Interaction between Antheridogens and Fatty Acids in Fern Spore Germination

ROSS B. PRINGLE
Cell Biology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario, Canada

ABSTRACT

Certain fatty acids in the C6 to C13 range, at concentrations as low as $10^{-4} \text{M}$, were found to inhibit the germinations of spores of the sensitive fern, *Onoclea sensibilis* L. The addition of gametophytic culture filtrates of the bracken fern, *Pteridium aquilinum* (L.) Kuhn, containing antheridogen A, was found to overcome this inhibition and allow the spores to germinate and the gametophytes to develop in a normal fashion. Some fatty acids were found to increase the antheridium-inducing potency of antheridogen A as much as 10-fold. An effect similar to this may promote the diecious reproduction of ferns.

Physiology of sex organ formation can be more readily investigated in ferns than in most other plants because the gametophytic generation can be grown in axenic culture under defined conditions of environment and nutrition. Döpp reported in 1950 that mature gametophytes of the bracken fern, *Pteridium aquilinum* (L.) Kuhn, secreted a substance (A-substanz) which induced newly germinated prothalli to form antheridia (1). This substance was later isolated and purified and named antheridogen A (11, 12). It was found to induce antheridia on a great many of the species of the family *Polypodiaceae* (1, 2, 6-8, 14).

Naf reported in 1959 that *Anemia phyllitidis* (L.) Swartz produced a second antheridogen (5). He discovered a third antheridogen from *Lygodium japonicum* (Thunb.) Swartz (6) and a fourth from *Onoclea sensibilis* (10). The term antheridogen is now used in a generic sense (10, 14).

Early work on the chemical characterization of antheridogen A was done shortly after the discovery of gibberellins, and gibberellic acid was compared to it and found to be different. However, in 1962 Schraudolf reported that gibberellin A1 was capable of inducing antheridia in certain species of *Schizaeaaceae* (13). Voeller showed that seven gibberellins, A1, A2, A4, A5, A7, A8, and A9, were capable of inducing antheridia in *A. phyllitidis* at a concentration of $5 \times 10^{-6} \text{ g/ml}$, but each was shown by thin layer chromatography not to be identical with the native antheridogen of *A. phyllitidis* (15). A general property which seems to differentiate antheridogens from gibberellins is stability to acid (11). Antheridogen A has been shown to be a carboxylic acid with a pKa, of about 5.0 (11). The carboxyl function is necessary for biological activity since this activity disappears on esterification and reappears after hydrolysis of the ester. The new antheridogen from *O. sensibilis* can be detected only after boiling for 10 min at pH 2 (10).

Certain long chain aliphatic fatty acids were found to produce a 3-fold increase in the potency of antheridogen A preparations (11). When representative organic acids of various classes were tested on *O. sensibilis* spores, it was found that naturally occurring straight chain aliphatic fatty acids inhibited germination at relatively low concentrations. The addition of dilute antheridogen A solutions would overcome this inhibition. A possible physiological role for these observations is suggested.

MATERIALS AND METHODS

Fern Spores. Sporophylls of *O. sensibilis* L. and *P. aquilinum* (L.) Kuhn were collected from various places in eastern Canada and the eastern United States. Spores were harvested by drying the fronds on large sheets of paper and sieving the ejected spores through fine nylon mesh. The dry spores were stored in tightly stoppered glass vials at 4°C, where they retained a high percentage (70 to 80%) of germinating ability for several years. In spite of its ubiquity, fertile fronds of bracken (*P. aquilinum*) were found only in a few locations.

Chemicals. Octadecen-2-transoic and octadecen-2-cisioic acids were gifts of Dr. M. Barbier, Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France. The remaining unusual fatty acids were purchased from K & K Laboratories, Plainview, New York. All acids were converted into solutions of their sodium salts before use.

Antheridogen A Preparations. *P. aquilinum* was grown from spores in liquid culture in 5-liter, Blake-type diphteria toxin bottles, containing 500 ml of fluid, and antheridogen A preparations were produced as previously described (11, 12). Antheridogen A content varied depending on supplements added and conditions of culture (14). In this report, culture filtrates that were not active at a dilution of more than 1:10,000 are considered to have low potency. Those that still possessed antheridium-inducing activity when diluted 1:30,000 are considered to have average potency, while those active when diluted 1:90,000 or more have high potency.

Assay Conditions. *O. sensibilis* spores (about 25 mg) were sterilized by first wetting with a solution of one drop 25% Aerosol OT in 10 ml of sterile water, then centrifuged and resuspended in a 10% solution of a freshly prepared commercial hypochlorite bleach (Javex). After a measured number of seconds, which varied depending on the amount of contamination present and with different batches of bleach, the spores were quickly centrifuged, washed twice by centrifuging in sterile water, and finally suspended in 10 ml of sterile water. One drop of this suspension was used to inoculate each 50 ml Erlenmeyer flask containing 10 ml of Moore's inorganic medium (as modified by Naf [4]) solidified with 0.9% agar. Flasks were kept at 22°C under con-

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1 Cell Biology Research Institute, Canada Department of Agriculture Publication 663.
tinuous illumination from cool white fluorescent lights (about 200 ft-c).

