# THE PHOSPHATE REQUIREMENT OF THE FERNS NOTHOLAENA COCHISENSIS AND NOTHOLAENA SINUATA

by

Cleon Duane Ferrell

---

A Thesis Submitted to the

COMMITTEE ON AGRICULTURAL BIOCHEMISTRY AND NUTRITION

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

1965

STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Cleon Duane Ferrell

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

A R Kemmerer 8/4/65
Arthur R. Kemmerer
Professor of Agricultural Biochemistry

Aug 5 1965
Date

## ACKNOWLEDGMENTS

The author expresses sincere gratitude:

To Dr. E. B. Kurtz, Jr. for the topic of this thesis.

To Drs. A. R. Kemmerer, K. Matsuda, and M. Zaitlin for counsel and guidance during the course of this study and in preparation of this thesis.

TABLE OF CONTENTS

# LIST OF TABLES

LIST OF FIGURES

ABSTRACT

In 1963 it was reported that N. cochisensis would grow equally well on both Knop's and Prantl's agar media while N. sinuata would grow only on the former medium. Since Prantl's medium lacks the phosphate ion and contains an excess of the sulfate ion, it was postulated that N. cochisensis has a very low phosphate requirement or is able to substitute the sulfate ion for the phosphate ion. The purpose of this thesis was to show the lowest level of phosphate required by both ferns and to show the influence of the sulfate ion on the amount of growth.

Experimentally, prothallia of both species were cultured on agar media that contained various amounts of phosphate and sulfate. Each agar medium was analyzed for phosphate, and the growth responses were determined by photographic and fresh weight studies.

When the results of these studies were obtained, it was found that N. sinuata, as well as N. cochisensis, would grow on Prantl's medium. In both cases the ferns grown on Prantl's medium had a lower fresh weight than those grown on Knop's medium. Under the experimental conditions used, both ferns required 0.8 mM $HPO_4$ and 2.7 mM $SO_4$ plus the other ions of Knop's solution for maximal growth. No prothallial growth was evident on media containing no phosphate and sulfate. Both fern species showed some growth on media containing no phosphate but high amounts of sulfate. On these media both ferns had larger cells than those

grown on media lacking phosphate and sulfate. When both ferns were grown on adequate amounts of phosphate, there was an increase in cell size and number.

# INTRODUCTION

In 1959 Hevly (6) reported that the xerophytic rock fern, Notholaena cochisensis Goodding, would grow equally well on both Knop's (2) and Prantl's (2) media; however, a sister species in the Notholaena complex, Notholaena sinuata (Lagasca ex Swartz) Kaulfuss, would die if grown on the latter medium. Knop's solution is a balanced nutrient solution while Prantl's solution lacks the phosphate ion and has an excess of sulfate ion((2). The results of Hevly's experiment indicate that the phosphate requirement of N. cochisensis is less than that of N. sinuata. Since there are no reports in the literature concerning the phosphate requirement of these ferns, an experiment was designed to determine the lowest level of phosphate ion necessary to sustain normal growth. In addition, there is a suggestion that sulfate may substitute partially for phosphate in plants (9). A study also was made to determine if growth of ferns could occur on phosphate deficient but sulfate containing media.

## REVIEW OF LITERATURE

The Notholaena ferns used in this thesis are usually found growing on north or west-facing slopes of rock ledges, cliffs, canyon walls, boulders, or on thin, rocky soils. N. cochisensis normally grows on calcareous sandstone or limestone in which the pH is neutral or slightly basic whereas N. sinuata grows on igneous and metamorphic rocks such as gneiss, quartsite, or granite in which the pH is neutral or slightly acidic (5,6). In these habitats, the ferns often are exposed to excesses or deficiencies of water and various minerals (6). Since the two fern species grow well under widely different and sometimes harsh conditions, their nutrient requirements may be very dissimilar. Hevly's studies with nutrient media cultures suggest that this is, in fact, the case; the phosphate requirement of N. cochisensis appears to be considerably less than N. sinuata.

Solution culture techniques have been used previously to assess the nutrient requirements of ferns. In 1920 Brown (2) reported that Biejernck's, Birner and Lucanus's, Knop's, Prantl's, and Sach's solutions were favorable for growth of the Polypodiacea ferns. She also stated that Knop's and Prantl's solutions were best suited for prothallia growth. Since both Brown and Hevly showed that some fern species could grow on phosphate deficient but sulfate containing media, it appears likely that some sulfate replacement of phosphate can occur, or there are phosphate-sulfate interactions which are not understood.

Various interactions of phosphate and sulfate have been shown to exist in plants. Coic, Lesaint, and Grandjean (3) found that although the grain and straw yields were less in phosphate deficient barley, the uptake of sulfate was greater than in plants grown on phosphate containing nutrient solutions. Also, in higher plants, Fujiwara and Torii (4) showed that when sulfate is lacking, the amount of phosphate is increased. On New Zealand pastures deficient in sulfate and phosphate Lamerink (10) found that the yield of cocksfoot could be increased by the addition of phosphate or sulfate, but timothy yields could be increased only with sulfate. By adding sulfate and phosphate together, large interactions that increased the herbage yield of white clover and ryegrass were observed. A similar effect of sulfate and phosphate was also observed by Takahashi et al. (12). They found that the two ions enhanced the stimulatory effect of gibberellin on germination of N. tabacum seeds. In view of the phosphate-sulfate interactions, it appears likely that sulfate can partially replace phosphate in higher plants.

To show that this substitution may exist, Wald (13) states that sulfate and phosphate are similar in that they have weak, wide-spaced bonds which are vulnerable to attack by other molecules that promote exchange reactions.

Kurtz and Waldon (9) studied the utilization of sulfate in compounds which normally contain phosphate. Using the oat plant, they have shown that a sulfated sugar(s) was formed when the oat was grown for three weeks on a phosphate deficient medium. Therefore, besides phosphorylated sugars, at least one sulfated sugar can exist in higher plants.

Sulfate has also been shown to occur in high energy compounds. Bandurski, Wilson, and Squires (1) showed that an enzyme system exists in yeast that combines sulfate with ATP[1] to form PAPS. This system was found to contain two enzymes: ATP sulfurase, which catalyzes the formation of APS from ATP and sulfate, and APS kinase, which uses ATP to convert APS to PAPS. Although the existence of high energy sulfate compounds has been shown in yeast and higher plants, substitution for high energy phosphate compounds has not yet been established.

In view of the findings that sulfate can be used in phosphorylated intermediates and in high energy compounds, one is led to speculate that sulfate may partially substitute for the phosphate requirement in some organisms. Therefore, in designing media 1 to 6 to determine the lowest phosphate level required by the ferns, amounts of $K_2HPO_4$ were omitted and equal amounts of $K_2SO_4$ were added to keep the amount of K constant and to show the influence of sulfate.

