

Growth and Development of the Megagametophyte of the  
Vascular Plant  
Selaginella (Lycopside) on Defined Media

by

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## Chapter I

### INTRODUCTION

The life cycle of the vascular plants involves an alternation of two morphologically different generations, the smaller and anatomically less complex gametophyte, and the larger, more complex sporophyte (Whittier, 1971; Foster and Gifford, 1974). The sporophyte generation begins with the fertilization of the egg cell to form a diploid zygote. Meiosis and the production of haploid spores initiate the gametophyte generation. Upon observing the general pattern of this life cycle, one might assume that ploidy level plays a major role in determining sporophyte versus gametophyte morphology. It follows from this that haploid growing tissue should develop into a gametophyte solely because the cells of this tissue contain 1 set of chromosomes instead of 2. However, Lang (1898), Manton (1950), Freeberg (1957), Bell (1958), Morlang (1967), Whittier (1965, 1971) and others (see White, 1971), working with homosporous vascular plants, have documented both naturally-occurring and experimentally-induced abnormalities in the life cycle that cast serious doubts on the presumed determinative role of the ploidy level. These abnormalities include apospory, the development of a gametophyte directly from, and comprised

of, diploid tissue, and apogamy, the development of a sporophyte directly from, and comprised of, haploid tissue. Thus, in some homosporous vascular plants growing tissue can develop as either sporophyte or gametophyte without regard to the ploidy level. The question remains as to what the determinants of these two different growth types might be.

W. H. Lang (1909) hypothesized that the normal alternation of morphologically dissimilar generations results from differences in the physical and chemical (nutritional) environments at the initiation of the two generations. Among the homosporous vascular plants the gametophyte begins as a single cell, the spore, which germinates by growing out of the sporoderm so that the first cell division occurs in an environment that is physically unconstrained, and in which there is only a small amount of nutrition initially available to the growing tissue. The sporophyte initiates as the zygote, physically constrained within the archegonium and having the benefit of a greater amount of nutrition available from the surrounding gametophyte tissue. Response to these two different sets of conditions, according to Lang, is gametophytic and sporophytic development, respectively. Lang proposed that the observed life cycle abnormalities resulted from modifications of these environmental factors. Many workers

have since attempted to find support for Lang's hypothesis through manipulation of physical and chemical factors under controlled conditions.

Experimental manipulation of physical constraint has included work by DeMaggio and Wetmore (1961). In an attempt to mimic the physical environment that confronts the spore they released the zygote and embryo of a fern, Todea barbara, from physical constraint by surgically removing them from the archegonium. The younger the embryo at the time of liberation the more delayed was normal sporophytic growth, while liberated zygotes gave rise only to 2-dimensional thallus-like structures that looked very similar to the early haploid or gametophytic plant. Apparently, by providing a physical environment like that of a germinating spore, DeMaggio and Wetmore have triggered gametophyte-like development. The reciprocal experiment of placing physical constraint on a germinating spore has not yet been done successfully (Bell, 1958).

The environment in which a plant develops also consists of a nutritional component. The first cell of each generation of the homosporous vascular plants faces a unique set of conditions. The spore has only its own nutritional reserves to draw from and, upon germination, the gametophyte must quickly photosynthesize or associate with a symbiotic

fungus to secure a source of nutrition. In these early stages of development the amount of available nutrients is likely to be low, and the tissue may develop in response to this condition. DeMaggio (1963) and Whittier (1978) have hypothesized that gametophyte morphology may be the result of a low nutritional (carbohydrate) level that does not allow for re-establishment of the more vigorous sporophytic pattern of growth. The zygote, on the other hand, is embedded within the tissue of the mature gametophyte, and development into an embryo and young sporophyte is presumably determined, at least partially, by a greater amount of nutrients. This may explain the observations that low levels of carbohydrate and/or light appear most effective for inducing apospory in cultured tissue (Morlang, 1967; Whittier, 1978), whereas high levels of carbohydrate and/or light are most effective for induction of apogamy (Bristow, 1962; Whittier, 1960, 1965). Whether induction of apogamy and apospory can be interpreted strictly in terms of the amount of energy available for growth is still not clear. Whittier (1975), for example, has determined that, when a basal level of carbohydrate is available, increasing the osmolarity of the medium with mannitol will increase the number of apogamous sporophytes produced. In this case sporophytic growth appears to be triggered by both energy and osmotic factors.

Response of homosporous plant tissue to experimental manipulation supports Lang's hypothesis. However, little work of this kind has used the heterosporous vascular plants. Heterospory refers to the production of two types of spores that differ in both size and potential. The megaspore contains the female megagametophyte which, in lower vascular plants and gymnospermous seed plants, is a complex organism consisting of nutritional reserve tissue, vegetative tissue, and reproductive tissue bearing archegonia which contain the egg cells. The microspore contains the male microgametophyte, of which most of the tissue is involved in production of sperm cells. Most of the heterosporous gametophytes develop from 1 cell to the mature organism within the confines of the sporoderm and, hence, are characterized as endosporic in their development. This is in contrast to the homosporous gametophytes, in which all cell divisions, growth, and development take place outside the sporoderm (exosporic). Thus, both sporophyte and gametophyte among the heterosporous plants are initiated in physically constrained environments. However, the cells surrounding the archegonium do yield to growth and possibly provide some nutrition or growth substance to the embryo. The spore wall is nutritionally inert and, except for the trilete end, is physically unyielding. Nutrition for cell

divisions, growth, and development of mega- and micro-gametophytes is provided by lipid, starch, and protein reserves incorporated into the young spores before the sporoderm is complete. Megaspores, especially, show marked expansion during this stage.

Though physical environments are similar at the beginning of each generation in the heterosporous vascular plants, the nutritional environments are different. For example, in the genus Selaginella the sporophyte is photosynthetic, providing this tissue with a carbohydrate-based type of nutrition. Neither male nor female gametophyte, on the other hand, appear to photosynthesize (though this has not been confirmed), nor have any relationships with fungal symbionts been reported (Foster and Gifford, 1974). Within the megagametophyte there is a large cavity in the basal end of the megaspore where abundant lipids are stored (Robert, 1971). This suggests that lipid is the major source of nutrition for the megagametophyte. Unlike the nutritional difference between homosporous generations of high versus low amounts of carbohydrate, there appears to be a more distinct difference in Selaginella. Here the sporophyte produces and uses carbohydrates while the gametophyte utilizes lipids. This difference can be taken advantage of to support or deny Lang's hypothesis. Can a heterosporous

gametophyte be experimentally manipulated to induce apogamy, as has been done so successfully with homosporous gametophytes, by supplemental nutrition?

Reasons for the neglect of the heterosporous plants, at least in terms of the seed plants, may involve the complexity of the relationship between the sporophyte and the permanently retained and fully enclosed megagametophyte, with respect to both physical constraint and nutritional status of the various tissues involved. Response to manipulation of environmental conditions in culture would likely prove very difficult to interpret in terms of Lang's hypothesis. Furthermore, the male gametophyte, or pollen, of these higher heterosporous plants offers little available tissue for cultural manipulation, although pollen callus tissue and regenerated haploid sporophytes have been obtained (Thomas and Davey, 1975).

The heterosporous lower vascular plants, e.g. Selaginella, however, exhibit 2 free-living generations, which may prove to be as useful for gaining an understanding of the alternation of dissimilar generations as has been the case with the homosporous plants. This work describes the experimental culture of the megagametophyte of 1 species of Selaginella on various types and concentrations of carbon sources.

Selaginella was selected for several reasons over other heterosporous lower vascular plants for several reasons. One was the terrestrial habit of Selaginella and, therefore, its easier propagation, maintenance and handling. A second reason was the many megaspores that this plant produces throughout the summer and fall. Thirdly, megagametophytes of two other species of Selaginella have previously been cultured successfully by Wetmore and Morel (1951). They observed that supplemental carbohydrate (glucose) produced megagametophytes twice as large as normal. The inclusion of a mixture of B vitamins with glucose produced growth that was continuous, giving rise to a callus-like mass of tissue covered with rhizoids and archegonia. No additional observations were provided by Wetmore and Morel (1951) on growth of Selaginella megagametophytes, nor did they supply any data or controls for their experiments. This present investigation utilized a third species of Selaginella, and extended the observations of Wetmore and Morel by providing additional treatments consisting of a variety of carbon sources in a range of concentrations, as well as control treatments for interpretations of the results.

## Chapter II

### MATERIALS AND METHODS

#### 2.1 MEDIA PREPARATION

Twenty types of culture media were prepared in June, 1981. All types contained Knudson's mineral salts (see Appendix A) and were solidified with 0.9% agar. Three sugars, glucose (G), sucrose (S), and trehalose (T), were utilized as the different carbohydrate substrates. Each sugar was mixed in concentrations of 1, 3, and 5% (w/v). A control treatment (K) without sugar was also prepared. These 10 types of nutritional media were also prepared with a mixture of B vitamins and growth factors (see Appendix A). This vitamin mixture was also used by Wetmore and Morel (1951). All chemicals were obtained from Sigma Chemical Co.

The mineral medium and agar was autoclave-sterilized. All sugars and vitamins were filter-sterilized using a 0.22 micrometer Millipore filter, and were then added to the cooled, sterile mineral medium. Media were then poured into quartered Petri dishes, and the dishes were sealed with strips of Parafilm and refrigerated.

## 2.2 COLLECTION AND INOCULATION OF MEGASPORES

Cuttings from one specimen of Selaginella martensii var. albovariegata were propagated in the VPI & SU biology greenhouse. Strobili were collected in late June, 1981, and air-dried overnight on paper towel. Released megaspores were collected by rolling them off the paper towel into a washing device described by Webster (1978). The washer was placed in a wire test-tube rack and lukewarm water was run slowly through it for 20 min to wash out microspores. The washer was immersed in distilled water 24 hrs to encourage germination of fungal or bacterial spores. All subsequent manipulations, including inoculation, were conducted in a laminar-flow hood. The washer was immersed two min in a freshly prepared solution of 25% Clorox bleach (v/v) in distilled water, with one drop of Tween 80 per 200 ml, to surface-sterilize the megaspores. The washer was rinsed twice in sterile distilled water for 30 sec. After the second rinse the megaspores were allowed to settle to one end of the washer as the water drained. The plastic cap containing the megaspores was removed and placed on the stage of a Nikon binocular dissecting microscope. Using fine forceps and sterile technique, megaspores were inoculated individually and randomly onto Petri dishes containing the various media over a period of several days.

Megaspores were visually selected for large size and lack of apparent defects, as preliminary studies indicated these were most likely to be viable. In the first several batches of Petri dishes 2 megaspores were inoculated per quarter dish, but this was changed to 3 megaspores per quarter in later batches. This was done, along with the use of quartered dishes, to prevent or slow the spread of any bacterial or fungal contamination. After inoculation each Petri dish was again sealed with a strip of Parafilm (which effectively reduced water loss from the agar medium). Dishes were labeled and placed in a reach-in growth chamber, where they were maintained for the duration of the experiment.

Since the sporophyte photosynthesizes, and the megagametophyte seems not to, consideration was given to the possibility that a reversion to sporophytic growth might hinge on the presence of a lighted environment. Bierhorst (1971) observed, and Webster (1967) showed, that zygotes remain dormant within megagametophytes until given adequate light, and only then will they grow and develop fully into sporophytes. Light ( $230 \text{ microeinsteins m}^{-2} \text{ sec}^{-1}$  photosynthetically active radiation - determined with a Li-Cor, Inc. photometer) was provided by cool fluorescent and incandescent bulbs on a 12 hr light-dark cycle.

Temperature was maintained at 27° and 25° C during the light and dark periods, respectively.

### 2.3 DATA COLLECTION

Megaspores were observed daily for 2 wk after inoculation, then twice a week. Germination was considered complete if the trilete suture showed any indication of having opened. Megaspores that were contaminated with either bacteria or fungi before germination were discarded. Megaspores that became contaminated after germination, though not included in growth analyses, were included in the analysis of percent germination.

A data sheet for each germinated megaspore recorded the dates of inoculation and germination, and camera lucida drawings of that megagametophyte at 2 wk intervals through the twelfth wk after germination. The drawings were made with a Wild M5A binocular dissecting microscope and camera lucida attachment. Megagametophytes were also occasionally photographed using a Wild Photoautomat MPS55 attachment.

Average number of days to germinate was calculated and compared among the different treatments. Since sample sizes differed between treatments, Duncan's Multiple Range Test was used to determine statistical significance at the 0.10 and 0.05 levels. Though the number of days to germinate

varied among megaspores within and between treatments, each megagametophyte was observed 12 wk from the day of its own germination.

Percent germinations were calculated by dividing the number of germinated megaspores by the number of uncontaminated megaspores inoculated, and were analyzed for significant differences using a Chi-square Test of Fitness at the 0.05 level.

Length/width measurements of megagametophytes were calculated from the camera lucida drawings. Average radius measurements were determined for each megagametophyte for each 2 wk period after germination. Volume of tissue was selected as a measure of differences in growth on the various treatments, since cubing the size measurements accentuated measured differences. Since most megagametophytes were spheroidal, the geometric conversion,  $V = 4/3 \text{ Pi} \times \text{radius}^3$ , was utilized. Average volume within each 2 wk period was calculated for each treatment, and these values were graphed. Average final volumes for all treatments were analyzed statistically with Duncan's Multiple Range Test at 0.10 and 0.05 levels.

## 2.4 ADDITIONAL NUTRITIONAL TREATMENTS

### 2.4.1 Sorbitol as an Osmotic Control

In December, 1981, six months after the initiation of the experiment, the importance of determining whether osmotic differences between the various concentrations of sugars could produce any of the observed responses became apparent. Sorbitol was selected as an osmotic agent rather than mannitol, which is also often used for this purpose (Whittier, 1975). Media containing 1, 3, and 5% sorbitol (Sb) with B vitamins were prepared in a similar manner as all previous media types, though in this case the sorbitol was autoclaved with the mineral salts solution. Sorbitol was obtained from Fisher Scientific Co. The B vitamin mixture, which generally enhanced growth, was also included in these treatments to facilitate germination, and possibly enhance potential differences between the three concentrations of sorbitol. A fourth treatment, 3% glucose with B vitamins, was run as a control to the sorbitol treatments. Response to this control treatment was compared to the earlier results on this medium, since there was concern that megaspores collected in December might respond differently from those collected in July.

#### 2.4.2 Repeat of Trehalose Treatments

A second set of trehalose treatments was prepared and megaspores inoculated in March, 1982. Media containing 1, 3, and 5% trehalose were prepared in an identical manner as before. Due to the lack of available growth chamber facilities at that time, however, Petri dishes were maintained on a laboratory benchtop. The amount of photosynthetically active radiation reaching the dishes at mid-day was 63 microeinsteins  $\text{m}^{-2} \text{sec}^{-1}$ . A control treatment of K medium was run concurrently with the trehalose treatments. Germination percentage, average number of days to germinate, and average final volume were analyzed and compared to the first trehalose treatments.

#### 2.5 CORRELATION ANALYSIS

Percent germination versus final volume, number of days to 50% germination versus final volume, and percent germination versus number of days to 50% germination were analyzed using a linear regression analysis to determine the degrees of correlation between these responses.

