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Factors Influencing Spore Germination and Early Gametophyte Development in *Anemia mexicana* and *Anemia phyllitidis*[1]

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ABSTRACT

Spores of *Anemia mexicana* Klotzsch and *Anemia phyllitidis* (L.) Swartz were tested comparatively to investigate the effects of various treatments on spore germination and early gametophyte development in light and darkness. The optimum pH for induction of spore germination is approximately 6. Both species have a minimum 8 hour light insensitive preinduction phase for spore germination. An additional 8 to 12 hours of light are needed to induce 50% germination in *A. phyllitidis* while at least 24 hours of light are needed for *A. mexicana* spores. *A. phyllitidis* has greater sensitivity to the four gibberellic acids tested (GA_3, GA_4, GA_7, and GA_{13}) than *A. mexicana* for induction of spore germination in darkness. In both species the greatest response was observed with GA_4 and GA_7. GA_{13} was clearly the least effective. Gametophytes of each species are 100 times more sensitive to their own antheridiogen than to the antheridiogen of the other species. AMO-1618 (1 millimolar), fenarimol (1 mM), and ancymidol (0.1 mM) had essentially no effect on light-induced germination. The latter two did, however, inhibit gametophyte development.

Light is required for spore germination in several species of ferns (8, 20, 26) and the involvement of phytochrome has been demonstrated (7, 12). In the schizaeaceous ferns *Anemia, Lygodium,* and *Mohria,* GA can substitute for the light requirement and also induce precocious antheridium formation (15, 22, 31). Weinberg and Voeller (32) 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,[3] an inhibitor of GA biosynthesis.

Some GAs have been shown to be more effective than others for induction of germination in darkness and precocious antheridium formation (23, 24, 28, 31). The antheridiogens from *A. phyllitidis* and *Anemia mexicana* have also been shown to stimulate germination in darkness and premature antheridium formation (14, 15, 18). The antheridiogen of *A. phyllitidis* has been chemically characterized as a GA-like compound with an unusual C/D ring arrangement (16) and the antheridiogen of *Anemia hirsuta* is identical to this compound (33). The major antheridiogen of *A. mexicana* is also a C-19 GA-like compound distinct from those previously described. It contains one hydroxyl group, a carboxyl group, one exocyclic methylene carbon, a lactone ring, and one unidentified double bond equivalent (19).

In addition to light and GA-like compounds, other factors including the pH of the culture medium (2), ethylene (6) and calcium availability (30) have been shown to influence the ability of spores to germinate and the subsequent growth of gametophytes (20, 25). In the present study, experiments were conducted to determine optimum germination conditions in order to use this as a bioassay for detection of compounds produced by gametophytes which stimulate spore germination in darkness.

Spores of *A. mexicana* and *A. phyllitidis* were also tested comparatively to investigate the effects of various treatments on spore germination and early gametophyte development in light and darkness. Spore germination is characterized by a rupturing of the spore coat and division of the spore cell. Spores of many ferns undergo 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.

MATERIALS AND METHODS

Plant Material and Culture. Spores of *Anemia 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 (21) except that the overnight spore presoak was omitted. The culture medium consisted of Parker's Macronutrients and Thompson's Micronutrients (9) with deletion of EDTA from the micronutrients and addition of 20 μl/L 1% FeCl. The medium was buffered with 50 mM Mes at pH 6 after this pH was determined to be optimum for germination. The pH of the culture medium was adjusted with 1.0 N NaOH or 1.0 N HCl. In studies with inhibitors of GA biosynthesis, 1% DMSO was used as a solvent.

Cultures were grown in darkness or under cool white fluorescent light bulbs (40 W, Sylvania, approximately 40 $\mu E\ cm^{-2}\ s^{-1}$) at 25 ± 2°C for 7 to 14 d. "Multiwell" tissue culture plates were used for germinating spores in different experimental conditions. Cultures were observed for evidence of spore germination and development. Photomicrographs of live gametophytes were obtained with a Zeiss inverted compound microscope using Nomarski optics. Semipermanent glass slides of fixed and stained germinating spores were made for determinations of percent germination (18).

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[3] Abbreviations: AMO-1618, 2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenylpiperidine-1-carboxylate; ancymidol, α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidine methyl alcohol; fenarimol, α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidine methyl alcohol.

