Plant Physiol. (1976) 57, 666-669

# Specificity of the Antheridogen from *Ceratopteris thalictroides* (L.) Brongn.[1]

Received for publication February 13, 1975 and in revised form October 11, 1975

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## ABSTRACT

**Characteristics of the fern antheridogen from *Ceratopteris thalictroides* (L.) Brongn. are investigated. These are: *(a)* determination of molecular size (it is readily dialyzable), *(b)* $pK_a$ (about 5), *(c)* movement in thin layer chromatography, and *(d)* ability to induce dark germination of fern spores. These four characteristics are compared to the same characteristics of three other antheridogens (antheridogens A and B or GA). Molecular size and $pK_a$ are similar, but, the antheridogens are separable from each other using thin layer chromatography. It was also shown that spore germination is not induced by the *Ceratopteris* antheridogen, even in its own spores, a characteristic not reported as shared by the other antheridogens. However, the inconsistency of spore germination as an assay for antheridogen is demonstrated. The presence of gametophyte-produced allelopathic substances is also shown.**

Hormonal induction of the male gametangium in ferns was first described by Dopp (3). He was able to induce antheridia precociously in *Dryopteris filix-mas* and *Pteridium aquilinum*. Such hormonal induction of fern antheridia is now a widely documented occurrence (see ref. 8, for review). Such an induction system is called an antheridogen system. An antheridogen system has recently been described for the fern, *Ceratopteris thalictroides* (13, 14). In addition to inducing antheridia, the *Ceratopteris* antheridogen also induces a male gametophyte morphology. In the absence of hormone, a hermaphroditic morphology develops. These two morphologies are mainly defined on the basis of the type(s) of gametes formed by the gametophytes (14). The present investigation details further characteristics of the *Ceratopteris* antheridogen (trivial name, antheridogen C) as well as comparing it to three other antheridogens. These other antheridogens are antheridogen A (from *Pteridium aquilinum*), B (from *Anemia phyllitidis*), and GA. (GA mimes the antheridogen B system [9, 15, 16], but antheridogen B is not the same compound as any described GA [4, 11].)

**Dark Germination.** Fern antheridogens may act to replace red light required in fern spore germination (see review in ref. 8). When an antheridogen initiates germination, it does so only in species where it also induces antheridia. For example, antheridogen A induces antheridia in *Anemia* but not in *Onoclea*. The same antheridogen induces *Anemia* but not *Onoclea* spores to dark-germinate. This phenomenon has been documented in a variety of fern species using antheridogens A (19), B (9, 18, 20), and GA (9, 18-20). Dark germination experiments were conducted utilizing *Ceratopteris* antheridogen and antheridogens A and B.

**Chemical Comparisons.** Molecular size (freely dialyzable) and $pK_a$ values (5.0) have been established for antheridogens A and B (12; Voeller, personal communication). Antheridogens A, B, and GA have also been tested for their relative movements in TLC systems. They have each been shown to have different $R_F$ values, indicating chemical distinctness (10, 17). Similar characteristics were established for the *Ceratopteris antheridogen.*

## MATERIALS AND METHODS

**Spore Sources.** Spores of *Ceratopteris thalictroides* (L.) Brongn. and *Anemia phyllitidis* (L.) Swartz were collected from completely homozygous plants (after method of ref. 6) grown in the U. of Mass. greenhouse. Spores of *Pteridium aquilinum* (L.) Kuhn were supplied by Dr. Bruce Voeller. *C. thalictroides* spores were initially from two sources (RML and 205). Source RML spores were utilized as *Ceratopteris* test organisms while source 205 spores served as *Ceratopteris* antheridogen source (see Table 2 of ref. 12).

**Culture Conditions.** Thompson's inorganic salts medium (5) was used throughout.

*Antheridogen Source Cultures.* These cultures were aseptically established using 5 mg spores/500 ml medium in 2000-ml cotton-stoppered flasks. Cultures were grown under continuous cool white fluorescent light (about 300 ft-c), at room temperature for 4 weeks, then harvested.