## 2.6 DETERMINING THE PRESENCE OF CHLOROPHYLL

Megagametophytes were examined for any indication of apogamy. There was a possibility that some component of sporophyte growth had been induced, which might be measured by chlorophyll production. While it is generally considered (Bierhorst, 1971; Foster and Gifford, 1974) that megagametophytes of Selaginella do not contain chlorophyll, a study was undertaken to detect chlorophyll-a by extracting pigments and analyzing by the sensitive fluorescence technique. Several megagametophytes from each of the majority of treatments were selected immediately after their last volume measurements. Megagametophytes within each treatment were pooled, and extracted in 2.5 ml spectral grade dimethyl-sulfoxide (DMSO) in glass vials for 18 hrs in the dark at room temperature. Sections of sporophyte stem, of the same plants from which the megaspores were collected, were also extracted to compare the relative amounts of chlorophyll-a present in megagametophytes. An equal volume of 90% spectral grade acetone was added to the vials. Extract solutions were centrifuged for 5 min at 600 x G, and decanted into fluorometer cuvettes. Fluorescence measurements were taken for each extract using a Turner Designs fluorometer. Each extract was acidified with two drops 50% HCl, mixed and, after 5 min, a second fluorescence

reading was taken. A decrease in fluorescence after acidification, beyond the immediate effect of slight dilution (accounted for in blank readings), was attributed to conversion of chlorophyll-a to pheophytin, indicating the presence of chlorophyll. Blank solutions were measured before and after acidification, and these background readings were subtracted from the actual extract readings. Remaining values of fluorescence, attributable to chlorophyll-a, were divided by the volume of tissue extracted in each treatment. Measurements of fluorescence per mm<sup>3</sup> of tissue were correlated to actual amounts of chlorophyll-a. These readings were used to construct a linear regression equation to convert readings from megagametophyte extracts to actual concentrations of chlorophyll-a (see Appendix D for all data). The chlorophyll-a used in producing the linear equation was obtained from Sigma Chemical Co.

## 2.7 CELL SIZE ANALYSIS

Megagametophytes cultured on K medium, and 1, 3, and 5% sorbitol with B vitamins were selected for observation and analysis of cell size. Megagametophytes that were large enough to remove the megaspore wall were selected after their last volume measurements, since this wall was an

impediment to adequate infiltration of plastic resins. They were fixed in 2.5% glutaraldehyde for 2 hrs at room temperature, washed three times for 90 min in phosphate buffer (pH 7.4), taken through an ethanol dehydration series of 50, 60, 70, 80, 90, 95, 100, 100, and 100% (30 min each step), and through a propylene oxide (po) series of 25, 50, 75, and 100% (30 min each step). Liquid Spurr's resin was added slowly over a period of 4 days. Through the removal of po/resin, and the addition of fresh resin, the concentration was gradually increased to 100%. After 24 hrs in 100% Spurr's resin, megagametophytes were flat-imbedded, since a consistent plane of sectioning was considered important. The resin was polymerized for 24 hrs at 60° C. The megagametophytes were cut out in plastic blocks using a jeweler's saw, and were mounted on dummy blocks so that sectioning of the tissue was from proximal to distal poles. Tissues often did not dehydrate sufficiently or infiltrate well enough, and this difficulty appeared due to the barriers of the megaspore wall, and a layer of mucous surrounding the tissues that slowed the penetration of fixatives, dehydrants, and resin. Megagametophytes that had not grown much beyond the megaspore wall were the most difficult to prepare. Robert (1971) had similar problems with his material, and also recognized the mucous layer as a

potential culprit. When possible he dissected the megaspore wall away from the living tissue, and this improved his results. In this work it was found that, by replacing an acetone dehydration series with both an ethanol and a propylene oxide series, results were much improved. Increasing the concentration of Spurr's resin slowly and gradually was also effective in producing better infiltration.

Since it was only necessary to observe cell walls in the analysis of cell size, megagametophytes that had been extracted in DMSO for fluorescence analysis also provided adequate material for sectioning and analysis. After the fluorescence analysis was completed, DMSO-extracted megagametophytes were taken into 100% acetone, and Spurr's resin was added slowly over a period of four days up to 100% concentration. These megagametophytes infiltrated more readily than live tissues, possibly due to the dissipation of the mucous layer. From this point the DMSO-extracted megagametophytes were handled in the same way as the previous group.

Megagametophytes were sectioned at 1.5 micrometers, using a Sorvall ultra-microtome and a glass knife. Sections were mounted on glass slides, stained with one percent toluidine blue O (Berwyn and Miksche, 1976) for five min on a

slide-warming tray, rinsed, dried, covered with mounting medium and a glass coverslip, and examined under a compound microscope.

A specific area of the megagametophyte was examined which was mid-way between the large cells forming on the periphery of the lipid reserve, and the much smaller cells associated with the reproductive tissue at top. Using a Leitz compound microscope with a camera lucida attachment, the cell walls in this area were outlined in pencil to fill in a pre-drawn square on paper. A stage micrometer was used in conjunction with the camera lucida attachment to determine the microscopic area covered by the square on the paper. The number of cells that occupied this area was determined for each megagametophyte, and an average number of cells per  $\text{mm}^2$  was determined for each treatment. The average cross-sectional area per cell ( $\text{mm}^2$ ) was determined for each treatment.

## 2.8 CELLULAR ORGANIZATION OF SELECTED MEGAGAMETOPHYTES

Megagametophytes sectioned for cell size analysis were also examined for general appearance of cells, presence and locations of archegonia, the status of the lipid reserve, and sites of cell divisions. Sections were photographed using a Leitz Wetzlar compound microscope with a Nikon

camera (Model M-35S) and a Nikon automatic photomicrographic attachment.

## Chapter III

### RESULTS

Apogamy was not induced in these experiments. The effects of the various nutritional treatments on megagametophyte growth and development evidenced themselves in varying rates and percentages of germination, final volume of tissue, and varying levels of chlorophyll-a.

#### 3.1 NUMBER OF DAYS TO GERMINATION

Addition of B vitamins increased the speed of germination for all treatments (see Appendix B for all data). This increase was significant at the 0.05 confidence level for all combined treatments with and without B vitamins, though within the individual treatments the difference was not always significant (Table 1).

Of the average number of days to germinate, among treatments without B vitamins, there was a range of responses from 10.0 days (3T) to 34.7 (5T). However, at the 0.05 confidence level there were no significant differences between any treatments, and at the 0.10 confidence level only treatments with the four most rapid responses (3T, 1S, 5G, 1G) were significantly different from the slowest treatment (5T) (Figure 1). Only one megaspore germinated on

TABLE 1

Differences in Average Number of Days to Germinate With and Without B Vitamins

| <u>Treatment</u>                          | <u>Confidence Level</u> |             |
|-------------------------------------------|-------------------------|-------------|
|                                           | <u>0.05</u>             | <u>0.10</u> |
| Combined Treatments<br>With and Without B | s.*                     |             |
| K+B vs K                                  | n.s.                    | n.s.        |
| 1G + B vs 1G                              | n.s.                    | s.          |
| 3G + B vs 3G                              | n.s.                    | n.s.        |
| 5G + B vs 5G                              | n.s.                    | n.s.        |
| 1S + B vs 1S                              | n.s.                    | n.s.        |
| 3S + B vs 3S                              | s.                      |             |
| 5S + B vs 5S                              | n.s.                    | n.s.        |
| 1T + B vs 1T                              | s.                      |             |
| 3T + B vs 3T                              | n.s.                    | n.s.        |
| 5T + B vs 5T                              | s.                      |             |

K = simple mineral salts medium,  
G = glucose, S = sucrose, T =  
trehalose, B = B vitamin mixture

\* - s. indicates significant difference  
at the specified confidence level.

3T and, due to the low sample size, this treatment could not be seriously compared with the others.

With B vitamins there was a smaller range for the average number of days to germinate, from 8.9 days (1T) to 22.4 (5S). There were several significant groupings at both the 0.10 and 0.05 confidence intervals, but there were also many overlaps between them (Figure 2).

Treatments without B vitamins tended to have greater variation in average number of days to germinate than treatments with B vitamins (Figure 3), i.e., germinations were more spread out over time. Figures 4 - 10 contrast the slower, unclustered germinations without B vitamins with the faster, more clustered germinations with B vitamins. Though the average number of days to germinate on K medium with and without B vitamins were not significantly different from each other (Figure 4), all other treatments clearly exhibited this distinct pattern.

There were no indications among treatments without B vitamins that responses were slower with increasing sugar concentrations. Among treatments with B vitamins, however, both sucrose and trehalose tended to exhibit patterns of slower germination with increasing concentration (Figure 2).

The sorbitol (Sb) control treatment, 3% G with B vitamins, germinated significantly faster than the first 3%

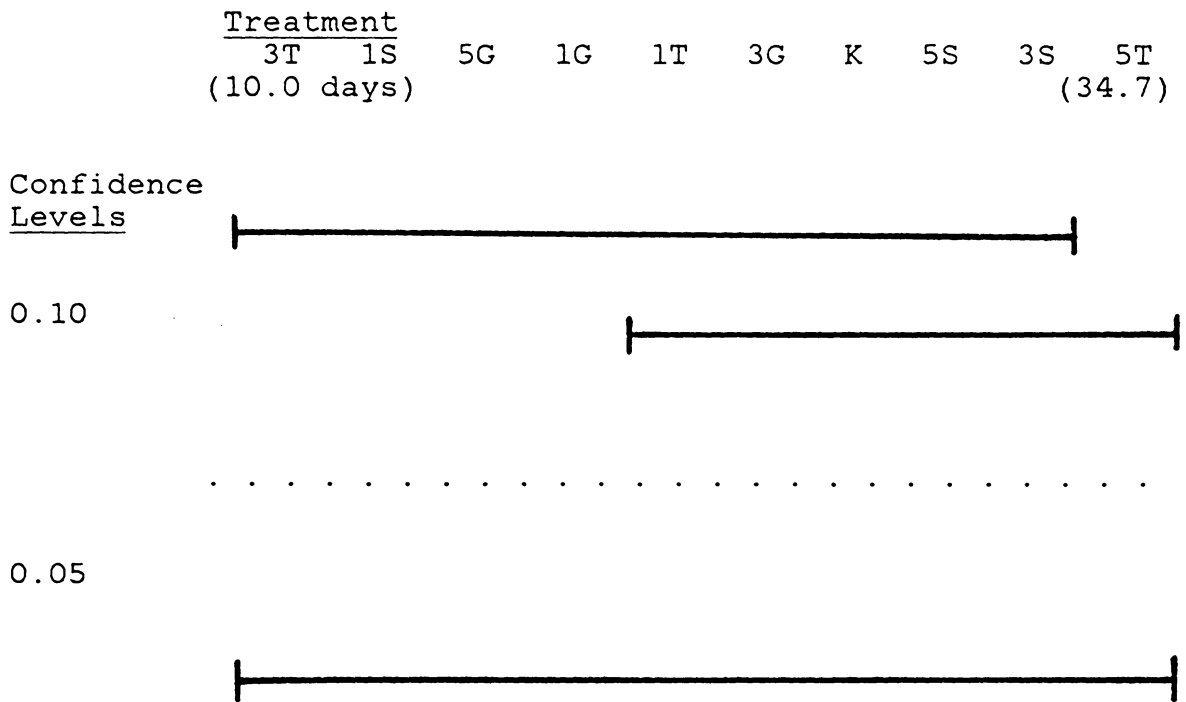


Figure 1: Differences in Average Number of Days to Germinate Without B Vitamins at 2 Confidence Intervals

Treatments sharing a common line are not significantly different at the specified confidence level.

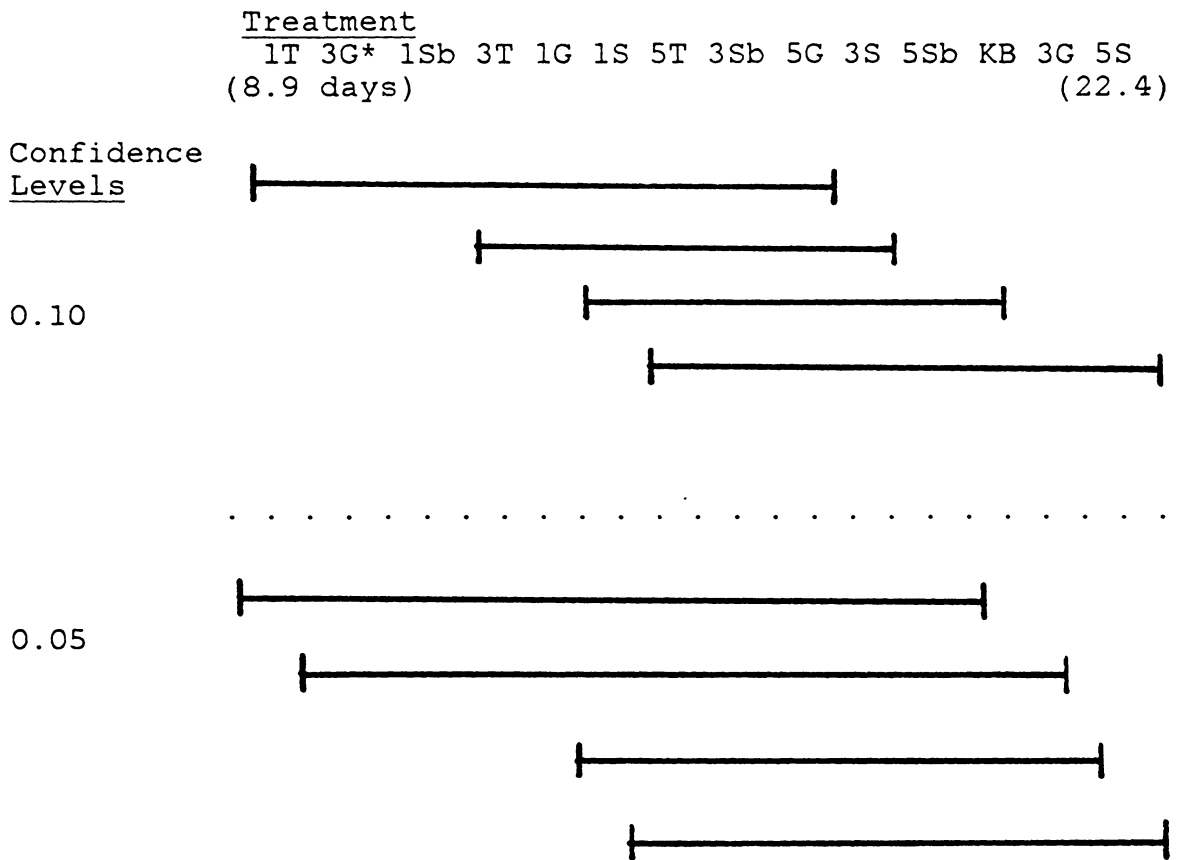
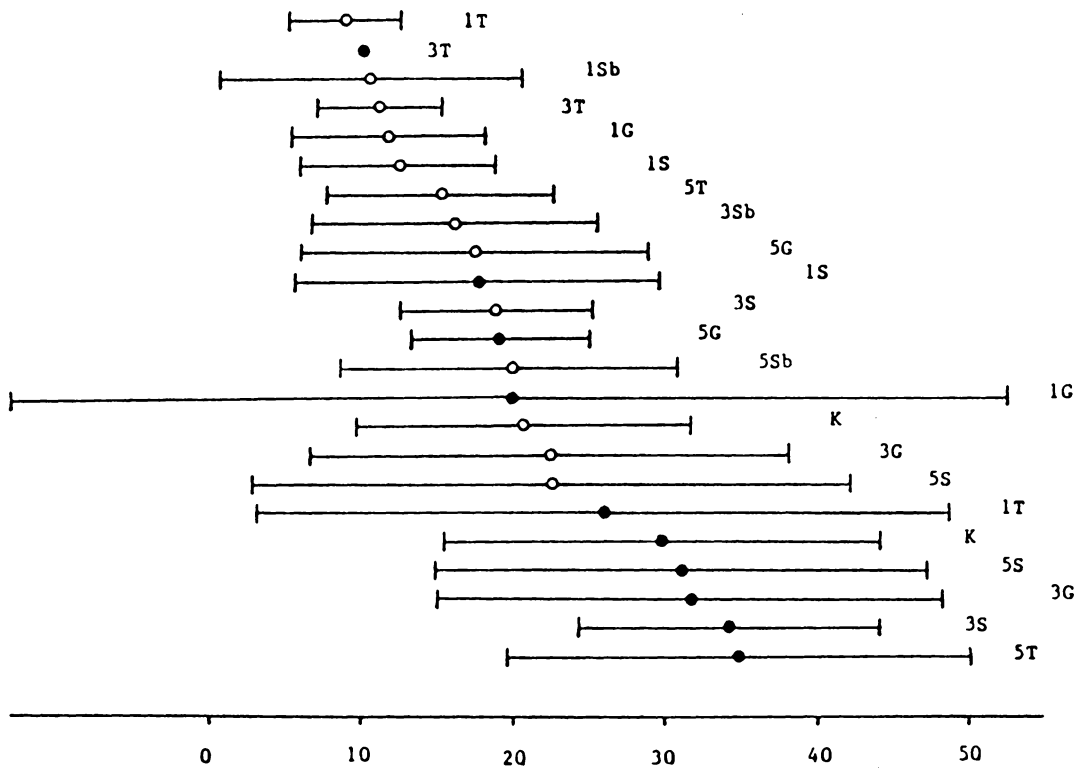


Figure 2: Differences in Average Number of Days to Germinate With B Vitamins

Sb = sorbitol treatments, \* = sorbitol control.