Biosynthesis and Extraction of Antheridiogen. Antheridiogens of *A. mexicana* and *A. phyllitidis* were obtained from gametophyte culture medium. Spores of *A. mexicana* and *A. phyllitidis* were sown separately onto the surface of 1% agar solidified medium. Plastic Petri plates (15 × 100 mm) containing 20 to 30 ml medium were used with 0.5 to 1.0 mg spores per plate. Eighteen plates were placed in a translucent plastic box and gametophytes were grown under continuous light. 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. The liquid was acidified to pH 2.5 with 1 N HCl and partitioned three times against one-third volume of ethyl acetate. The ethyl acetate was evaporated under vacuum to near dryness and the residue was dissolved in methanol and applied to precoated TLC plates (0.25 mm silica gel) and chromatographed 15 cm in diisopropyl ether:acetic acid (95:5, v/v). Plates were divided into 10 fractions and the silica gel was eluted with methanol. Aliquots of these samples were used for the bioassay to determine the R_F of the antheridiogen.

Chemicals. Authentic standards of GA_4 and GA_7 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 gifts from Eli Lilly & Co. GA_3 was purchased from Sigma Chemical Co. AMO-1618 was purchased from Calbiochem Co.

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 7.5 is seen in Figure 1. Maximum germination occurred in the range of 5.0 to 6.5 for both species. Spores of *A. mexicana* are more sensitive to changes in pH in darkness than in light. There is a sharp decline in percent germination below pH 5.5 in darkness, whereas in light, a decline occurs below pH 4.5. Spore germination in *A. phyllitidis* was quite similar in light and dark grown cultures (Fig. 1B), decreasing below pH 5.

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 protonemal growth. The absence of GA did not affect germination in the light. Control cultures in the absence of buffer indicated that the buffer had no influence on germination or subsequent gametophyte growth. With 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. Results of experiments designed to elucidate the timing and duration of light required for germination under ordinary fluorescent lights are illustrated in Figure 2. Spores were given various light treatments for 3 d and then all were placed in darkness for an additional 7 d to allow germination. Spores of *A. phyllitidis* appear to be insensitive to light during the first 8 h of imbibition and require greater than 8 h of subsequent light to induce 50% germination. This requirement can be met by either one continuous treatment at any time after the first 8 h or by additive treatments. It is not known how long the spores remain viable after imbibition, but spores which have been in darkness for 7 d germinate and grow when placed in light. Spores of *A. mexicana* also have a light insensitive phase (Fig. 2). A longer duration of light (24–32 h) is needed in this species for 50% spore germination.

GA Sensitivity. Four different gibberellins (GA_3, GA_4, GA_7, and GA_{13}) were tested to determine differences in sensitivity to the GA for induction of spore germination in darkness and precocious antheridium formation. In one experiment, percent spore germination was determined 7 d after spore sowing. In a second experiment, percent germination was determined 11 d after spore sowing. Relative response to the GA was similar in both experiments, however, spores of *A. mexicana* demonstrated slightly higher percent germination after 11 d. *A. phyllitidis* spores are more sensitive to the GAs than those of *A. mexicana* for induction of germination in darkness (Fig. 3). 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 and GA_7 than to GA_3 or GA_{13}. *A. mexicana* responded only minimally to 1.0 mM GA_{13} (highest concentration tested). Using 50% spore germination for comparison, *A. phyllitidis* spores were approximately 1000 times more sensitive to GA_4 and GA_7 than were spores of *A. mexicana*.

Spores which germinated in darkness in response to GA gave rise to gametophytes which were filamentous in shape (Fig. 4). Chloroplast development occurred in darkness and few (less than

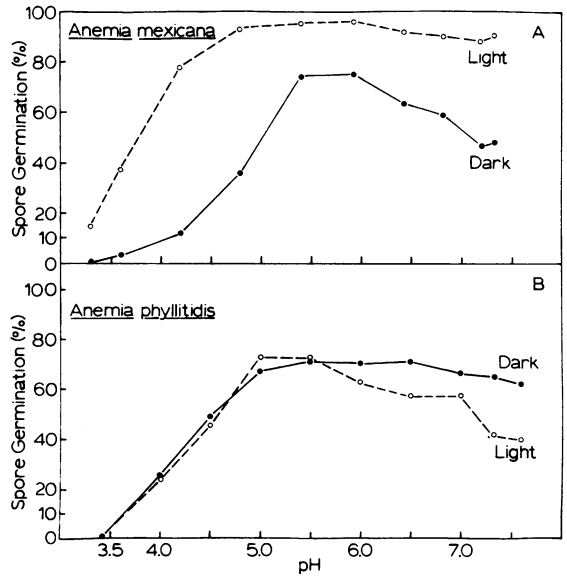


FIG. 1. Effect of pH of the culture medium on GA_3-induced spore germination in darkness in *A. mexicana* (1.5 mM GA_3) and *A. phyllitidis* (0.15 mM GA_3). Media contained 50 mM Mes. Percent spore germination determined 7 d after sowing spores. No germination occurred in darkness in the absence of GA. Points represent mean values from 3 replicate samples. The SE was less than 10.