## EXPERIMENTAL METHODS

The media used in this study were designed to determine the lowest phosphate level required by the ferns and to show the influence of sulfate. Large quantities of nutrient agar media were prepared, and 10 ml aliquots containing 0.2 g agar were transferred to 2 x $\frac{1}{2}$ inch petri dishes. All solutions except No. 11 contained, in each liter, 2 ml of Kurtz's minor

---

[1] Abbreviations used are: PAPS, adenosine-3' -phosphate-5'-phosphosulfate; APS, adenosine-5'-phosphosulfate; $P_i$, orthophosphate; ATP, adenosine triphosphate.

element solution (8) and 1 drop of 1% $FeCl_3$. The pH values of each solution were adjusted to 7.5 with 2N NaOH. Eleven different solutions were used, and the compositions are the following:

Solution 1 (Knop's solution)

| Salt | Concentration of Salt | |
|---|---|---|
| | g/liter | mM |
| $MgSO_4$ | 0.25 | 2.1 |
| $Ca(NO_3)_2.4H_2O$ | 1.00 | 4.2 |
| KCl | 0.12 | 1.6 |
| $K_2HPO_4$ | 0.25 | 1.4 |
| 0.65 ml 2 N NaOH | | 1.2 |
| Total | | 10.5 |

Solution 2

| Salt | Concentration of Salt | |
|---|---|---|
| | g/liter | mM |
| $MgSO_4$ | 0.25 | 2.1 |
| $Ca(NO_3)_2.4H_2O$ | 1.00 | 4.2 |
| KCl | 0.12 | 1.6 |
| $K_2HPO_4$ | 0.20 | 1.2 |
| $K_2SO_4$ | 0.05 | 0.3 |
| 0.50 ml 2 N NaOH | | 1.0 |
| Total | | 10.4 |

Solution 3

| Salt | Concentration of Salt | |
|---|---|---|
| | g/liter | mM |
| $MgSO_4$ | 0.25 | 2.1 |
| $Ca(NO_3)_2.4H_2O$ | 1.00 | 4.2 |
| KCl | 0.12 | 1.6 |
| $K_2HPO_4$ | 0.15 | 0.8 |
| $K_2SO_4$ | 0.10 | 0.6 |
| 0.40 ml 2 N NaOH | | 0.8 |
| Total | | 10.1 |

Solution 4

| Salt | Concentration of Salt | |
|---|---|---|
| | g/liter | mM |
| $MgSO_4$ | 0.25 | 2.1 |
| $Ca(NO_3)_2.4H_2O$ | 1.00 | 4.2 |
| KCl | 0.12 | 1.6 |
| $K_2HPO_4$ | 0.10 | 0.6 |
| $K_2SO_4$ | 0.15 | 0.8 |
| 0.30 ml 2 N NaOH | | 0.6 |
| Total | | 9.9 |

Solution 5

| Salt | Concentration of Salt | |
|---|---|---|
| | g/liter | mM |
| $MgSO_4$ | 0.25 | 2.1 |
| $Ca(NO_3)_2.4H_2O$ | 1.00 | 4.2 |
| KCl | 0.12 | 1.6 |
| $K_2HPO_4$ | 0.05 | 0.3 |
| $K_2SO_4$ | 0.20 | 1.2 |
| 0.20 ml 2 N NaOH | | 0.4 |
| Total | | 9.8 |

Solution 6

| Salt | Concentration of Salt | |
|---|---|---|
| | g/liter | mM |
| $MgSO_4$ | 0.25 | 2.1 |
| $Ca(NO_3)_2.4H_2O$ | 1.00 | 4.2 |
| KCl | 0.12 | 1.6 |
| $K_2SO_4$ | 0.25 | 1.4 |
| 0.10 ml 2 N NaOH | | 0.2 |
| Total | | 9.5 |

Solution 7 (Prantl's solution)

| Salt | Concentration of Salt | |
|---|---|---|
| | g/liter | mM |
| $MgSO_4$ | 0.50 | 4.2 |
| $K_2SO_4$ | 0.70 | 4.0 |
| NaCl | 0.23 | 4.0 |
| $CaSO_4.2H_2O$ | 0.70 | 4.0 |
| $NH_4NO_3$ | 0.01 | 0.2 |
| 0.05 ml 2 N NaOH | | 0.1 |
| Total | | 16.5 |

Solution 8

| Salt | Concentration of Salt | |
|---|---|---|
| | g/liter | mM |
| $MgSO_4$ | 0.25 | 2.1 |
| $Ca(NO_3)_2.4H_2O$ | 1.00 | 4.2 |
| KCl | 0.12 | 1.6 |
| $K_2HPO_4$ | 0.25 | 1.4 |
| $Na_2SO_4$ | 0.17 | 1.2 |
| 0.60 ml 2 N NaOH | | 1.1 |
| Total | | 11.6 |

Solution 9

| Salt | Concentration of Salt | |
|---|---|---|
| | g/liter | mM |
| $MgSO_4$ | 0.25 | 2.1 |
| $Ca(NO_3)_2.4H_2O$ | 1.00 | 4.2 |
| KCl | 0.12 | 1.6 |
| $K_2HPO_4$ | 0.25 | 1.4 |
| $K_2SO_4$ | 0.25 | 1.4 |
| 0.75 ml 2 N NaOH | | 1.5 |
| Total | | 12.2 |

Solution 10

| Salt | Concentration of Salt | |
|---|---|---|
| | g/liter | mM |
| $MgCl_2.6H_2O$ | 0.68 | 3.4 |
| $Ca(NO_3)_2.4H_2O$ | 1.00 | 4.2 |
| KCl | 0.22 | 2.9 |
| 0.10 ml 2 N NaOH | | 0.1 |
| Total | | 10.6 |

Solution 11

One liter deionized water and 0.1 ml 2 N NaOH

Table 1 shows a comparison of the total salt and ion concentrations of the nutrient media. Medium 1 is Knop's solution plus agar and is used as the first medium in a concentration series that has varying amounts of phosphate. Since Hevly (6) reported that both ferns grew well on Knop's medium, its phosphate concentration, 1.4 mM, was the maximum used in this study.

Media 2 to 6 represent the other media in the concentration series of varying amounts of phosphate; they were used to determine the lowest level of phosphate required for maximum growth. In their preparation the amounts of $K_2HPO_4$ and $K_2SO_4$ were regulated to maintain a constant potassium concentration, and the pH values were adjusted to pH 7.5. As shown in Table 1, the phosphate concentration was ultimately lowered to 0.0 mM in solution 6, but the sulfate concentration was increased to 3.5 mM.

Medium 7 is Prantl's solution plus agar. This medium was prepared to confirm Hevly's statement (5, 6) that N. sinuata dies and N. cochisensis lives when grown on it. It differs from media 1 to 6 in that it has no phosphate, but its sulfate content is a value 3 to 4 times higher than in the former solutions.

In this experiment media 8, 9, 10, and 11 were used as control media. Media 8 and 9 contain 3.5 mM sulfate with excess Na and K. By adding $Na_2SO_4$ and $K_2SO_4$ to medium 1, these media were prepared to show that any possible changes in the amount of growth on media 1 to 6 was not due to a harmful effect of increasing amounts of sulfate.