Serial dilutions of the sodium salts of the acids to be tested were incorporated into the medium before sterilization by autoclaving for 15 min at 20 lbs pressure. Experiments were performed in triplicate. Germination could be detected in 2 or 3 days as a green “bloom” of young prothalli covering the surface of the agar. When the gametophytes were several millimeters wide, antheridogen activity was assayed as described by Näf (8). Germination was judged to be inhibited if no growth appeared after 2 weeks of incubation.

RESULTS AND DISCUSSION

The effect of naturally occurring, normal (straight chain), saturated, monobasic fatty acids on the germination of *O. sensibilis* spores is shown in Table I. The lower members of the homologous series, up to 4 carbon atoms (butyric), had no effect on germination or subsequent growth at millimolar concentrations. Starting with C₁₂ (capric) and ascending the homologous series to C₁₈ (capric), each member inhibited germination completely at a concentration of 5 × 10⁻³ M or higher. Except for valeric acid, this inhibition of germination was reversed by adding diluted culture filtrate of *P. aquilinum*. A 1:10,000 dilution of culture filtrate of average antheridogen A potency when mixed with 10 times the minimum inhibitory dose of these acids permitted normal germination and growth and development of gametophytes. Trimethyl acetic acid, a branched chain isomer of valeric acid, was also found to inhibit germination at 10⁻³ M. This inhibition was reversed by *P. aquilinum* culture filtrate.

The higher molecular weight, naturally occurring, even carbon homologues, C₁₂, C₁₄, C₁₆, and C₁₈, were found to be still more powerful inhibitors, inhibiting germination completely at a concentration of 1 × 10⁻³ M or higher. Again, a 1:10,000 dilution of *P. aquilinum* culture filtrate of average potency was able to overcome 10 times the inhibitory dose of these four acids.

The effect of some simple, unbranched, unsaturated acids in the same range of chain length is shown in Table II. For the most part, the unsaturated acids did not inhibit spore germination as did their saturated analogues. An exception was found in the case of the C₁₈ acids, oleic (mono-unsaturated) and linoleic (di-unsaturated). Even in this case, a shift in the position of the double bond removed the inhibition. The two synthetic C₁₈ α,β-unsaturated acids which are isomers of oleic acid were not inhibitory. Another α,β-unsaturated acid, traumatic acid (wound hormone), did not show any inhibition compared with its C₁₂ saturated counterpart, lauric acid. The addition of diluted culture filtrate of *P. aquilinum* was found to reverse the inhibition produced by oleic and linoleic acids, in the same manner as the reversal of the inhibition caused by the saturated analogue, stearic acid.

With the exception of oleic and linoleic acids, this unusual property of inhibiting fern spore germination seems to be confined to the simple, monobasic, aliphatic carboxylic acids of medium or high molecular weight. Table III lists a number of representative acids without effect at concentrations below 10⁻³ M. Although the list is by no means exhaustive, it includes commonly occurring members of dibasic, tribasic, amino, hydroxy, sulfhydryl, and aromatic classes. None of these acids was found to influence the potency of antheridogen A preparations. In 10⁻³ M indoleacetic acid, germination and growth were much slower than normal.

<table>
<thead>
<tr>
<th>Acid</th>
<th>No of Carbon Atoms</th>
<th>Lowest Concentration to Completely Inhibit Germination</th>
<th>Behavior on Adding Antheridogen A</th>
</tr>
</thead>
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<tr>
<td>Formic</td>
<td>1</td>
<td>No effect at 10⁻³ M</td>
<td>Reversal of inhibition</td>
</tr>
<tr>
<td>Acetic</td>
<td>2</td>
<td>No effect at 10⁻³ M</td>
<td>Reversal of inhibition</td>
</tr>
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<td>Propionic</td>
<td>3</td>
<td>No effect at 10⁻³ M</td>
<td>Reversal of inhibition</td>
</tr>
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<td>Butyric</td>
<td>4</td>
<td>No effect at 10⁻³ M</td>
<td>No reversal of inhibition</td>
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<tr>
<td>Valeric</td>
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</tr>
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<td>Caproic</td>
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<td>5 × 10⁻³ M</td>
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<td>Heptanoic</td>
<td>7</td>
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<td>Caprylic</td>
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<td>Pantonic</td>
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<td>Capric</td>
<td>10</td>
<td>5 × 10⁻³ M</td>
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<td>Lauric</td>
<td>12</td>
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<td>Stearic</td>
<td>18</td>
<td>1 × 10⁻³ M</td>
<td>Reversal of inhibition</td>
</tr>
</tbody>
</table>

Table II. Effect of Unsaturated Fatty Acids on Germination of *O. sensibilis* Spores

The effect of some simple, unbranched, unsaturated acids on germination of *O. sensitibilis* spores is shown in Table II. The lower members of the homologous series, up to 4 carbon atoms (butyric), had no effect on germination or subsequent growth at millimolar concentrations. Starting with C₁₂ (valeric) and ascending the homologous series to C₁₈ (capric), each member inhibited germination completely at a concentration of 5 × 10⁻³ M or higher. Except for valeric acid, this inhibition of germination was reversed by adding diluted culture filtrate of *P. aquilinum*. A 1:10,000 dilution of culture filtrate of average antheridogen A potency when mixed with 10 times the minimum inhibitory dose of these acids permitted normal germination and growth and development of gametophytes. Trimethyl acetic acid, a branched chain isomer of valeric acid, was also found to inhibit germination at 10⁻³ M. This inhibition was reversed by *P. aquilinum* culture filtrate.

