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Effects of inorganic nitrogen sources on spore germination and gametophyte growth in *Botrychium dissectum*

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Abstract. The effects of variations in nitrogen source upon spore germination and gametophyte growth of the eusporangiate fern *Botrychium dissectum* forma *obliquum* were investigated. Spore germination and early gametophyte growth were directly related to the oxidation level of the supplied nitrogen source. Nitrate and nitrite inhibited spore germination and at concentrations above 0.035 mol m^{-3} prevented it entirely. Ammonium promoted germination well above the levels attained on media without nitrogen. Concentrations of ammonium greater than 0.035 mol m^{-3} often resulted in germination above 90%. The growth of young gametophytes from spores was reduced on media without nitrogen or on media with high concentrations of nitrate. Ammonium stimulated the growth of both young and older gametophytes. However, older gametophytes were able to grow on media containing nitrate as the sole nitrogen source. We conclude that a reduced nitrogen source is necessary for spore germination and early growth of *Botrychium* gametophytes. This requirement has several ecological implications which may be related to the distribution of these ferns and the establishment of mycorrhizal associations.

Key-words: Botrychium dissectum forma *obliquum* (Muhl.) Fernald; Ophioglossaceae; grape fern; spore germination; gametophyte growth; nitrogen metabolism; nitrate; ammonium.

Introduction

The subterranean gametophytes of the Ophioglossales differ from the photosynthetic gametophytes characteristic of the Filicales in several aspects. Gametophytes of the Ophioglossales are white, non-photosynthetic, and tuberous. They are sustained in nature by mycorrhizal fungi of an endotrophic type. The subterranean habit has made developmental studies of these gametophytes difficult. However, gametophytes can be grown in axenic culture if the nutrient medium contains a carbon source (Whittier, 1972; Gifford & Brandon, 1978).

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These are slow growing organisms and efforts have been made to accelerate their development in axenic culture. Increasing the amount of sugar available to the gametophytes promotes gametophyte growth (Whittier, 1984). The more-recently employed nutrient media improve growth and appear to be appropriate for gametophyte development because sexual reproduction has taken place in culture (Whittier, unpublished data).

The cultural conditions which promote the development of *Botrychium* gametophytes do not appear to be the most favourable for spore germination. The highest germination percentages in axenic culture for ophioglossaceous spores have been obtained with *Botrychium dissectum* forma *obliquum*. The germination, however, did not exceed 19% after 4 months in culture (Whittier, 1973). There were no observable structural differences among the spores sown. None were aborted or collapsed and all had nuclei and cytoplasmic contents. It appeared, from visual inspection, that all should have the ability to germinate under appropriate conditions.

Because variations in the carbon source have no significant effect on germination (Whittier, 1984), other modifications to the nutrient media were made in an attempt to promote germination. Preliminary data suggested that alterations of the nitrogen source might improve germination. The aim of this investigation, therefore, was to determine, for *Botrychium*, whether or not the form of nitrogen was important for spore germination and if it would have an effect on the growth of gametophytes in axenic culture.

Materials and methods

Spores of *Botrychium dissectum* forma *obliquum* (Muhl.) Fernald were obtained from fertile spikes collected in Nashville, TN, U.S.A. Voucher specimens of the plants are on deposit at the Vanderbilt University Herbarium.

The spores were surface sterilized with 20% Clorox and suspended in sterile water by the method of Whittier (1973). The surface-sterilized spores were sown on 15 cm^3 of nutrient medium in 100 × 20 mm screw cap culture tubes. Because previous studies

have shown that the germination of spores of the Ophioglossales occurs in the dark only (Whittier, 1973; Gifford & Brandon, 1978), the cultures were maintained in darkness at 24 ± 1 °C for a period of 7 weeks.

The basic nutrient medium used was a modification of Knudson's solution of mineral salts (Knudson, 1922) which allowed for variations in the nitrogen source. This medium contained 1.7 mol m^{-3} Na_2SO_4, 0.5 mol m^{-3} $MgSO_4 \cdot 7H_2O$, 2.1 mol m^{-3} $CaCl_2$, and 0.7 mol m^{-3} K_2HPO_4. The mineral component of the medium was completed with FeEDTA and minor elements (Whittier, 1973). For most experiments, the medium was solidified with 0.8% agar and contained 0.5% sucrose. Unless indicated otherwise, the pH of the medium was adjusted to 6.1 prior to autoclaving. After autoclaving, the pH of the medium averaged 5.8.