Table 1

Comparison of Total Salt and Ion Concentrations of the Nutrient Media (mmoles/liter)

| Sol. No. | Salt | Na | K | Ca | Mg | $NO_3$ | $HPO_4$ | $SO_4$ | Cl |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 10.5 | 1.2 | 4.4 | 4.2 | 2.1 | 8.4 | 1.4 | 2.1 | 1.6 |
| 2 | 10.4 | 1.0 | 4.6 | 4.2 | 2.1 | 8.4 | 1.2 | 2.4 | 1.6 |
| 3 | 10.1 | 0.8 | 4.4 | 4.2 | 2.1 | 8.4 | 0.8 | 2.7 | 1.6 |
| 4 | 9.9 | 0.6 | 4.4 | 4.2 | 2.1 | 8.4 | 0.6 | 2.9 | 1.6 |
| 5 | 9.8 | 0.4 | 4.6 | 4.2 | 2.1 | 8.4 | 0.3 | 3.3 | 1.6 |
| 6 | 9.5 | 0.2 | 4.4 | 4.2 | 2.1 | 8.4 | 0.0 | 3.5 | 1.6 |
| 7 | 16.5 | 4.1 | 8.0 | 4.0 | 4.2 | 0.2 | 0.0 | 12.2 | 4.0 |
| 8 | 11.6 | 3.5 | 4.4 | 4.2 | 2.1 | 8.4 | 1.4 | 3.5 | 1.6 |
| 9 | 12.2 | 1.5 | 7.2 | 4.2 | 2.1 | 8.4 | 1.4 | 3.5 | 1.6 |
| 10 | 10.6 | 0.1 | 2.9 | 4.2 | 3.4 | 8.4 | 0.0 | 0.0 | 9.7 |
| 11 | 0.1 | 0.1 | --- | --- | --- | --- | --- | --- | --- |

Medium 10 was used to demonstrate growth responses when both sulfate and phosphate are absent. Similarly, medium 11 was used to show growth responses on agar when no nutrients were added.

Using the method of Kitson and Mellon (7), phosphate analyses were performed on the media and the spores. This method was selected because the sensitivity is 1 ppm, the color reaction is stable, and interference from other ions in the medium is negligible. To prepare a nutrient medium for analysis, a 25 ml sample of the nutrient solution containing agar was pipetted into a crucible. Ten replicate analyses were run from each nutrient medium. After carefully drying the water from the samples in the crucibles by placing them in a drying oven, the samples were ignited and ashed at 500°. The contents of the crucibles were taken up in 10 ml of 1 N $HNO_3$ and quantitatively transferred to 100 ml volumetric flasks. The flasks were diluted to volume, and 25-35 ml aliquots containing 10-200 µg $HPO_4$ were transferred to 50 ml volumetric flasks. Five milliters each of 7.5 N $HNO_3$, 0.02 M $NH_4VO_3$, and 0.07 M $(NH_4)_6Mo_7O_{24}.4H_2O$ were added in order, and the flasks were brought to volume with deionized water and shaken. After one-half hour, the absorbance of the sample was determined with a Beckman DU spectrophotometer at the wavelength of maximum absorption, 365 mµ. The results were compared to standard phosphate solutions, prepared and analyzed at the same time. An example of the standard phosphate curve is shown in Figure 1.

The results of the phosphate analyses on the media containing agar are shown in Table 2. In all cases the phosphate found by analyses was nearly equal to the theoretical amount added by the nutrient solution alone.

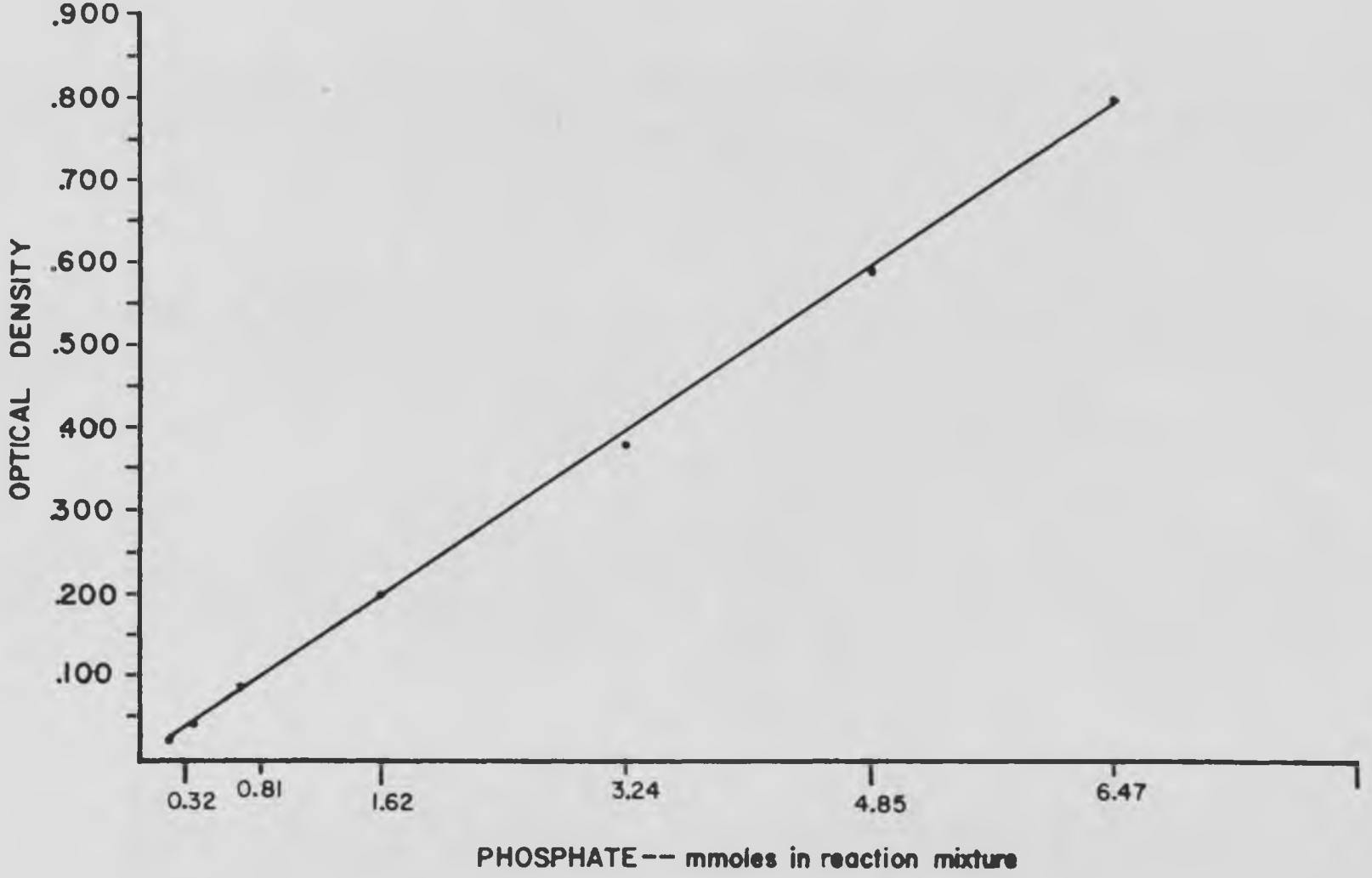


Figure 1. Standard Curve for Phosphate Analyses by the Kitson-Mellon Method.

