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Comprehensive investigation of spore germination and antheridiogen chemistry in Anemia mexicana Klotzsch

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COMPREHENSIVE INVESTIGATION OF SPORE GERMINATION AND
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Comprehensive investigation of spore germination

and antheridiogen chemistry in

Anemia mexicana Klotzsch

by

Joan Elizabeth Nester

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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In Charge of Major Work

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For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1985

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DEDICATION

I dedicate this dissertation to
my Grandfather: John B. Nester,
my Grandmother: Mary B. Hons,
my parents: Esther M. and John J. Nester
and to my entire family:
both blood and volunteer--good people.

ABBREVIATIONS

AMO-1618	2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenylpiperidine-1-carboxylate
ancymidol	α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidine methyl alcohol
BSTFA	bis(trimethylsilyl)trifluoroacetamide
n-BuOH	butanol
C_6H_6	benzene
CH_2N_2	diazomethane
$CHCl_3$	chloroform
cm	centimeter
EDTA	ethylenediaminetetraacetic acid
EtOAc	ethyl acetate
fenarimol	α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidine methyl alcohol
FID	flame ionization detector
HMG	3-hydroxy-3-methyl-glutaryl
GA	gibberellic acid
GAs	gibberellins
GC/FT-IR	gas chromatography/Fourier transform-infrared spectrometry
GC/MS	gas chromatography/mass spectrometry
^{1}H NMR	proton nuclear magnetic resonance
HOAc	acetic acid
kV	kilovolts
µE	micro Einstein

M^+	molecular ion, parent ion
MeOH	methanol
MES	2(N-morpholino)ethanesulfonic acid
mM	millimolar
mm	millimeter
MVA	mevalonic acid
SEM	scanning electron microscopy
Si gel	silica gel
s (time)	second
s (IR)	strong
temp.	temperature (C)
TLC	thin-layer chromatography
TMCS	trimethyl chlorosilane
TMSi	trimethylsilyl ether
w (IR)	weak

GENERAL INTRODUCTION

Ferns are a group of vascular plants with two distinct free-living generations, the sporophyte and the gametophyte. In the majority of ferns, the sporophyte is the dominant generation in the life cycle. It is composed of roots, stems and leaves. The stems are often compact and subterranean. The leaves of different fern genera vary in size from small leaves of some _Trichomanes_ sp. (several mm) to large vining leaves of some _Lygodium_ sp. which have active apical growth and may reach a length of 20 m (Bierhorst, 1971). Ferns grow in a variety of habitats and have different ecological nitches. _Pteridium_ _aquilinum_ is terrestrial and is considered a weed in many locations. Tree ferns can grow several meters tall when in tropical areas while some species of _Pellaea_ and _Notholaena_ are adapted for a dry environment with leaves 10 to 15 cm long possessing pinnae only 0.2 to 0.5 cm in length. Thus, ferns make up a diverse group of plants.

The sporangia which will contain the spores are formed on the leaves, usually on the underside in clusters. A spore mother cell will undergo meiosis to give rise to 4 spores. At maturity, these spores are dehydrated and the sporangium opens releasing the spores to be dispersed in the wind.

When a spore reaches a suitable environment, each will germinate and begin to grow into a gametophyte. Moisture is required for germination and many species also require a red light treatment to stimulate this event. After germination, the gametophyte continues to grow, developing a flat, heart-shaped prothallus. Rhizoids are formed at the base near the original spore coat and they are single, elongate cells, similar to root hairs and function in anchorage and absorption. After becoming heart-shaped, the gametophyte may form antheridia and/or archegonia. In the presence of water, the antheridium will open releasing the multiflagellated sperm. The archegonium neck opens up releasing the neck canal contents and the sperm swims to an archegonium where fertilization occurs, forming the zygote. Subsequent development of the sporophyte, thus completes the cycle.

The gametophyte of ferns is a morphologically simple plant but physiologically and hormonally, it is quite complex. A hormonal response which has been studied from the morphological, taxonomic and physiological viewpoints is the antheridiogen. An antheridiogen was first detected by Döpp in 1950. When he sowed spores of Pteridium aquilinum on culture medium,

which previously had mature Pteridium gametophytes growing on the surface, the newly developing gametophytes prematurely formed antheridia. From his studies, he concluded that a substance produced by the mature gametophytes had been present in the medium and this compound had stimulated the precocious antheridium formation. Other workers began to study the physiology of this compound and in 1961 Pringle termed it an antheridogen. Later Näf (1969) referred to it as an antheridiogen, an etymologically preferred derivation.

Antheridiogen is synthesized by gametophytes with notched (organized) meristems and these gametophytes are presently or will be archegoniate. Thus, antheridiogen is synthesized by female gametophytes and the compound diffuses into the culture medium causing other asexual gametophytes to be antheridiate.

Since 1950, the antheridiogen of P. aquilinum has been shown to be effective with greater than 28 species of ferns in six different families (Näf et al., 1975). This compound has been characterized as a complex carboxylic acid (Pringle, 1961) but since that time, little structural information has been obtained.

An obvious lack of sensitivity to Pteridium antheridiogen occurs with the schizaeaceous ferns, Anemia and Lygodium. The schizaeaceous ferns are not

sensitive to the antheridiogen of Pteridium or any of
the antheridiogens from the other groups of ferns except
from their own family. It was soon found that Anemia
phyllitidis produced an antheridiogen which was
different from that of Pteridium (Näf, 1959; 1968).
Schraudolf (1962) discovered that gibberellic acid (GA)
could substitute for antheridiogen, inducing premature
antheridium formation. This indicated that the
antheridiogen of A. phyllitidis may be related to or is
a GA. GA had virtually no effect on gametophytes
sensitive to Pteridium antheridiogen. No authentic
compounds have been found which can substitute for the
Pteridium antheridiogen.

The antheridiogen of Anemia was found to have a
second biological activity, namely the ability to
substitute for the light requirement for germination
(Näf, 1966). Red light is an absolute requirement for
spore germination in the schizaeaceous ferns. The
induction of spore germination in the dark has been
shown to be 30 times more sensitive to antheridiogen
than the premature induction of antheridia (Näf, 1966;
Endo et al., 1972).

In 1971, Nakanishi et al. determined the chemical
structure of the A. phyllitidis antheridiogen. It is

very similar to a C-19 gibberellin with 2 hydroxyl groups and one ring double bond in an unusual C/D ring arrangement. The antheridiogen of Anemia hirsuta is identical to that of A. phyllitidis and it was concluded that in this case structural diversity existed at the genus level (Zanno et al., 1972). It had previously been shown chromatographically and biologically that the antheridiogen of A. phyllitidis and Lygodium japonicum were different (Näf, 1959; 1960; 1968). In 1979, Yamane and his coworkers identified GA_9-methyl ester as an antheridiogen of L. japonicum. Thus, two closely related genera of ferns were found to have distinct antheridogens, Lygodium with a gibberellin and Anemia with a gibberellin-like structure containing an unusual C/D ring arrangement.

In 1969, Weinberg and Voeller detected a GA-like compound synthesized during light-induced spore germination in A. phyllitidis. It was concluded that this compound was synthesized during germintion since AMO-1618, an inhibitor of GA biosynthesis caused a decrease in germination. It was unknown if the compound synthesized at germination was antheridiogen.

The present study focuses on spore germination and antheridiogen chemistry in Anemia mexicana Klotzsch (Fig. 1). Anemia mexicana is native to Texas and
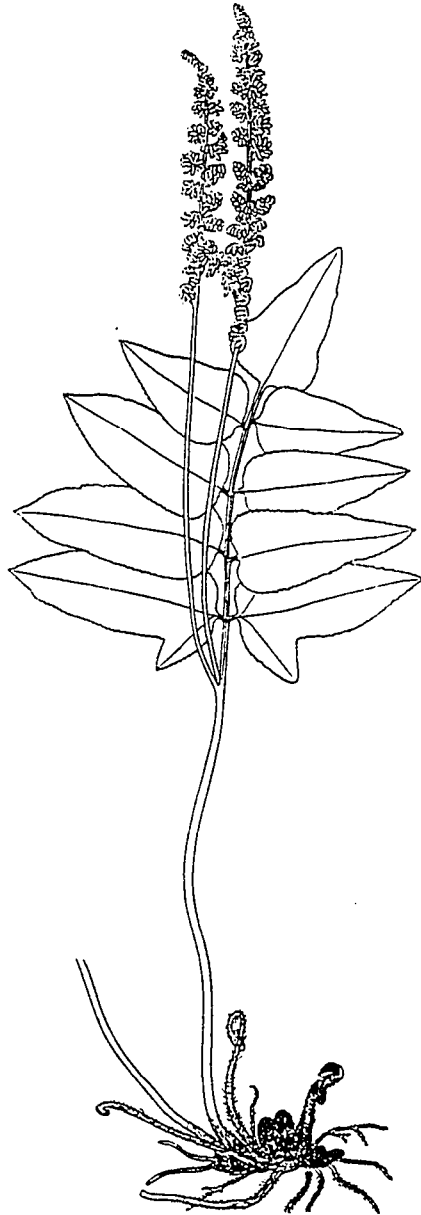
Fig. 1. Sporophyte of <u>Anemia</u> <u>mexicana</u> (from Correll, 1955)

Mexico. The genus <u>Anemia</u> is characterized by having the sporangia localized on the basal pair of pinnae. In <u>A.</u> <u>mexicana</u> the leaves are once pinnately compound and the fertile basal pair of pinnae are erect and usually longer than the remainder of the blade.

Previous studies had indicated an antheridiogen was synthesized by archegoniate gametophytes (Nester and Schedlbauer, 1982) but it was unknown if this antheridiogen was identical to that of <u>A. phyllitidis</u>.

In this study, several aspects of spore germination and antheridiogen chemistry in <u>A. mexicana</u> were studied with some comparisons with <u>A. phyllitidis</u>. There were three main objectives:

1. To describe spore germination using both scanning electron microscopy (SEM) and light microscopy. Very few studies have utilized SEM to describe germination and to obtain a three-dimensional view of spore germination. There was also a question of the cell division pattern during spore germination in <u>Anemia</u> (Raghavan and Huckaby, 1980; Schraudolf, 1981) and this question was also addressed. Raghavan and Huckaby (1980) had found that all nine species of <u>Anemia</u> which they studied had the same type of cell division pattern and they suggested that this could be used as a taxonomic character, but Schraudolf (1981) observed a

different pattern in his studies with A. phyllitidis.
Thus, it was important to study another species of
Anemia.

2. To test the effects of various treatments on spore
germination in both A. mexicana and A. phyllitidis. The
pH optimum for GA_3-induced germination was determined in
order to optimize germination conditions. The quantity
of light required for germination was also studied. In
order to obtain evidence concerning the synthesis of a
GA-like compound at germination, precursors and
inhibitors of GA biosynthesis were tested to determine
their effect on spore germination and early gametophyte
development in the light and dark.

3. To chemically characterize the A. mexicana
antheridiogen using combined gas chromatography/mass
spectrometry and gas chromatography/infrared
spectrometry. It was unknown if all antheridiogens from
the genus Anemia were identical or dissimilar. This
information would help determine if structural diversity
of antheridiogens exists at the species or genus level
in ferns.

PAPER I. MICROSCOPIC STUDY OF SPORE GERMINATION

AND EARLY GAMETOPHYTE DEVELOPMENT IN

ANEMIA MEXICANA KLOTZSCH

ABSTRACT

Spore germination and early gametophyte development in the fern _Anemia mexicana_ Klotzsch is described using both scanning electron microscopy of intact plants and light microscopy of sectioned germinating spores and young gametophytes. The combination of both of these techniques allows a better interpretation of fern spore germination and development. Germinating spores rupture along the triradiate mark with protrusion of the rhizoid and protonemal cells. From light microscopic observations of serial sections of germinating spores, the spore cell undergoes two successive asymmetric cell divisions. The first-formed cell differentiates into a rhizoid. The second cell division gives rise to the protonemal cell. The rhizoid elongates and its nucleus migrates from the base to a central position in the cell at maturity. The protonemal cell divides again to form the photosynthetic prothallus. The spore cell may undergo a third asymmetric cell division forming a small cell which remains almost entirely within the spore coat. The two-dimensional prothallus initiates a lateral meristem which eventually shifts to a terminal position.

INTRODUCTION

Morphological studies of spore germination and gametophyte development in ferns have primarily utilized light microscopy of whole mounts of gametophytes (Atkinson and Stokey, 1964; Nayar and Kaur, 1971). Some investigators have studied sectioned materials both with light microscopy and transmission electron microscopy (Gantt and Arnott, 1965; Beisvåg, 1970; Fraser and Smith, 1974; Cran, 1979). Few studies have used scanning electron microscopy (SEM) to describe fern spore germination.

Fern spore morphology has been described in detail with SEM for many groups such as the Cyatheaceae (Gastony, 1974; 1979; Gastony and Tryon, 1976). Several scanning electron micrographs of gametophytes have been published (Elmore and Adams, 1976; Huckaby and Raghavan, 1981; Huckaby et al., 1981; Von Aderkas and Cutter, 1983; Whittier and Peterson, 1984), but development has not been followed with this technique. This paper describes spore germination and early gametophyte development in Anemia mexicana Klotzsch, utilizing both SEM of gametophytes and light microscopy of sectioned spores.

The germinating spore in Anemia undergoes two asymmetric cell divisions resulting in the formation of

three cells. One of these cells is the original spore cell, one differentiates into the first rhizoid and the third develops into the protonemal cell. Two different cell division patterns have recently been described in Anemia (Raghavan and Huckaby, 1980; Schraudolf, 1981). This study focuses on the cell division pattern during germination and deals with spore germination and early gametophyte development using SEM and light microscopy.