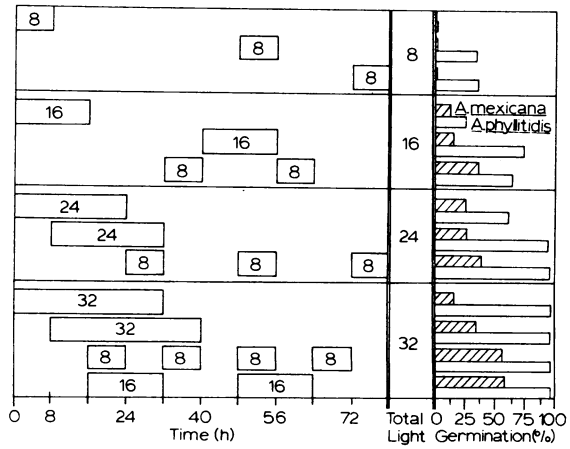


FIG. 2. Light-induced spore germination in *A. phyllitidis* and *A. mexicana* using both continuous and alternating light and dark periods. Boxes indicate hours in the light. Percent germination represented for *A. phyllitidis* with open bars, *A. mexicana* with slashed bars. Cultures were given light and dark treatments directly after spore sowing for 3 d and then all cultures were placed in darkness an additional 7 d.

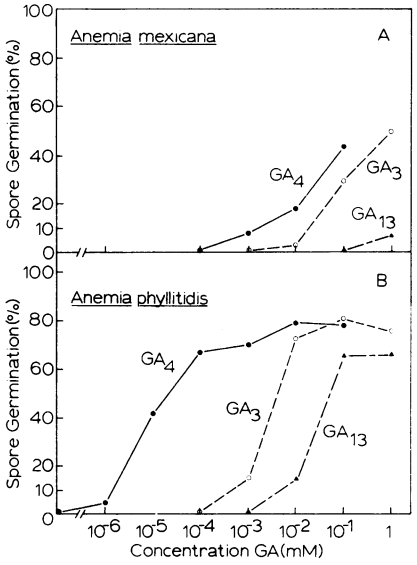


FIG. 3. Percent spore germination in darkness of *A. mexicana* and *A. phyllitidis*, induced by GA_4, GA_3, and GA_{13} at different concentrations. Media contained 50 mM Mes at pH 6.0. Percent spore germination determined 7 d after sowing spores. The SE was less than 10.

10%) of the gametophytes initiated antheridia 7 d after spore sowing.

Since spores of both species germinate readily in light, GAs have no effect. However, both species responded similarly to GA in the antheridium induction bioassay 13 d after spore sowing. The gametophytes were again most sensitive to GA_7 and GA_4, yielding 45% antheridiate gametophytes in 0.01 mM GA_4 or GA_7. 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} (1.0 mM).

Each antheridiate gametophyte (induced by GA) had one or more antheridia by 13 d after sowing. In the absence of added GA, antheridium formation normally does not occur until the gametophyte has developed an organized meristem (greater than 19 d after spore sowing for *A. mexicana* grown on solid culture medium [17]). Antheridiate gametophytes were often filamentous and branched (Fig. 5), whereas those gametophytes without antheridia were single filaments in *A. phyllitidis* (Fig. 6) or spatulate in shape in *A. mexicana* (Fig. 7).

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 darkness. Figure 10 illustrates the distribution of biological activity on plates chromatographed in diisopropyl ether:acetic acid (95:5). The primary *A. mexicana* antheridiogen has an R_F of 0.7; that of *A. phyllitidis* is 0.1. Results show that each species had an optimal 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 (data not shown).