Table 2

Phosphate Content of Nutrient Media[a] (mM)

| Media No. | Theoretical in medium (mM) | Analyses of samples[b] (mM) |
|---|---|---|
| 1 | 1.43 | 1.42 |
| 2 | 1.15 | 1.16 |
| 3 | 0.86 | 0.89 |
| 4 | 0.58 | 0.52 |
| 5 | 0.29 | 0.27 |
| 6 | 0.00 | 0.05 |
| 7 | 0.00 | 0.03 |
| 8 | 1.44 | 1.55 |
| 9 | 1.44 | 1.41 |
| 10 | 0.00 | 0.04 |
| 11 | 0.00 | 0.02 |

[a] Nutrient media indicates nutrient solution plus agar.

[b] Indicates average amount of 10 replicates on the medium.

For example, 1.43 mmoles $HPO_4$ was added to each liter of medium 1; the average amount found was 1.42 mM. As shown in Table 2, media 6, 7, 10, and 11 contained no added phosphate, but small amounts were found. For example, in medium 6, 0.04 mM was found. This small amount of phosphate can probably be attributed to errors in analytical procedures.

Since the spores themselves may have high levels of endogenous phosphorus, their contribution to the medium was also determined. When 45.8 mg of N. cochisensis spores, representing approximately 2800 spores, were analyzed for phosphate, 0.233 mmoles $HPO_4$ was found. By using 58 as the average number of germinating spores present in each dish, it was calculated that $4.1 \times 10^{-3}$ mM $HPO_4$ was contributed by the spores. Therefore, the amount of phosphate contributed by N. cochisensis is insignificant.

Following a method described by Hevly (6), spores of both Notholaena species were obtained from fertile fronds that were collected in the Tucson area. The spores were placed in vials and stored in the dark to prevent premature germination (5, 6). The ferns were identified by Dr. Hevly and are on file at the University of Arizona Herbarium.

A method of spore sterilization (14) was obtained from the literature and modified. By using disposable transfer pipettes, approximately 100 mg of spores were placed on glass wool in the bottom of a 7 x $\frac{1}{2}$ inch chromatographic column that had been sterilized previously. The glass wool allowed liquids to drain but did not allow the spores to pass through. To prevent clumping, the spores were treated by immersing the column in sterile 0.1% Tween 80 for 20 minutes. This was followed

by exposing the spores to 30% Chlorox[1] for 20 minutes. The spores were then rinsed once with sterile, deionized water and finally suspended in 35 ml of sterile, deionized water. Sterile petri dishes, containing different nutrient agar media, were inoculated with 0.3 ml of the spore suspensions. This gave an average number of 58 and 65 germinating spores for N. cochisensis and N. sinuata, respectively. After inoculation the petri dishes were kept in a culture room, maintained at 30°, and received continuous light intensities of 700-900 ft-c from banks of cool white, fluorescent lights. The light intensities were measured with a Weston 603 photometer. Sterile, deionized water (0.2-0.3 ml) was added at least once a week to maintain the moisture content of the agar media. Since the light intensities varied from 700-900 ft-c on the growing table, the dishes were rearranged after each addition of water.

The fresh weight of the prothallial contents of each dish was determined by first loosening the prothallia from the agar with water. When the prothallia were freed from the agar, they were collected on 4 cm Whatman No. 1 filter paper in a small Buchner funnel, removed with a spatula or tweezers, and placed in a small, tared vial. The surface of the agar media and filter papers were checked with a dissecting microscope to be sure that all prothallia and their rhizoids were added to the vial. If the prothallia or the vial had drops of water on their surface, the fresh weight was not measured. Once the initial prothallial weights

---

[1] Chlorox is the commercial name for 5.25% NaOCl (by weight).

were obtained, fresh weight measurements were made every 5 days until the thirty-fifth day after germination.

The morphological changes which occurred during growth were recorded on 35 mm slide pictures, taken through a 10X lens of a Zeiss 2083594 GFL microscope. The pictures were taken on Kodachrome II color slide film, using a Zeiss Ikon camera set at 1/25 of a second. A Zeiss LTKL Nr transformer set at 8 was used to control the light source, and the emitted light was filtered with a blue filter.

## RESULTS AND DISCUSSION

To avoid microbial growth, the agar was autoclaved, and the spores were sterilized with a disinfectant prior to germination. A 20 minute treatment with 30% Chlorox was found to be the maximum allowable treatment of the spores. The treatment removed most of the mold, bacterial, and algal contamination from the spore surface but did not eliminate the imperfect fungi Alternaria, Diplodia, and Sporotrichum. The Chlorox treatment also had a harmful effect on the spores since some did not germinate. In addition, the amount of growth of the prothallia was less than when spores were given shorter exposures to Chlorox.

When the spores of both Notholaena species were placed on nutrient media and exposed to continuous light intensities of 700 to 900 ft-c, germination occurred on the sixth day after inoculation. In both species the morphological changes which occurred after germination were similar to those of the bracken fern Pteridium aquilinum (11). After imbibing water, the spores ruptured, and a green, unseptate filament and one rhizoid emerged. Within a few days the filament became septate, and other rhizoids formed on it. Isolated prothallia would undergo further development by producing a platelet from the filament. These platelets continued to develop in an irregular shape because of continuous, high light intensities (5, 6). When the prothallia were crowded, the filaments did not develop into platelets.

The results of fresh weight studies showed that N. cochisensis and N. sinuata responded similarly to different nutrient media. This is shown in Figures 2, 3, 4, and 5. For both species a fifteen day period after the onset of germination was required before prothallial weights could be obtained on media 1, 2, 3, 4, 7, 8, and 9. With the exception of medium 7, Prantl's medium, these media had at least 0.6 mM phosphate and 2.1 mM sulfate. In contrast a 20 day period was needed on media 5 and 6, which contained 3.3 and 3.5 mM sulfate and 0.3 and 0.0 mM phosphate, respectively, before either species yielded sufficient prothallia for weighing. On media 10 which has no sulfate or phosphate and media 11 which has no nutrients, the prothallial weights were insufficient for determination even after growing 35 days.

The results from media 1 and 7 differ in two ways from those of Hevly (6), who reported that N. cochisensis would grow equally well on both Knop's and Prantl's agar medium and N. sinuata would not grow on Prantl's medium. First, N. cochisensis did not grow equally well on Knop's and Prantl's media, and, secondly, N. sinuata did grow when placed on Prantl's medium.

When spores were placed on media 10 and 11, the prothallia germinated at the same time as those grown on more balanced media. The amount of growth on medium 11 shows that certain nutrients required for normal growth are absent in agar alone while the amount of growth on medium 10 shows that phosphate and sulfate cannot both be excluded from the agar medium.