MATERIALS AND METHODS

Anemia mexicana Klotzsch spores were collected from
plants growing in southwest central Texas and were
stored at 4 C. Voucher specimens are deposited in the
Iowa State University Herbarium. Spores were sown
aseptically and gametophytes were cultured as described
previously (Klekowski, 1969; Schedlbauer, 1976; Nester
and Schedlbauer, 1981). A 1% calcium hypochlorite
solution was used as the sterilant and the plants were
grown at approximately 26 C under cool white fluorescent
light (ca. 40 μE s^{-1} cm^{-2}).

For SEM, pieces of agar (3-4 mm cubes) with
germinating spores or developing gametophytes were
removed from the plates, fixed, dehydrated, and critical
point dried as described earlier (Nester, 1985). Agar
pieces with gametophytes were mounted onto brass discs
with collodial silver paste. Nongerminated spores were
also observed by scattering these onto the surface of
metal tape. All materials for SEM were sputter coated
with gold palladium and observed with a JEOL JSM 35 SEM
at 15 kV.

Germinating spores were embedded in resin for light
microscopic studies. Attempts to fix and embed dry
spores or imbibed spores with unruptured spore coats
were unsuccessful, as neither osmium tetroxide (OsO_4)

nor resin penetrated the spore coat. Thus, only germinating spores with ruptured spore coats were studied.

Spores were germinated on the surface of agar-solidified medium in 100 x 15 mm plastic petri dishes and were fixed while on the plates. After fixation, they were rinsed from the plates into glass vials. Some spores were germinated in liquid medium (without agar) and grown in glass vials which were later used for fixation and processing of the gametophytes. Liquid culture medium was buffered at pH of 6.0 with 0.05 M 2(N-morpholino)ethanesulfonic acid (MES). All gametophytes were fixed with a mixture of 3 % glutaraldehyde and 2 % paraformaldehyde in 0.05 M MES (pH 6.0) buffered liquid medium. Germinating spores in vials were fixed and processed directly in the initial growing vials. Spores of A. mexicana settle to the bottom of the vial after each change in solution, thus centrifugation of the liquids was not necessary. Gametophytes were postfixed with OsO_4, rinsed with buffered liquid medium and gradually dehydrated in an ethanol series. After the gametophytes were in 100% ethanol, they were slowly infiltrated with L. R. White resin and cast in aluminum dishes. These were placed in

a 60 C drying oven for curing of the resin.

Serial sections (1-2 µm thick) of germinating spores and young gametophytes were obtained using an LKB III Ultramicrotome. Sections were stained with methylene blue/Azure II. Glass slides containing the material were observed with an Olympus BH-2 model microscope and photographed.

RESULTS

Spores of A. mexicana are tetrahedral with
relatively smooth parallel ridges ornamenting the
surface (Figs. 1, 2). Some spores have knob-like
projections on the ridges and the parallel ridges near
the triradiate mark are variable in shape and
orientation (Fig. 3). The surface of some spores
appears smooth (Fig. 1) while on others it is more rough
in texture (Figs. 2, 3).

Three to four days after sowing, spores begin to
germinate. The first evidence of germination is the
rupturing of the spore coat along the triradiate mark
(Fig. 4). A thin rough outer coating may begin to crack
(Fig. 4, arrow) and eventually this layer may begin to
flake off of the spore coat revealing a smooth
subsurface (Fig. 5). In many developing gametophytes,
the spore coat remains constricted about the protruding
gametophyte cells (Fig. 6). The rhizoid cell elongates
while the protonemal cell divides to form the two-celled
protonema (Fig. 7). The original spore cell remains
within the spore coat.

Serial sections of a single germinating spore, in a
plane nearly perpendicular to the triradiate mark, show
that the spore cell has undergone one asymmetric cell
division prior to or shortly after initial rupturing of

Figs. 1-7.   Scanning electron micrographs of spores and
             young gametophytes of _Anemia_ _mexicana_

        1.   Proximal view of spore with triradiate
             mark. x650

        2.   Distal view of spore with parallel
             ridges. x700

        3.   Spore with knob-like projections of
             ridges. x660

        4.   Germinating spore, arrow indicates
             cracking of thin outer coating. x860

        5.   Surface of germinating spore in which
             thin rough outer coating has been
             partially removed. x8600

        6.   Germinating spore with elongate rhizoid.
             x580

        7.   Young gametophyte with two protonemal
             cells and a rhizoid. Arrow indicates
             cell wall between two protonemal cells.
             x580
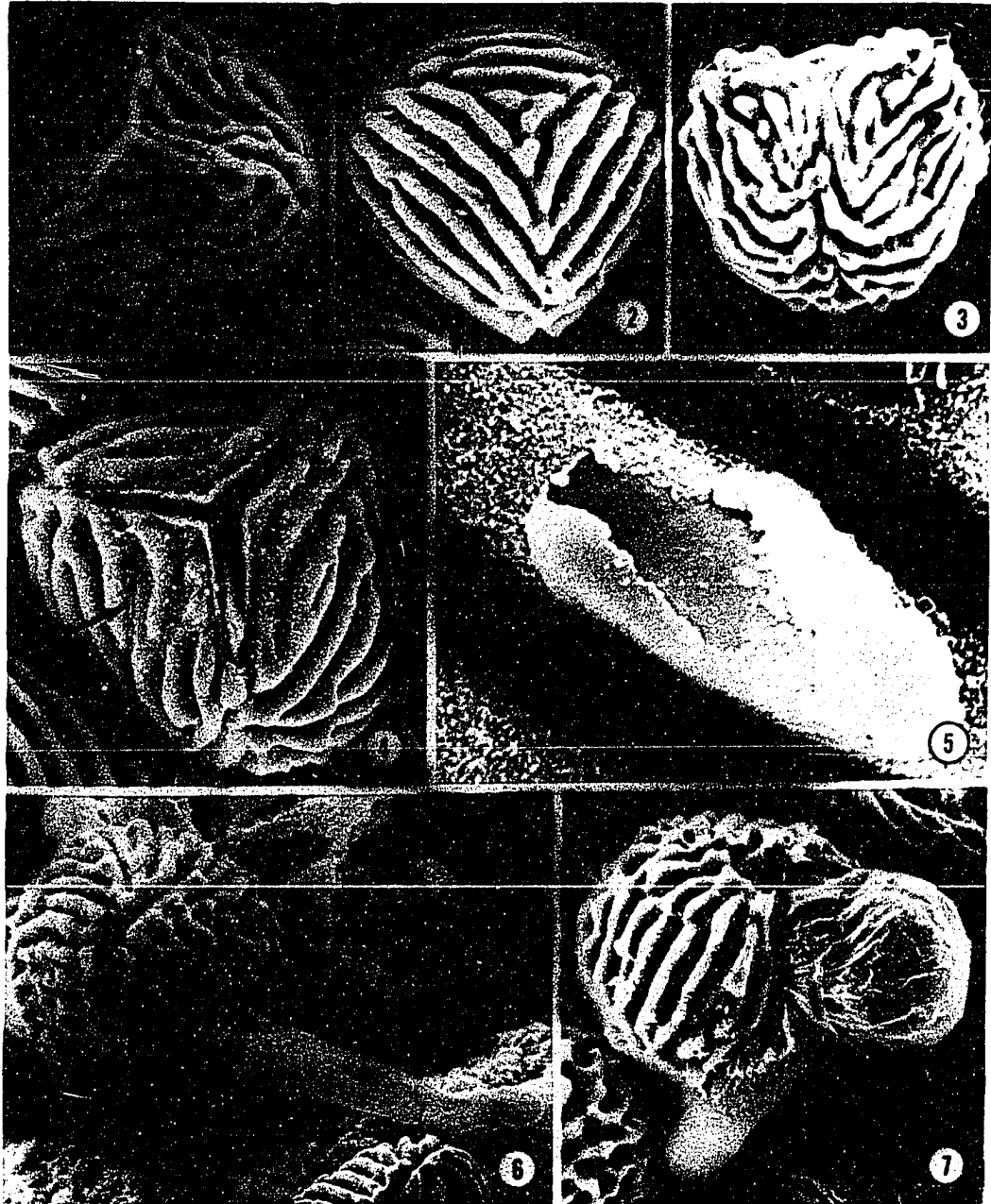
the spore coat (Figs. 8-12). This cell division occurred near the triradiate mark. The cytoplasm of the smaller of the two cells (R) appears to be almost devoid of chloroplasts and becomes the rhizoid (Figs. 9, 10). Dense cytoplasm surrounds 1) the nucleus of this new cell and 2) the area between the two nuclei and the opening of the spore coat (Figs. 10, 11). The larger spore cell (Figs. 11, 12) is composed primarily of lipid (L) material and protein bodies (PB).

The larger cell undergoes a second asymmetric division giving rise to the first protonemal cell. As seen in serial sections of a different germinating spore in Figs. 13-16, this second division is in a plane perpendicular to the first. The spore cell nucleus is in mitosis with condensed chromosomes seen in Fig. 15 (plane of cell division indicated by the arrow). The majority of the chloroplasts will be present in the new cell after division (Figs. 15, 16).

After this cell division, the gametophyte is composed of three cells, the first rhizoid, first protonemal cell and the larger original spore cell within the spore coat. The spore cell nucleus remains near the protonemal cell wall after the second cell division (Figs. 17, 18).

The protonemal cell next expands (Fig. 18) and

Figs. 8-19. Light micrographs of germinating spores of
Anemia mexicana

8-12. Serial sections of spore cell which
has undergone one asymmetric cell
division. Plane of sectioning is
almost perpendicular to triradiate
mark. R is first rhizoid cell, S is
spore cell with chloroplasts (chl).
Spore cell is composed primarily of
lipid material (L) and dark staining
protein bodies (PB). x400

13-16. Serial sections of a different spore
cell which is undergoing a second
asymmetric cell division; sections
through chromsomes (chr) are seen.
W indicates region of cell wall
separating rhizoid from spore cell.
Plane of cell division is indicated
by the arrow in Fig. 15.
Chloroplasts (chl) are more abundant
in cytoplasm of cell which will
become first protonemal cell. x450

17. Three-celled gametophyte before
expansion, composed of rhizoid (R),
protonemal cell (P) and spore cell
(S). Nucleus of spore cell remains
near wall of protonemal cell. x460

18. Three-celled gametophyte after
expansion of protonemal cell and
before elongation of rhizoid.
Protonemal cell (P) has many
chloroplasts surrounding centrally
located nucleus. x380

19. Older gametophyte with spore cell
which has undergone a third
asymmetric cell division forming a
third cell (arrow). x340
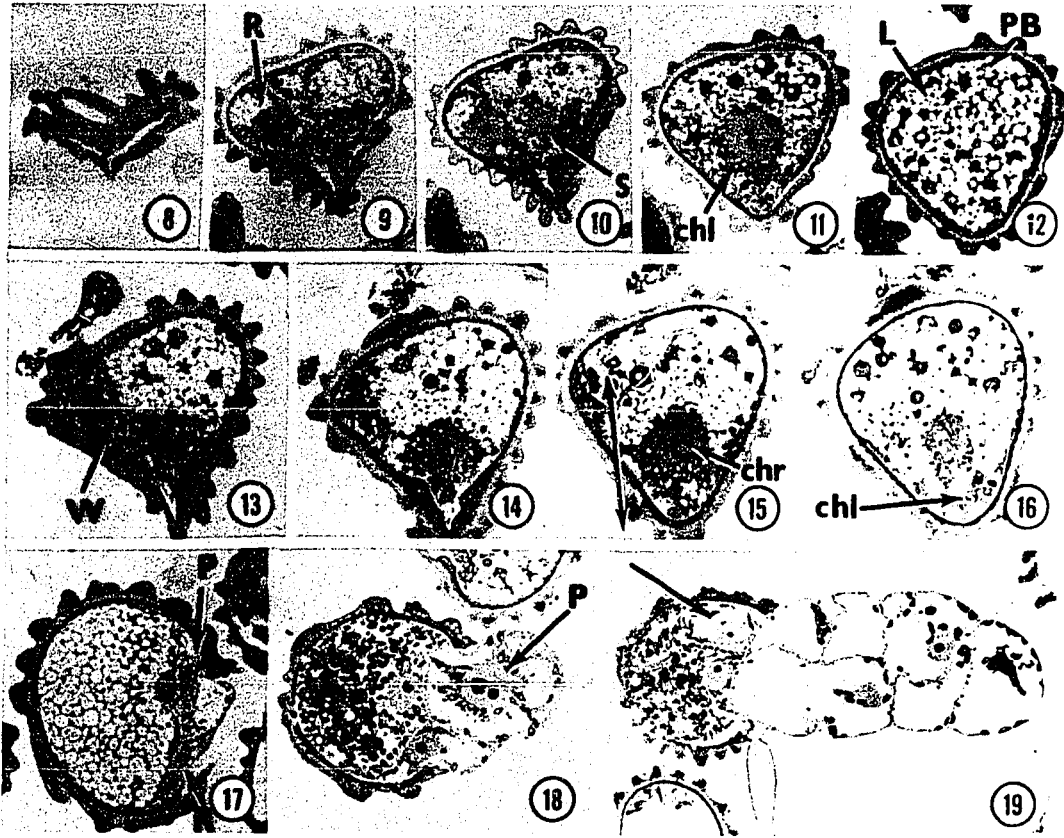
dense cytoplasm with chloroplasts surrounds the central
nucleus and is present at the cell tip. The rhizoid is
almost devoid of chloroplasts and the nucleus is
initially located near the spore cell. As the rhizoid
elongates, the nucleus migrates from the base to a
central position in the cell. The cytoplasm is
concentrated near the tip of the rhizoid and its base
becomes highly vacuolate.