Average Number of Days to Germinate

Figure 3: Average Number of Days to Germinate  $\pm 1$  Standard Deviation

● = without B vitamins, ○ = with B vitamins.

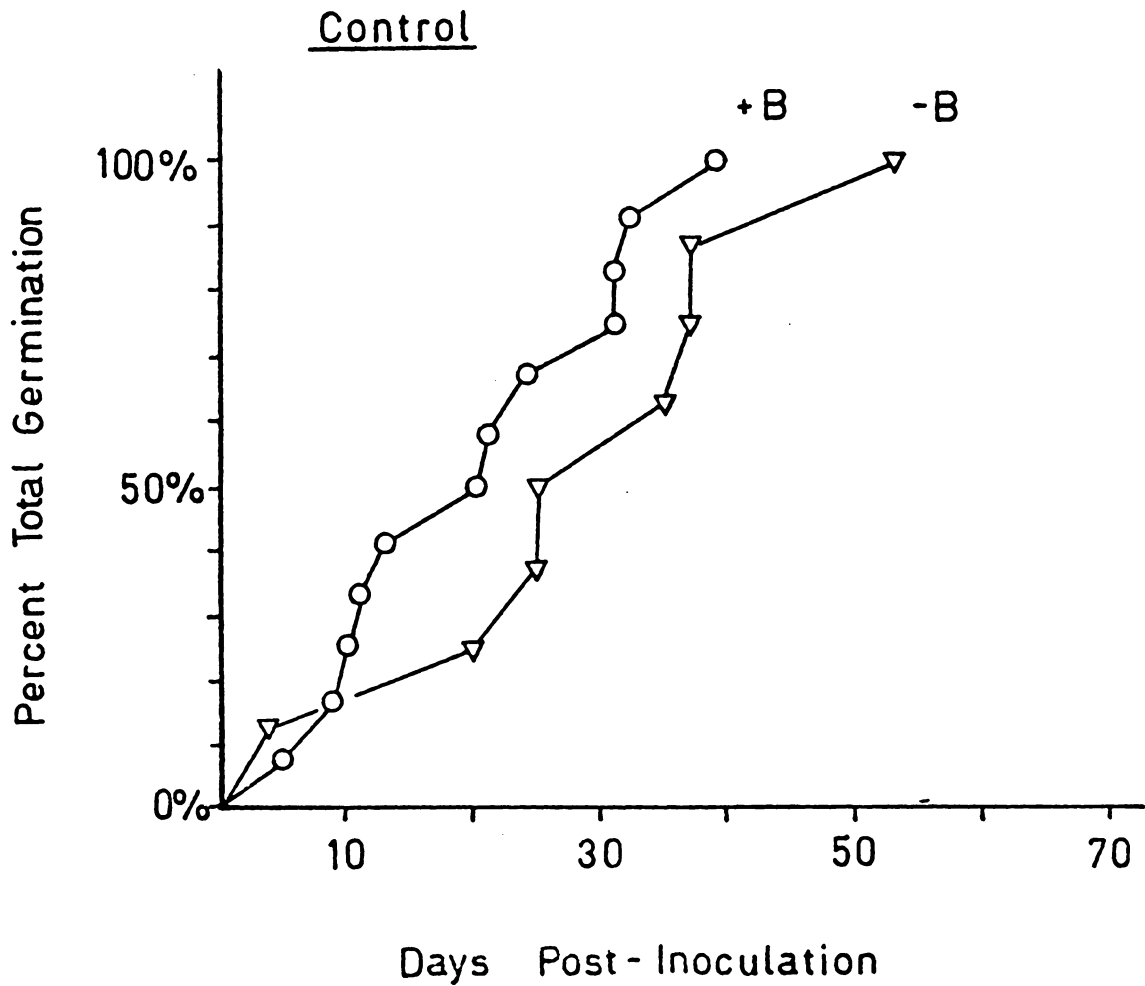


Figure 4: Time to Reach 100% Germination - Control  
(Knudson's Medium With and Without B Vitamins)

Each point on the graph represents one germinated megaspore.

G with B vitamin treatment (Figures 2 and 11) at the .05 confidence level. This suggests that megaspores collected in December were not responding the same as those collected in June. The sorbitol treatments were included in Figure 2, though, and statistically there were few differences between them and other treatments with B vitamins. As with the sugar and B vitamin treatments, germinations were relatively rapid, and tightly clustered in time. This was particularly evident on 1% Sb with B vitamins (Figure 12). Sorbitol treatments also exhibited slower germinations with increasing concentration (Figure 2).