Figures 2 and 3 show that N. cochisensis and N. sinuata grew similarly on nutrient media 1 to 3. Medium 1, Knop's medium, is a balanced nutrient medium with phosphate and sulfate contents of 1.4 mM and 2.1 mM, respectively. Medium 3 is similar to medium 1 except that the phosphate and sulfate concentrations are 0.8 and 2.7 mM, respectively. The fact that the growth of prothallia was approximately the same on these media suggests the level of phosphate in medium 3 is not limiting growth in this study.

Figures 2 and 3 also show that the amount of growth of both ferns on medium 4 was less than on media 1 to 3. In this medium the phosphate and sulfate contents were 0.6 mM and 2.9 mM, and the amounts of other ions were not varied from those on medium 1. Since approximately the same number of spores of each species were used per dish, it may be concluded that the requirements for phosphate of the two species are the same. The fact that growth on medium 4 was less than on medium 3 shows that sulfate cannot stoichiometrically replace phosphate. Medium 4 contains 0.6 mM phosphate and 2.9 mM sulfate whereas medium 3 contains 0.8 mM $HPO_4$ and 2.7 mM $SO_4$.

Figures 2 and 3 also show that the amounts of prothallial growth on media 5 and 6 are even less than on medium 4 and far less than on medium 1. Again, both species responded similarly. Media 5 and 6 differ from medium 1 by having 0.3 and 0.0 mM phosphate, respectively, instead of 1.4 mM phosphate; they also differ by having 3.3 and 3.5 mM sulfate, respectively, instead of 2.1 mM sulfate. From the amount of growth obtained on media 5 and 6, it may be concluded again that sulfate cannot

stoichiometrically replace phosphate. However, there was some growth on these media whereas no measurable growth was found using medium 10 which had all other ions except phosphate and sulfate. Since media 5 and 6 differ from medium 10 primarily in that they contain different amounts of sulfate for growth, there may be a partial replacement of phosphate by sulfate.

Figures 4 and 5 show both ferns responded quite well to medium 7, Prantl's medium. This medium differs from media 1 to 6 in that other ions, in addition to phosphate and sulfate, were varied. It is somewhat similar to the composition of medium 6 in that it has no phosphate, but the sulfate content is considerably higher than in the latter medium. When the amount of growth on Prantl's medium is compared to that of medium 10, the conclusion is similar to that obtained when media 6 and 10 are compared: the addition of sulfate causes a large increase in the amount of growth.

When the spores of both ferns were placed on media 8 and 9, the amount of growth was equal to that obtained from medium 1, as shown in Figures 4 and 5. Medium 8 contains the same ion content as medium 1 except that the Na content is 3.5 mM instead of 1.2 mM and the sulfate content is 3.5 mM instead of 2.1 mM. Since the amounts of growth on medium 8 and 9 were the same as on medium 1, excess sulfate is not harmful in the presence of excess Na or K. These results also show that the decrease in amounts of growth on media 4, 5, 6, and 7 is due to omission of amounts of phosphate in the medium and not due to changes in the Na or sulfate contents.

The morphological changes which occurred during growth are shown in Figure 6. Plate 1 shows a N. cochisensis prothallium on medium 1 twenty-five days after germination. After careful observation with a dissecting microscope, prothallia from media 2, 3, 8, and 9 were found to be indistinguishable from those on medium 1. The prothallia appear as broad platelets with many rhizoids.

Plates 2 and 3 of Figure 6 show prothallia of N. cochisensis and N. sinuata, respectively, on medium 4 twenty-five days after germination. Although the plates do not show equal growth of both species, microscopic examination revealed that N. cochisensis was not distinguishable from N. sinuata when their prothallia were compared. Prothallia of both ferns grown on medium 4, which contains 0.6 mM $HPO_4$, had about the same cell number as those grown on medium 1, but the cells were smaller. This suggests that cell enlargement rather than cell division was hindered by the low phosphate content.

Plate 4 of Figure 6 shows N. cochisensis prothallia on medium 7, Prantl's medium, twenty-five days after germination. The platelets are not quite as wide as those on media 1 and 4, and the cell number is less than those of prothallia grown on media 1 and 4. The cell size of prothallia on medium 7 is similar to those of prothallia from medium 4. Since the fresh weight studies showed that the decrease in amount of growth on media 4 and 7 was due to omission of phosphate, it may be concluded that adequate amounts of phosphate also increase the number of cells in a prothallium.

As shown in Plate 5 of Figure 6, N. cochisensis prothallia grown on media 5 and 6 are more filamentous than plate-like twenty-five days after germination. Prothallia grown on these media have approximately the same cell number as medium 7, but the cells are slightly smaller. The decrease in amount of growth on these media from that on medium 1 was shown to be a lack of phosphate. Therefore, the smaller increases in cell size and number can be attributed to the lack of phosphate. But, since the prothallial weights for both ferns were greater on medium 7 than on media 5 and 6, it may be concluded that sulfate also can influence cell enlargement.

Plate 6 of Figure 6 shows N. cochisensis prothallia on medium 10 thirty-five days after germination. Prothallia from medium 11 were not found to be significantly different from those on medium 10. In the absence of phosphate and sulfate the prothallia failed to develop a platelet or filament. The cell number in prothallia of both species grown on media 10 and 11 is not significantly different from that of prothallia grown on medium 7, but the cells were very small. This agrees with a conclusion derived in the previous paragraph; sulfate can influence cell enlargement.

In summary, the photographic studies and microscopic observations have been used to show that when prothallia of both ferns are grown on media containing adequate phosphate, the cell size and number does not decrease. The addition of sulfate to a phosphate-sulfate deficient medium also caused the cell size to be increased.