The higher molecular weight, naturally occurring, even carbon homologues, C₁₂, C₁₄, C₁₆, and C₁₈, were found to be still more powerful inhibitors, inhibiting germination completely at a concentration of 1 × 10⁻³ M or higher. Again, a 1:10,000 dilution of *P. aquilinum* culture filtrate of average potency was able to overcome 10 times the inhibitory dose of these four acids.

The effect of some simple, unbranched, unsaturated acids in the same range of chain length is shown in Table II. For the most part, the unsaturated acids did not inhibit spore germination as did their saturated analogues. An exception was found in the case of the C₁₈ acids, oleic (mono-unsaturated) and linoleic (di-unsaturated). Even in this case, a shift in the position of the double bond removed the inhibition. The two synthetic C₁₈ α,β-unsaturated acids which are isomers of oleic acid were not inhibitory. Another α,β-unsaturated acid, traumatic acid (wound hormone), did not show any inhibition compared with its C₁₂ saturated counterpart, lauric acid. The addition of diluted culture filtrate of *P. aquilinum* was found to reverse the inhibition produced by oleic and linoleic acids, in the same manner as the reversal of the inhibition caused by the saturated analogue, stearic acid.

With the exception of oleic and linoleic acids, this unusual property of inhibiting fern spore germination seems to be confined to the simple, monobasic, aliphatic carboxylic acids of medium or high molecular weight. Table III lists a number of representative acids without effect at concentrations below 10⁻³ M. Although the list is by no means exhaustive, it includes commonly occurring members of dibasic, tribasic, amino, hydroxy, sulfhydryl, and aromatic classes. None of these acids was found to influence the potency of antheridogen A preparations. In 10⁻³ M indoleacetic acid, germination and growth were much slower than normal.

The factor in the culture filtrates of *P. aquilinum* responsible for reversing the fatty acid inhibition of *O. sensibilis* spore germination seems to be present at a concentration roughly parallel to the concentration of antheridogen A. However, it is not certain, at this point, that the factor is identical with antheridogen A, since not enough pure material was available to perform a definitive experiment.

The antheridium-inducing potency of culture filtrates was enhanced by the addition of dilute solutions of fatty acids, as previously reported (11). It was found that only those acids capable of inhibiting germination had this effect and that the effect was most pronounced at a concentration of acid about one-tenth that needed to inhibit germination. The effect was also found to vary with the potency of the culture filtrate tested. With

Table III. Acids without Effect on Germination of *O. sensibilis* Spores

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<tr>
<th>Acid</th>
<th>Oxalic</th>
<th>Aspartic</th>
<th>Glutamic</th>
<th>Glycerophosphoric</th>
<th>Thiglycolic</th>
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culture filtrates of low antheridogen A potency the activity was
generally doubled. With those of average potency the increase
was 3- to 6-fold. In three cases involving C16 and C18 acids, the
activity of a culture filtrate of high potency was increased more
than 10 times. In one experiment, the antheridium-inducing
potency of pure antheridogen A was increased by a subinhibitory
dose of lauric acid.

Although fatty acids have a widespread distribution in plants,
it is not known if such substances are functional inhibitors of fern
spore germination under natural conditions. A possible physio-
logical role for this interaction between fatty acids and anther-
dogen could be to regulate the germination of fern spores. Although
O. sensibilis is self-fertile, functional antheridia on young gameto-
phies, under natural conditions, release spermatids which fertilize
the archegonia on separate older gametophytes. This occurs
because, in the development of the individual prothallus, anther-
idia formation stops when archegonia formation starts. Naf has
shown that developing prothalli of O. sensibilis quickly become
insensitive to antheridogen A (4). They fail to form antheridia
even when supplied with antheridogen A at a concentration
15,000 times higher than the concentration necessary to induce
antheridia in prothalli just 2 days younger.

Thus, successful induction of antheridia and subsequent suc-
cessful fertilization depend on the spores which will form the male
gametophytes germinating later than those spores which produce
the receptive female gametophytes. This could be achieved by
antheridogen from mature prothalli stimulating the germination of
inhibited spores which would form prothalli sensitive to the
same antheridogen.

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the Laboratory of Plant Morphogenesis, Manhattan College), and Dr. Bruce Voeller,
for encouragement and stimulating discussions.

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