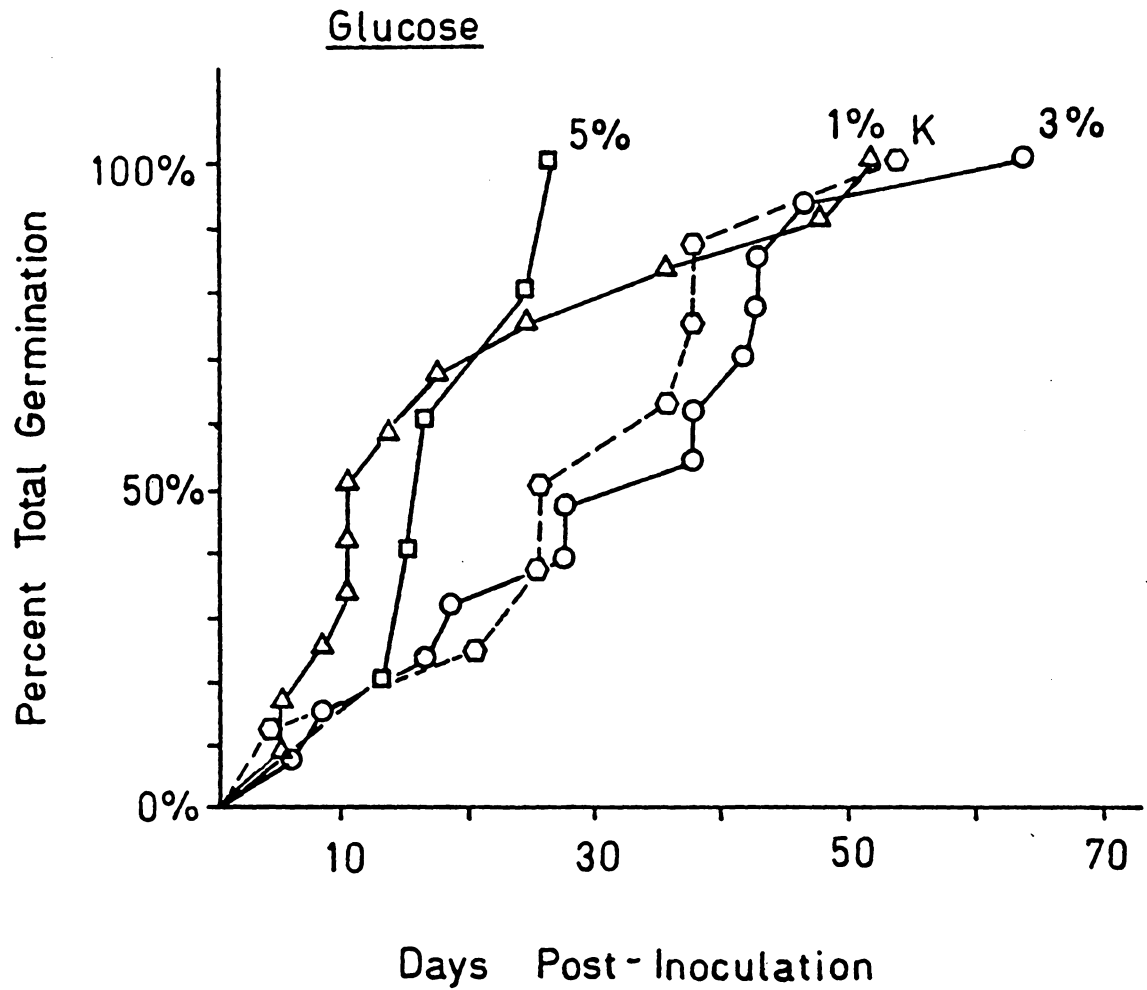


Figure 5: Time to Reach 100% Germination - Glucose

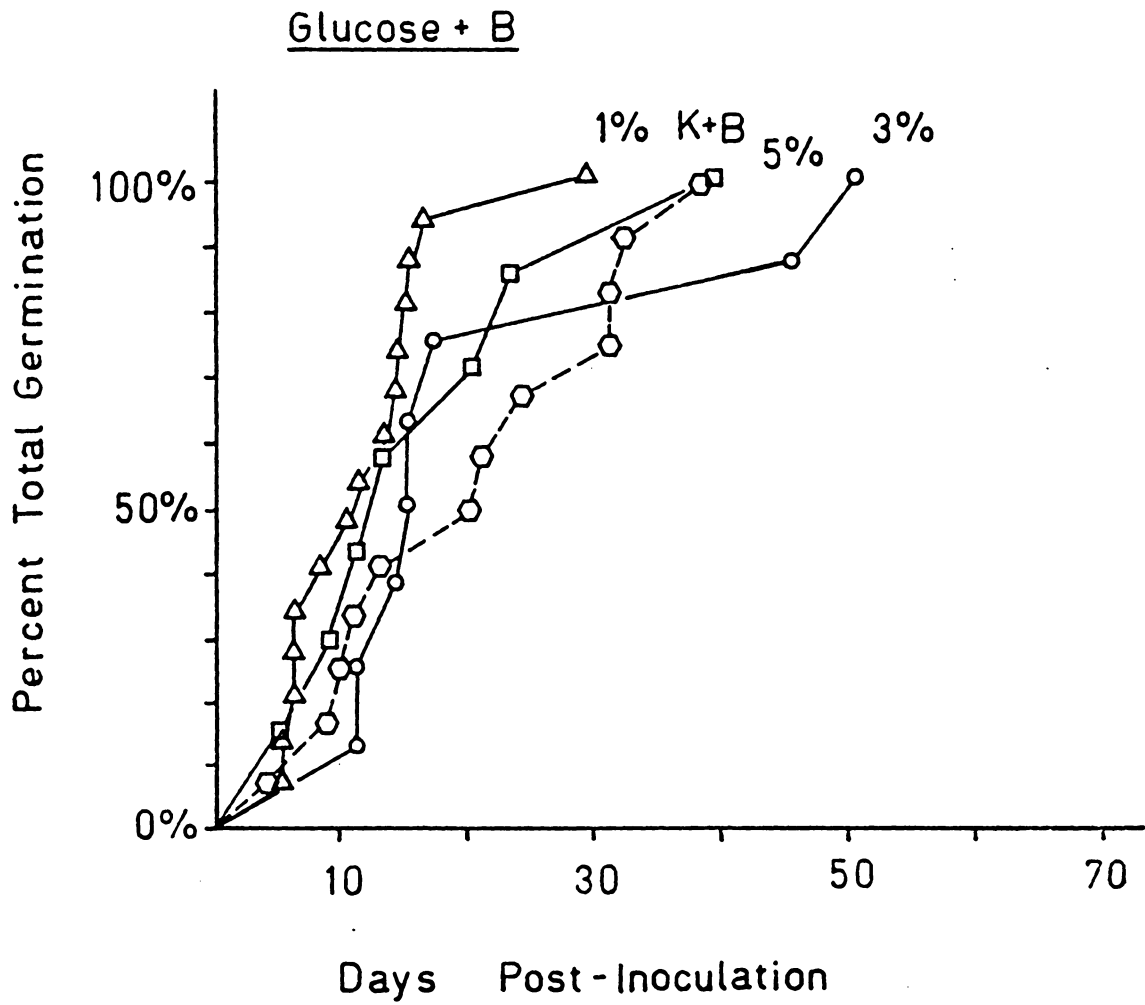


Figure 6: Time to Reach 100% Germination - Glucose With B Vitamins

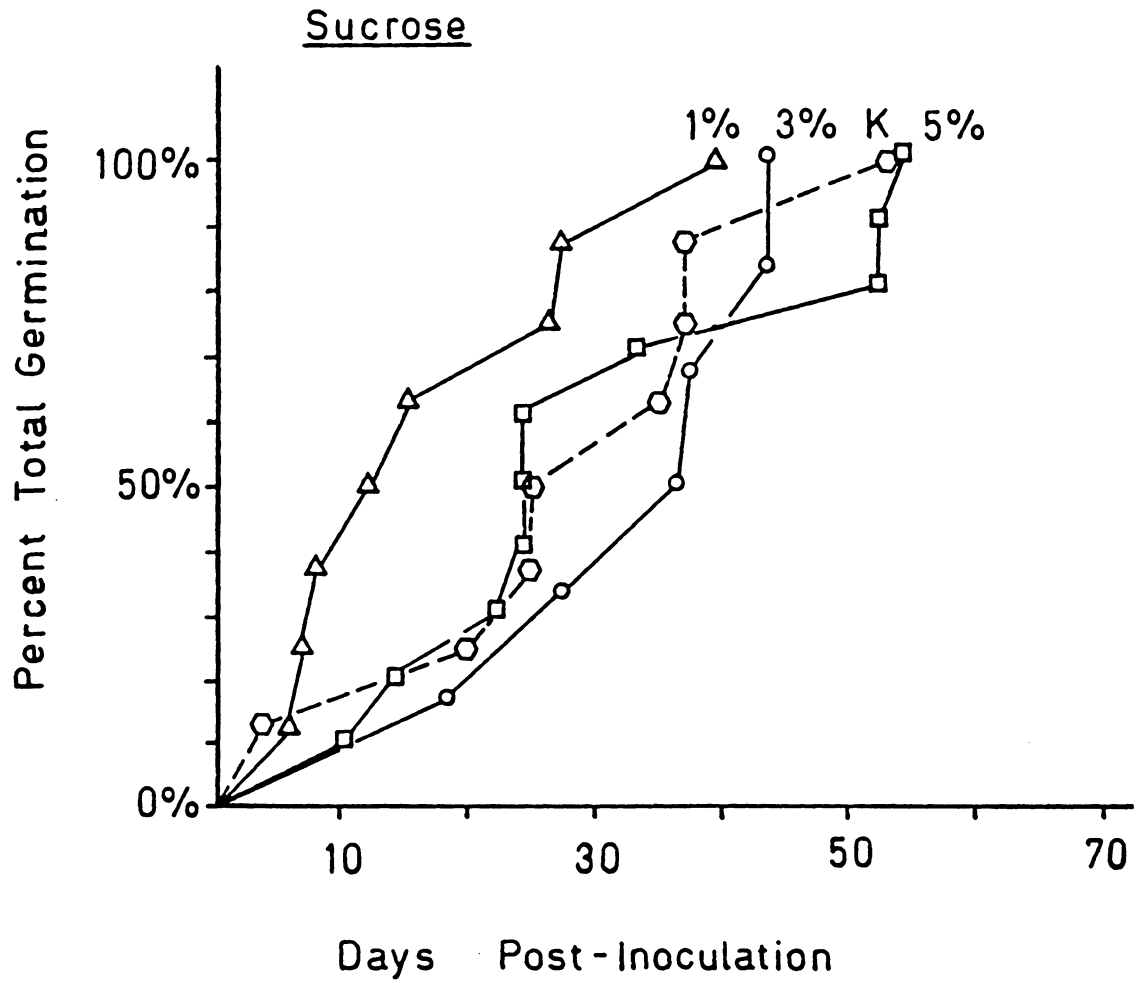


Figure 7: Time to Reach 100% Germination - Sucrose

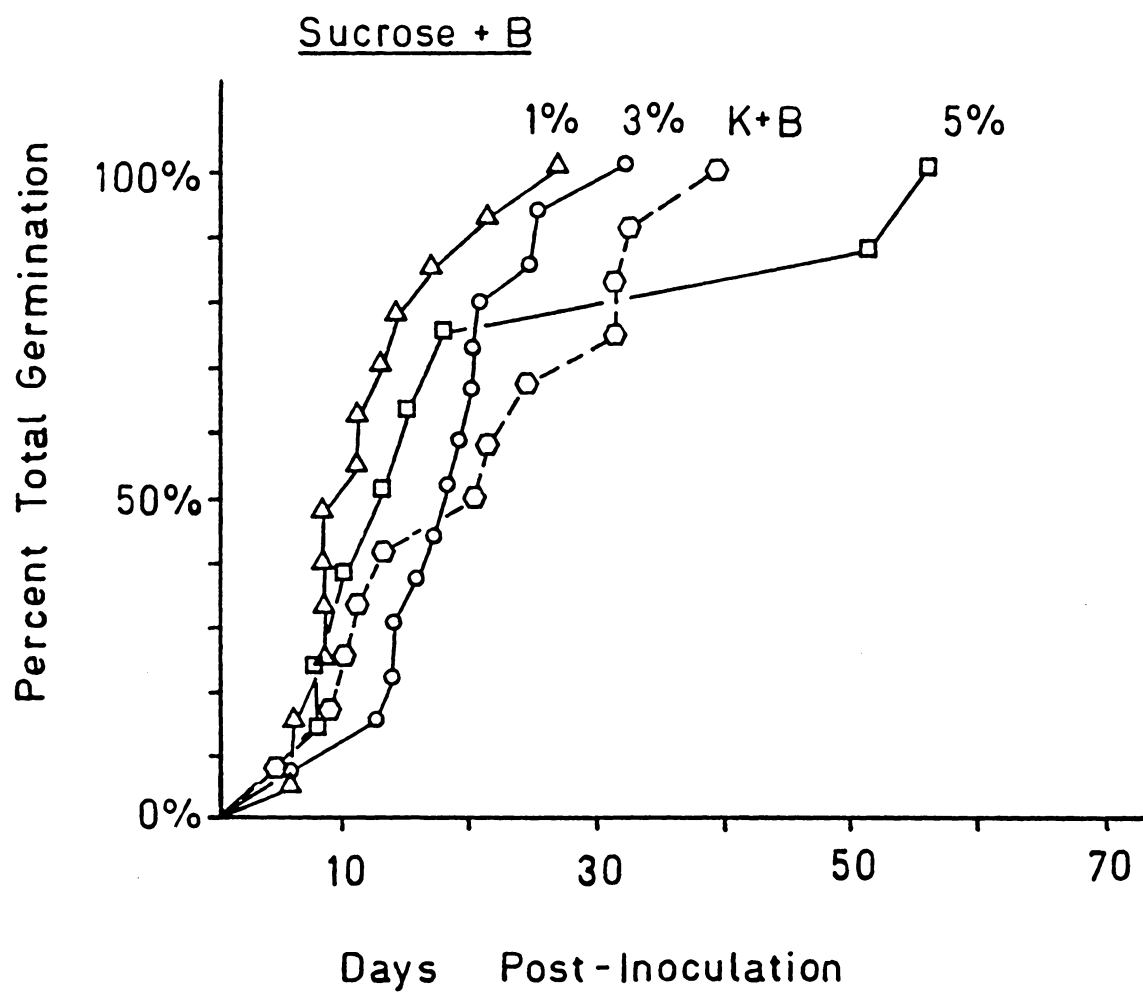


Figure 8: Time to Reach 100% Germination - Sucrose With B Vitamins

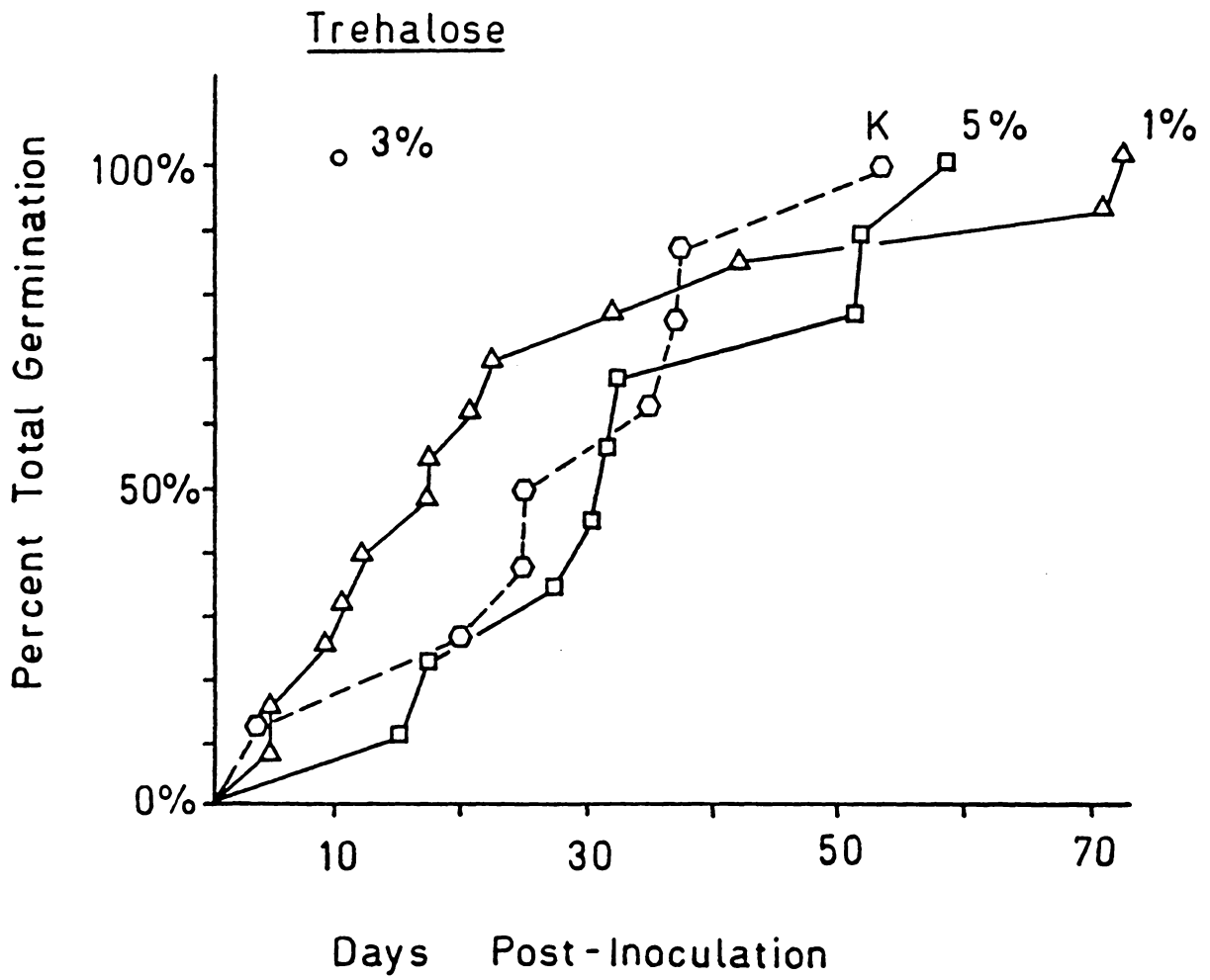


Figure 9: Time to Reach 100% Germination - Trehalose

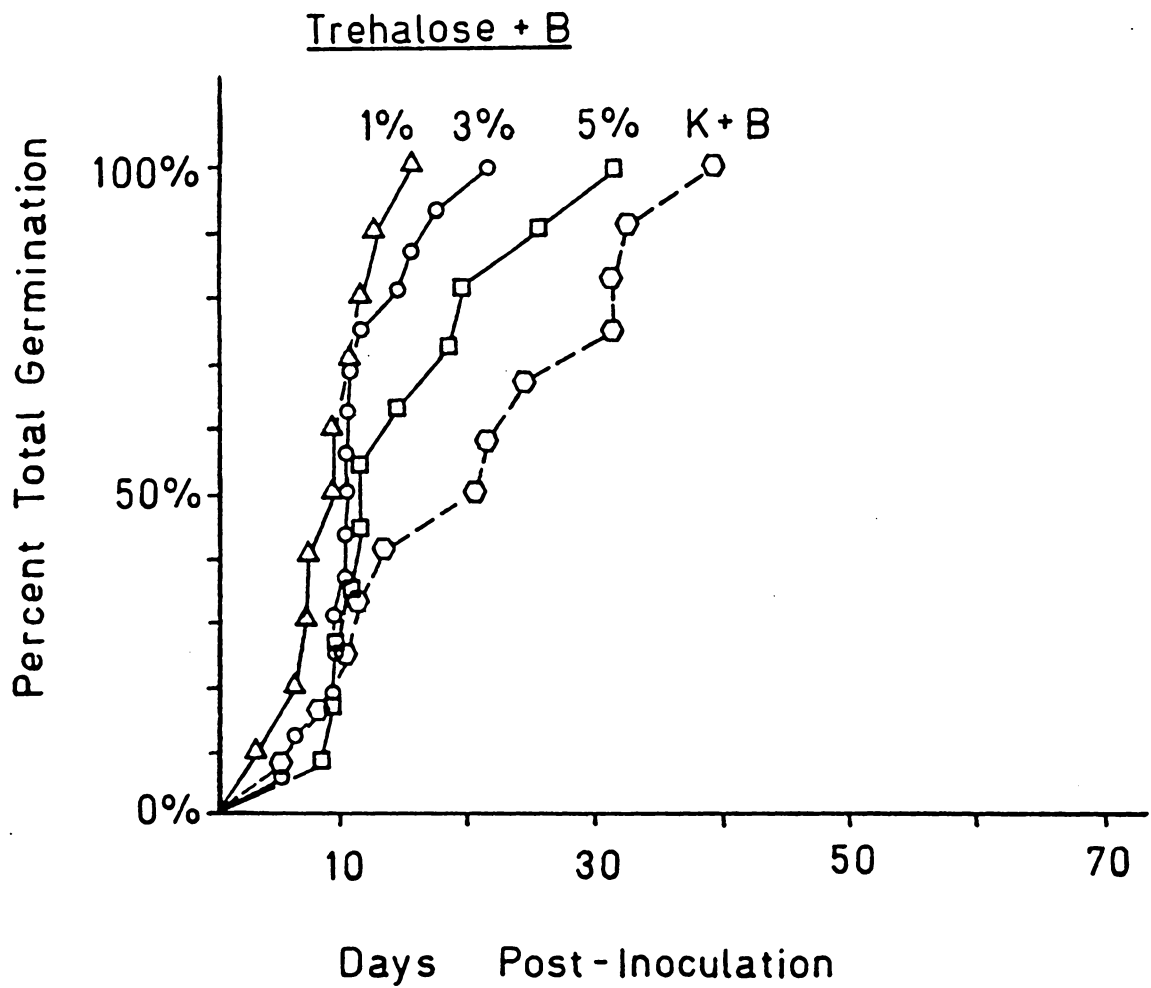


Figure 10: Time to Reach 100% Germination - Trehalose With B Vitamins

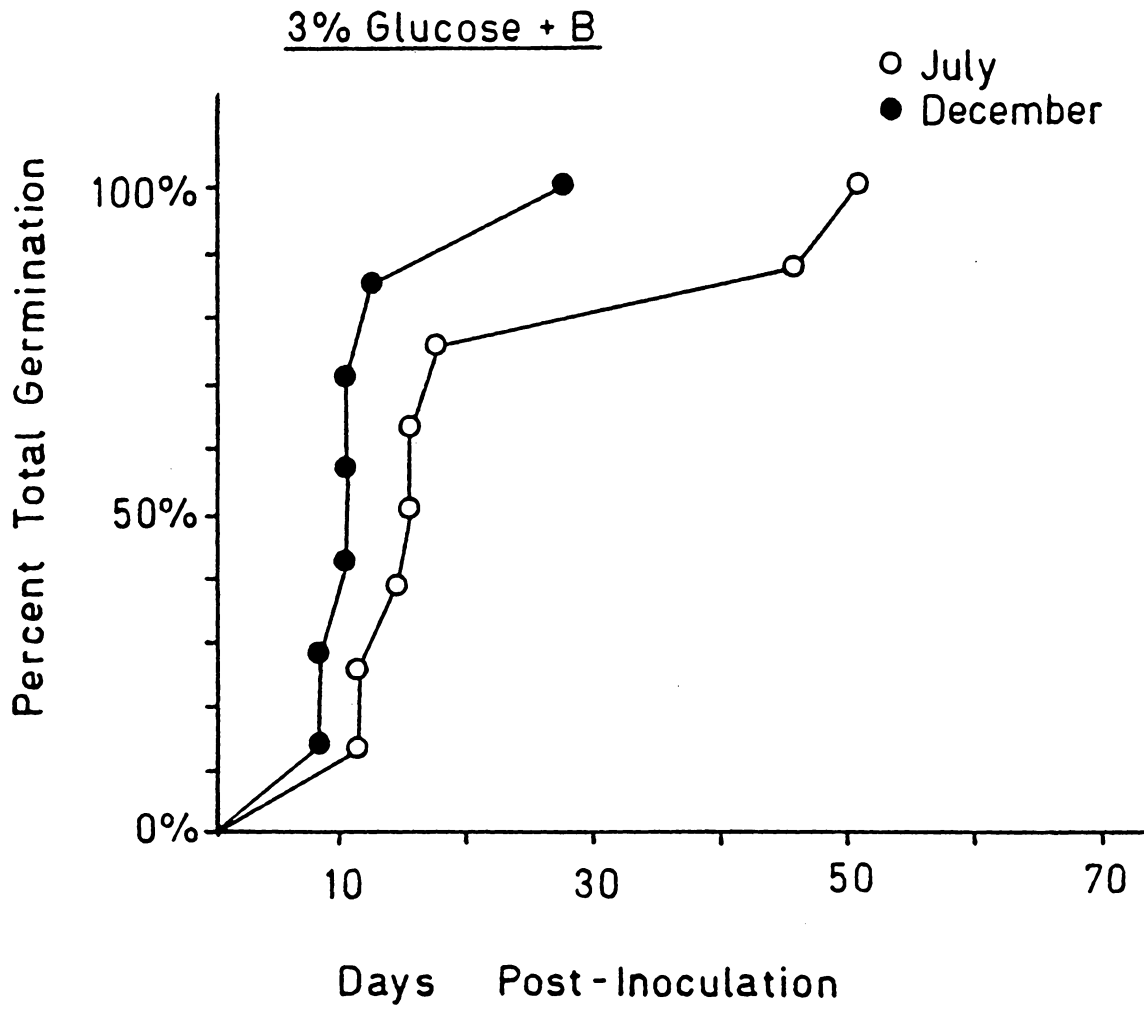


Figure 11: Time to Reach 100% Germination - 3% Glucose With B Vitamins (1st and 2nd Experiments)