Inhibitors of GA Biosynthesis. Three known inhibitors of GA biosynthesis (AMO-1618, fenarimol, and ancymidol) were tested for their effects on light-induced germination and subsequent gametophyte development. Spores were placed in three different light treatments and 1% DMSO (final concentration) was used as a solvent for the inhibitors. In one treatment the spores were given 24 h of light and then placed in darkness for 9 d. In the second treatment, spores were given 48 h of light and subsequently placed in darkness. In the third treatment, spores were given continuous light for 10 d (Table I). Under continuous light and with 48 h light, none of these inhibitors had any effect on germination, but ancymidol and fenarimol inhibited protonemal growth at 1.0 mM. Under suboptimal light conditions (24 h), spore germination was reduced by approximately 50% in *A. phyllitidis* by 1.0 mM ancymidol and fenarimol.

These two inhibitors both affected protonemal growth and development at 0.1 mM, although ancymidol seemed to be more active. At this concentration of ancymidol, gametophytes of both species were morphologically abnormal. Protonemal cells of gametophytes of *A. phyllitidis* treated with 0.1 mM ancymidol expand in width instead of in length (Fig. 8) (compare with Fig. 6) resulting in a more rounded shape. After 14 d in light, gametophytes were still more three-dimensional in shape than normal gametophytes. Cell division was not inhibited (Fig. 9). Normal growth and development occurred with lower concentrations of ancymidol.

In an attempt to determine if GA_3 can overcome the inhibition of growth and change in morphology of gametophytes caused by ancymidol, spores were sown in culture medium containing 1.0 or 0.1 mM ancymidol (in 1% DMSO) and GA_3. The addition of GA_3 (1.0, 0.1 mM) did not reverse the effect of ancymidol on developing gametophytes.

