Hickok and R.J. Scott. 1988. Characterization and genetic analysis of antheridiogen-insensititive mutants in the fern Ceratopteris. Bot. J. Linn. Soc. 96:371-379.

Warne, T.R. and R.M. Lloyd. 1981. Inbreeding and homozygosity in the fern Ceratopteris pteridoides (Hooker) Hieronymus (Parkeriaceae), Bot. J. Linn. Soc. 83:1-13.

Warne, T.R., G.L. Walker and L.G. Hickok. 1986. A novel method for surface-sterilizing and sowing fern spores. Amer. Fern J. 76:187-188.

Wayne, R. and P.K. Helper. 1984. The role of calcium ions in phytochrome-mediated germination of spores of Onoclea sensibilis L. Planta 160:12-20.

Weinberg, E.S. and B.R. Voeller. 1969. Induction of fern spore germination. Proc. Nat. Acad. Sci. 64(3):835-842.

Wilkie D. 1963. Genetic analysis of variation in the bracken prothalus. J. Linn. Soc., Bot. 58:333-336

Yamane, H., N. Takahashi, K. Takeno, and M. Furuya. 1979. Identification of gibberellin A_9 methyl ester as a natural substance regulating formation of reproductive organs in Lygodium japonicum. Planta. 147:251-256.

Yamane, H. K. Nohara, N. Takahashi and H. Schraudolf. 1987. Identification of antheridic acid as an antheridiogen in Anemia rotundifolia and Anemia flexuosa. Plant Cell Physiol. 28(7):1203-1207.

Zanno, P.R., M. Endo, K. Nakanishi, U. Naf and C. Stein. 1972. On the structural diversity of fern antheridiogens. Naturwissenschaften 59:512.

APPENDIX

Table A-1. Various Characteristics of Gametophytes of Different Spore Collections Grown in Darkness on 0.2% CAPE Supplemented Medium.

Spore Collection[a]	x% G[b]	x Length[c]	x A#[d]
H	93±3	1,005±180	15.4±3.6
H	91±4	1,350±255	15.5±4.2
H	93±3	1,172±216	18.0±3.3
176D	88±2	899±104	2.6±1.2
176D	93±4	938±117	2.9±1.5
176D	90±6	1,013±133	3.0±1.3
57.45	69±7	1,408±141	1.8±0.9
57.45	61±10	1,157±148	2.2±0.9
57.45	80±7	1,273±124	1.6±0.8
HaC23	95±1	1,831±158	0.1±0.4
HaC23	88±2	1,553±142	0.4±0.7
H1761-12	96±2	1,312±114	7.9±2.9
H1761-52	90±4	1,364±126	4.1±1.5
H1761-58	57±4	800±129	17.7±3.6
H1761-58	83±3	869±123	15.5±2.1
H1761-100	44±6	995±149	11.5±2.4
H1761-100	63±5	999144	11.4±3.1
H1761-118	94±2	1,252±122	2.6±1.0
H1761-166	85±3	1,263±111	10.8±3.0
H1761-173	78±2	1,287±130	10.3±2.1
H1761-173	89±3	1,092±156	11.0±1.8
H1761-177	95±2	1,642±173	5.4±2.4
H1761-179	96±2	1,415±134	16.0±3.1
H1761-196	86±3	1,146±108	12.0±2.9
H1761-198	99±2	1,194±144	15.9±2.7
F1(176D X H)1	93±1	1,037±222	9.6±6.6
F1(H X 176D)1	87±4	1,012±279	10.0±5.9
F1(HaC23 X 176D)1	84±2	1,239±251	6.3±7.3
F1(57.45 X 176D)1	90±2	1,153±229	5.9±6.6
F1(57.45 X HaC23)1	93±3	1,398±214	4.3±6.8

[a]Data for one spore collection may be given two or more times because two or more experiments were performed for several collection.

[b]x% G indicates percent germination ± the standard deviation of the sample (N = 3) following the dark-growth period.

[c]x Length[b] indicates the average gametophyte length in um ± the standard deviation of the sample, following dark-growth. (N = 50 - 375).

[d]x A# indicates the average antheridia number ± the standard deviation of the sample, following dark-growth. (N = 50 - 375).

VITA

Rodney J. Scott was born in Birmingham, Michigan, on March 24, 1961. In 1979, he graduated from Manatee High School in Bradenton, Florida. He received an Associate in Arts degree from Manatee Junior College in Bradenton, Florida in the summer of 1981. In the following fall, he began attending the University of West Florida in Pensacola, Florida. In the fall of 1982, he dedicated his life to the Lord. He received his Bachelor of Science degree in Biology from the University of West Florida in 1983. In the fall of 1983, he came to the University of Tennessee, Knoxville to pursue a Master's of Science degree in Botany. On December 28, 1985 he married Donna L. Sarfert. In 1986, he received the Master's of Science degree from the University of Tennessee. He is presently looking forward to becoming a father within the next several weeks and to receiving his Doctor of Philosophy degree in Botany soon thereafter. In the fall of 1989, he will assume the duties of an Assistant Professor of Biology at Wheaton College in Wheaton, Illinois.