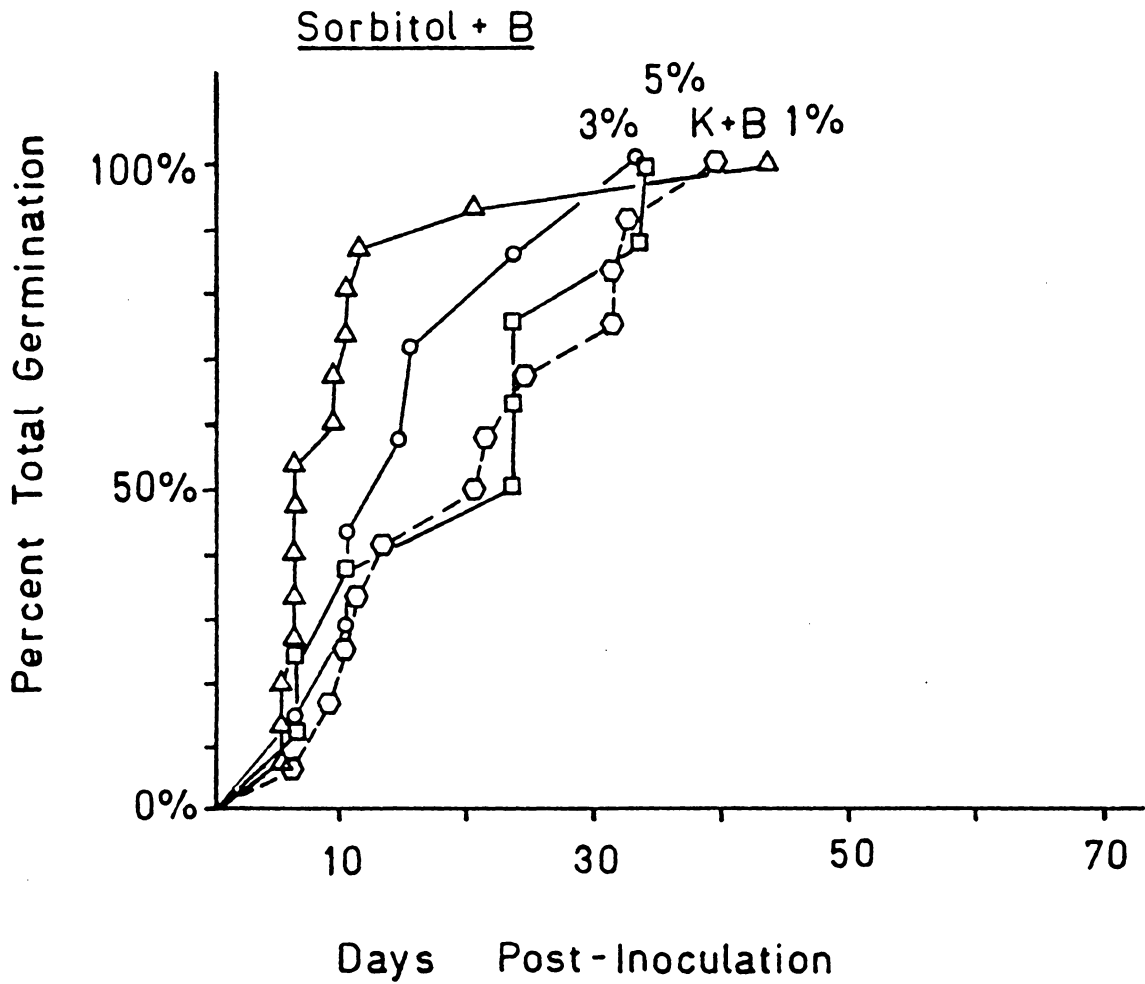


Figure 12: Time to Reach 100% Germination - Sorbitol With B Vitamins

### 3.2 PERCENT GERMINATION

In all cases % germination was higher on sugar treatments when B vitamins were included (Figure 13, Table 2-b) (see Appendix C for all data). Statistically, though, differences were not significant on several sugar treatments (1G, 5S, 1T, 5T) (Table 2-a).

Without B vitamins % germination for all glucose was significantly higher than for sucrose, though neither were significantly different from trehalose (Table 2-e). Comparisons of concentrations of each sugar type producing the highest % germination (1T, 1G, 5S) indicated that there were no significant differences between them (Table 2-f). Without B vitamins only 1 and 3% glucose, and 1% trehalose, enhanced % germination significantly above that on K medium (Table 2-c). Germination without B vitamins was lowest on 3% trehalose and sucrose, though these were not significantly lower than on K medium (Table 2-c).

As with speed of germination, the addition of B vitamins produced the most marked enhancement of % germination. Significant enhancement occurred on K medium and several sugar treatments with B vitamins (Table 2-a). Within the treatments with B vitamins, sugars that produced enhancements of % germination significantly higher than K medium with B vitamins included 1 and 5% percent glucose,

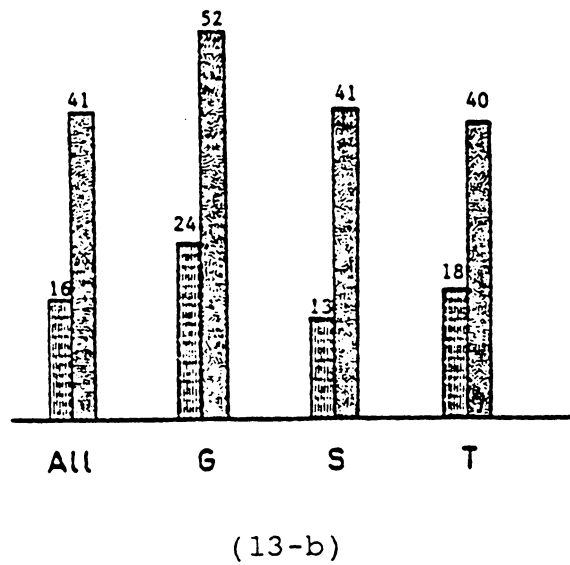
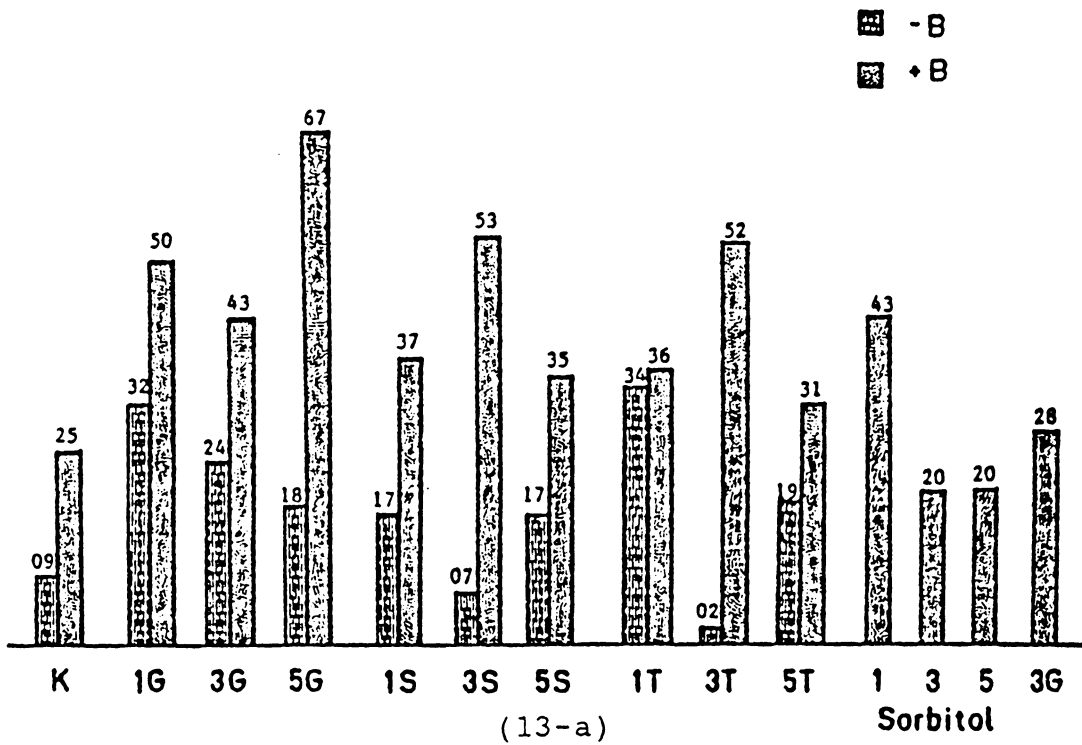


Figure 13: Percent Germination Among Treatments With and Without B Vitamins

TABLE 2

Differences in % Germination Among All Treatments at the  
0.05 Confidence Level

a) Individual Sugar Treatments

|           |           |
|-----------|-----------|
| K+B > * K | 3S+B > 3S |
| 1G+B = 1G | 5S+B = 5S |
| 3G+B > 3G | 1T+B = 1T |
| 5G+B > 5G | 3T+B > 3T |
| 1S+B > 1S | 5T+B = 5T |

b) Combined B vs Non-B

All +B &gt; All Non-B

|         |
|---------|
| G+B > G |
| S+B > S |
| T+B > T |

c) K vs Sugars

|        |      |
|--------|------|
| K < 1G | = 5S |
| < 3G   | < 1T |
| = 5G   | = 3T |
| = 1S   | = 5T |
| = 3S   |      |

d) K+B vs Sugars With B

|            |        |
|------------|--------|
| K+B < 1G+B | = 5S+B |
| = 3G+B     | = 1T+B |
| < 5G+B     | < 3T+B |
| = 1S+B     | = 5T+B |
| < 3S+B     |        |

e) Total Sugars

|       |
|-------|
| G > S |
| G = T |
| S = T |

f) Highest Percent Germination on Each Sugar

1T = 1G = 5S

\* = indicates a significantly higher % germination  
at the 0.05 confidence level.

Table 2 (cont'd)g) Total Sugars With B

$$G+B = S+B = T+B$$

h) Highest Percent  
Germination on Each  
Sugar With B Vitamins

$$5G+B = 3S+B = 3T+B$$

i) Sorbitol With B  
and Control vs  
Sugars with B

$$3G+B \text{ (1st)} = 3G+B \text{ (2nd)}$$

$$G+B > Sb+B$$

$$S+B > Sb+B$$

$$T+B > Sb+B$$

j) Highest Percent  
Germination on  
Sorbitol With B  
and Sugars With B

$$5G = 1Sb$$

$$3S = 1Sb$$

$$3T = 1Sb$$

and 3% percent sucrose and trehalose (Table 2-d). The highest % germination occurred on 5% glucose with B vitamins, and the most remarkable enhancements occurred with the addition of B vitamins to 3% trehalose and sucrose (Figure 13). The total combined % germination for each of the three sugars with B vitamins were not significantly different from each other (Table 2-g), nor were there significant differences between concentrations of sugar types with B vitamins exhibiting the highest % germinations (5G+B, 3S+B, 3T+B) (Table 2-h).

Percent germination of the control for the sorbitol treatments, 3% glucose with B vitamins, was not significantly different from the original treatment of this type (Table 2-i). Therefore, a comparison of % germination between the sorbitol treatments and the other treatments with B vitamins could be made. Total % germinations for glucose, sucrose, and trehalose with B vitamins were significantly higher than for the sorbitol treatments (Table 2-i). Comparison between concentrations of sorbitol and sugars with B vitamins exhibiting the highest % germinations indicated that these differences were not significant (Table 2-j).

### 3.3 DIFFERENCES IN FINAL VOLUME

Average final volume for all treatments with B vitamins ( $13.2 \times 10^{-2} \text{ mm}^3$ ) was significantly higher than for those without B vitamins (5.4) at the 0.05 confidence level (see Appendix B for all data). All treatments with B vitamins achieved greater final volumes than corresponding treatments without B vitamins, though these differences were not all significant (Table 3) (see Figure 52 a-e for examples of megagametophytes exhibiting normal and enhanced growth). Notably, the addition of B vitamins to all three concentrations of sucrose produced significant growth enhancement at the 0.05 confidence level.

Without B vitamins, final volume ranged from  $2.32 \times 10^{-2} \text{ mm}^3$  (3T) to 9.87 (1T). At the 0.05 confidence level 1% trehalose produced a significantly higher final volume than any of the other treatments (Figure 14). Megagametophytes cultured on 1, 3, and 5% sucrose and 3 and 5% trehalose, tended to achieve smaller final volumes than those on K medium, though these differences were not significant (Figure 14). All three glucose treatments tended to produce megagametophytes with greater final volumes than those grown on K medium, although these differences were also not significant.

TABLE 3

Differences in Average Final Volume With and Without B Vitamins

| <u>Treatment</u> | <u>Confidence Level</u> |             |
|------------------|-------------------------|-------------|
|                  | <u>0.05</u>             | <u>0.10</u> |
| Total +B vs -B   | s.*                     |             |
| KB vs K          | n.s.                    | n.s.        |
| B1G vs 1G        | s.                      |             |
| B3G vs 3G        | n.s.                    | n.s.        |
| B5G vs 5G        | n.s.                    | n.s.        |
| B1S vs 1S        | s.                      |             |
| B3S vs 3S        | s.                      |             |
| B5S vs 5S        | s.                      |             |
| B1T vs 1T        | n.s.                    | n.s.        |
| B3T vs 3T        | n.s.                    | n.s.        |
| B5T vs 5T        | s.                      |             |

\* s. indicates significant difference at the specified confidence level.

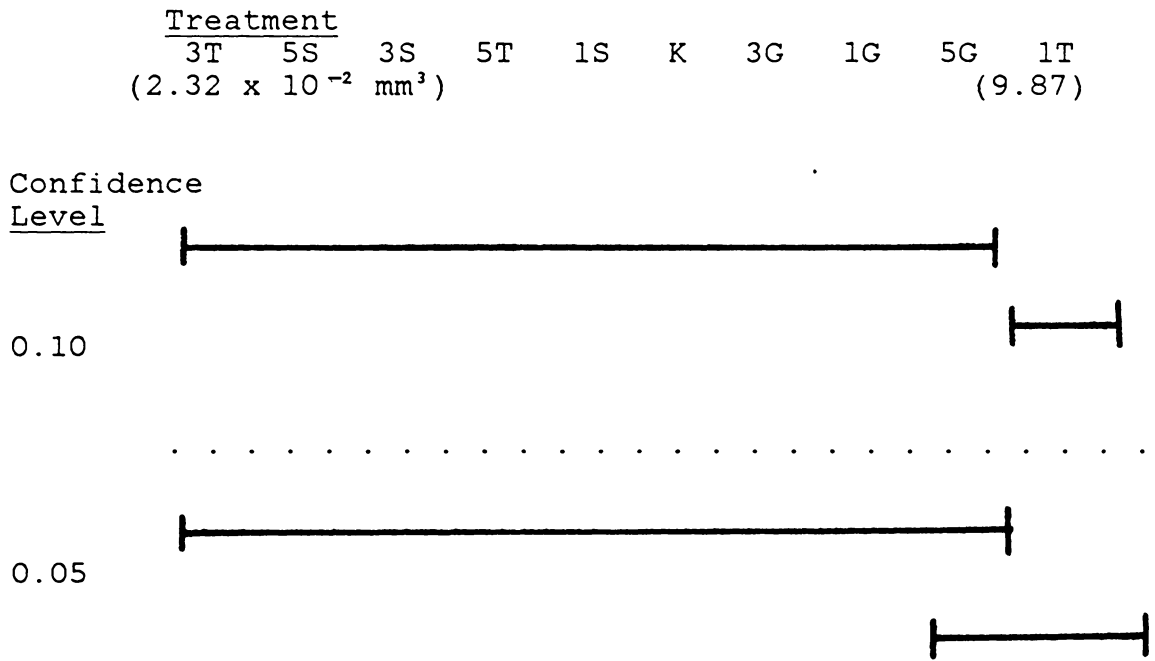


Figure 14: Differences in Final Volume for Treatments Without B Vitamins

Treatments sharing a common line are not significantly different at the specified confidence level.

Among treatments with B vitamins, average final volume had a wider range than among those without B vitamins; from  $4.86 \times 10^{-2} \text{ mm}^3$  (K+B) to 20.04 (5G+B). There were significant differences between several groups of treatments with B vitamins, though there was much overlap between them (Figure 15). At the 0.05 confidence level, average final volumes on 1 and 5% glucose with B vitamins, and 1 and 3% trehalose with B vitamins were significantly higher than on K+B.

Volume increases for each treatment through the 12 wk growth period are presented in graphs and camera lucida drawings in Figures 16 through 46.