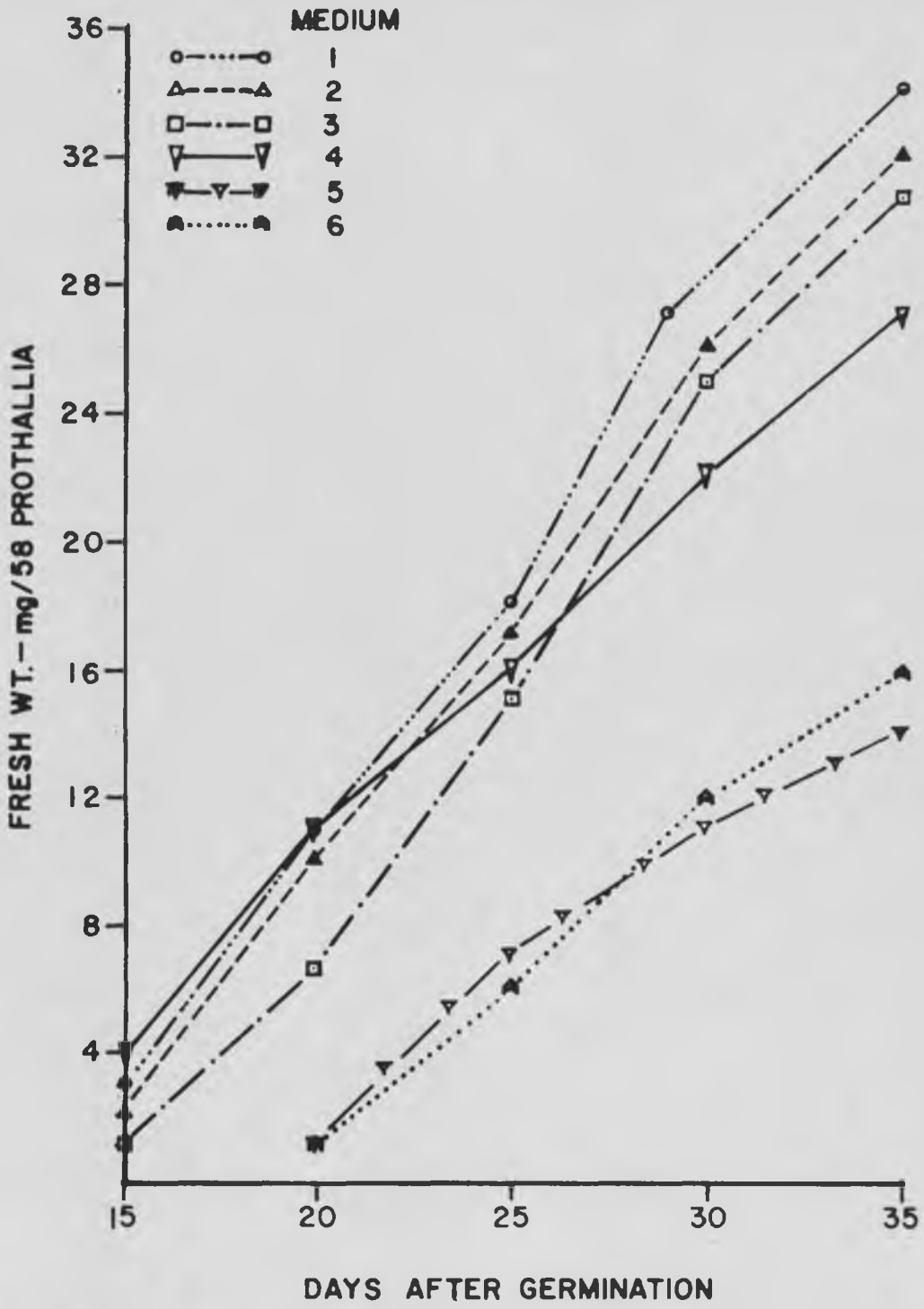


Figure 2. The fresh weight of N. cochisensis grown on various amounts of phosphate.

All media contain 4.4 mM K, 4.2 mM Ca, 2.1 mM Mg, 8.4 mM $NO_3$ and 1.6 mM Cl. The Na, $HPO_4$, and $SO_4$ concentration (mM) of the media are respectively: Medium 1, 1.2, 1.4, 2.1; Medium 2, 1.0.1.2, 2.4; Medium 3, 0.8, 0.8, 2.7; Medium 4, 0.6, 0.6, 2.9; Medium 5, 0.4, 0.3, 3.3; Medium 6, 0.2, 0.0, 3.5.

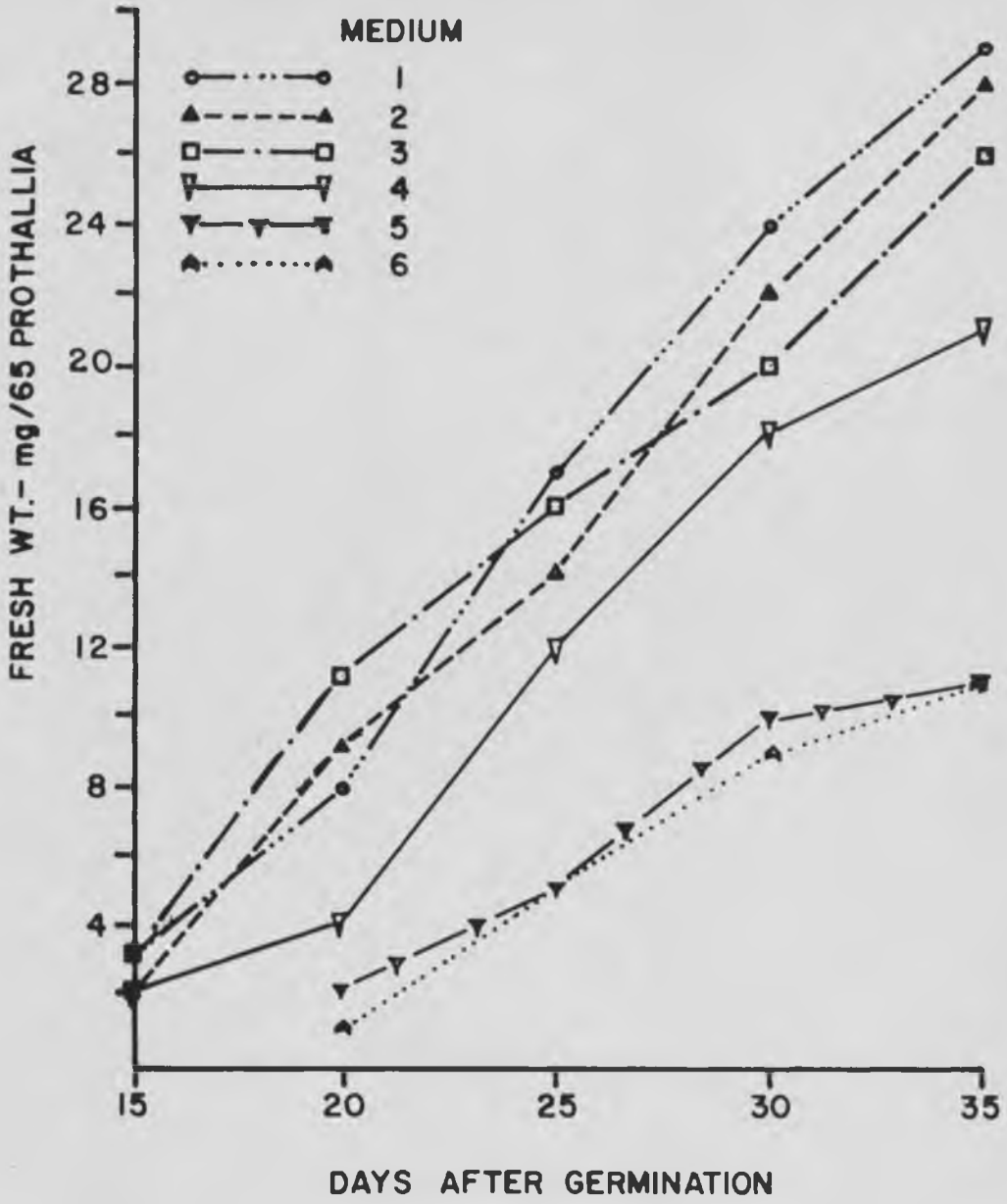


Figure 3. The fresh wieght of N. sinuata grown on various amounts of phosphate.