The spore cell in gametophytes with greater than
three prothallial cells may divide asymmetrically a
third time to form a cell which appears to remain almost
entirely within the spore coat (Fig. 19, arrow). Such
cells, as observed from serial sections of gametophytes,
did not originate from a prothallial cell. This third
cell has chloroplasts but it is not known whether it
will undergo further divisions. The other prothallial
cells are highly vacuolate. Their chloroplasts occur
near the cell wall, and dense cytoplasm and chloroplasts
surround the nucleus.

The protonemal cells continue to divide forming the
prothallus. The gametophyes become two-dimensional
(Fig. 20) through cell divisions perpendicular to the
plane of previous cell divisions. Two-dimensional
gametophytes develop a lateral, organized meristem (Fig.
20, arrow). This meristem is composed of elongate cells

Figs. 20-23.   Scanning electron micrographs of
               gametophytes of *Anemia mexicana*

          20.   Two-dimensional gametophyte with newly
                initiated lateral meristem (arrow).
                x320

          21.   Slightly rough surface of elongate
                rhizoid. x2900

          22.   Larger gametophyte with lateral
                meristem (arrow). x210

          23.   Gametophyte with notched meristem
                (arrow). x110
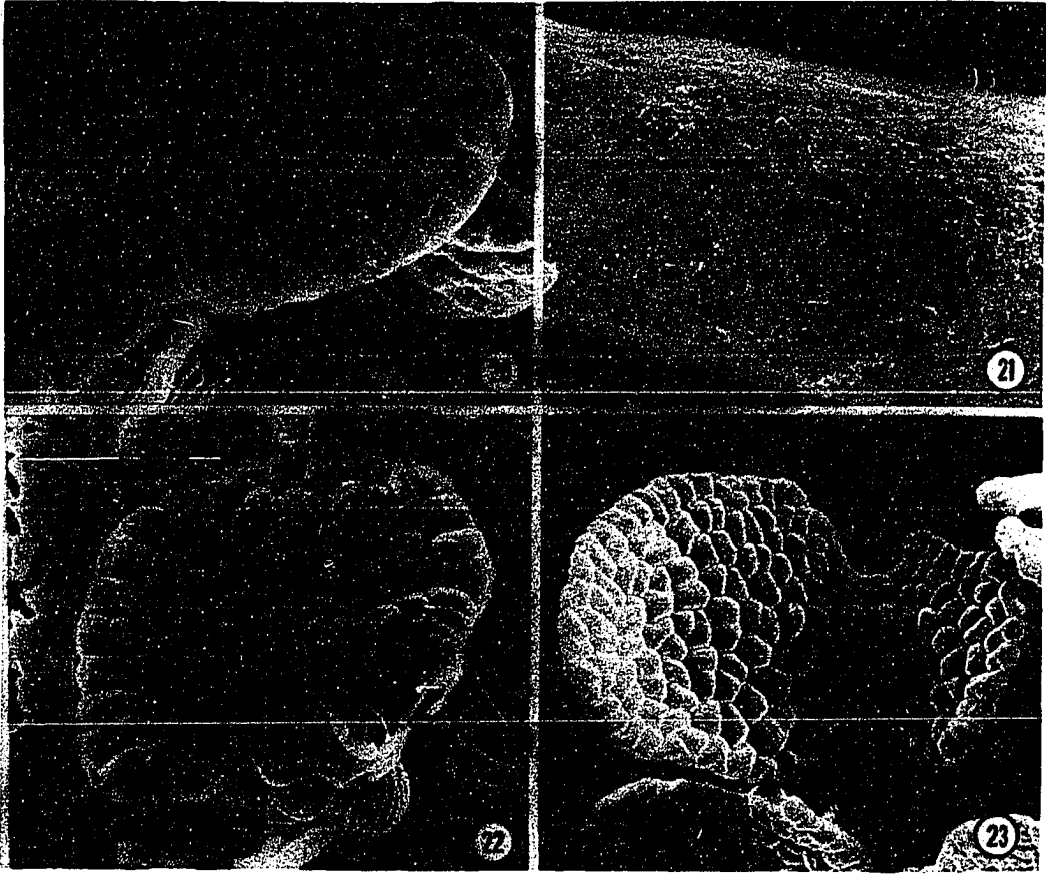
along the margin. Rhizoids are formed from the basal cells of the prothallus. The surface of these cells is slightly rough in texture (Fig. 21).

The meristem remains in a lateral position as the gametophyte grows (Fig. 22). Through production of wing cells to both sides, this lateral meristem eventually shifts to a more terminal position (Fig. 23) with continued growth of the gametophyte. Cells at the base of the gametophyte become much larger than those of the wings. Wings of the gametophyte remain one cell layer thick while the midrib becomes several cell layers thick. More rhizoids are initiated from prothallial cells at the base of the gametophytes.

DISCUSSION AND CONCLUSIONS

Spores of A. mexicana are like those described
previously by Mickel (1981) with two minor differences.
The knob-like projections of the ridges had. not
previously been described. These structures and the
irregular pattern of the ridges add variability to the
spore morphology. Spores of Anemia subgenus Anemia have
large rod-shaped projections or appendages on the ridges
(Hill, 1979). These structures are different in
morphology from those observed in spores of A. mexicana,
which is a member of the subgenus Anemiorrhiza.

Some spores of A. mexicana have a smooth coat while
others have a thin rough layer. Mickel (1981) also
observed a distinct outer layer in spores of the
subgenus Anemiorrhiza. This outer layer is not a
perine. It is also different from the surface of spores
of Anemia subgenus Coptophyllum, which have small spine-
like structures on the ridges (Hill, 1977).

Attempts to fix and embed ungerminated spores, both
dry and imbibed, were unsuccessful in this study. Other
workers have also described this problem (Gullvåg, 1969;
Beisvåg, 1970; Cran, 1979).

Serial sections through spores reveal that when the
spore coat has ruptured along the triradiate mark, the
spore cell has already divided at least once (Figs. 8-12).

Unless serial sections are made of germinating spores, misinterpretations may be made, as individual sections may appear to be of undivided cells (Fig. 11).

The degree of spore coat opening during germination varies. This feature is not easily observable with whole mounts or sectioned material. In some gametophytes, the spore coat remains highly constricted even after the rhizoid and protonemal cell have grown out of the spore coat (Fig. 6). In other gametophytes, the growth of the protonemal cell appears to force the spore coat open (Fig. 7).

There has been some disagreement concerning the cell division pattern during spore germination in Anemia. Raghavan and Huckaby (1980) studied three Anemia sp. and concluded that the first asymmetric division of the spore cell gives rise to a cell which develops into the protonemal cell while the second division of the spore cell gives rise to a cell which differentiates into the rhizoid. They suggested that since this trait was common among the three Anemia sp. they studied, it could be used as a taxonomic character. Schraudolf (1981) presented evidence that in Anemia phyllitidis, the reverse order of cell division and differentiation occurs. The cell division pattern

during spore germination in <u>A. mexicana</u> agrees with that described by Schraudolf (1981) for <u>A. phyllitidis</u>. The first division of the spore cell gives rise to the rhizoid and the second gives rise to the protonemal cell. Spores germinated in liquid medium or on the surface of agar-solidified medium were studied and both gave similar, consistent results.

Lipid and protein bodies are common storage products in nongreen fern spores (Gantt and Arnott, 1965; Gullvåg, 1969; Beisvåg, 1970). Gemmrich (1977) determined that lipid composed 56 % of the dry weight of spores of <u>A. phyllitidis</u>. In <u>Polypodium vulgare</u>, larger lipid bodies were observed near the basal end of the spore (Fraser and Smith, 1974). Lipid and protein bodies vary in shape and size in the germinating spore of <u>A. mexicana</u>, with no consistent arrangement or gradation. During germination, these storage products are metabolized by the cell (Raghavan, 1976; Gemmrich, 1977).

Schraudolf (1981) noted that in <u>A. phyllitidis</u>, the spore cell nucleus remains near the wall of the protonemal cell for a long period of time after the second cell division. This was also observed consistently in germinating spores of <u>A. mexicana</u>. From his studies of nucleic acid and protein synthesis in <u>A.</u>

phyllitidis, Raghavan (1976) observed that the spore cell remained metabolically active after the second cell division. As evidenced by the occasional formation of a third cell, this was also observed in this study.

Raghavan and Huckaby (1980) described the formation of a second rhizoid in the young gametophyte of Mohria coffrorum. In this fern, the spore cell underwent a third asymmetric cell division directly after the first two divisions. In A. mexicana, the spore cell may undergo a third asymmetric cell division after the gametophyte has become greater than three protonemal cells large. This third cell does not appear to differentiate into a rhizoid. It remains within the spore coat or may protrude slightly. Although it has chloroplasts, the function of this cell is unknown.

Wada and Staehelin (1981), using freeze fracture techniques, observed a waxy surface coat on the protonemal cells of Adiantum capillus-veneris. They concluded this material was structurally equivalent to the cuticle of other plant cells. This waxy coating was not present on the rhizoid. The surface of the rhizoid of A. mexicana does not appear to have a cuticle-like coating either but possesses a slightly rough texture (Fig. 21). Smith (1972) and Dyer and Cran (1976) have

demonstrated that the rhizoid has specific characteristics involving wall structure and absorption properties which are directly related to its function for absorption and anchorage. Thus, the lack of waxy material on the rhizoid surface is important for its function whereas the presence of the material on the prothallial cells reduces the loss of water vapor from the gametophyte (Wada and Staehelin, 1981). In this study, it is not known what effect the fixation/dehydration process may have on any surface coatings of the prothallial or rhizoid cells.

The two-dimensional gametophyte of A. mexicana with an organized meristem, as viewed with SEM, is similar to that previously described (Nester and Schedlbauer, 1981).

Spore germination observed with SEM allows a better understanding of how the spore coat opens during germination and also how the gametophyte emerges and grows out of the spore coat. Results from SEM and light microscopy of sectioned material complement one another, allowing a better interpretation of the germination phenomenon and gametophyte growth in ferns.

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PAPER II. FACTORS INFLUENCING SPORE GERMINATION AND

EARLY GAMETOPHYTE DEVELOPMENT IN

ANEMIA MEXICANA AND ANEMIA PHYLLITIDIS

ABSTRACT

Spores of <u>Anemia mexicana</u> and <u>Anemia phyllitidis</u>
were tested comparatively to investigate the effects of
various treatments on spore germination and early
gametophyte development in the light and dark.
Experiments were performed to determine optimum
germination conditions in order to use this as a
bioassay for the detection of compounds (produced by
the gametophytes) which stimulate spore germination in
the dark. Treatments included a range of culture medium
pH values, different light periods and the addition of
antheridiogen of <u>A. mexicana</u> or <u>A. phyllitidis</u> to the
culture medium. The medium was also supplemented with
GA_3, GA_4, GA_7, GA_{13}, mevalonic acid, kaurene, kaurenoic
acid, AMO-1618, fenarimol or ancymidol. The results
show that the optimum pH is approximately 6. A minimum
of 16 to 24 hours of light is needed to induce 50 %
germination and an 8 hour preinduction phase is
required. The spores of these two ferns differ in their
sensitivities to the four GAs in a bioassay for
induction of spore germination in the dark. <u>Anemia
phyllitidis</u> has greater sensitivity than <u>A. mexicana</u>.
In both species the greatest response was observed with
GA_4 and GA_7 (approximately equal) which were more
effective than GA_3. GA_{13} was clearly the least

effective. For premature induction of antheridia in the light by the GAs, both species were equally sensitive, with the same order of GA sensitivity as for GA-induced spore germination. Gametophytes of each species are 100 times more sensitive to their own antheridiogen than to the antheridiogen of the other species. Mevalonic acid, kaurene and kaurenoic acid did not induce germination in the dark. AMO-1618 (1 mM) had essentially no effect on light-induced germination or gametophyte growth. Fenarimol (1 mM) also had no effect on germination but inhibited gametophyte development. Ancymidol (0.1 mM) did not inhibit spore germination in the light or GA_3-induced germination in the dark but caused gametophytes to be round and abnormal in morphology. Ancymidol also inhibited GA_3-induced premature antherium formation in the light.

INTRODUCTION

Light is required for spore germination in several species of ferns (reviews by Sussman, 1965; Howland and Edwards, 1979; and Raghavan, 1980) and the involvement of phytochrome has been demonstrated (Mohr, 1956; Raghavan, 1971; Furuya, 1977; Grill and Schraudolf, 1981). In the schizaeaceous ferns Anemia, Lygodium, and Mohria, gibberellic acid (GA) can substitute for the light requirement and also induce precocious antheridium formation (Schraudolf, 1962; Näf, 1966, Weinberg and Voeller, 1969a; Voeller, 1971). Weinberg and Voeller (1969b) have suggested that a GA-like germination-stimulating compound is synthesized in response to the light treatment in Anemia phyllitidis. In their studies with suboptimal light treatments, spore germination was reduced by AMO-1618, an inhibitor of GA biosynthesis.

Some GAs have been shown to be more effective than others for the induction of germination in the dark and precocious antheridium formation (Schraudolf, 1964; 1966; Voeller, 1964; Weinberg and Voeller, 1969a). The antheridiogen from Anemia phyllitidis has also been shown to stimulate germination in the dark and premature antheridium formation (Näf, 1959; 1966). This antheridogen has been chemically characterized as a GA-like compound with an unusual C/D ring arrangement

(Nakanishi et al., 1971) and the antheridiogen of A. hirsuta is identical to this compound (Zanno et al., 1972). The antheridiogen of Anemia mexicana has been partially characterized as a C-19 GA-like compound with one hydroxyl group, one ring double bond, and a lactone ring (Nester et al., in prep.).