Figure 16 presents the average growth for each substrate type, which was greatest on trehalose and glucose with B vitamins. Sucrose and sorbitol with B vitamins produced megagametophytes of lesser volume. Average final volumes on K medium with and without B vitamins were not significantly different; both treatments produced little enhancement. As with germination rates, the addition of B vitamins produced significant enhancement only in conjunction with sugars.

Figure 17 presents growth of megagametophytes on the concentration of each substrate that had the greatest effect. Notably, 1% trehalose without B vitamins produced enhanced growth comparable to 5% sucrose and 1% sorbitol

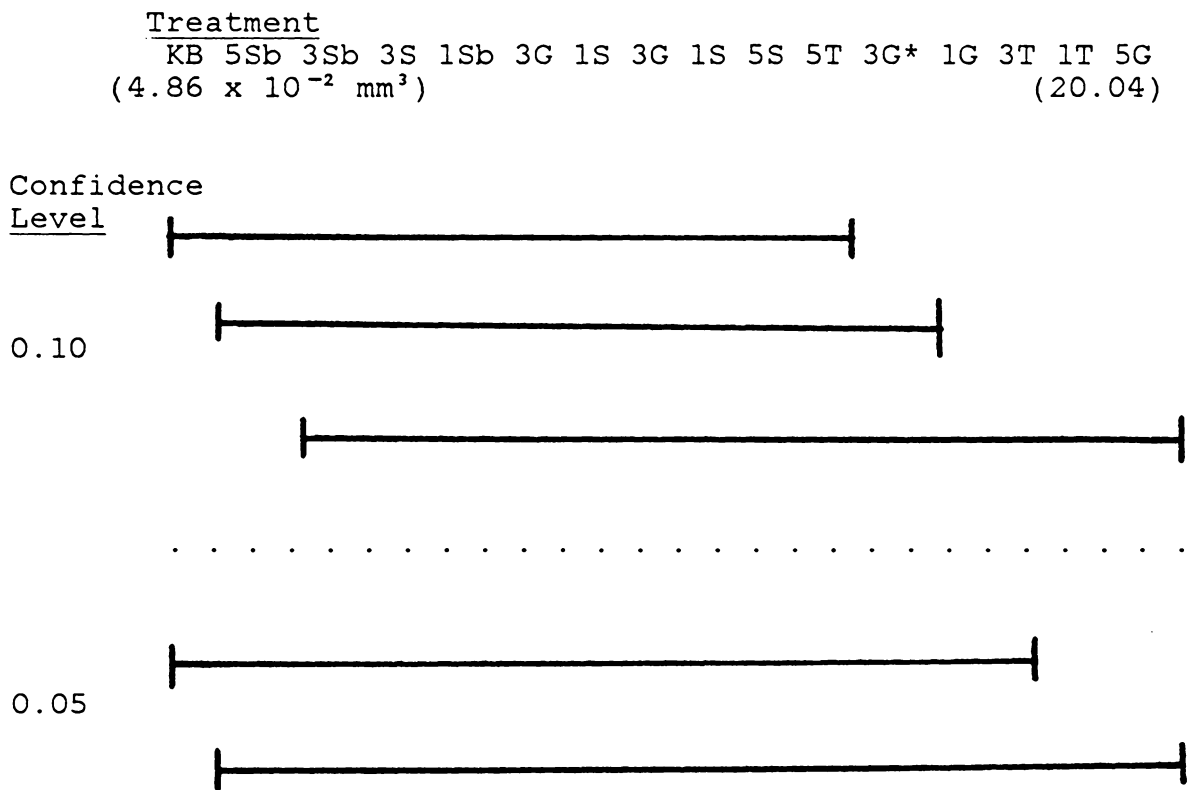


Figure 15: Differences in Final Volume for Treatments With B Vitamins

\* = sorbitol control.

Average  
Growth on All Substrate Types

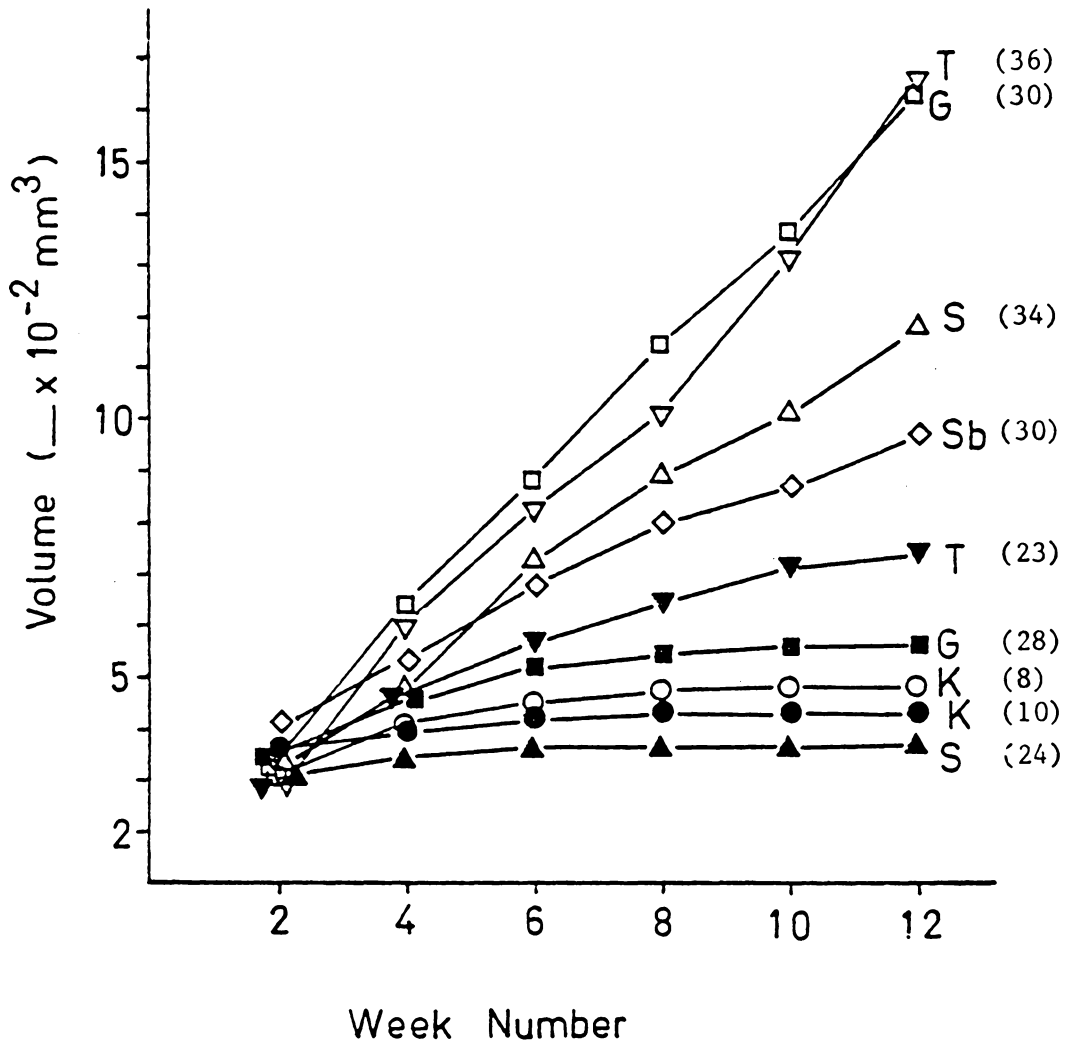


Figure 16: Average Growth on Each Substrate Type

● = without B vitamins, ○ = with B vitamins, numbers in parentheses represent sample sizes.

with B vitamins. Maximum growth response on glucose was slightly greater than on K medium, and the maximum volume achieved on sucrose was slightly below that of K medium.

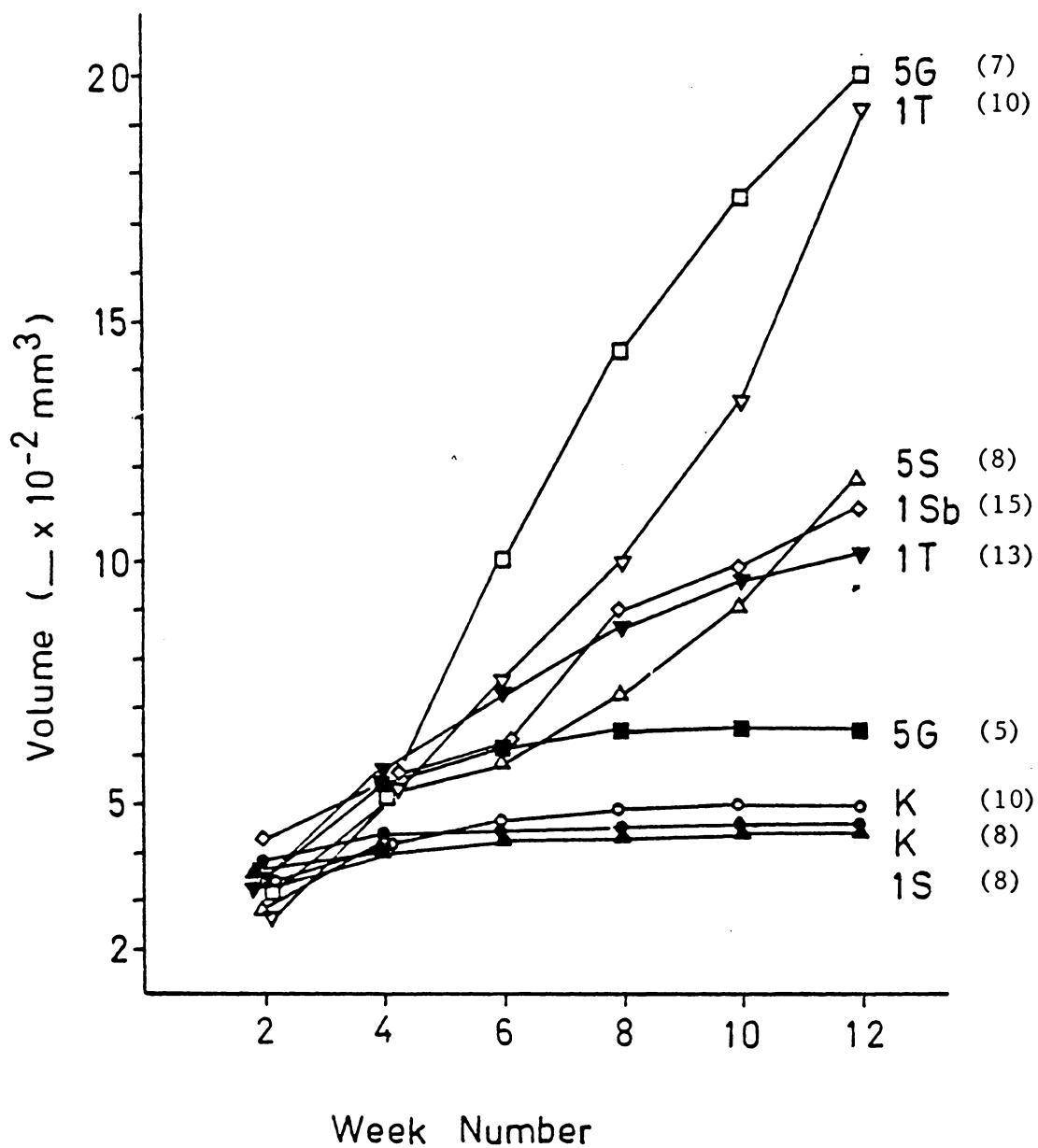


Figure 17: Highest Growth on Each Substrate Type

● = without B vitamins, ○ = with B vitamins.

### 3.3.1 Growth on K Medium With and Without B Vitamins

Figure 18 presents growth curves for K medium with and without B vitamins. Growth response was low in both treatments and leveled off in both by the eighth week. Figures 19 and 20 depict megagametophytes cultured on K medium with and without B vitamins for the entire 12 wk period. Both treatments produced normal growth responses resulting in protrusion of only the proximal surface of megagametophyte tissue from between the trilete flaps. There was little rhizoid development, and archegonia, were occasionally observed (Figures 19-a and 20-a). In all cases the surface of the tissue presented a textured, firm appearance, and color varied from white to pale yellow.

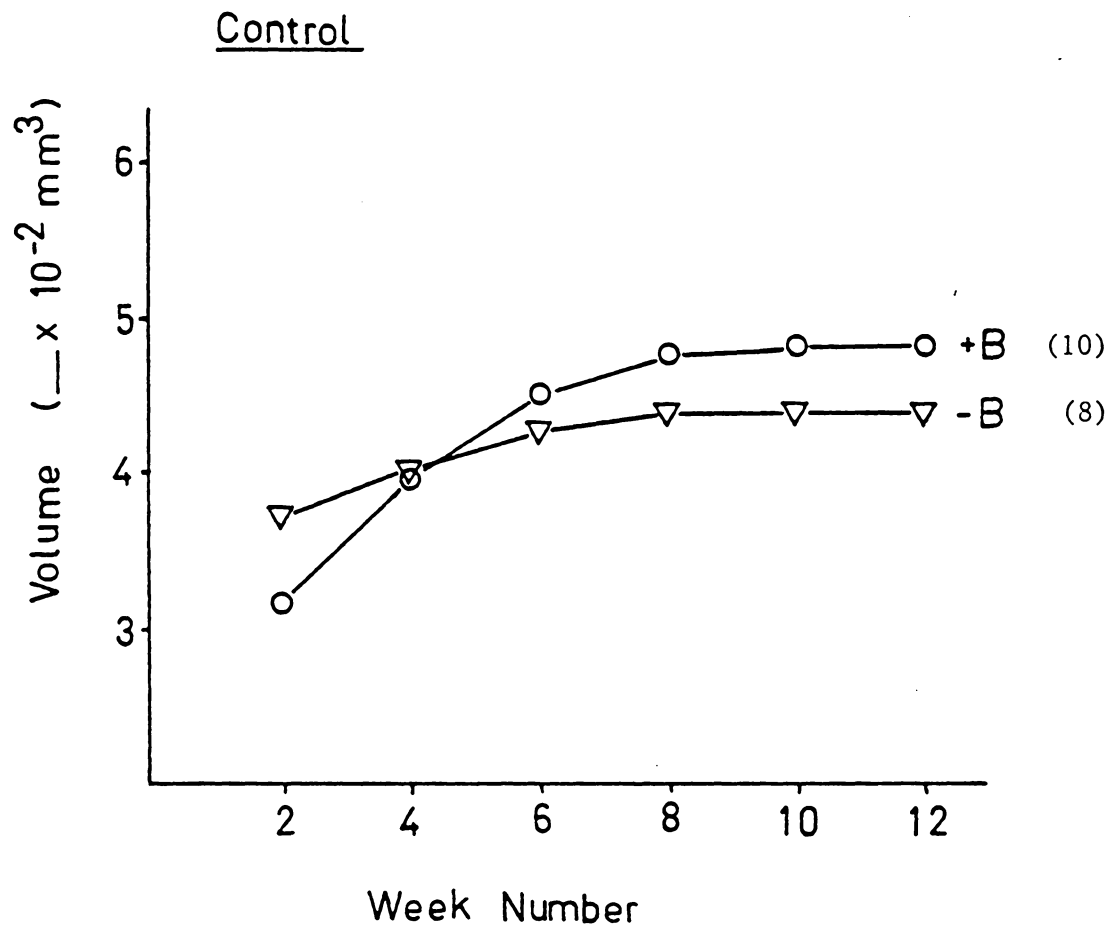


Figure 18: Growth on K Medium With and Without B Vitamins

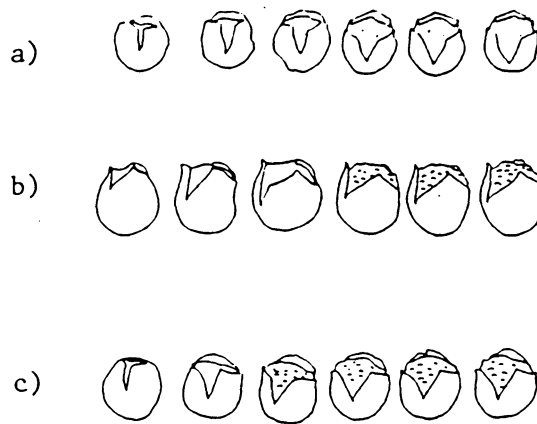


Figure 19: Response of Megagametophytes Grown on K Medium

a = K(3)IIin\* , b = K(10)Iin, c = K(7)IIIout, scale bar = 0.5 mm. Drawings were made at 2 wk intervals from germination.