All media contain 4.4 mM K, 4.2 mM Ca, 2.1 mM Mg, 8.4 mM $NO_3$ and 1.6 mM Cl. The Na, $HPO_4$, and $SO_4$ concentrations (mM) of the media are respectively: Medium 1, 1.2, 1.4, 2.1; Medium 2, 1.0,1.2, 2.4; Medium 3, 0.8, 0.8, 2.7; Medium 4, 0.6, 0.6, 2.9; Medium 5, 0.4, 0.3, 3.3.; Medium 6, 0.2, 0.0, 3.3.

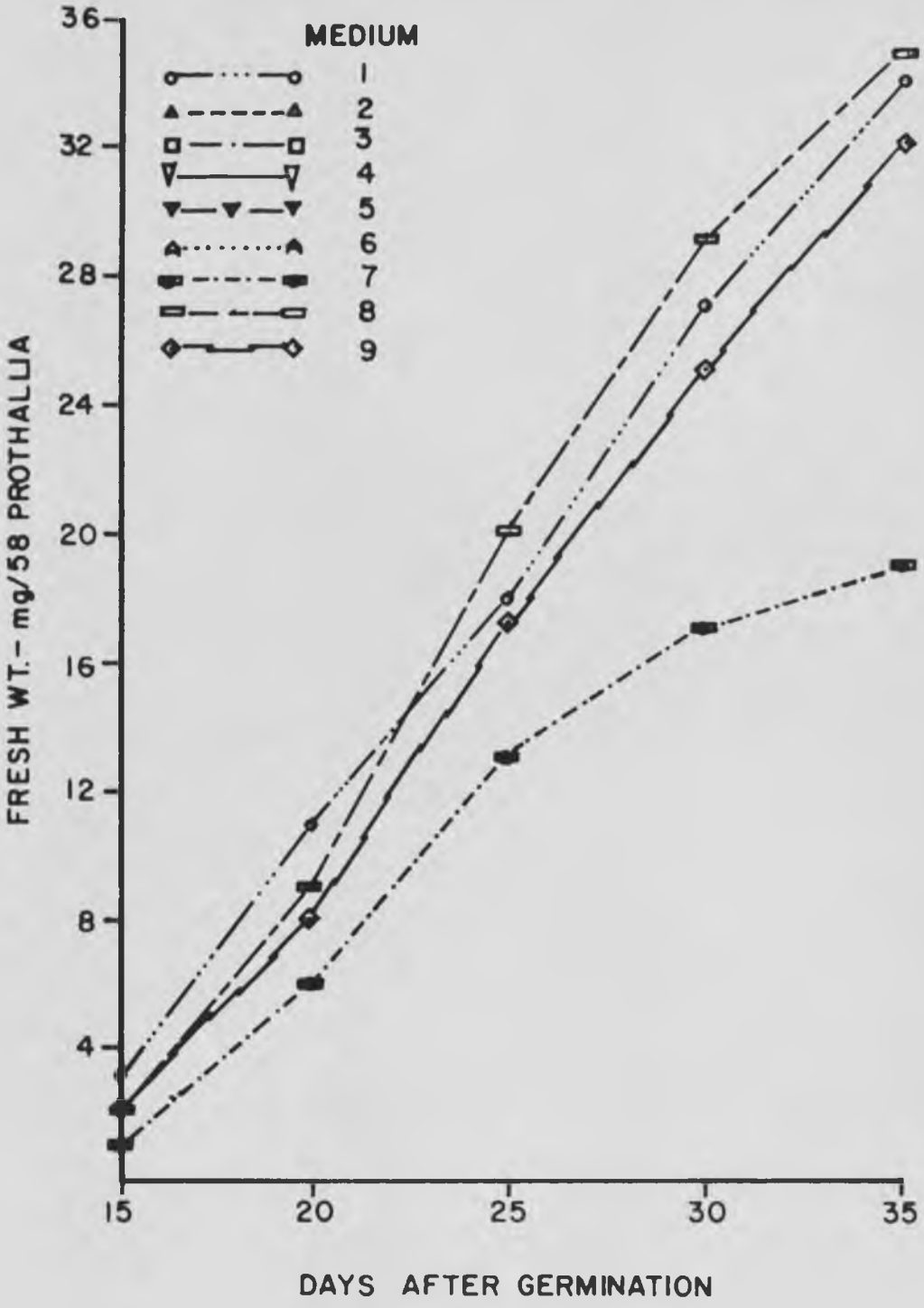


Figure 4. The fresh weight of N. cochisensis grown on media containing a deficiency or an excess of phosphate or sulfate.

Media 7, 8, 9, and 10 contain the following concentrations of ions (mM) respectively: Na, 4.1, 3.5, 1.5, 0.1; K, 8.0, 4.4, 7.2, 2.9; Ca, 4.0, 4.2, 4.2, 4.2; Mg, 4.2, 2.1, 2.1, 3.4; $NO_3$, 0.2, 8.4, 8.4, 8.4; Cl, 4.0, 1.6, 1.6, 9.7. The $HPO_4$ and $SO_4$ concentrations of the media (mM) are: 0.0, 1.4, 1.4, 0.0 and 12.2, 3.5, 3.5, 0.0. Medium 11 contains no nutrients. No growth curves were obtained for media 10 and 11.