In addition to light and GA-like compounds, other factors including the pH of the culture medium, ethylene (Edwards and Miller, 1972; Fisher and Miller, 1975) and calcium availability (Wayne and Hepler, 1984) have been shown to influence the ability of spores to germinate and the subsequent growth of gametophytes (reviews by Raghavan, 1980; and Smith, 1979). Spore germination is characterized by a rupturing of the spore coat and division of the spore cell. The spore of many ferns undergoes two asymmetric cell divisions. One division gives rise to a cell which will develop into a rhizoid while the other division of the spore cell gives rise to a protonemal cell which will continue to divide to form the prothallus. The germination event is distinct from gametophyte development which involves the subsequent growth of the protonemal cell.

In the present study, spores of Anemia mexicana and Anemia phyllitidis were tested comparatively to

investigate the effects of various treatments on spore

germination and early gametophyte development in the

light and dark. Also experiments were designed to

determine optimum germination conditions in order to use

this as a bioassay for the detection of compounds

produced by the gametophytes which stimulate spore

germination in the dark.

MATERIALS AND METHODS

Spores of A. phyllitidis (L.) Swartz were collected from plants grown in the Botany Greenhouse at Iowa State University. Anemia mexicana Klotzsch spores were collected from plants growing in south central Texas. Voucher specimens of both plants are deposited in the Botany Department Herbarium at Iowa State University.

Spores of both species were sterilized with 1 % calcium hypochlorite and sown into liquid culture medium as described previously (Schedlbauer, 1976). The overnight spore presoak was omitted. The culture medium consisted of Parker's Macronutrients and Thompson's Micronutrients (Klekowski, 1969) with the deletion of the EDTA from the micronutrients and the addition of 20 μL/L 1 % FeCl. The medium was buffered with 0.05 M MES (2-(N-morpholino)ethanesulfonic acid) at pH 6 after this was determined to be optimum for germination.

Cultures were grown in the dark or under cool white fluorescent light bulbs (app. 40 μE s^{-1}cm^{-2}) at 25 $\pm$ 2 C for 7 to 14 days, after which the number of germinating spores out of 200 was determined (Nester and Schedlbauer, 1982). "Multiwell" tissue culture plates were used for germinating spores in different experimental conditions. Photomicrographs were obtained with a Zeiss inverted compound microscope.

Antheridiogen of <u>A. mexicana</u> and <u>A. phyllitidis</u> was
obtained from gametophyte culture medium as described
elsewhere (Nester et al., in prep.) and partially
purified by thin-layer chromatography (di-isopropyl
ether-acetic acid, 95:5).

Authentic standards of GA_4, GA_7 and kaurene were
kindly supplied by Dr. R. K. Clark, Abbott Laboratories;
GA_{13} was a gift from Dr. Broadbent, Imperial Chemical
Industries; ancymidol and fenarimol were from Eli Lilly
and Co.; kaurenoic acid was from Dr. P. R. Jeffries,
Dept. of Organic Chemistry, University of Western
Australia, Nedlands, Western Australia. GA_3 and MVA
were purchased from Sigma Chemical Co., St. Louis, MO.
AMO-1618 was purchased from Rainbow Chemicals.

RESULTS

pH optimum

The influence of pH of the culture medium on induction of spore germination by GA_3 over a range from 3.0 to 8.0 is seen in Fig. 1. Maximum germination occurred in the range of 5.0 to 6.5 for both species. In replicate cultures with decreasing concentrations of GA_3 (0.1, 0.01 mM), the maximum response of A. mexicana spores declined for dark-grown cultures (data not shown). But at all three concentrations in the light, the percent germination was similar to that at 1.5 mM GA_3. Spores of A. mexicana are more sensitive to changes in pH in the dark than in the light since there is a sharp decline in percent germination below pH 5.5 in the dark whereas in the light, a decline occurs below pH 4.5.

Spore germination in A. phyllitidis was quite similar for the three GA_3 concentrations and the light grown cultures (Fig. 1B). In this species a decrease in percent germination occurred at pH 7.5. This effect was greater in the light-grown cultures at all GA_3 concentrations.

For both species, growth was inhibited at pH 4.5 and below, with most germinating spores only rupturing along the triradiate mark and exhibiting very little
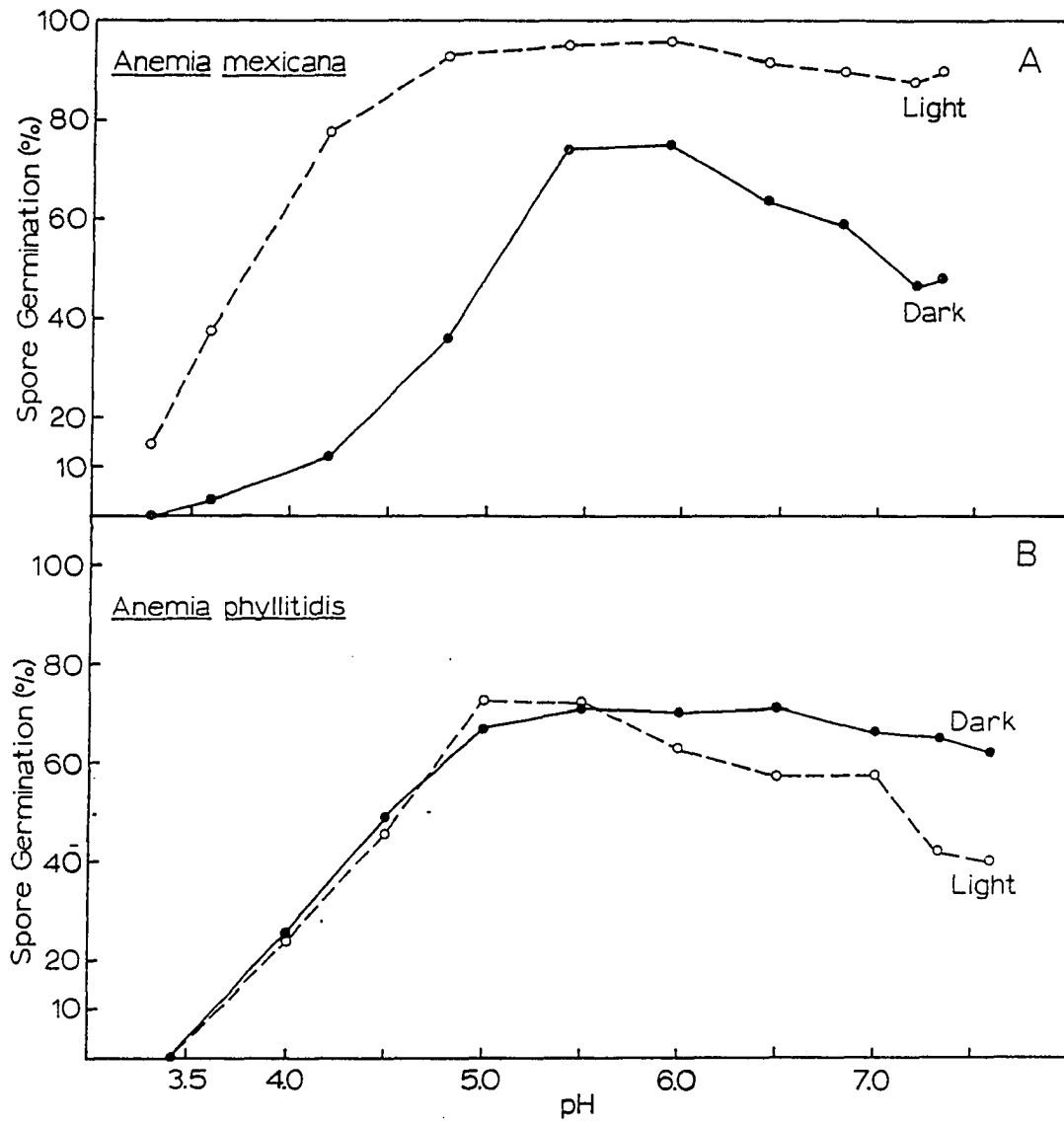
Fig. 1. Effect of pH of the culture medium on GA$_3$-induced spore germination in the dark in A. mexicana (1 mM GA$_3$) and A. phyllitidis (0.1 mM GA$_3$). Media contained 0.05 M MES. Percent spore germination determined 7 days after sowing spores

protonemal growth. Control cultures in the absence of
MES (0.05 M) indicated that the buffer had no influence
on germination or subsequent gametophyte growth (data
not shown). With the buffered medium, the pH of the
medium did not change during germination and subsequent
growth. Based upon these results, the culture medium
was buffered at pH 6 in all subsequent experiments.

Light requirement

Experiments designed to elucidate the timing and
duration of light required for germination are
illustrated in Fig. 2. Spores were given various light
treatments for three days and then all were placed in
the dark for an additional four days to allow
germination. Spores of A. phyllitidis appear to be
insensitive to light during the first 8 hours of
imbibition and require greater than 16 hours of
subsequent light to induce 50 % germination. This
requirement can be met by either one continuous
treatment at any time after the first eight hours or by
additive treatments. It is not known how long the
spores remain viable after imbibition, but spores which
have been in the dark for seven days are able to
germinate and grow when placed in the light (data not
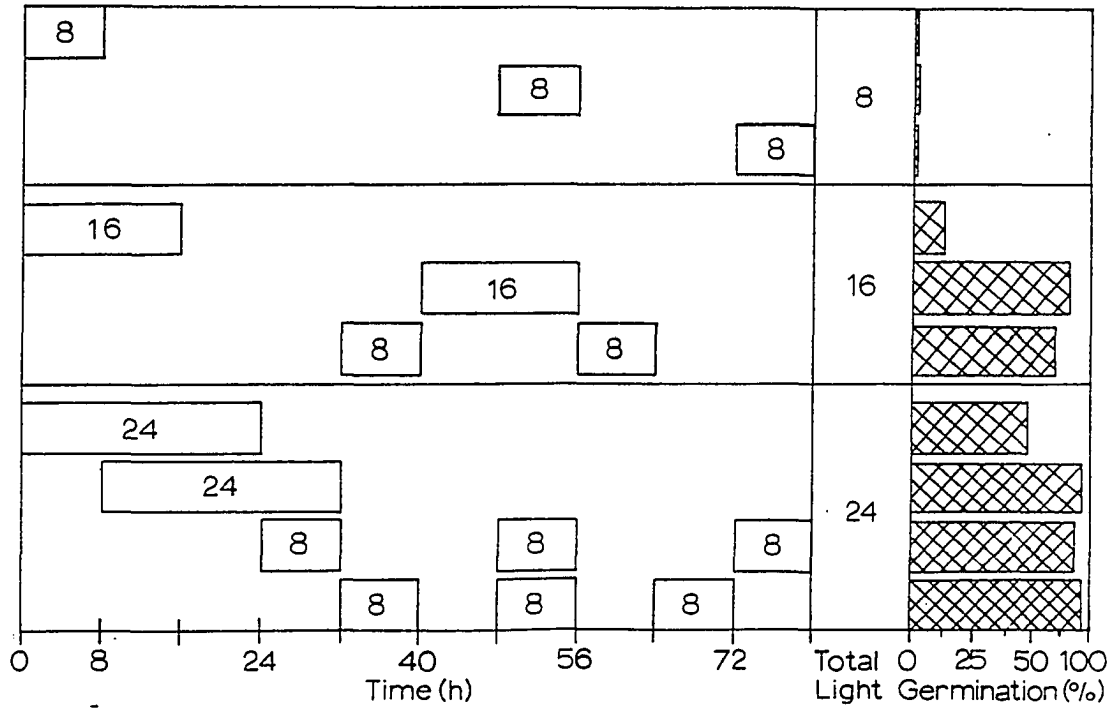shown). Spores of A. mexicana were also tested and

Fig. 2. Light-induced spore germination in
A. phyllitidis using both continuous and
alternating light and dark periods. Boxes
indicate hours in the light. Cultures
were given the light and dark treatments
directly after spore sowing for three days
and then all cultures were placed in the
dark an additional 4 days

similar results were obtained although percent germination was reduced in these experiments.

GA sensitivity

Four different gibberellins (GA_3, GA_4, GA_7, and GA_{13}) were tested to determine differences in sensitivity to the GAs. As seen in Fig. 3, A. phyllitidis spores are more sensitive to all GAs than A. mexicana spores for induction of germination in the dark. In both species, GA_7 and GA_4 gave essentially identical results, so only GA_4 is presented. Both species showed greater sensitivity to GA_4 than GA_3 or GA_{13}. Anemia mexicana responded only minimally to 1.0 mM GA_{13} (the highest concentration tested). Using 50 % spore germination for comparison, Anemia phyllitidis spores were approximately 1000 times more sensitive to GA_4 and GA_7 than were spores of A. mexicana.

Spores which germinated in the dark in response to GA gave rise to gametophytes which were filamentous in shape (Fig. 4). Chloroplast development occurred in the dark and less than 10 % of the gametophytes initiated antheridia 7 days after spore sowing.