\* - notation designates a specific megagametophyte. See Appendix B.

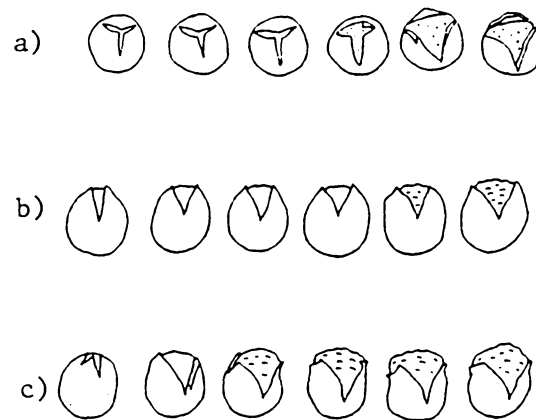


Figure 20: Response of Megagametophytes Grown on K Medium With B Vitamins

a = K+B(4)IVin, b = K+B(3)IIin, c = K+B(3)IIout,  
scale bar = 0.5 mm.

### 3.3.2 Growth on Glucose With and Without B Vitamins

Figure 21 presents growth on glucose as compared to K medium, and though average final volume was not significantly different between any of these treatments, there was a tendency for 1 and 5% percent glucose to enhance growth over either K medium or 3% glucose. Growth leveled off by the tenth week for all concentrations of glucose. Figures 22 - 24 depict a range of responses to glucose. There was definite enhancement of growth in comparison to that on K medium. Several megagametophytes grew entirely out of their megaspore walls, and spherical, callus-like masses of tissue developed, which frequently exhibited rough textures with fissures and folds. Color of the tissue, as on K medium, ranged from white to pale yellow, and there was only slight rhizoid development. One of the megagametophytes (Figure 22-c) grown on 1% percent glucose was covered with archegonia from the tenth week on.

Figure 25 displays the greatly enhanced growth that occurred on glucose with B vitamins in comparison to K+B medium. Average final volumes on the three concentrations of glucose with B vitamins were not significantly different from each other, though 1 and 5% glucose with B vitamins were significantly greater than on K+B medium (Figure 15). Unlike glucose without B vitamins, growth did not level off

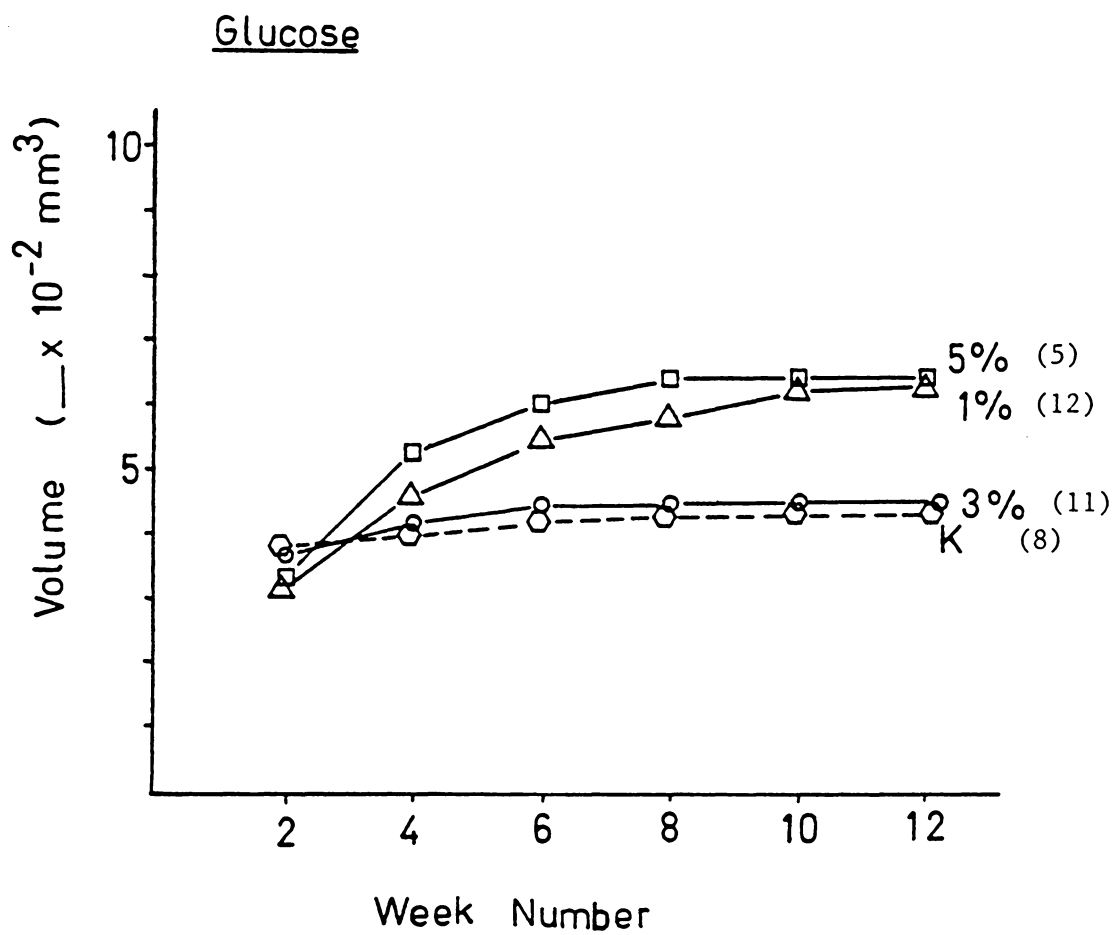


Figure 21: Growth on Glucose

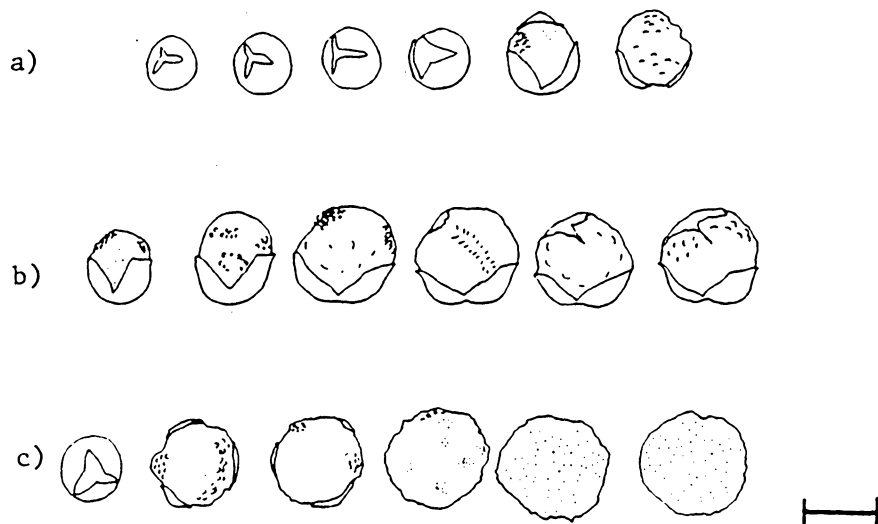


Figure 22: Response of Megagametophytes Grown on 1% Glucose

a = 1G(3)Iin, b = 1G(2)IVout, c = 1G(6)IIin,  
scale bar = 0.5 mm.

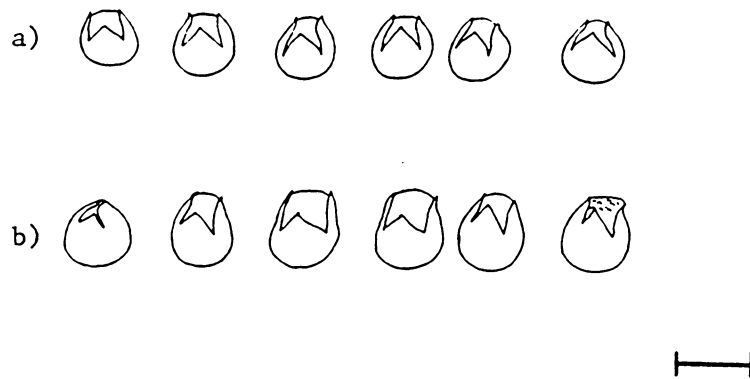


Figure 23: Response of Megagametophytes Grown on 3% Glucose  
a = 3G(2)IVout, b = 3G(10)Iout, scale bar = 0.5 mm.

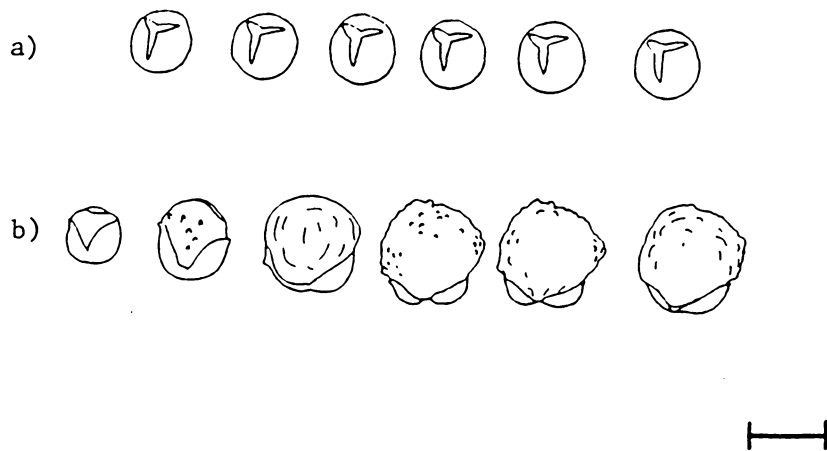


Figure 24: Response of Megagametophytes Grown on 5% Glucose  
a = 5G(7)IIIout, b = 5G(7)IVin, scale bar = 0.5 mm.

on any of the glucose treatments with B vitamins. Figures 26 - 28 depict the greatly enhanced growth that occurred on glucose with B vitamins. In most cases megagametophytes outgrew their megaspore walls, and continued to grow to large sizes. Megagametophyte tissue was often highly textured and rough, exhibiting fissures and folds, and archegonia. Tissue color was often more deeply yellow than on other treatments.

Average final volume on the sorbitol control, 3% glucose with B vitamins, was not significantly different from the first 3% glucose treatment with B vitamins, and a megagametophyte from the sorbitol control treatment was included in Figure 27. This megagametophyte became quite large, and rhizoid development was unusually pronounced. The megagametophyte depicting growth on 5% glucose with B vitamins (Figure 28) exhibited the greatest increase in size of any megagametophyte on any treatment. Final size was so large that volume data for this individual was not included in the statistical analysis with the other megagametophytes. This megagametophyte was marked by a very folded and sculpted surface, and by a bright yellow color.

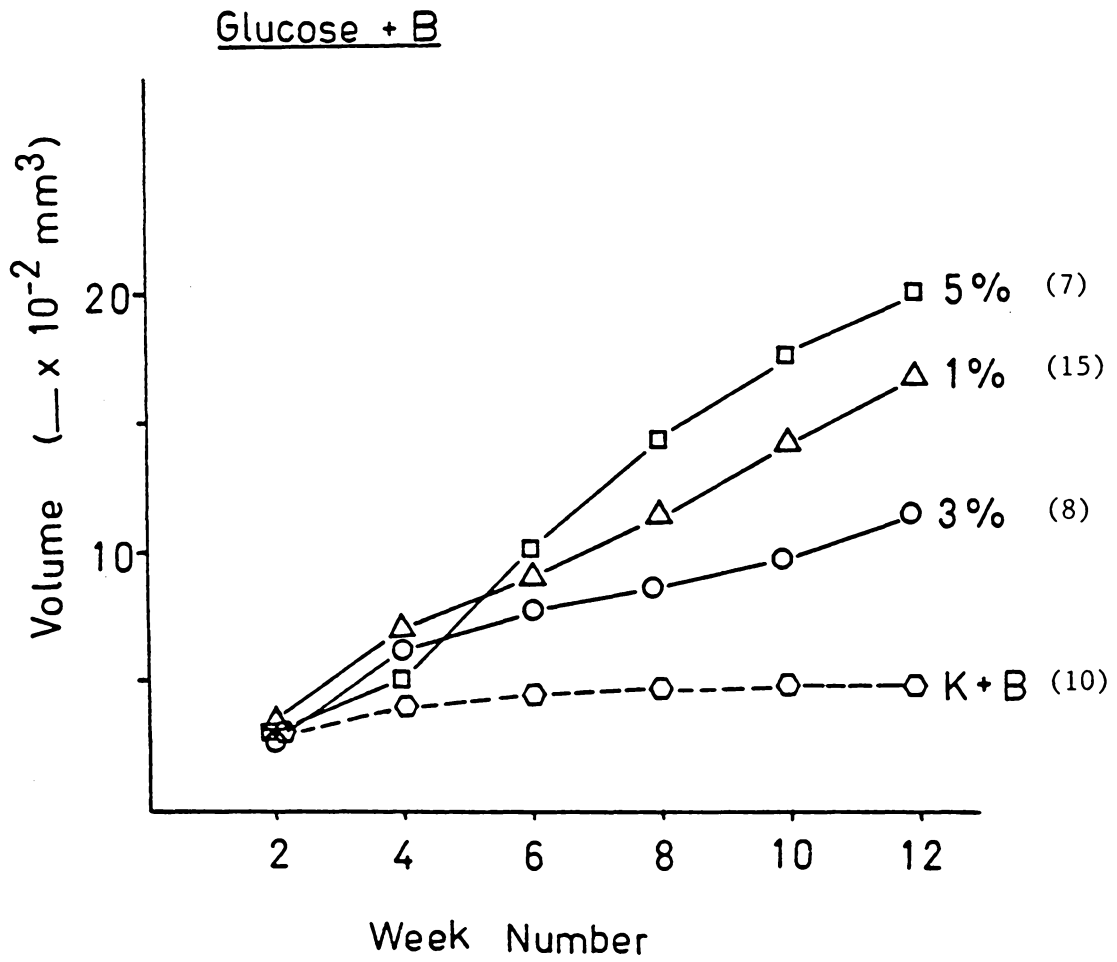


Figure 25: Growth on Glucose With B Vitamins



Figure 26: Response of Megagametophytes Grown on 1% Glucose With B Vitamins

a = 1G+B(2)IIout, b = 1G+B(4)IVout, scale bar = 0.5 mm.

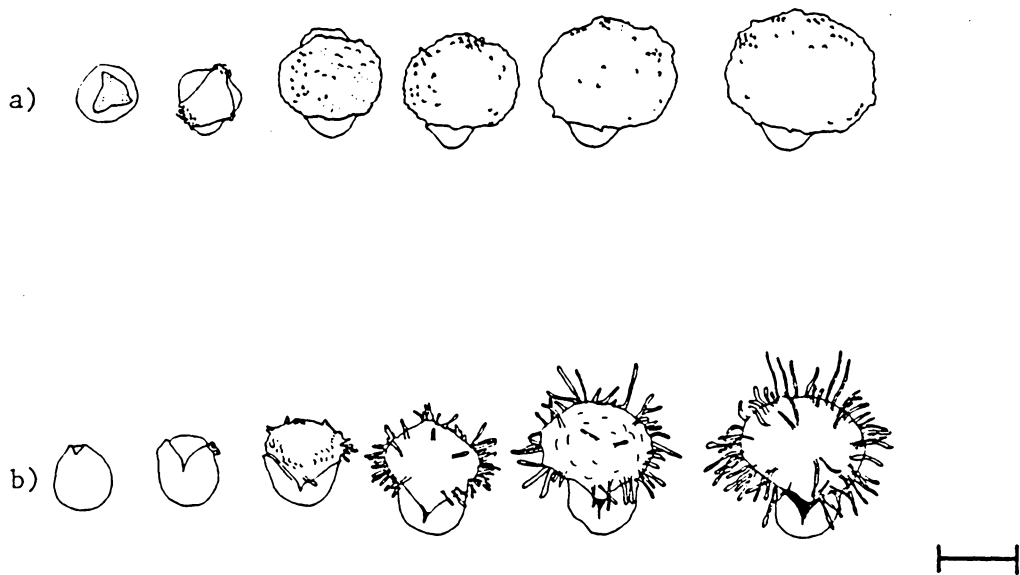


Figure 27: Response of Megagametophytes Grown on 3% Glucose With B Vitamins

a = 3G+B(4)Iout, b = sorbitol control-  
3G+B(6)IIImid, scale bar = 0.5 mm.