*Bioassays.* Aseptic multispore cultures were again used. Each employed 3 drops of sterile spore suspension (1 mg spores/2 ml tap water). Cultures were established in dilution series. Antheridogen-containing medium was Millipore-filtered and diluted into autoclaved, fresh medium (dark germination assays) or diluted into fresh medium before autoclaving ($pK_a$ and dialysis assays). Cultures were grown under continuous red light ($pK_a$, dialysis and most chromatography assays) or continuous darkness (dark germination and some chromatography assays) for approximately 10 days, at room temperature. Cultures were examined microscopically for antheridium-inducing or dark germination-promoting abilities. The dark germination cultures were kept closed during observations. transferred to continuous red light for 2 weeks, when they were examined for spore germination and presence of antheridiate gametophytes.

**Spore Sterilization and Sowing.** A quantity of spores was placed in the bottom of a 15-ml glass centrifuge tube. Wetting agent (2 drops Tween 20/100 ml distilled $H_2O$) was added. The spores were shaken into a suspension. Within 2 min the suspension was centrifuged, and the supernatant was removed. The spores were resuspended in sterile, distilled $H_2O$. The suspension was again centrifuged, supernatant was removed, and the spores were suspended overnight in sterile tap water. The overnight soaking caused fungal spores to germinate, while the fern spores remained closed. The tap water was removed after cen-

[1] This work is based in part on a dissertation presented in partial fulfillment of the degree of doctor of philosophy at the University of Massachusetts, Amherst.

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trifugation, and the spores were suspended in 1% calcium hypochlorite with *Ceratopteris* spores, 5% with *Pteridium* and *Anemia* spores. The following steps were carried out aseptically. Spores were sterilized for a total of 4 min, shaken occasionally, centrifuged, and resuspended in sterile distilled $H_2O$. They were centrifuged and resuspended in an inoculating solution of sterile tap water.

**Antheridogen Preparation.** Media from multispore cultures were filtered to remove any gametophytes. These media were then flash-evaporated to 10% original volume for dialysis and dark germination studies or to 5% for $pK_a$ and TLC studies. These solutions were directly used as antheridogen-containing media in the dialysis and dark germination studies.

**Dialysis.** Twenty-five ml of antheridogen-containing *Ceratopteris* medium was placed in a 20 cm long, 2 cm wide piece of dialysis tubing, with ends double tied. The filled tubing was placed in a beaker with 50 ml of distilled $H_2O$. The beaker contents were stirred slowly for 2 hr on a magnetic stirrer. The water was decanted off and saved. Two more washes were carried out, the first for 2 hr, the second overnight. The combind washes were saved as the "diffusate." The wash procedure was carried out at 15 C. Contents of the dialysis tubing was then removed and brought up to the same volume as the diffusate and labeled "retenate." An equal volume of original concentrated medium was brought up to the same final volume and labeled "whole medium." This was used as the reference medium to note initial antheridium-inducing activity of the solution within the dialysis tubing. Multispore cultures were used to test the hormone content of the diffusate and retenate in comparison to the hormone activity of the whole medium.

**Determination of $pK_a$.** Concentration *Ceratopteris* medium was partitioned twice with ethyl acetate (1/2 volume each partitioning) at three pH values, 3, 5, and 8 (the pH of the starting medium was 5.5; 0.5 M NaOH and 1 N and HCl was used to adjust pH). Thus, six different solutions resulted, two per pH value. Each of these aqueous and ethyl acetate solutions was then tested for its antheridogen activity.

**Thin Layer Chromatography.** Silica gel thin layer sheets (Eastman Kodak) were used after activating at 110 C for 15 min. The solvent system used was diisopropyl etherglacial acetic acid (95:5, v/v) (7). Antheridogen A, B, and C solutions spotted were ethyl acetate phases from partitionings at pH 3. Several concentrations of each were used. Material was spotted 2 cm apart, 2 cm from the bottom of the sheet, and 2 cm from either edge. The solvent was allowed to move approximately 10 cm from the origin. The chromatograph was then dried (allowing solvent to evaporate; about 30 min). Bioassays were established by incorporating squares of chromatograph (backing and silica gel) into fresh liquid cultures (5 ml/glass Petri dish). Ten or 20 squares were produced from the area above each spot. The cultures were then autoclaved and aseptically sown. They were grown under continuous red light (for *Pteridium* and *Ceratopteris* assays) or continuous darkness (for induction of germination in *Anemia* assays).