The experiments involved supplementing the basic nutrient medium with various nitrate, nitrite, or ammonium salts. In most cases, sodium nitrate and/or ammonium chloride at concentrations ranging from 0.0035–3.5 mol m^{-3} were employed. Several replicates were carried out for each treatment and experiments were repeated at least once. The percentage of germination was determined by observing one thousand or more spores from each experimental treatment.

Gametophyte growth was measured in two ways. For young gametophytes growing from the spores, the length, in micrometers, of 30 gametophytes was measured and an average was calculated to determine the gametophyte growth for each treatment. With older gametophytes, the length of the gametophytes was measured at the beginning and end of the experiment and the average per cent increase in length was used as a measure of growth. A medium without nitrogen was included in most experiments to demonstrate whether or not the nitrogen source promoted gametophyte growth above the limited amount which occurs on media without nitrogen. Differences in gametophyte growth for similar treatments in separate experiments are related to variations in experiment duration, which ranged from 3 to 5 months, and/or age of the spores.

The data on gametophyte growth are expressed as means $\pm$ the standard error of the mean (SE). Analysis of variance and the Tukey test (Zar, 1984) were used to determine if the differences among the means were statistically significant.

These are the basic procedures and conditions which were used to determine effective nitrogen sources for spore germination and gametophyte growth. Any exceptions or modifications to these procedures, made in some experiments, are discussed with their respective results.

Table 1. Effects of various nitrate and ammonium sources on the germination of *Botrychium dissectum* spores after 7 weeks in culture

Nitrogen source	Concentration (mol m^{-3})	Germination (%)
$Ca(NO_3)_2$	1.75	<0.01
KNO_3	3.5	0.0
NH_4NO_3	3.5	12.5
$(NH_4)_2SO_4$	1.75	84.5
NH_4 tartrate	3.5	48.1
NH_4Cl	3.5	78.5

Results

The effects of various nitrogen sources upon the germination of *Botrychium* spores are presented in Table 1. The nitrogen sources were supplied at concentrations which gave 3.5 mol m^{-3} nitrate or ammonium. In the case of ammonium nitrate, a 3.5 mol m^{-3} concentration of both nitrogen forms was supplied. Media supplemented with nitrate salts resulted in low percentages of germination whereas ammonium salts gave higher values. Providing the spores with ammonium nitrate resulted in a germination value lower than for ammonium alone and higher than for nitrate alone.

To test the influence of nitrate or ammonium on germination further, media with various concentrations of $NaNO_3$ and NH_4Cl were employed. In addition, a medium without nitrogen was included to determine the level of germination in the absence of exogenous nitrogen. All concentrations of nitrate resulted in low germination percentages (Table 2). The level of germination on media containing nitrate (<2%) was less than that on the medium without nitrogen. Except for the lowest ammonium concentration, germination on the media with ammonium exceeded that on the media with nitrate and the medium without nitrogen. The medium containing the highest ammonium concentration (3.5 mol m^{-3}) resulted in a germination percentage of over 90%.

In a separate experiment, the effect of nitrite was tested on germination. Media containing 0.0035–3.5 mol m^{-3} sodium nitrite were used. Germination percentages were essentially the same as for media with nitrate and less than that on the medium without nitrogen (data not shown).

Experiments longer than 7 weeks were carried out to study the effects of nitrate and ammonium on gametophyte growth. Length of the gametophytes in micrometers was used as the measure of growth. Because the spores germinated and gametophytes started to grow on media without nitrogen, both

Table 2. Effects of varying concentrations of nitrate or ammonium on the per cent germination of *Botrychium dissectum* spores after 7 weeks in culture

	Concentration (mol m^{-3})				
Nitrogen source	0	0.0035	0.035	0.35	3.5
$NaNO_3$	10.0	1.1	1.4	1.3	0.4
NH_4Cl	—	8.3	32.5	63.7	93.0

Table 3. Growth of *Botrychium dissectum* gametophytes after 3.5 months in culture and supplied with either nitrate or ammonium