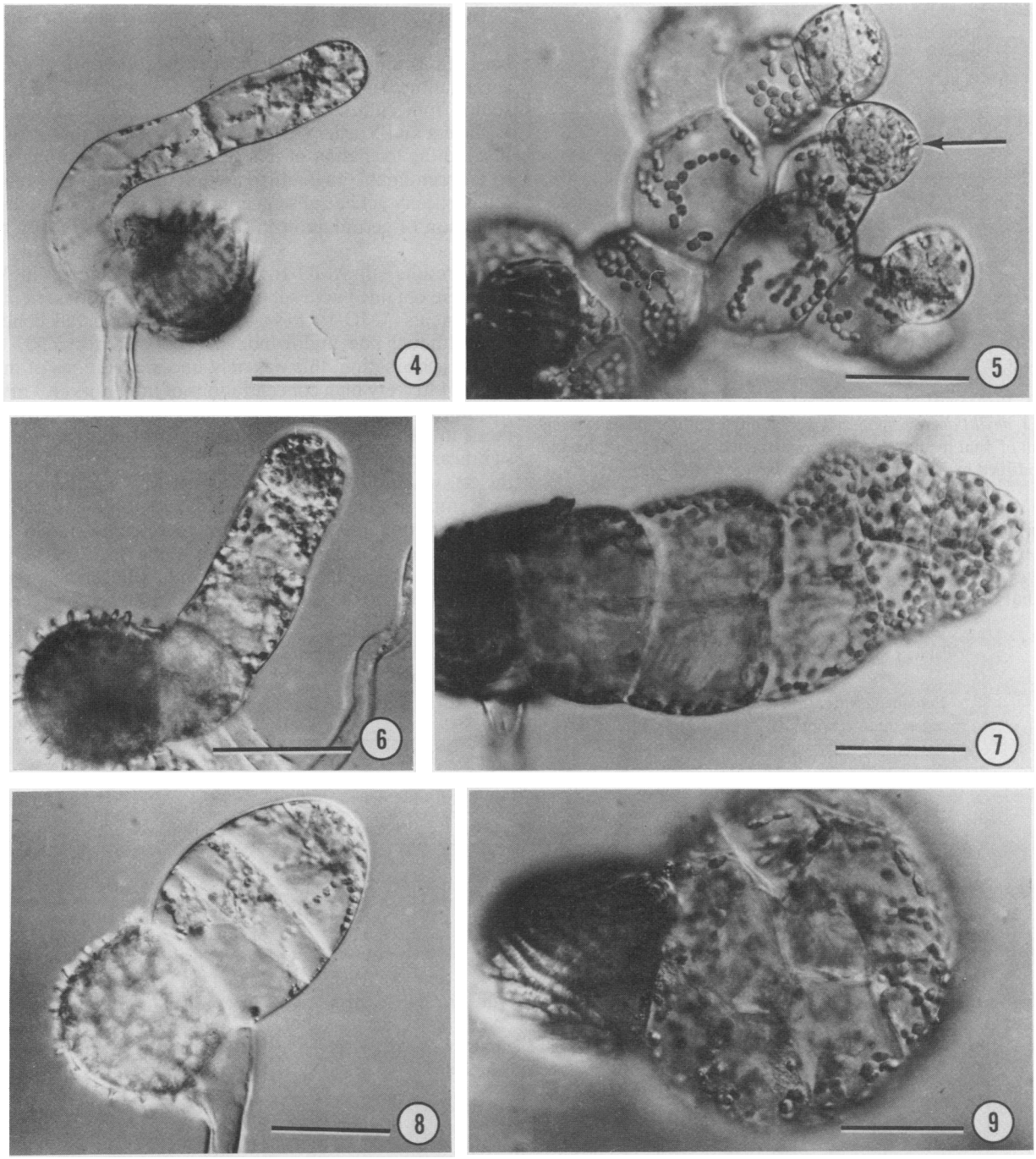
DISCUSSION

GA_3-induced spore germination in darkness 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 (32), 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. However, other authors using different species of ferns, have reported pH optima for germination and growth between 5.0 and 7.0 (11, 29). Our results are consistent with the latter (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 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 (4).

The germination process in *Onoclea sensibilis* has been described in three phases: (a) the preinduction phase when spores develop maximum photosensitivity but are insensitive to light, (b) the photoinduction phase when they are most sensitive to red light, and (c) the postinduction phase when the photoproduct triggers processes which will eventually lead to germination (1, 27). These three phases are also present in both *Anemia* species examined in this study. With the cultural conditions of this study, *A. phyllitidis* exhibited a preinductive phase of approximately 8 h and a photoinductive phase of 8 to 12 h. In *A. mexicana* there is a similar 8 h preinductive phase, but 24 to 32 h of light are required to induce greater than 50% spore germination. These light periods 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 in inducing germination. Similar responses have also been reported in other ferns (8). Results from this study also indicate that spores can remain photosensitive for at least 64 h after imbibition and possibly as long as 7 d without a decrease in viability.

Spores of *A. mexicana* and *A. phyllitidis* have different sensitivities to GA for induction of germination in darkness. Spores of *A. phyllitidis* are 100 to 1000 times more sensitive to the GA tested than are spores of *A. mexicana*. In both species GA_4 and GA_7 were more active than GA_3. GA_{13} was less active than GA_3. These results with GA_4, GA_7, and GA_3 agree with previous results



FIGS. 4 to 9. Photomicrographs of gametophytes of *A. mexicana* and *A. phyllitidis*. All bars = 50 μm.

FIG. 4. GA_3-induced germinating spore of *A. mexicana* with elongate protonemal cells. Germinated in darkness with 0.1 mM GA_3, 13 d after spore sowing (×400).

FIG. 5. Antheridiate gametophyte of *A. mexicana* grown in light with 0.1 mM GA_3. Arrow indicates an antheridium, 13 d (×380).

FIG. 6. Filamentous gametophyte of *A. phyllitidis* grown in light without GA added, 8 d (×420).

FIG. 7. Spatulate gametophyte of *A. mexicana* grown in light without GA added, 13 d (×400).

FIG. 8. Gametophyte of *A. phyllitidis* grown in medium containing 0.1 mM ancymidol, 8 d (×370).

FIG. 9. Gametophyte of *A. mexicana* grown in medium with 0.1 mM ancymidol, 13 d (×370).

for *A. phyllitidis* (31).

In contrast to the results on GA-induced germination, gametophytes of both species had similar sensitivities to GA for premature formation of antheridia. The relative responses to the four different GA were, however, similar to the responses for germination in darkness. These results are in agreement with earlier studies (23, 24, 28). 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.

Zanno *et al.* (33) had determined that the antheridiogens of *A. phyllitidis* and *A. hirsuta* were identical and concluded in that case that structural diversity of antheridiogens existed at the genus level. From the chromatographic evidence presented here

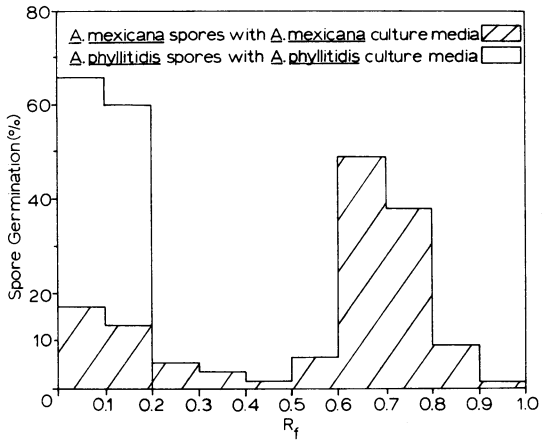


FIG. 10. Percentage of dark-induced spore germination in *A. mexicana* and *A. phyllitidis* in response to gametophyte culture media extracts (equivalent of 5 ml media). Media were extracted with ethyl acetate and partially purified by TLC with diisopropyl ether:acetic acid (95:5, v:v).