Gibberellic acid (Eastman Organic Chemicals; 80+% GA), 1 mg in 1 ml of ethyl acetate, was also chromatographed using the same solvent system. The GA-spotted chromatograph portions were developed with a spray of ethanol and concentrated $H_2SO_4$ (95:5 v/v) and the location of GA noted under UV light (7). Several concentrations of GA were used.

## RESULTS

**Dark Germination.** Antheridogens A, B, and C were tested for their ability to induce dark germination of *Ceratopteris, Pteridium,* and *Anemia* spores. Spore viability and antheridogen activity were checked by subsequent growth of the same cultures under continuous red light, when both spore germination and presence of antheridiate gametophytes were noted. The *Ceratopteris* antheridogen did not induce dark germination in any of the spores tested, even its own. Antheridogen C activity (the induction of antheridia on *Ceratopteris* gametophytes only) was verified in the medium used. These data and those presented in Table I show only spores of *Anemia* respond to added (*Anemia*) antheridogen by dark germinating.

**Chemical Comparisons by $pK_a$.** The concentration aqueous culture medium containing antheridogen C was partitioned with ethyl acetate to determine the approximate acidic disassociation constant of the *Ceratopteris* antheridogen. This experiment is based on the observation that both antheridogens A and B have a $pK_a$ value of 5.0.

The partitioning of the *Ceratopteris* antheridogen was done at three pH levels: 3, 5, and 8. For each pH, two solutions, one aqueous, the other ethyl acetate, resulted and were tested in dilution series for antheridogen activity (*i.e.* ability to induce male gametophyte morphology). The $pK_a$ of the *Ceratopteris* antheridogen (Fig. 1) is near pH 5. This is the pH at which the molecule is equally distributed (as noted by antheridogen activity) between the aqueous, polar and the acetate, nonpolar phases.

**Chemical Comparisons by Thin Layer Chromatography.** A means of comparing the chemical structure of molecules is by the use of chromatography. Using a solvent system already known to separate antheridogens B and GA (17), the present experiment chromatographed antheridogens A, B, C, and GA. If the four antheridogens migrate different distances in the same solvent system, then the molecules are chemically distinct. The move-

Table I. *Effect of Antheridogen C on Dark Germination of Spores and Antheridia Induction in Gametophytes of Anemia phyllitidis and Pteridium aquilinum*

| Test[1] | No. Spores Germinating/Total | | Antheridia-induced? |
|---|---|---|---|
| | Dk[2] | Lt[2] | |
| P × P, 50 | 0/50[3] | 0/50 | NG[4] |
| 20 | 0/20 | 12/64 | +[4] |
| 10 | 0/20 | 15/74 | + |
| 5 | 0/100 | 6/63 | + |
| 0 | 0/100 | 20/90 | −[4] |
| C × P, 50 | 0/20 | 12/64 | − |
| 20 | 0/100 | 30/115 | − |
| 10 | 0/20 | 33/121 | − |
| 5 | 0/20 | 13/69 | − |
| A × A, 50 | 27/85 | 50/100[3] | + |
| 20 | 109/149 | 50/100 | + |
| 10 | 81/159 | 50/100 | + |
| 5 | 67/99 | 50/100 | + |
| 0 | 0/100 | 5/58 | − |
| C × A, 50 | 0/100[3] | 0/100[3] | NG |
| 20 | 0/200 | 50/100 | − |
| 10 | 0/250 | 1/100 | − |
| 5 | 0/150 | 50/100 | − |

[1] Letters refer to first, antheridogen source (A = *Anemia,* C = *Ceratopteris,* P = *Pteridium*), second to spore source. The number after the comma refers to the % concentration of antheridogen-media used (i.e., 50 = 50%). Thus, C × C, 50 is a measure of the effect a 50% dilution of *Ceratopteris* antheridogen on *Ceratopteris* spores.