	Concentration (mol m^{-3})				
Nitrogen source	0	0.0035	0.035	0.35	3.5
	Gametophyte length (μm) $\pm$ SE*				
$NaNO_3$	97.3 ± 5.3^{a}	126.4 ± 8.0^{b}	99.5 ± 4.1^{a}	94.4 ± 5.2^{a}	—
NH_4Cl	93.6 ± 2.7^{a}	111.5 ± 3.9^{ab}	125.6 ± 5.2^{b}	186.4 ± 7.9^{c}	222.4 ± 14.4^{d}

*Values followed by the same letter show no significant difference at $P < 0.05$.

experiments included media without nitrogen as controls. These results are presented in Table 3. Growth on the media containing ammonium was promoted above that on the medium without nitrogen. The increase in gametophyte length was statistically significant except in the case of the lowest concentration of ammonium (0.0035 mol m^{-3}). There was a small but statistically significant increase in gametophyte growth on the medium with the lowest nitrate concentration (0.0035 mol m^{-3}). The growth on media with 0.035–0.35 mol m^{-3} nitrate was not significantly different from that on the medium without nitrogen. There was almost no germination on the medium with 3.5 mol m^{-3} nitrate, therefore, no effect was made to measure these gametophytes.

Because ammonium and nitrate appeared to have opposing influences on the germination of *Botrychium* spores, the effects of media containing both ammonium and nitrate were examined. The selection of the concentrations of nitrogen employed were based on the earlier experiments. All combinations of the three highest concentrations of ammonium and all the nitrate concentrations were tested for their effects on spore germination. In addition, gametophyte length was measured to determine what effects the combined nitrogen sources had on gametophyte growth.

The results of this experiment are presented in Table 4. The maximum germination percentage was obtained with the medium containing 3.5 mol m^{-3} ammonium. Additions of nitrate to this medium reduced the level of germination. This reduction of germination was proportional to the concentration of nitrate. Germination was affected in a similar manner by the addition of nitrate to media with lower concentrations of ammonium.

The growth of the gametophytes on these media showed considerable variation. The longest gametophytes were obtained on the medium with 3.5 mol m^{-3} ammonium or with 3.5 mol m^{-3} ammonium + 0.0035 mol m^{-3} nitrate. The addition of higher concentrations of nitrate to any of the media with ammonium reduced gametophyte growth. The smallest increments of growth occurred on media supplemented with 3.5 mol m^{-3} nitrate.

In order to investigate the possibility that autoclaving the nitrogen was having an effect on germination of the spores or growth of the gametophytes, filter-sterilized media were prepared. These media, which contained ammonium, nitrate, or lacked nitrogen, had the same pH, 5.6, after sterilization. The media with the filter-sterilized nitrogen gave the same responses (Table 5) as observed for media with autoclaved nitrogen. Germination percentages were low on media containing nitrate and higher on media containing ammonium. Gametophyte growth occurred on the media with ammonium whereas the medium without nitrogen had reduced germination and very slow gametophyte growth.

It has been reported that the absorption of nitrate and ammonium can be influenced by the external pH (Nightingale, 1937). Therefore, we examined germi-

Table 4. Germination percentages and gametophyte lengths (μm) $\pm$ SE* of *Botrychium dissectum* spores grown for 3.5 months on media containing various concentrations of both ammonium and nitrate

Nitrate concentration (mol m^{-3})	Ammonium concentration (mol m^{-3})		
	3.5	0.35	0.035
3.5	11.2% 111.3 ± 5.0^{fghi}	5.3% 76.5 ± 3.8^{ijk}	0.3% 53.9 ± 1.1^{k}
0.35	46.0% 175.7 ± 9.2^{abc}	16.3% 102.9 ± 4.2^{fghij}	1.5% 73.1 ± 4.5^{ijk}
0.035	55.2% 192.3 ± 8.5^{ab}	47.1% 128.5 ± 6.7^{def}	3.3% 73.6 ± 3.2^{ijk}
0.0035	82.6% 202.4 ± 11.9^{a}	54.4% 153.9 ± 7.1^{cde}	20.0% 118.6 ± 5.2^{fgh}
0.0	93.1% 200.3 ± 11.8^{a}	90.7% 161.9 ± 6.5^{bcd}	30.9% 123.5 ± 4.2^{efg}

*Values followed by the same letter show no significant difference at $P < 0.05$.