Gametophytes germinated in the light with GA also formed antheridia prematurely (Fig. 5). Each antheridiate gametophyte had one or more antheridia by 13 days after sowing. In the absence of added GA,
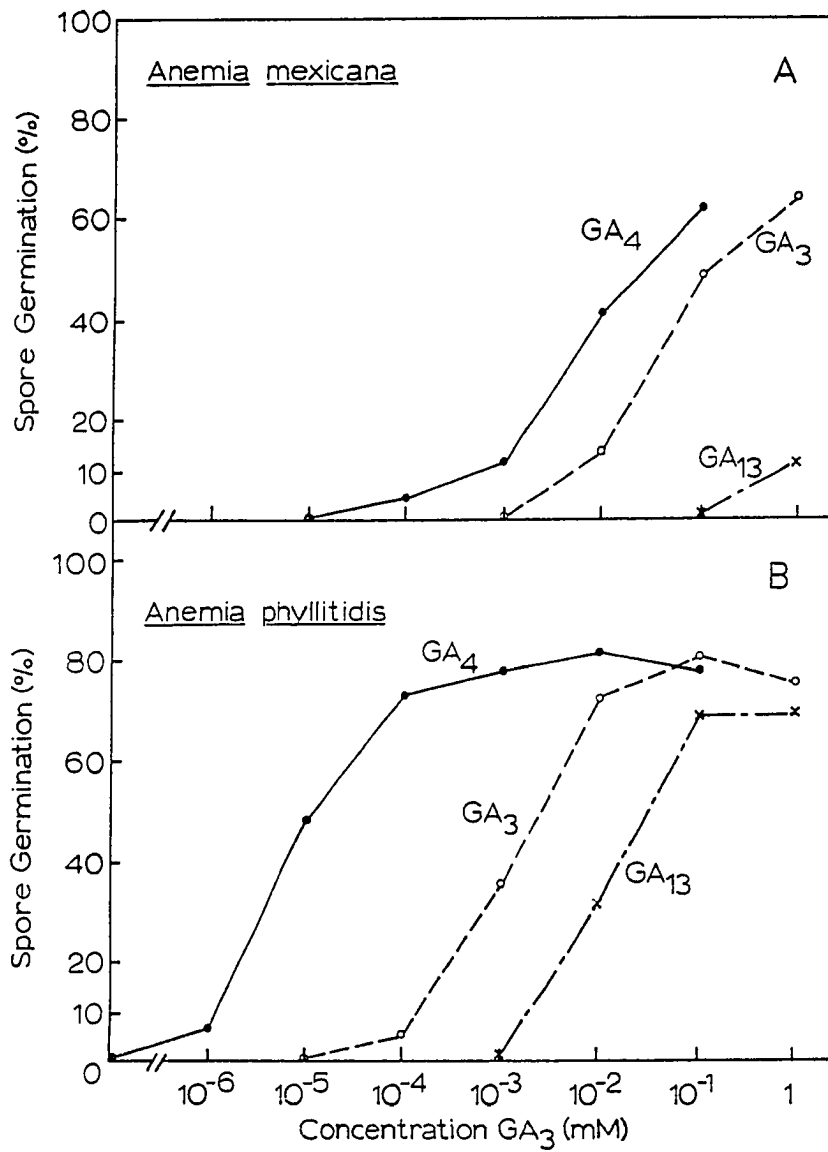
Fig. 3. Percent spore germination in the dark of
A. mexicana and A. phyllitidis, induced by GA_4,
GA_3 and GA_{13} at different concentrations.
Media contained 0.05 M MES at pH 6.0

Figs. 4-10. Photomicrographs of gametophytes of A.
             mexicana and A. phyllitidis

        4.   GA$_3$-induced germinating spore of A.
             mexicana with elongate protonemal
             cells. Germinated in the dark with
             0.1 mM ·GA$_3$, 13 days after spore sowing,
             x400

        5.   Antheridiate gametophyte of A. mexicana
             grown in the light with 0.1 mM GA$_3$.
             Arrow indicates an antheridium,
             13 days, x380

        6.   Filamentous gametophyte of
             A. phyllitidis grown in the light
             without GA added, 8 days, x420

        7.   Spatulate gametophyte of A. mexicana
             grown in the light without GA added,
             13 days, x400

        8.   Gametophyte of A. phyllitidis grown in
             medium supplemented with 0.1 mM
             ancymidol, 8 days, x370

        9.   Gametophyte of A. mexicana grown in
             medium supplemented with 0.1 mM
             ancymidol, 13 days, x370
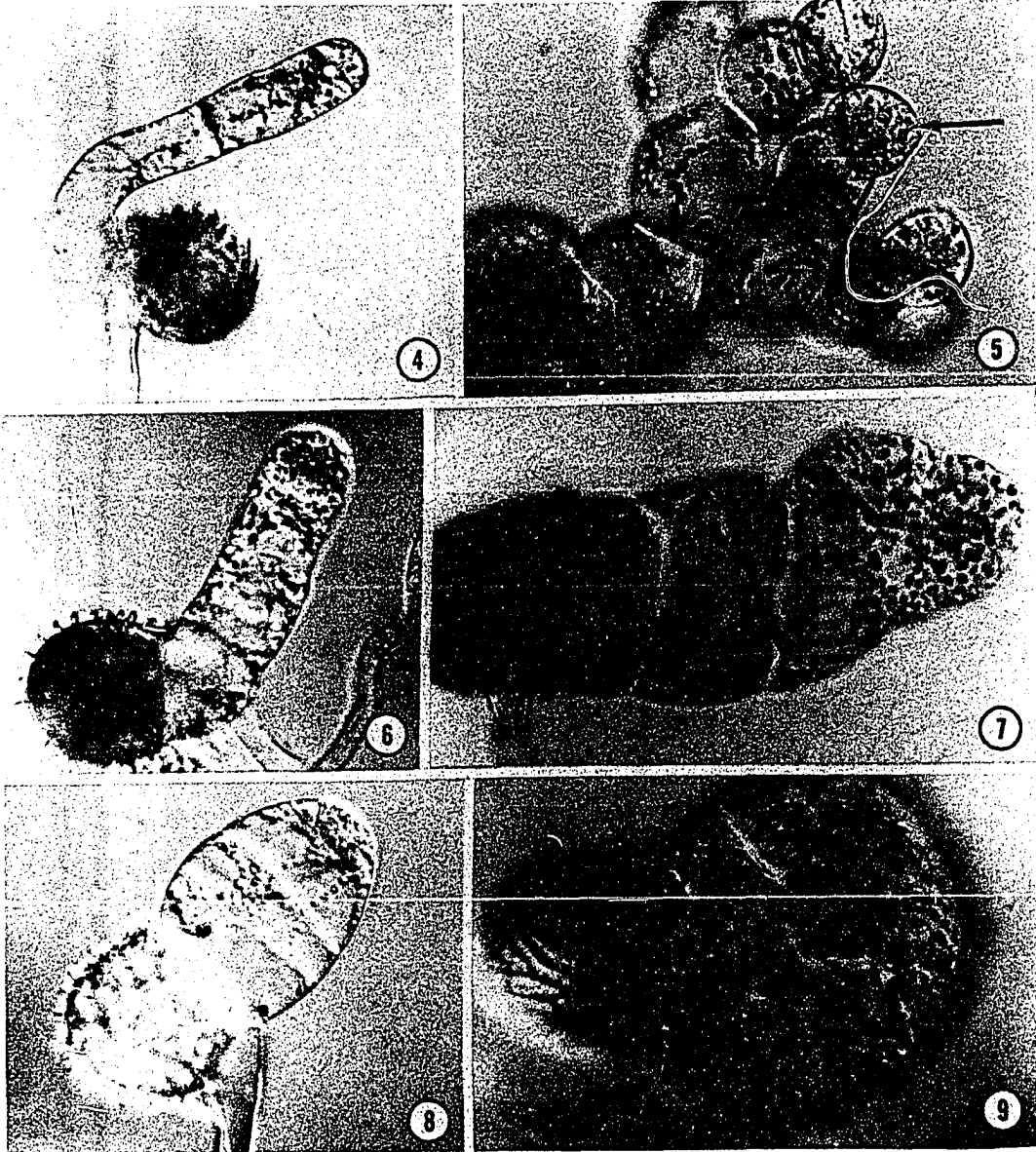
antheridium formation normally does not occur until the
gametophyte has developed an organized meristem [greater
than 19 days after spore sowing for A. mexicana (Nester
and Schedlbauer, 1981)]. The antheridiate gametophytes
were often filamentous and branched, whereas those
gametophytes without antheridia were single filaments in
A. phyllitidis (Fig. 6) or spatulate in shape in A.
mexicana (Fig. 7).

Both species had similar sensitivities to GAs for
the antheridium induction bioassay. The gametophytes
were most sensitive to GA_7 and GA_4 yielding 45 %
antheridiate gametophytes in 0.01 mM of these two GAs.
No gametophytes had initiated antheridia at this
concentration of GA_3 or GA_{13}. Only 15% of either
species initiated antheridia with the highest
concentration of GA_{13} (1 mM).

Sensitivity to antheridiogen

Antheridiogens of A. mexicana and A. phyllitidis,
obtained from culture medium extracts and partially
purified by TLC, were assayed for the induction of spore
germination in the dark. The results show that each
species had a high response to its own antheridiogen
(extract from equivalent of 5 ml culture medium); A.
mexicana 50 %, A. phyllitidis 65%. One hundred times
more extract was necessary from both species to elicit a

germination response in the other species.

Precursors and inhibitors of GA biosynthesis

In preliminary experiments with three known precursors of GAs, mevalonic acid (MVA), kaurene and kaurenoic acid were tested for their effects on spore germination in the dark. None of these compounds stimulated germination at the concentrations tested (up to 1 mM for MVA and kaurene, and up to 0.5 mM for kaurenoic acid).

Similarly, three known inhibitors of GA biosynthesis (AMO-1618, fenarimol and ancymidol) were tested for their effects on light-induced germination and subsequent gametophyte development. None of these compounds affected light-stimulated germination. AMO-1618 had no effect on germination and only slightly inhibited gametophyte development at the highest concentration (1 mM). Fenarimol (1 mM) also had no effect on spore germination but totally inhibited protonemal growth; at 0.1 mM it minimally decreased growth.

Gametophyte development in both species was affected by ancymidol, although spore germination was not inhibited. In the presence of 1 mM ancymidol A. phyllitidis spores germinated in the light but the

spores had only ruptured with little or no protonemal growth. In A. mexicana spores, limited growth occurred at this concentration. The gametophytes of both species were abnormal in morphology at 0.1 mM ancymidol. As seen in Fig. 8 of a gametophyte of A. phyllitidis, protonemal cells expand in width instead of in length (compare with Fig. 6) resulting in a more round shape. Normal growth and development occurred with lower concentrations of ancymidol. After 14 days in the light, gametophytes were still more three-dimensional in shape than normal two-dimensional gametophytes. Cell division was not inhibited (Fig. 9).

In an attempt to determine if GA_3 can overcome the ·inhibition of growth and change in morphology of the gametophytes caused by ancymidol, spores were sown in culture medium containing both ancymidol (1.0, 0.1 mM) and GA_3 (1.0, 0.1 mM). GA_3-induced germination in the dark was not inhibited by ancymidol but growth was influenced as described for cultures in the light. Thus, GA_3 did not overcome the effect of ancymidol on developing gametophytes.

As noted above, GA_3 can precociously induce the formation of antheridia (Fig. 5). In the presence of 1.0 mM or 0.1 mM ancymidol, this GA-induced antheridium formation in the light was inhibited.

DISCUSSION

GA$_3$-induced spore germination in the dark in A.
phyllitidis and A. mexicana is sensitive to the pH of
the culture medium. In this study, optimum germination
occurred at pH 5.0 to 6.5 with substantial reduction in
percent germination below pH 4.5. In a previous work by
Weinberg and Voeller (1969b), it was reported that GA$_3$-
induced germination in A. phyllitidis was pH-dependent
with an optimum between pH 3.5 and 5.0, especially with
lower concentrations of GA$_3$. Other authors using
different species of ferns (review by Miller, 1968; von
Aderkas and Cutter, 1983), have reported pH optima for
germination and growth between 5.0 and 7.0. These pH
values are consistent with the present results for
germination in the light (Fig. 1).

Growth of the germinating spores was inhibited at
pH values below 4.5. Although the germination process
was not deterred in some spores, subsequent elongation
of the protonema did not occur, indicating an inhibitory
effect on growth. GA$_3$ could not overcome this
inhibitory effect. Similar growth inhibition at low pH
has been observed in other ferns (Courbet, 1955).

The germination process in Onoclea sensibilis has
been divided into three phases: 1) preinduction phase
when the spores develop maximum photosensitivity but are

insensitive to light, 2) photoinduction phase when they are most sensitive to red light and 3) post induction phase when the photoproduct triggers processes which will eventually lead to germination (Towill and Ikuma, 1975; Chen and Ikuma, 1979). These three phases are also present in both Anemia species. A preinductive phase of approximately 8 hours and a photoinductive phase of 16 to 24 hours is required to induce greater than 50 % spore germination. This light period can be either continuous or interrupted by dark periods of different lengths. This is not a photoperiodic response since both continuous or intervals of light are effective for inducing germination. This type of light response has also been reported in other ferns (reviews by Dyer, 1979 and Howland and Edwards, 1979).

Imbibed but non-induced spores of A. phyllitidis have been shown to have a high rate of transcription and translation (Fechner and Schraudolf, 1984). These spores are preparing for the photosensitive phase. Results from this study indicate that spores can remain photosensitive for at least 64 hours after imbibition and possibly as long as 7 days without a decrease in viability (Nester, unpublished observation).

Spores of A. mexicana and A. phyllitidis

demonstrate differences in sensitivity to GAs for dark induction of germination. Spores of A. phyllitidis are 100 times more sensitive to the GAs tested than are spores of A. mexicana. These results with GA_4, GA_7, and GA_3 agree with previous results for A. phyllitidis (Weinberg and Voeller, 1969a).

Gametophytes of both species had similar sensitivities to the GAs for premature formation of antheridia. The relative responses to the four different GAs were similar to the responses for germination in the dark. The greatest activity was with GA_7 and GA_4 and the least was with GA_{13}. These results are in agreement with earlier studies (Schraudolf, 1964; 1966; Voeller, 1964). GA_{13} is a C-20 GA with three acid groups, native to the fungus Gibberella fujikuroi. It is not surprising that the fern spores are less sensitive to it than to C-19 GAs.

Spores of A. phyllitidis and A. mexicana are more sensitive to their own antheridiogens than to that of the other species. This is expected since the antheridiogens of these two species of Anemia are different compounds (Nakanishi et al., 1971; Nester et al., in prep.). Zanno et al. (1972) had determined that the antheridiogens of A. phyllitidis and Anemia hirsuta were identical and concluded that in that case,

structural diversity of antheridiogens existed only at the genus level. From chemical evidence (Nester et al., in prep.) and the present results on biological activity, structural diversity at the species level is apparent.