[2] Dk = counts made after 10 days in the dark: Lt = counts made on the same cultures after 2 weeks subsequent growth under continuous red light.

[3] Numbers are roughly calculated; small area of field of view counted, that number multiplied by approximate number of such areas in the field of view, this figure was then rounded off.

[4] NG = no germination. + = all gametophytes antheridiate, none with notch meristem; − = no antheridiate plants or mixture of antheridiate plants (antheridia younger in development than in + response) and plants with notch meristems.

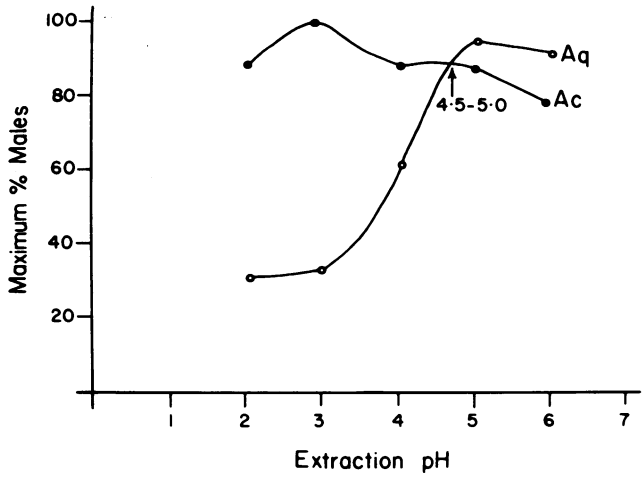


FIG. 1. Atheridogen activity of *Ceratoperis thalictroides* multispore culture medium. Medium partitioned with ethyl acetate giving aqueous (Aq) and ethyl acetate (Ac) phases.

ments of native antheridogens (A, B, and C) were monitored using bioassays. GA was localized by direct observation using UV light (7). When bioassay or fluorescence results were compared, it was found that each antheridogen had a different $R_F$. Each antheridogen was run at several different concentrations, to obtain minimal trailing. $R_F$ values obtained were: GA, 1.9; antheridogen A, 3.0 to 3.5; antheridogen B, 2 to 3; and antheridogen C, 9.5 to 10.

**Molecular Size.** Dialysis tubing will permit the diffusion of relatively small molecules (up to approximately 2000 mol. wt) such as antheridogens A, B, or GA and many other biologically active molecules. To determine if antheridogen C was of similar molecular size, it was subjected to dialysis. This procedure was carried out in a cold room to minimize possible degradation of antheridogen. The "diffusate" is the solution in which dialysis tubing was washed (=distilled $H_2O$, at start). The "retenate" is the contents of the tubing after washing, brought up to the same volume as the diffusate. The "whole medium" is the starting solution of *Ceratopteris* antheridogen-containing medium, without washing brought up to the same final volume as the diffusate and retenate solutions. Bioassays demonstrated that the repeated washes (3×) resulted in the movement of most antheridogen from the dialysis tubing (retenate) to the washing medium (diffusate). Thus, antheridogen C, like the other known antheridogens, is readily diffusible.

## DISCUSSION

The present study provides further information on the characteristics of antheridogen C. This allows one to understand more fully the nature of antheridogen C and to compare it to other antheridogens (*e.g.* antheridogens A, B, and GA).

The results of the studies reported here show antheridogen C to be a compound of relatively small molecular size (*i.e.* it is freely dialyzable) and to have a disassociation constant near 5 (Fig. 1). In these characteristics, antheridogen C is similar to other antheridogens. In other characteristics, it is different.