Table 5. Spore germination and gametophyte growth of *Botrychium dissectum* spores after 4 months on filter sterilized media

	Nitrogen source and concentration (mol m^{-3})				
	$NaNO_3$			NH_4Cl	
	3.5	0.35	No nitrogen	3.5	0.35
Germination (%)	0	0	1.6	90.4	88.5
Gametophyte length (μm) $\pm$ SE*	—	—	61.0 ± 1.8^a	144.8 ± 6.9^b	142.3 ± 5.8^b

*Values followed by the same letter show no significant difference at $P < 0.05$.

nation on media with a range of pH values after sterilization. Media containing either nitrate (3.5 mol m^{-3}) or ammonium (3.5 mol m^{-3}) and having six pH values in equal increments from 6.5–4.0 were formulated. Germination was less than 0.01% on the nitrate media and above 90% on the ammonium media (data not shown).

Because a variety of ammonium and nitrate salts were employed in our experiments, it suggested that the cations, other than ammonium, and the anions, other than nitrate, were having little effect on germination. In order to confirm this point, media containing excess chloride (3.5 mol m^{-3} KCl or 1.75 mol m^{-3} $CaCl_2$) or excess sodium (3.5 mol m^{-3} NaCl or 1.75 mol m^{-3} Na_2SO_4) in the basic medium without nitrogen were formulated. Germination percentages on these media were essentially the same as those obtained previously on media lacking nitrogen.

The growth of older gametophytes on various nitrogen sources was examined to determine if it was similar to that of young gametophytes. Equivalent-sized gametophytes ($\simeq$ 1 mm) from 8-month-old cultures containing both ammonium and nitrate were measured and transferred to media containing ammonium, nitrate, or both ammonium and nitrate. A medium without nitrogen was included as a control. At the end of the experiment, the gametophytes were remeasured and the per cent increase in length was calculated. These results are presented in Table 6. All gametophytes on media containing a nitrogen source showed increases in length greater than those grown on the medium without nitrogen. The greatest increase in length was obtained on the medium containing both ammonium and nitrate.

Table 6. Per cent increase in length $\pm$ SE* of 8-month-old *Botrychium dissectum* gametophytes transferred to media containing various nitrogen sources for 5 months

Nitrogen source†			
None	Nitrate	Ammonium	Both
166.5 ± 9.1^a	526.0 ± 32.0^b	518.9 ± 24.9^c	695.7 ± 38.4^c

*Values followed by the same letter show no significant difference at $P < 0.05$.

†[ammonium] = 12.5 mol m^{-3}; [nitrate] = 12.5 mol m^{-3}; experiment with both ammonium and nitrate supplied 6.3 mol m^{-3} of each.

Gametophyte growth on the nitrate medium was, however, essentially the same as that on media containing ammonium. In this experiment with older gametophytes, the nitrate concentrations were higher than those which were found to inhibit the growth of young gametophytes. In longer experiments, *Botrychium* gametophytes transferred to media with nitrate as the sole nitrogen source have remained alive and continued to grow for up to 2 years (data not shown).

Discussion

In this investigation, we have shown that the nitrogen composition of nutrient media strongly affects the germination of *Botrychium dissectum* spores. Generally, less than 11% of the spores germinate on a medium without nitrogen. Nitrate and nitrite inhibit germination and at higher concentrations can prevent it. Ammonium (0.035–3.5 mol m^{-3}) promotes germination above the level occurring on the medium without nitrogen. On media containing the higher concentrations of ammonium, 90% or more of the spores germinate. Mixtures of ammonium and nitrate, which give intermediate levels of germination, demonstrate the opposing influences that ammonium and nitrate have on germination.

The influences that ammonium and nitrate have on germination are not related to methods of media sterilization, pH differences, or the ions supplied in conjunction with ammonium or nitrate. Media with filter-sterilized ammonium or nitrate at the same pH gave the same percentages of germination as autoclaved media. Variations in the initial pH levels of the media containing ammonium or nitrate did not alter the germination percentage. Chloride, the anion usually supplied with ammonium, did not promote germination and sodium ions, usually supplied with nitrate, did not inhibit germination.