The nature of the light response causing germination is unknown. Since GAs and antheridiogen can substitute for light, it has been assumed that light induces the formation of a GA-like germination stimulating compound. Isolation of such a substance from culture medium of A. phyllitidis was reported by Weinberg and Voeller (1969b). Furthermore, they reported that AMO-1618, an inhibitor of kaurene biosynthesis blocked the formation of this germination stimulator. Several approaches to confirm this result in the present study have been unsuccessful. First, MVA, kaurene and kaurenoic acid did not stimulate spore germination in the dark in A. mexicana and A. phyllitidis. If the regulatory step controlled by light is prior to the formation of MVA, such as HMG CoA reductase, we would expect MVA to stimulate germination. If the regulated step is prior to kaurene, as suggested by the reported inhibition of germination by AMO-1618, kaurene ought to stimulate germination. And, if the

regulatory step involves the oxidation of kaurene, kaurenoic acid may substitute for GA. None of these compounds had any effect on spore germination. Furthermore, in our experiments AMO-1618 and ancymidol, a rather specific inhibitor of kaurene oxidation, did not inhibit light-induced spore germination. Finally, preliminary attempts to isolate a substance from the medium of light-germinated spores and the spores themselves, which would subsequently induce spore germination in the dark, have also been unsuccessful.

The reasons for the differences between these results and those of Weinberg and Voeller (1969b) are unknown and will require further investigation. It seems unlikely that the different pH and the suboptimal light conditions they used could account for these differences. One possibility is that these precursors and inhibitors did not penetrate the spore coat. Fern spores have been and continue to be difficult to prepare for microscopic studies because the spore coat is impermeable to some fixatives and many embedding chemicals (Gullvåg, 1969; Beisvåg, 1970; Cran, 1979). GA and antheridiogen apparently are able to penetrate the spore coat since these two compounds can stimulate germination in the dark, but the ability of precursors or inhibitors of GA biosynthesis to penetrate the spore

coat is unknown. Use of radioactively labelled compounds and other penetrating solvents may be useful in addressing these questions.

Although the inhibitors did not affect germination per se, the effects of ancymidol and fenarimol on the subsequent growth and development of gametophytes are quite interesting. Fenarimol (1.0 mM) inhibited gametophyte growth, and ancymidol drastically influenced the morphology of developing gametophytes. Fenarimol is an analog of ancymidol and can inhibit gibberellin biosynthesis in the fungus, Gibberella fujikuroi (Coolbaugh et al., 1982a). This compound did not inhibit growth in peas (Coolbaugh et al., 1982b) and in the present study it did not inhibit spore germination. Concentrations lower than 0.1 mM had no effect on growth of the gametophytes.

Ancymidol, an inhibitor of ent-kaurene oxidation in peas and wild cucumber (Coolbaugh et al., 1982a) affected the morphology of Anemia gametophytes at 0.1 mM. Gametophytes of both species were round in shape (Figs. 8, 9), whereas those in cultures without ancymidol were filamentous (A. phyllitidis, Fig. 6) or spatulate (A. mexicana, Fig. 7). Montague (1975) demonstrated inhibition of GA-induced elongation of

Avena stems with the addition of 1.0 mM ancymidol.
Cells from epidermal peels of the internodes of GA_3 and
ancymidol-treated tissue resulted in cells which had
more lateral expansion than untreated tissues. He
compared this cytological effect with the effect caused
by colchicine. In Anemia, a similar expansion in cell
width resulted and this gametophyte morphology could be
compared with that resulting from treatments with
colchicine (Mehra, 1952; Smith, 1979). However, a major
difference between the effect of colchicine and that of
ancymidol is the inhibition of cell division with
colchicine and lack of this inhibition with ancymidol.
Cell division occurs with 0.1 mM ancymidol, but cells do
not differentially expand in length as do those of
untreated gametophytes.

Anemia mexicana spores are less sensitive to
ancymidol than A. phyllitidis spores. One mM ancymidol
almost entirely inhibited growth and expansion of
protonemal cells of A. phyllitidis, although spore coats
had ruptured. At this same concentration, expansion of
the protonema occurred in A. mexicana. This expansion
and growth was less than the growth in 0.1 mM ancymidol.

GA_3 did not overcome the growth effects of
ancymidol, either in light-induced or GA-induced spore
germination and subsequent gametophyte growth. Also,

GA_3-induced premature antheridium formation was inhibited by ancymidol. It is not known if gametophytes older than 14 days could overcome the effect of ancymidol. In peas, ancymidol (0.1 mM) inhibited internode elongation and this inhibition could not be overcome with 0.1 mM GA_3 (Coolbaugh et al., 1982b).

In summary, we have shown that the optimum pH for germination of spores of A. mexicana and A. phyllitidis is approximately pH 6; these spores from the two species have different sensitivites to various GAs and natural antheridiogens; they are both sensitive to light with requirements for incubation before the light treatment and a minimum of 16 hours of light to stimulate germination. At the present time we have no evidence to indicate that a GA-like compound is synthesized at germination, as proposed by Weinberg and Voeller (1969b), although this cannot be ruled out since it is not known if the inhibitors successfully penetrated the spore coat. Further work needs to be done on the elucidation of this proposed germination substance.

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PAPER III.   MASS SPECTROMETRIC AND INFRARED
             SPECTROMETRIC CHARACTERIZATION OF AN
   ANTHERIDIOGEN OF ANEMIA MEXICANA

ABSTRACT

An antheridiogen of Anemia mexicana has been
characterized by combined gas chromatography/mass
spectrometry and gas chromatography/Fourier transform-
infrared spectrometry. It is a C-19 gibberellin-like
compound with one carboxyl group, a methylene carbon, a
lactone ring, one hydroxyl group, and one ring double
bond. It is not GA$_5$, GA$_7$, GA$_{31}$, or GA$_{62}$. It is
different from the antheridiogens of Anemia phyllitidis,
Anemia hirsuta and Lygodium japonicum.

INTRODUCTION

An antheridiogen was first described by Döpp (1950) as a compound, present in Pteridium aquilinum gametophyte culture medium, which hastened the onset of antheridium formation in this fern. Antheridiogen activity has since been detected in many different genera of ferns including Ceratopteris thalictroides (Schedlbauer and Klekowski, 1972), Bommeria sp. (Haufler and Gastony, 1978), Lygodium japonicum (Näf, 1966), and Anemia mexicana (Nester and Schedlbauer, 1982). The biological activity and specificity of antheridiogens has been studied in several species (see review by Näf et al., 1975). In addition to inducing precocious antheridium formation, antheridiogens can substitute for the light requirement for spore germination in some ferns (Näf, 1966), including A. mexicana (Nester and Schedlbauer, 1982). Gibberellic acid (GA_3) can also induce precocious antheridia formation and spore germination in the dark in Anemia phyllitidis and other ferns in the Schizaeaceae (Schraudolf, 1962). The vegetative propagules (gemmae) of Vittaria also form antheridia in response to GA_3 (Emigh and Farrar, 1977).

An antheridiogen of A. phyllitidis has been characterized as a gibberellin-like compound with two hydroxyl groups and one ring double bond (1) (Nakanishi
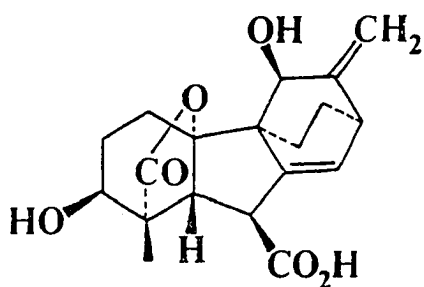
1
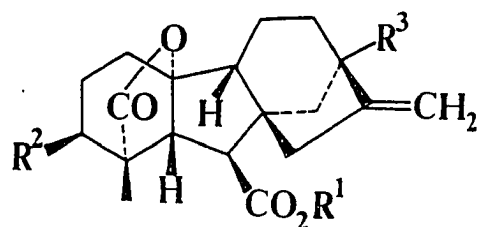
2a $R^1 = Me$, $R^2 = R^3 = H$
2b $R^1 = Me$, $R^2 = R^3 = OH$
2c $R^1 = Me$, $R^2 = OH$, $R^3 = H$
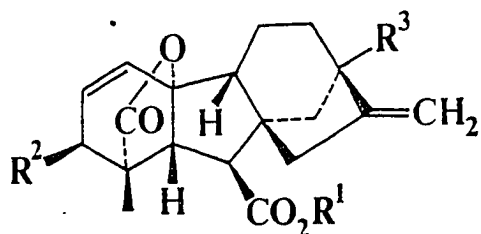
3a $R^1 = Me$, $R^2 = R^3 = OH$
3b $R^1 = Me$, $R^2 = OH$, $R^3 = H$
3c $R^1 = R^3 = H$, $R^2 = OH$
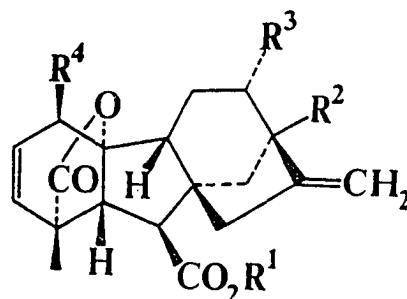
4a $R^1 = Me$, $R^2 = OH$, $R^3 = R^4 = H$
4b $R^1 = R^3 = R^4 = H$, $R^2 = OH$
4c $R^1 = R^2 = R^4 = H$, $R^3 = OH$
4d $R^1 = R^2 = R^3 = H$, $R^4 = OH$
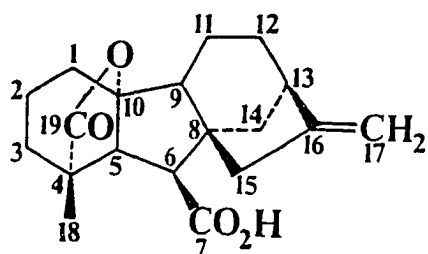
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et al., 1971).   It is distinct from the known
gibberellins by virtue of its unusual C/D ring
arrangement.   Zanno et al. (1972) determined that the
antheridiogens of A. phyllitidis and Anemia hirsuta are
identical and they concluded that, at least in this
case, structural diversity of antheridiogens did not
exist at the species level.   Yamane et al. (1979)
reported that an antheridiogen of L. japonicum is GA_9-
methyl ester (2a).   The present paper describes an
antheridiogen of A. mexicana which is different from the
antheridiogens of A. phyllitidis,   A. hirsuta, and L.
japonicum.

RESULTS AND DISCUSSION

Antheridiogen was isolated from 3-month-old
cultures of gametophytes of <u>A. mexicana</u> and partitioned
from acidic aqueous medium into ethyl acetate. After
preparative TLC, fractions were bioassayed for the
induction of spore germination in the dark. A major
band of biological activity was detected at R_f 0.7 (50%
germination resulting from the equivalent of extract
from 5 ml of culture medium), while lesser activity (16%
germination) was detected at R_f 0.1. Similar extracts
of culture medium from gametophytes of <u>A. phyllitidis</u>
yielded a single band of biological activity at R_f 0.1.
The specificity and cross reactivity of the
antheridiogens of the two species are described
elsewhere (Nester and Coolbaugh, in prep.)

Further purification of the major biologically
active fraction from <u>A. mexicana</u> (R_f 0.7) by TLC
followed by capillary GC/MS of methylated fractions
resulted in a major peak at 17.20 min. The mass
spectrum of the presumptive antheridiogen methyl ester
yielded a molecular ion at <u>m/z</u> 344 as shown in Fig. 1.
The <u>m/z</u> 344 ion was determined by high resolution MS to
correspond to $C_{20}H_{24}O_5$; similarily the <u>m/z</u> 223 ion
corresponds to $C_{17}H_{19}$. Common fragments include $[M-15]^+$,
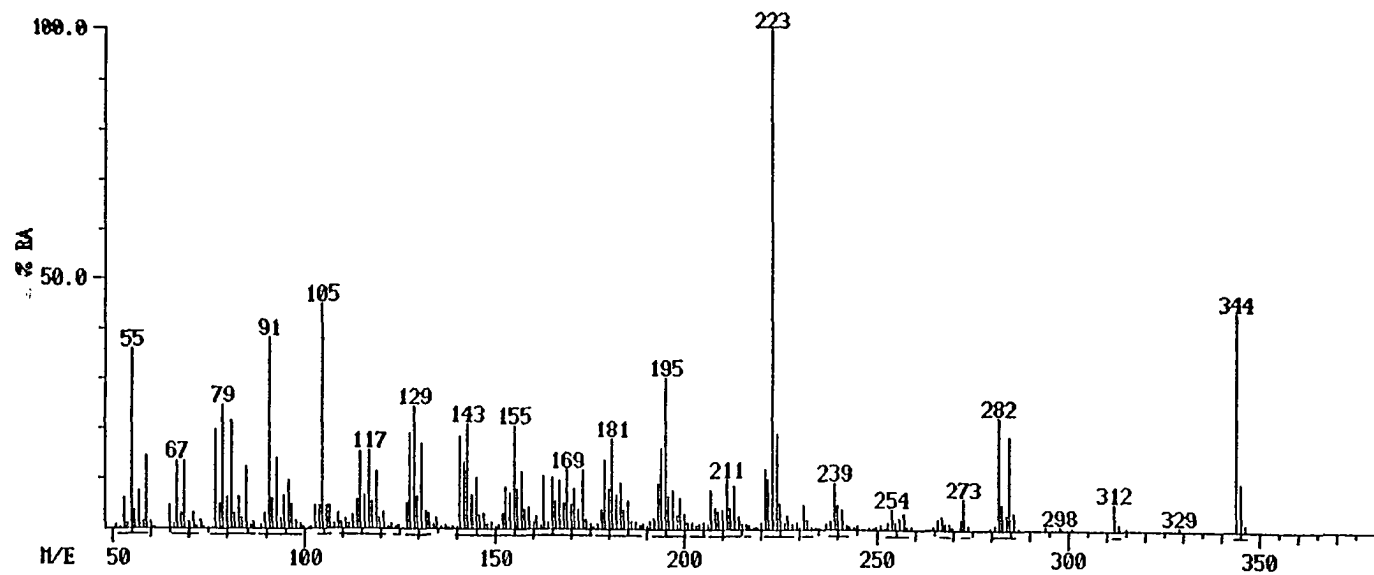$[M-32]^+$, $[M-59]^+$, $[M-62]^+$, and $[M-121]^+$. The mass

Fig. 1. Mass spectrum of *Anemia mexicana* antheridiogen, methyl ester derivative

spectrum of the methyl ester TMSi derivative of the antheridiogen was also obtained (Fig. 2; ret. time of 17.15 min.). This compound has an apparent molecular ion at $\underline{m/z}$ 416 with fragments at $[M-32]^+$, $[M-59]^+$, $[M-131]^+$, and $[M-134]^+$. The fragmentation pattern is similar to those of other methyl ester and methyl ester TMSi ether derivatives of GAs (Binks et al., 1969; Takahashi et al., 1969). The GC retention times are within the range of methyl ester and methyl ester TMSi ether derivatives of authentic gibberellins (16 to 26 min.). These data are indicative of a C_{19} GA-like compound with one carboxyl group, a lactone ring, a single hydroxyl group and one ring double bond.