Another general antheridogen characteristic is its ability to promote dark germination of fern spores. The *Ceratopteris* antheridogen does not promote dark germination even in its own spores. It is unlike all the other reported antheridogens. That is, in species where an antheridogen acts to induce antheridia, the same antheridogen (where it has been tested) will also promote dark germination of that species' spores (9, 18–20). Antheridogen C fails to promote dark germination even of its own spores. (It is possible that other collections of *Ceratopteris thalictroides* spores will be found that can be induced to dark-germinate. If this proves to be true, then dark-germination will be of variable use as an antheridogen C assay.) The data in Table I show that, in this system, only antheridogen B acts to promote dark germination of its own *(Anemia)* spores. Both *Pteridium* and *Ceratopteris (thalictroides)* spores have been reported to dark-germinate without added antheridogen (see review in ref. 8). Under the present conditions, neither dark-germinated, even with its own antheridogen present in the medium. The data in Table I also show both species' spores to be viable and their antheridogens active. I concluded that there is some difference between the test system used in this study and those which have achieved dark germination. The differences may be one of temperature, age of spores used, or some other factor(s).

The data presented in Table I show a general tendency for spore germination (in the light) to be inhibited at high concentrations of the antheridogen-containing medium. Since this medium is a concentrate of old culture medium, it may contain secreted substances other than antheridogen. These substances would also be concentrated during flash-evaporation to be present in the medium in which the spores are germinating. The data in Table I show evidence of the secretion by ferns of growth-inhibiting substances. Reports also exist of a fern *(Thelypteris)* sporophyte producing such substances. These substances (thelypterins A and B) inhibit fern gametophyte and oat coleoptile growth (1, 2). Growth inhibition data (Table I) may also be the result of allelopathic substances, in this instance, gametophyte-produced.

The lack of interspecific activity in the promotion of dark germination does not aid in the taxonomic placement of the Parkeriaceae (to which the one genus, *Ceratopteris,* belongs). The uncertain affinity of this family to either the Pteridaceae (contains *P. aquilinum*, source of antheridogen A) or the Anemiaceae (to which *A. phyllitidis,* source of antheridogen B, belongs) has been reviewed elsewhere (13).

**Thin Layer Chromatography.** The chromatography of Antheridogens A, B, C, and GA enables one to demonstrate the chemical distinctions of the four antheridogens (each has different $R_F$ values). The movement of antheridogen C at or near the solvent front may indicate it is being carried along as a complex with another molecule, *e.g.* a fatty acid. Each of the antheridogens is prepared in the same manner, thus, each should be able to complex with fatty acids if present. The generic uniqueness of antheridogens A, B (or GA), and C, shown in this study, is further strengthened by antheridium-inducing data (13), again showing lack of intergeneric activity among the three families. The dark germination results are not conclusive as an antheridogen test. Molecular size and $pK_a$ data indicate all the antheridogens may belong to a similar group of compounds, though many biologically active molecules fall in this same range. Only antheridogen B and its miming antheridogen GA (several are active [16]) have been fully characterized, the former as a diterpenoid compound, closely related in chemical structure to $GA_3$ (14, 11). Several concentrations of antheridogen C were also chromatographed with GA. When this chromatograph was developed so that a gibberellin would fluoresce (under UV light), only the known GA fluoresced. Thus, antheridogen C did not fluoresce like a GA. This lack of fluorescence may reflect the low level of antheridogen C present in the medium. Although all the antheridogens move to different areas in TLC and lack intergeneric activity in the induction of antheridia and possibly in the promotion of dark germination, they all produce the same phenomena (antheridium induction and (most) the promotion of dark germination) and have similar molecular sizes and $pK_a$ values. Also, the only characterized native antheridogen, antheridogen B, is very similar in chemical structure to the only other fully characterized (though "artificial") antheridogen GA. Thus, antheridogens seems to be a group of molecules with similar

chemical properties and biological activities. The familial distinctness noted in biological activity and chromatography is probably the result of the length of time the three families have been evolving as separate taxa.

*Acknowledgment*—The author wishes to thank E. J. Klekowski, Jr. for his assistance in this research and in the reading of this manuscript. Thanks also go to B. R. Voeller for his assistance with the techniques used in this study.

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