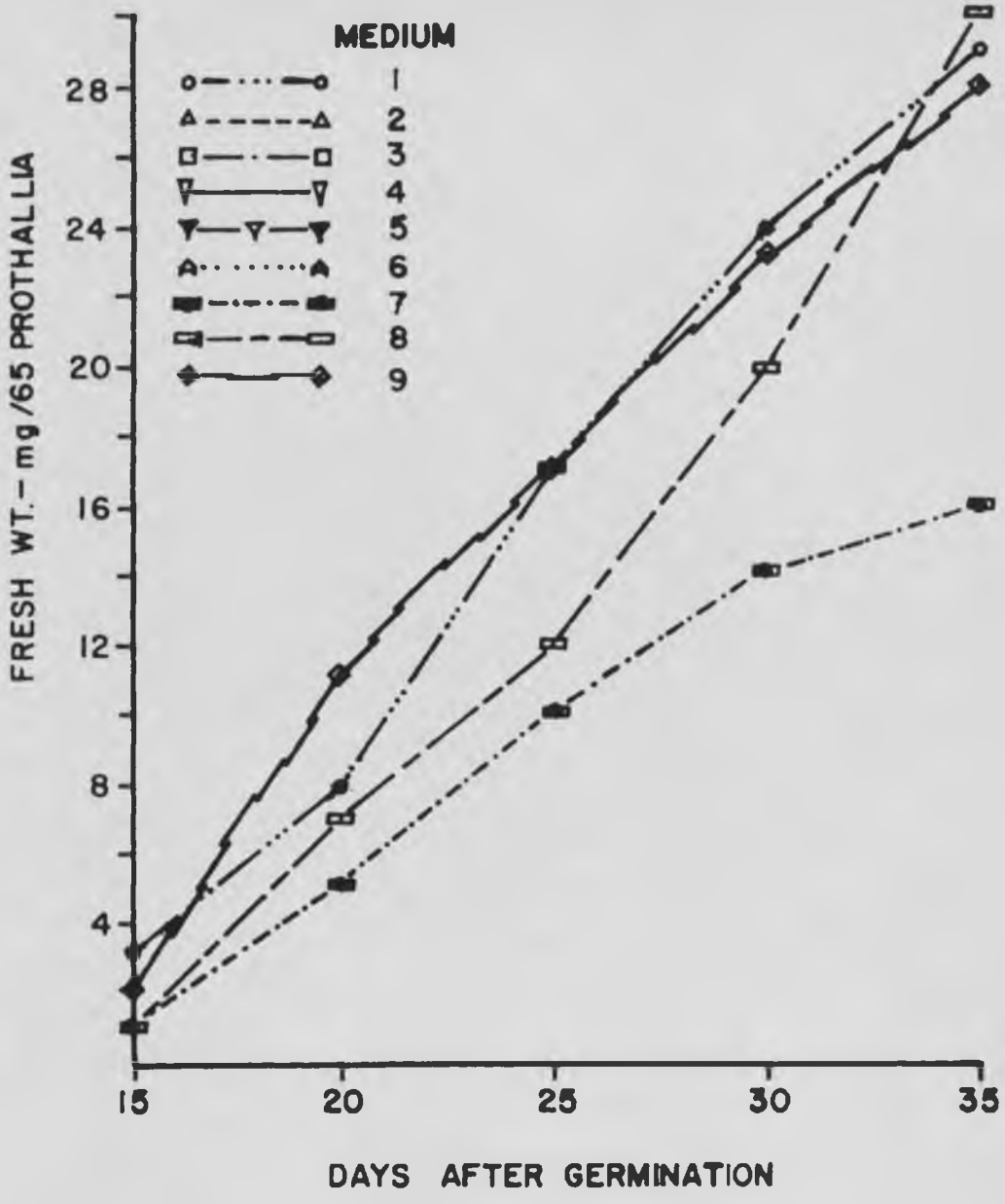


Figure 5. The fresh weight of N. sinuata grown on media containing a deficiency or an excess of phosphate or sulfate.

Media 7, 8, 9, and 10 contain the following concentrations of ions (mM) respectively: Na, 4.1, 3.5, 1.5, 0.1; K, 8.0, 4.4, 7.2, 2.9; Ca, 4.0, 4.2, 4.2, 4.2; Mg, 4.2, 2.1, 2.1, 3.4; $NO_3$, 0.2, 8.4, 8.4, 8.4; Cl, 4.0, 1.6, 1.6, 9.7. The $HPO_4$ and $SO_4$ concentrations of the media (mM) are: 0.0, 1.4, 1.4, 0.0, and 12.2, 3.5, 3.5, 0.0. Medium 11 contains no nutrients. No growth curves were obtained for media 10 and 11.

Plate 1. N. cochisensis prothallium on medium 1 twenty-five days after germination.

Plate 2. N. cochisensis prothallium on medium 4 twenty-five days after germination.

Figure 6. Plates of N. cochisensis and N. sinuata prothallia grown on different agar media.

Plate 3. N. sinuata prothallia on medium 4 twenty-five days after germination.

Plate 4. N. cochisensis prothallia on medium 7 twenty-five days after germination.

Figure 6.-- Continued.

Plate 5. N. cochisensis prothallia on medium 5 twenty-five days after germination.

Plate 6. N. cochisensis prothallia on medium 10 thirty-five days after germination.

Figure 6.-- Continued.

## SUMMARY

Prothallia of Notholaena cochisensis and Notholaena sinuata ferns were grown from spores and cultured on agar media that contained various levels of phosphate and sulfate. Photographic and fresh weight studies were used to measure their growth responses.

Using these studies, it was possible to show that the lowest level of phosphate required by the ferns is 0.8 mM in the presence of 2.7 mM sulfate and the other ions of Knop's solution.

When prothallia of both ferns were grown on media with low amounts of phosphate, the cell number and size would decrease. The addition of sulfate to phosphate-sulfate deficient media would increase the cell size.

In concluding, Hevly (5, 6) reported that N. cochisensis would grow equally well on Knop's and Prantl's media; however, N. sinuata would grow well on Knop's medium but would not grow on Prantl's medium. These results were not verified; it was found that N. cochisensis did not grow equally well on both media, and N. sinuata would grow for at least two months if placed on Prantl's medium. The results obtained in this study do not rule out the possibility that sulfate can substitute for phosphate; in fact, they suggest that sulfate can substitute for phosphate, but further work is needed to establish this hypothesis as a fact.

## LITERATURE CITED


1. Bandurski, R. S., L. G. Wilson, and C. L. Squires. 1956. The mechanism of active sulfate formation. J. Amer. Chem. Soc. 78:6408-9.
2. Brown, D. 1920. The value of nutrient solutions as culture media for fern prothallia. Torreya 20:76-83.
3. Coic, Y., C. Lesaint, and M. Grandjean. 1963. The influence of phosphorus deficiency on the mineral composition of barley at harvest. Ann. Physiol. Vegetable 5 (4):277-80. Chem. Abstr. 60:15089h. 1963.
4. Fujiwara, A., and K. Torii. 1961. Physiology of sulfate on higher plants. I. Effect of sulfur deficiency on metabolism of higher plants. Tohoku J. Agr. Res. 12 (3):277-90. Biol. Abstr. 39 (1):619 #7720. 1962.
5. Hevly, R. H. 1959. The taxonomy of the Notholaena sinuata complex. Master's thesis. University of Arizona, Tucson.
6. Hevly, R. H. 1963. Adaptations of cheilanthoid ferns to desert environments. J. Ariz. Acad. Sci. 2 (4):164-75.
7. Kitson, R. E., and M. G. Mellon. 1944. Colorimetric determination of phosphorus as molybdivanadophosphoric acid. I & E Chem. (Anal. ED.) 16 (6):379-82.
8. Kurtz, E. B., and A. M. Mac Ewan. 1954. Introductory Plant Physiology Laboratory Manual, 2nd ed. Burgess, Minneapolis.

9. Kurtz, E. B., and H. B. Waldon. 1960. A new role for sulfur in higher plants. J. Ariz. Acad. Sci. 1:102-3.

10. Lammerink, J. 1962. Interactions of phosphate and sulfate on irrigated pasture. New Zealand J. Agr. Res. 5:450-9.

11. Steeves, T. A., I. M. Sussex, and C. R. Partanen. 1955. In vitro studies on abnormal growth of prothallia of the bracken fern. Am. J. Bot. 42:232-44.

12. Takahashi, N., T. Yamada, T. Hashimoto, and J. Yamaki. 1962. Effect of inorganic compounds on dark germination of tobacco seeds induced by gibberellin. Bot. Mag. 75:49-55. Intl. Abstr. Biol. Sci. 28:596. 1963.

13. Wald, G. 1963. In: Horizons in Biochemistry. M. Kasha and B. Pullman, eds. p. 127-42. Academic Press, N.Y.

14. Whittier, D. P. 1964. The effect of sucrose on apogamy in Cyrotomium falcatum presl. Am. Fern J. 54:20-24.