Binks et al. (1969) have stated that a fragment at $\underline{m/z}$ 129 from a methyl ester TMSi ether derivative was evidence for a hydroxyl group bonded to carbon number 3 (5) in the gibberellin ring system (numbering system according to Rowe, 1968). This is a possible location for the hydroxyl group in this unknown compound, although the intensity of this fragment is low compared with other fragments. Mass spectra of the antheridiogen of A. mexicana indicate that it is not GA_5 (4b), GA_7 (3c), GA_{31} (4c), or GA_{62} (4d), which are known monohydroxy C-19 gibberellins with one ring double bond. Mass spectra of authentic samples of GA_5 and GA_7 were
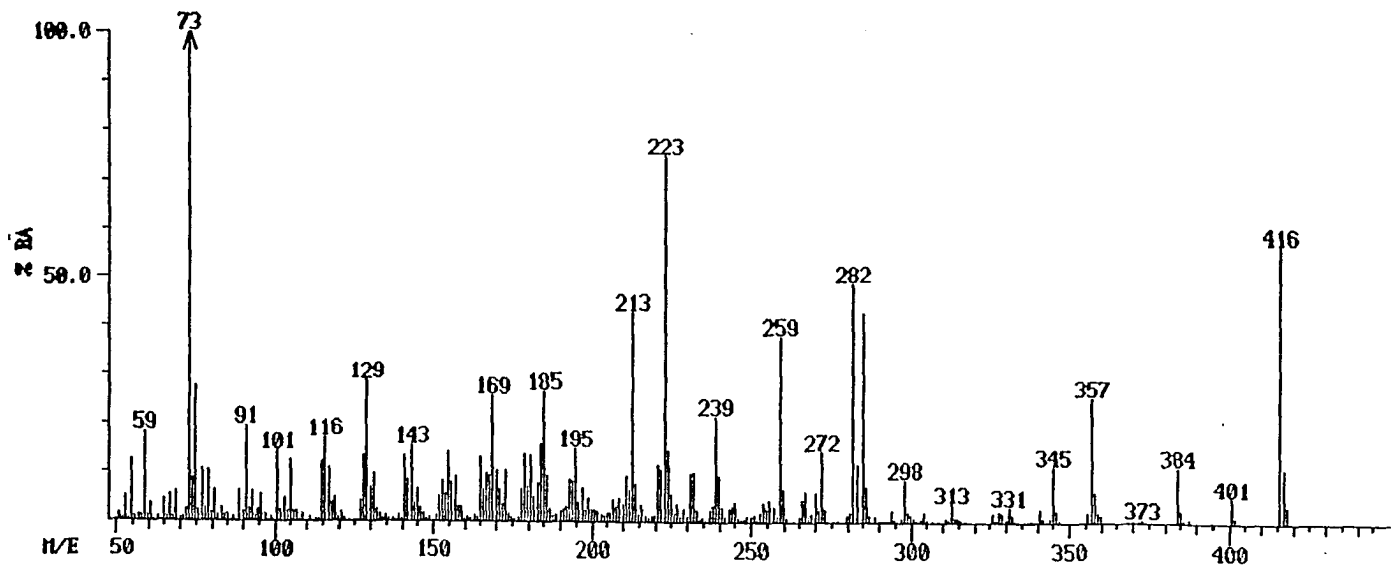
Fig. 2. Mass spectrum of Anemia mexicana antheridiogen, methyl ester TMSi derivative

obtained for comparative purposes. The compound is not
GA$_{31}$ since the published spectrum of the methyl ester
derivative lists distinct fragments at m/z 300, 294,
284, and 222 (Murofushi et al., 1973), which are not
characteristic of the antheridiogen. The published
mass spectrum for GA$_{62}$ methyl ester has notable
fragments at m/z 282 and 214 (Kirkwood and MacMillan,
1982) but does not have fragments at m/z 312 and 285,
which are present in the antheridiogen mass spectrum.

The antheridiogen of A. phyllitidis was also
studied for comparative purposes (Figs. 3, 4). The
retention time of the methyl ester derivative was 21.67
min. while that of the methyl ester TMSi ether was 25.13
min. The mass spectrum of the methyl ester yielded a
molecular ion at m/z 360 and that of the methyl ester
TMSi ether at m/z 504. These are consistent with the
structure (1) described by Nakanishi et al. (1971). The
A. phyllitidis antheridiogen was not identified in the
A. mexicana fraction of R$_f$ 0.1 demonstrating biological
activity. Further purification of this faction will be
necessary for identification of the biologically active
compound.

The IR spectrum of the methyl ester of A. mexicana
antheridiogen is presented in Fig. 5. This spectrum was
obtained by GC/FT-IR vapor phase spectrometry and is
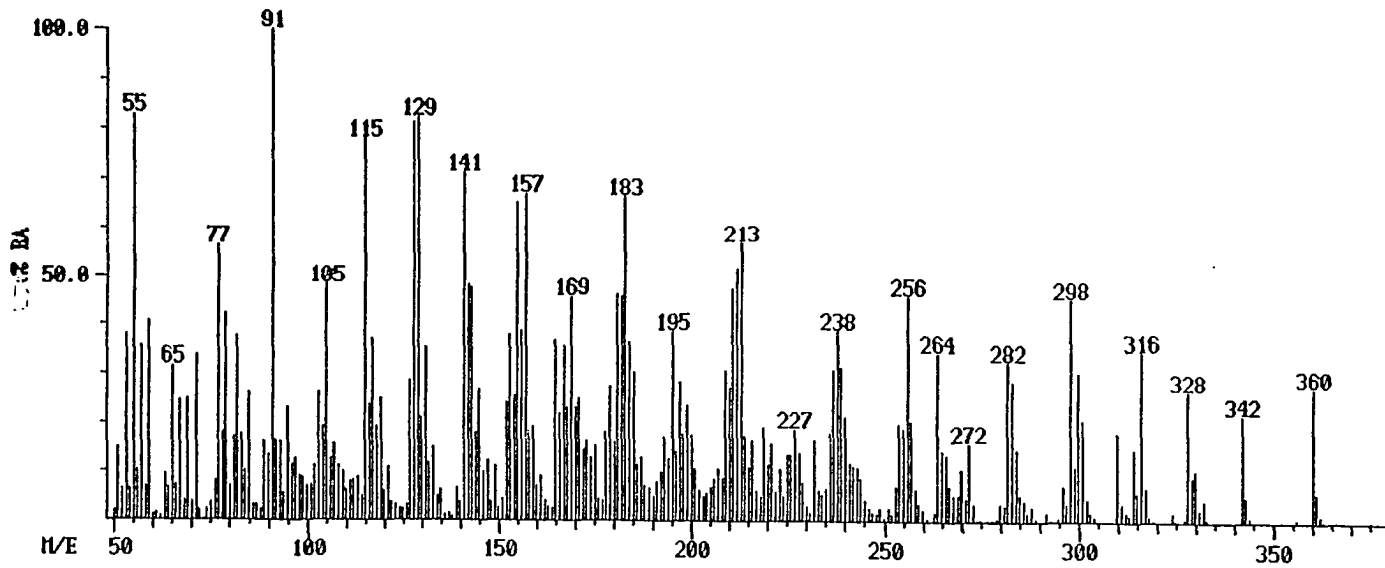
Fig. 3. Mass spectrum of <u>Anemia</u> <u>phyllitidis</u> antheridiogen,
methyl ester derivative
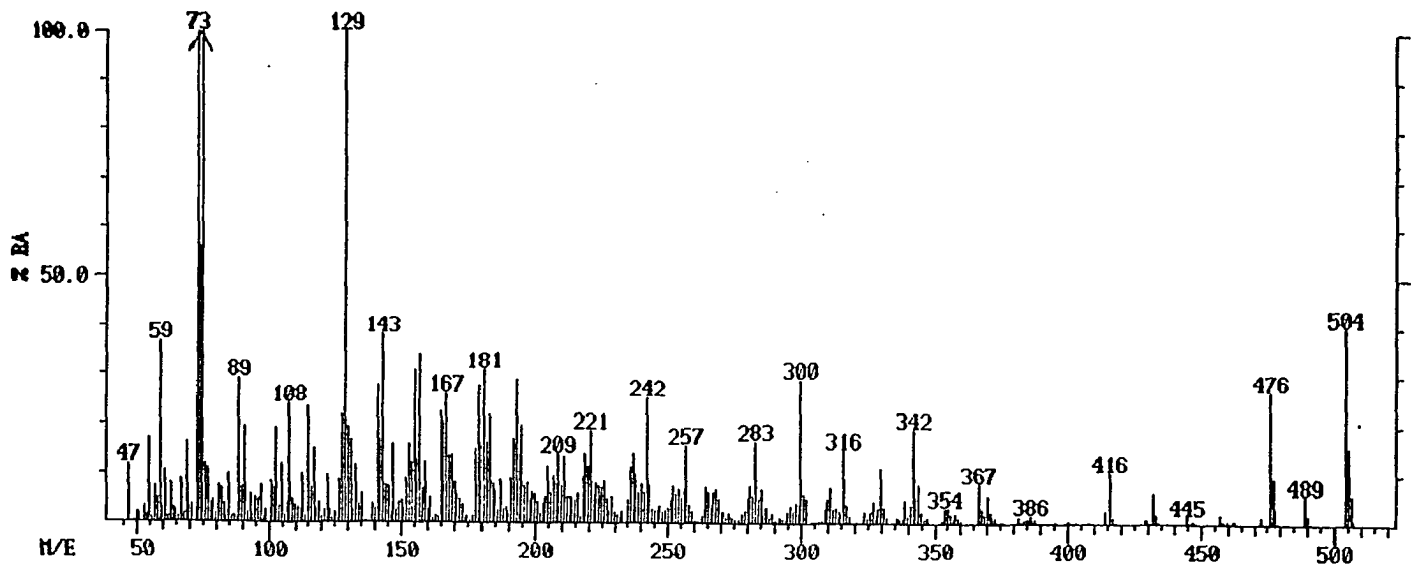
Fig. 4.   Mass spectrum of <u>Anemia</u> <u>phyllitidis</u> antheridiogen,
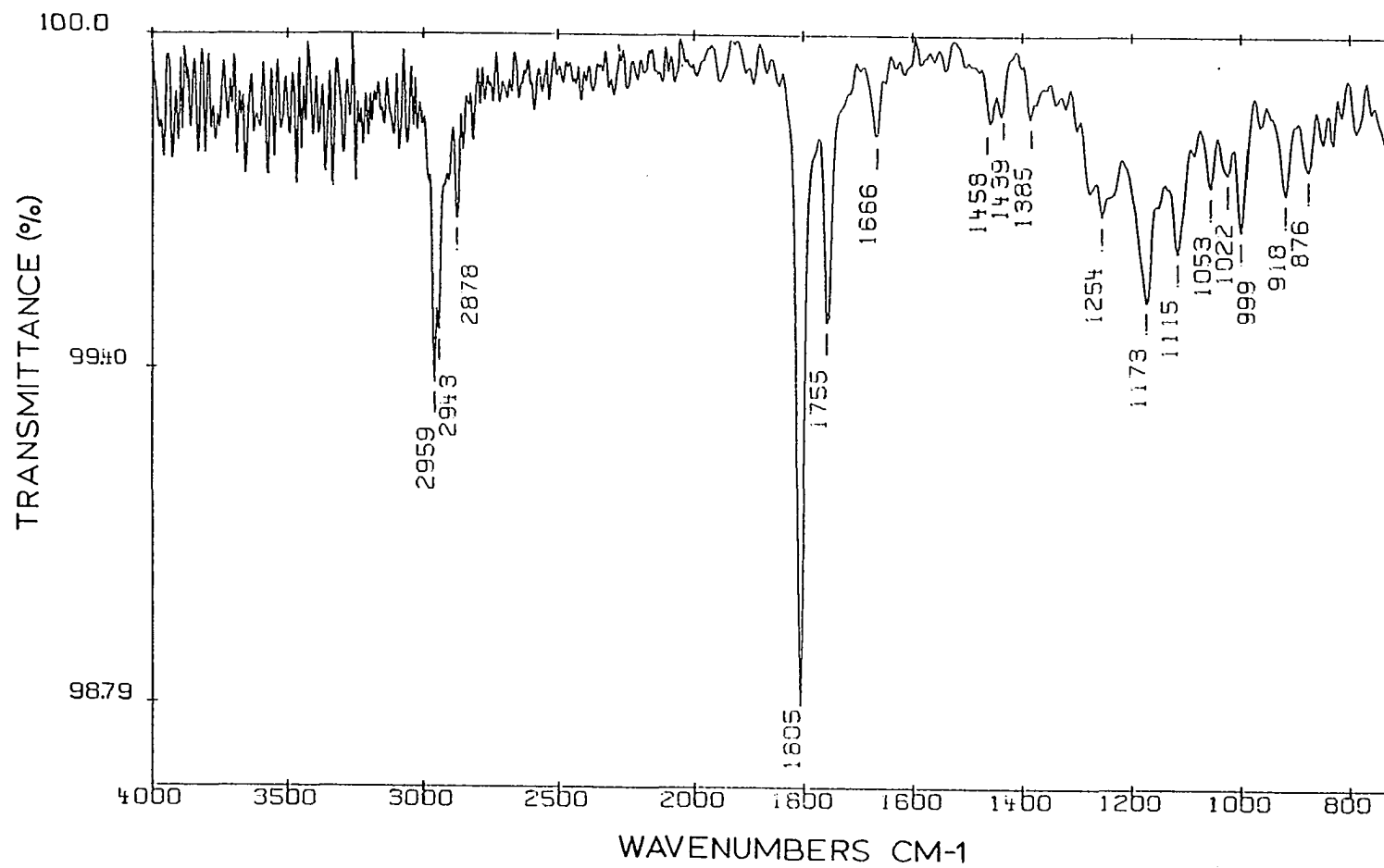   methyl ester TMSi derivative