Ammonium gave improved germination of *Botrychium* spores over the nitrogen sources previously employed (Whittier, 1973, 1984). The highest germination percentage obtained previously on Knudson's solution of mineral salts, which contains both ammonium and nitrate, was 18.9% after 4 months (Whittier, 1973). With ammonium alone, the best germination in this study was 93% in 7 weeks which is almost a five-fold increase in less than half the time.

The growth of young gametophytes from spores was greatest on media containing ammonium. Nitrate, except at low concentrations, did not promote early development. In an experiment examining the antagonistic effects of nitrate and ammonium on early gametophyte growth, nitrate was added to media with ammonium concentrations which promote germination and growth. Except at the lowest concentrations of nitrate, which were without effect on growth, nitrate inhibited early gametophyte growth in the presence of ammonium.

Older gametophytes showed an ability to utilize nitrate at concentrations which were inhibitory to the growth of young gametophytes. In a 5-month experiment, the gametophytes supplied with nitrate showed a greater growth increase than those on media without nitrogen and essentially the same growth as gametophytes on media containing only ammonium. In some experiments, the gametophytes have continued to grow for up to 2 years on media containing only nitrate.

The ability of the gametophytes to utilize nitrate effectively appeared to change with age. Concentrations of nitrate which either did not promote or inhibited the growth of young *Botrychium* gametophytes promoted the growth of older gametophytes. This increased growth suggests that the older gametophytes are able to use nitrate to satisfy their nitrogen requirements for growth. Similar observations have been made with orchid seedlings. Raghavan & Torrey (1964) reported that *Cattleya* seedlings require ammonium for their early development. However, under appropriate conditions, the later growth of these seedlings could be rapid on media containing nitrate. In addition, Burgeff (1936) concluded that reduced nitrogen, ammonium, was the optimal nitrogen source for the non-photosynthetic phase of young orchid seedlings. *Botrychium* gametophytes appear to exhibit a similar condition.

The non-photosynthetic gametophytes of pteridophytes depend on mycorrhizal fungi for their organic nutrition. These gametophytes can be considered parasitic on the fungi (Lewis, 1973a; Wagner, Wagner & Beitel, 1985). Since the carbon nutrition of these gametophytes is heterotrophic, it is possible that other aspects of their metabolism are heterotrophic as well. Lewis (1973b) postulated that higher plants which are associated with mycorrhizal fungi are heterotrophic for nitrogen. In support of this postulation, it has been demonstrated that mycorrhizal fungi supply amino acids to the roots of some trees (Lewis, 1975). It is possible that the nitrogen requirements of *Botrychium* gametophytes in nature are met with organic nitrogen from their mycorrhizal fungi. If the mycorrhizal fungi supply amino acids to the gametophytes under natural conditions, then they would be expected to show an increased or exclusive ability to assimilate reduced forms of nitrogen. Under such circumstances, it would not be surprising for young gametophytes to initially have a reduced ability to assimilate nitrate.

There are certain plants which prefer ammonium as opposed to nitrate for their nitrogen source (Haynes & Goh, 1978). In some of these plants nitrate can have toxic effects. For *Vaccinium*, the toxicity appears to be related to a reduced activity of nitrate reductase in their tissues (Foy, Chaney & White, 1978). It is possible that the inhibitory effect that nitrate has on spore germination and early gametophyte development in *Botrychium* may be related to deficiencies in nitrate reductase as well.

Photosynthetic fern gametophytes typically show developmental abnormalities when ammonium is supplied as the nitrogen source (Miller, 1968). However, ammonium is an excellent nitrogen source for the non-photosynthetic, mycorrhizal gametophytes of *Psilotum* in axenic culture (Whittier, 1989). With *Psilotum*, nitrate inhibits germination but ammonium does not stimulate germination as it does with *Botrychium*. Nitrate has a greater inhibitory effect on the growth of *Psilotum* gametophytes and these gametophytes have difficulty in developing the ability to assimilate nitrate for growth.

Ammonium may play a role in the germination of *Botrychium* spores in nature. Ammonium in the soil would be expected to stimulate the germination of buried spores. Root exudation, the ammonification of organic matter and animal excreta could increase the level of ammonium in the soil. From these or other soil processes, sufficient ammonium could be present in certain areas of the soil to stimulate the germination of *Botrychium* spores. Thus, it is conceivable that ammonium availability may be an important factor in determining when and where *Botrychium* spores germinate in the soil.

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