(Fig. 10) it is apparent that the antheridiogens of *A. phyllitidis* and *A. mexicana* are different and structural diversity at the species level does occur. Furthermore, spores of *A. phyllitidis* and *A. mexicana* are more sensitive to their own antheridiogens than to that of the other species.

The nature of the light response causing germination is unknown. Since GA 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 (32). They also reported that AMO-1618 blocked the formation of this germination stimulator. We have so far been unable to confirm these results either by isolation of a biologically active substance in light-germinated spores or by inhibition of germination in light by inhibitors of GA biosynthesis.

The results with AMO-1618 reported by Weinberg and Voeller (32) were obtained with suboptimal light conditions. In our own experiments AMO-1618 was ineffective under all conditions. And, although ancymidol and fenarimol did effect a 50% reduction in germination, this was only under conditions of minimal light (24 h), very high concentrations of inhibitors (1.0 mM), and was not reversible by GA. Since ancymidol blocks GA biosynthesis in peas after 10 μM soil drench treatments, we doubt the significance of the present result with 1.0 mM ancymidol. Nevertheless, we cannot rule out the possibility that the spore coats were impermeable to the inhibitors in the present study. Fern spores are difficult to prepare for microscopic studies because the spore coat is impermeable to some fixatives and many embedding chemicals (5). GA and antheridiogen apparently are able to penetrate the spore coat since these two compounds can

Table I. *Effects of GA-Biosynthesis Inhibitors on Spore Germination and Early Gametophyte Development in A. phyllitidis* and *A. mexicana*

Percent germination and growth characteristics were recorded 10 d after sowing. Light treatments commenced upon sowing.

Treatment		Effect			
		A. phyllitidis		*A. mexicana*	
Light period	Inhibitor	Germination[a]	Growth	Germination[a]	Growth
h	*mM*				
240	AMO-1618				
	0.1	High	Filamentous	High	Spatulate
	1.0	High	Filamentous	High	Spatulate
	Ancymidol				
	0.1	High	Round	High	Round
	1.0	High	No growth	High	No growth
	Fenarimol				
	0.1	High	Filamentous	High	Spatulate
	1.0	High	Severely inhibited	High	Severely inhibited
48	AMO-1618				
	0.1	High	Filamentous	Low	Filamentous
	1.0	High	Filamentous	Low	Filamentous
	Ancymidol				
	0.1	Medium	Round	Low	Round
	1.0	Medium	No growth	Low	No growth
	Fenarimol				
	0.1	High	Filamentous	Low	Filamentous
	1.0	High	Inhibited	Low	Severely inhibited
24	AMO-1618				
	0.1	Medium	Filamentous	None	
	1.0	Medium	Filamentous	None	
	Ancymidol				
	0.1	High	Round	None	
	1.0	Medium	No growth	None	
	Fenarimol				
	0.1	High	Inhibited	None	
	1.0	Medium	No growth	None	

[a] Spore germination greater than 70% (high), 40 to 60% (medium), less than 25% (low).

stimulate germination in darkness, but the ability of inhibitors of GA biosynthesis to penetrate the spore coat is unknown. DMSO was used to facilitate penetration of the chemicals into spores but the effectiveness of this solvent is unknown. Use of radioactively labeled inhibitors will be useful in addressing these questions.

Although the inhibitors did not affect germination *per se*, the effects of ancymidol on subsequent growth and development of gametophytes are quite interesting. Ancymidol, an inhibitor of *ent*-kaurene oxidation in peas and wild cucumber (3), affected the morphology of *Anemia* gametophytes at 0.1 mM. Gametophytes of both species were round in shape (Figs. 8 and 9), whereas those in cultures without ancymidol were filamentous (*A. phyllitidis*) (Fig. 6) or spatulate (*A. mexicana*) (Fig. 7). Montague (13) 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 (10, 25). 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.

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 sensitivities 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 h 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 (32), although this cannot be ruled out on the basis of our negative results. Further work needs to be done on the elucidation of this proposed germination substance.

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