Fig. 5.   Vapor phase IR spectrum of <u>Anemia</u> <u>mexicana</u> antheridiogen,
methyl ester derivative

similar to those of methyl esters of authentic GA_1 (2b), GA_3 (3a), GA_4 (2c), GA_5 (4a), GA_7 (3b), and GA_9 (2a) which were obtained for comparison. The following wavenumbers indicate significant functional groups: 1805 [γ lactone (Nyquist, 1984)], 1755 [methoxy carbonyl (Nyquist, 1984)], and 1666 [exocyclic methylene (from condensed phase IR spectra; Koshimizu et al., 1968)]. Lack of a strong OH-stretching in the spectra is the result of very little intermolecular hydrogen bonding in the vapor phase, especially at temperatures greater than 180° (Nyquist, 1984). Our spectra were obtained at a GC oven of 280° and light pipe temperature of 250°.

Preliminary ^{1}H NMR studies indicate the antheridiogen of A. mexicana does not have an A-ring arrangement as in GA_7 which has a Δ 1,2 double bond and a hydroxyl group at carbon number 3. These studies indicate that the single protons attached to carbon atom numbers 5 and 6 [(5) for a gibberellin ring system] are doublets and are only coupled to each other.

The results above lead to three major conclusions: 1. The primary antheridiogen of A. mexicana is not the same as the antheridogen from A. phyllitidis, A. hirsuta or L. japonicum; therefore, in this case, variability exists at the species level; 2. GC/MS, high resolution MS and GC/IR lead to the conclusion that the

antheridiogen is a C-19 gibberellin-like compound with one carboxyl group, a methylene carbon, a lactone ring, one hydroxyl group, and one ring double bond; but it is not GA_5, GA_7, GA_{31} nor GA_{62}; and 3. The determination of the arrangement of the C/D rings, the position of the hydroxyl group and ring double bond will require further analysis.

EXPERIMENTAL

Plant material

Spores of A. mexicana Klotzsch were collected in
July 1983 from plants growing near Garner State Park in
southwest central Texas. Fertile pinnae with ripe
spores were removed from the plants and placed in
glassine envelopes. Upon drying, the spores were
released from the sporangia and collected. Spores of A.
phyllitidis (L.) Swartz were collected from greenhouse-
grown plants in the same manner. Voucher specimens of
both species are deposited in the Botany Department
Herbarium at Iowa State University, Ames, Iowa.

Biosynthesis of antheridiogen

Antheridiogen is synthesized by gametophytes and
diffuses into the culture medium. Spores of A. mexicana
and A. phyllitidis were sown separately onto the surface
of 1% agar solidified mineral medium [Parker's
macronutrients, Thompson's micronutrients (Klekowski,
1969); EDTA was omitted and 20 µL 1% $FeCl_3$ was added].
Spores were sterilized in 1% calcium hypochlorite and
0.5-1.0 mg of spores were sown aseptically (Schedlbauer,
1976) onto each plastic petri plate (15 x 100 mm)
containing 20 to 30 mL medium. Eighteen plates were
placed in a translucent plastic box. Gametophytes
were grown under cool white fluorescent lights (app.

$40 \ \mu E \ s^{-1} cm^{-2}$) at a temp. of 26° for 3 months.

Extraction and initial purification

After 3 months, gametophytes were removed from the surface of the agar medium. The medium was frozen, thawed, and filtered to remove the liquid from the agar. For each batch of media (1-24 L), the liquid was acidified to pH 2.5 with 1 N HCl. The acidified aq. portion was partitioned (x3) against one-third vol. of EtOAc. The EtOAc was evaporated under vacuum to near dryness and the residue was dissolved in MeOH and applied to prep. TLC plates (2 mm, Si gel) and chromatographed 15 cm in iso-propyl ether-HOAc (95:5). Plates were divided into 10 fractions and the Si gel was eluted (x3) with MeOH. Aliquots of these samples were used for the bioassay to determine the R_f of the antheridiogen.

Bioassay for antheridiogen

A bioassay for the induction of spore germination in the dark was used to monitor fractions through the purification steps. This assay is more sensitive to antheridiogen and more rapid than the induction of precocious antheridia formation in A. mexicana and A. phyllitidis . An aliquot of each sample in MeOH was dissolved in liquid culture medium containing 0.05 M MES

adjusted to pH 6.0. Spores were sown onto the liquid in
"MultiWell" tissue culture plates and replicate plates
were placed in the dark or under cool white fluorescent
lights for 7-10 days. The cultures in the dark were
observed for evidence of spore germination and semi-
permanent glass slides (Hoyer's mountant, fixative with
acid fuchsin stain) were made. From these slides,
germinated and ungerminated spores were counted to
determine percentage of germination (Nester and
Schedlbauer, 1982).

Chromatography

Additional purification of active TLC fractions
was conducted by TLC in EtOAc-CHCl$_3$-HOAc (75:25:5) and
C$_6$H$_6$-$\underline{n}$-BuOH-HOAc (70:25:5). Antheridiogen activity was
again monitored by bioassay. Portions of the TLC
fractions were methylated with ethereal CH$_2$N$_2$,
evaporated and resuspended in MeOH. Authentic samples
of GA$_1$, GA$_3$, GA$_4$, GA$_5$, GA$_7$, and GA$_9$ were also
methylated. Samples were then analyzed with a Hewlett-
Packard Model 5890 GC linked with a Hewlett-Packard 5790
Mass Selective Detector. The GC was equipped with a
J & W Scientific DB-1 (30 m x 0.25 mm i.d.) WCOT fused
silica capillary column. The carrier gas, He, was at a
flow velocity of 25 cm/sec. The temp. program was from
50° to 200° at 20°/min, continuing to 250° at 10°/min.

A split ratio of 40:1 was used. The injector temp. was 220°; the transfer line was at 280°.

To form the methyl ester TMSi ether derivatives of authentic GAs and antheridiogens, the methylated samples were dried, dissolved in pyridine, and then treated in closed vials at room temp. with BSTFA containing 1% TMCS.

Combined GC/MS

Methyl ester and methyl ester TMSi ether derivatives were analyzed with a Finnigan 9610 gas chromatograph linked with a Finnigan 4000 mass spectrometer. Samples were injected onto a J & W Scientific DB-1 (22 m) WCOT fused silica capillary column. The flow velocity of He was 60 cm/sec with a splitless injection (0.33 min). Injector temp. was 250°. The temp. program described above was used. Positive ions were formed and detected at 70eV with 1 scan/sec.

High resolution MS

The elemental compositions of ions at m/z 344 and m/z 223 of the methylated A. mexicana antheridiogen were determined with a Kratos MS-902 mass spectrometer by direct probe analysis. Peak matching was obtained with perfluorotributylamine as the reference compound.

Combined GC/FT-IR

The antheridiogen of A. mexicana and authentic GAs were analyzed as their methyl ester derivatives. An IBM 98 Series IR spectrometer was connected to a Hewlett Packard 5700 Series GC for this analysis. The GC was equipped with a DB-1 (15 m x 0.25 mm i.d.) WCOT fused silica capillary column with a He carrier gas flow velocity of 30 cm/sec. The injector temp. was 250° and the column was programmed from 50° to 280° at 20°/min. Splitless injection was used. The capillary column was connected to a gold-lined light pipe (250°) inside the IR. The other end of the light pipe was connected to a 0.5 m portion of uncoated capillary column (250°) which was connected to an FID. This allowed the GC, in addition to the IR, to monitor the retention times and separation of the components of the mixture.

Antheridiogen of A. mexicana

Methyl ester derivative ([M]$^+$ at m/z 344.16177; calc for $C_{20}H_{24}O_5$: 344.16238; [M-121]$^+$ at m/z 223.14861; calc for $C_{17}H_{19}$: 223.14867); GC/MS m/z (rel. abundance): 344 (44), 312 (5), 285 (19), 282 (23), 273 (6), 254 (4), 239 (9), 231 (5), 223 (100), 211 (10), 195 (30). IRν_{max} cm^{-1}: 1805 s, 1755 s, 1666 w. Methyl ester TMSi ether derivative. GC/MS m/z (rel. abundance): 416 [M]$^+$ (57), 401 (5), 384 (11), 357 (25), 345 (11), 331 (3),

313 (4), 298 (8), 285 (43), 282 (49), 272 (14), 259 (38), 239 (21), 223 (75), 129 (29), 73 (100).

Antheridiogen of A. phyllitidis

Methyl ester derivative. GC/MS $\underline{m/z}$ (rel. abundance): 360 $[M]^+$ (28), 342 (22), 328 (27), 316 (35), 300 (31), 298 (45), 282 (32), 272 (16), 264 (34), 256 (46), 238 (39), 227 (19), 213 (57), 195 (39), 183 (66), 157 (67), 141 (71), 129 (82), 128 (81), 115 (78), 91 (100). Methyl ester TMSi ether derivative. GC/MS $\underline{m/z}$ (rel. abundance): 504 $[M]^+$ (6), 489 (1), 476 (4), 432 (1), 416 (2), 367 (1), 342 (3), 330 (2), 316 (2), 300 (5), 283 (3), 257 (2), 242 (4), 221 (3), 193 (5), 181 (5), 143 (6), 129 (15), 75 (23), 73 (100).

$\underline{^1H}$ $\underline{NMR}$

Underivatized A. mexicana antheridiogen and authentic GA_7, GA_3, and GA_4 were analyzed with a Brucker 300 MHz instrument. Chemical shift values were measured in CD_3OD with CD_3OD as the internal standard referenced to TMS.

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GENERAL CONCLUSIONS

Several conclusions have been made from this study:

1. During spore germination in A. mexicana, the spore cell undergoes two asymmetric cell divisions. The first division gives rise to the rhizoid and the second gives rise to the protonemal cell. This cell division pattern is identical to that described by Schraudolf (1981) for A. phyllitidis and contrasts with the pattern described by Raghavan and Huckaby (1980) in which the first-formed cell developed into the protonemal cell and the second one into the rhizoid.

In A. mexicana the spore cell may undergo a third division after the gametophyte is several cells large and this third cell remains almost entirely within the spore coat. The function of this third cell is unknown but it demonstrates that the spore cell remains active for a length of time after germination.

SEM allowed a better three-dimensional view of germination and when complemented with light microscopy, the internal as well as the external events of spore germination could be described.

2. Spores of A. mexicana and A. phyllitidis have a pH optimum for germination between 5 and 6.5. After an eight hour photoinsensitive phase, spores of both species require at least 16 hours of light to induce 50 %

spore germination.

Anemia phyllitidis is more sensitive to GAs for the induction of germination in the dark. The relative sensitivity of the two ferns to the GAs is essentially the same with GA_4 and GA_7 equally effective and exhibiting the highest activity, GA_3 intermediate in activity and GA_{13} the least effective. Each species is 100 times more sensitive to its own antheridiogen than to the antheridiogen of the other species.

Mevalonic acid, kaurene and kaurenoic acid did not stimulate spore germination in the dark. Inhibitors of GA biosynthesis, AMO-1618, fenarimol and ancymidol did not inhibit light-induced germination. At 1 mM AMO-1618 had very little influence on early gametophyte growth while at this concentration, fenarimol totally inhibited gametophyte growth. Ancymidol at 0.1 mM caused gametophytes to develop abnormally. These gametophytes expanded in width more than in length, resulting in a round morphology instead of long and filamentous in A. phyllitidis and spatulate in A. mexicana. No evidence was obtained to indicate a GA-like compound is synthesized during germination. Further work with large quantities of newly germinating spores will be necessary for detection and characterization of this

compound if it is present.

   3.   The antheridiogen of <u>A. mexicana</u> is different
from the antheridiogen of <u>A. phyllitidis</u>; thus, in this
case, structural diversity exists at the species level.
This antheridiogen is a C-19 GA-like compound with one
hydroxyl group, one lactone ring, one carboxylic acid
group, an exocyclic methylene carbon and one ring double
bond.   It has been concluded from mass spectral data,
that it is not GA_5, GA_7, GA_{31}, or GA_{62}.   Further
analysis with ^1H NMR, ^{13}C NMR and X-ray crystallography
(if possible) will be required to determine the complete
structure of this new antheridiogen.

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