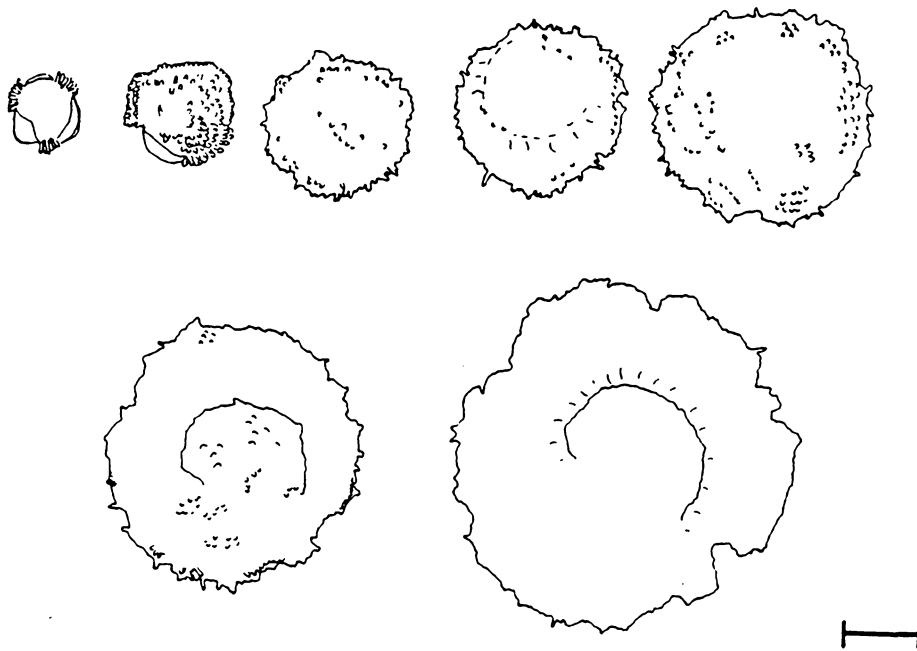


Figure 28: Response of Megagametophytes Grown on 5% Glucose With B Vitamins

5G+B(4)IIIout, scale bar = 0.5 mm.

### 3.3.3 Growth on Sucrose With and Without B Vitamins

Growth on all three concentrations of sucrose without B vitamins was depressed below that on K medium (Figure 29). Average final volume tended to decrease with increasing concentration. Though these differences were not statistically significant by themselves, the appearance of the tissue supports the idea that this sugar inhibited megagametophyte growth and development. Figures 30 - 32 depict megagametophytes grown on sucrose. These exhibited weak growth with little development of tissue. Most visible tissue appeared to lack firmness, appeared watery, and was always colored white. There was also little development of rhizoids, and apparent archegonia were rare (Figure 30-a).

One of the most notable observations in these experiments is the ability of B vitamins to greatly enhance growth on sugars, or concentrations of sugars, that produced poor responses otherwise. Growth on sucrose with B vitamins (Figure 33) is a case in point. All three concentrations of sucrose with B vitamins produced greatly enhanced growth of megagametophytes, with no indication that growth was leveling off by the end of the 12 wk period. Average final volumes on all concentrations, though, were not significantly different from that on K+B, which was a reflection of the amount of variation of response within

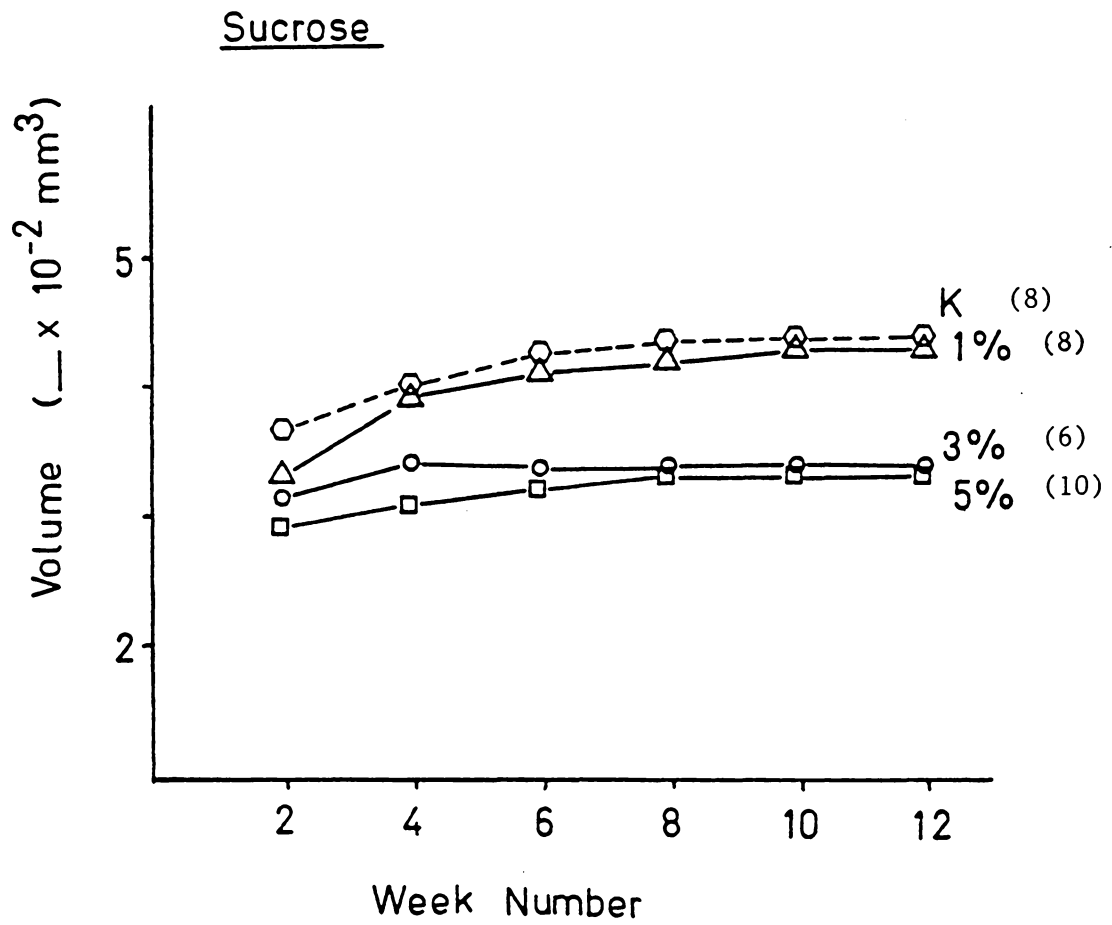


Figure 29: Growth on Sucrose

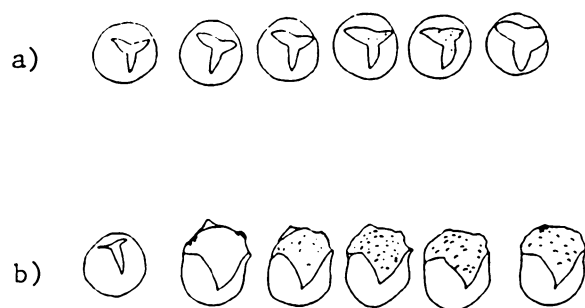


Figure 30: Response of Megagametophytes Grown on 1% Sucrose  
 a = 1S(4)IVin, b = 1S(5)IIout, scale bar = 0.5 mm.

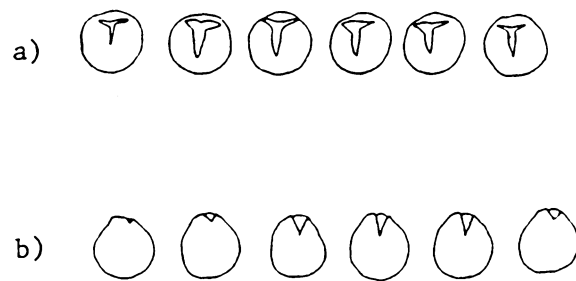


Figure 31: Response of Megagametophytes Grown on 3% Sucrose

a = 3S(5)IVout, b = 3S(8)IVin, scale bar = 0.5 mm.

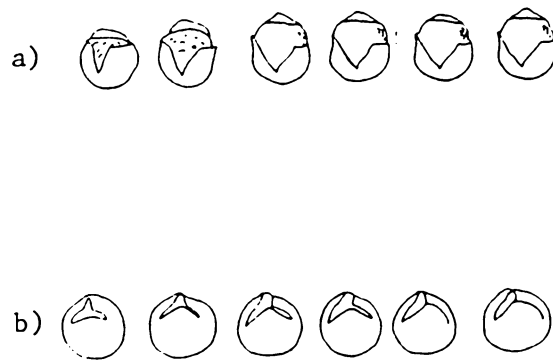


Figure 32: Response of Megagametophytes Grown on 5% Sucrose

a = 5S(4)IVin, b = 5S(4)Iout, scale bar = 0.5 mm.

these treatments. Though growth of several megagametophytes was greatly enhanced, others grew only to normal sizes. Figure 34 depicts the enhancement of growth that occurred on all concentrations of sucrose with B vitamins. Several megagametophytes grew out of their megaspore walls, and the tissues exhibited a textured and firm appearance, with no indication of the weak growth response observed on sucrose treatments without B vitamins. There was also some rhizoid formation, and color of the tissue ranged from white to yellow.

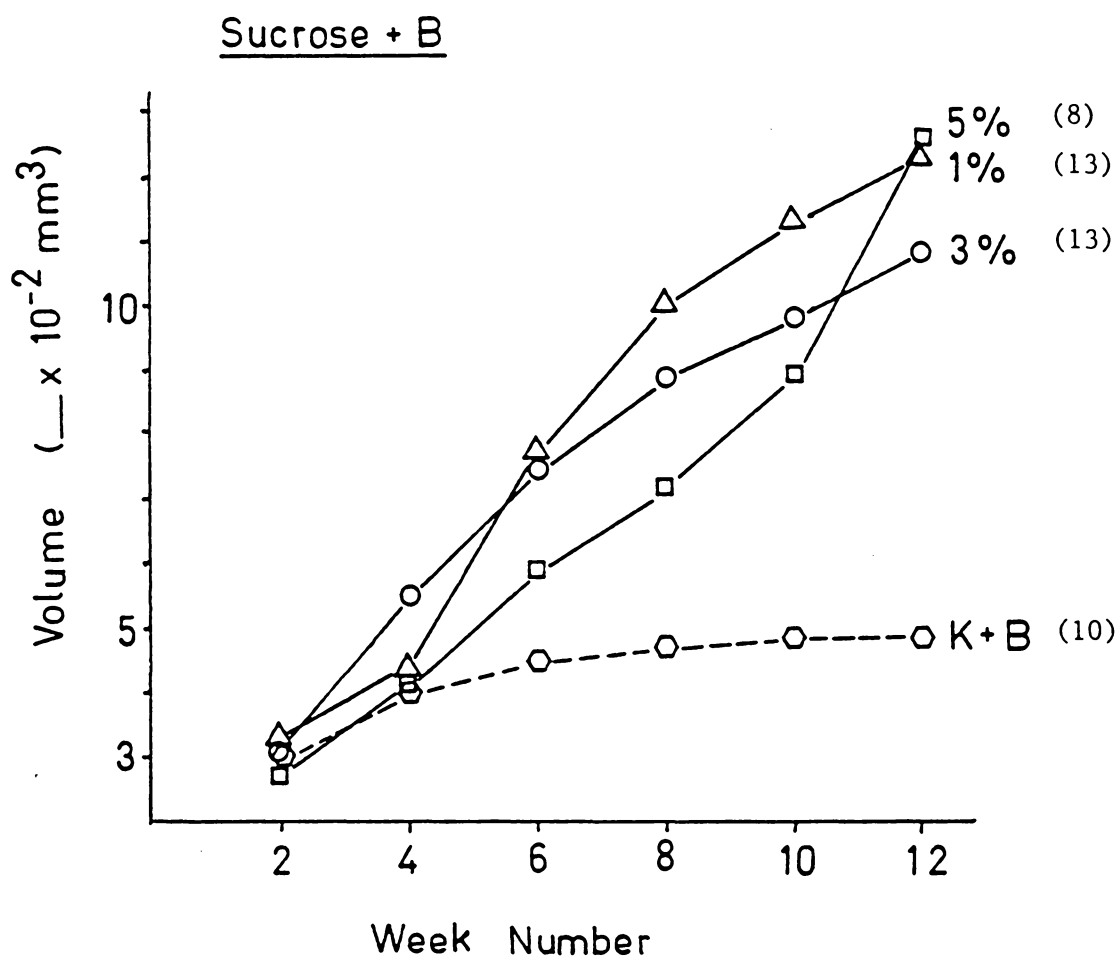


Figure 33: Growth on Sucrose With B Vitamins

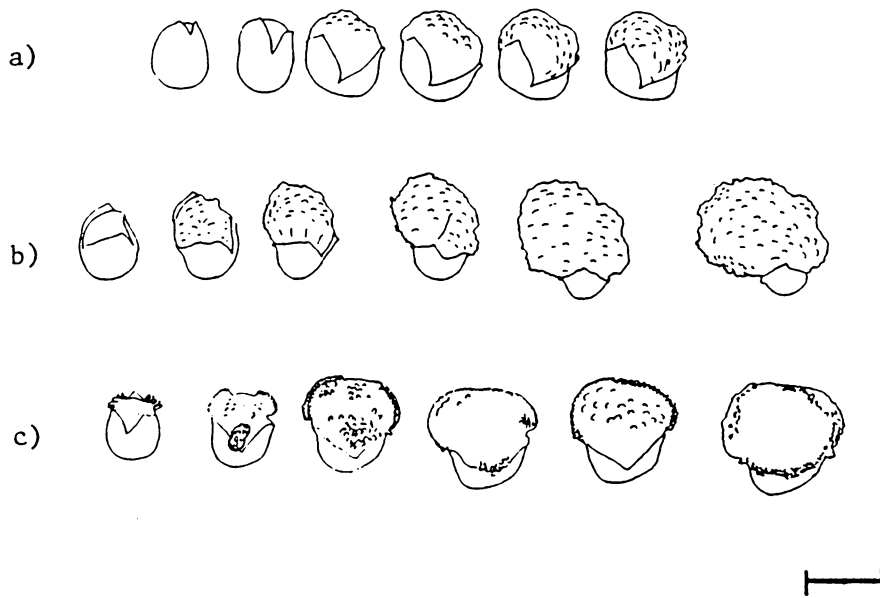


Figure 34: Response of Megagametophytes Grown on 1, 3, and 5% Sucrose With B Vitamins

a = 1S+B(7)Iin, b = 3S+B(3)Iout, c = 5S+B(4)IIIin, scale bar = 0.5 mm.

#### 3.3.4 Growth on Trehalose With and Without B Vitamins

Figure 35 presents the response of megagametophytes to trehalose treatments without B vitamins. Growth was greatly enhanced on 1% trehalose, but was depressed on 3 and 5%. The lack of growth on 3 and 5% is reminiscent of the poor growth on sucrose treatments. Differences in average final volume between K medium, and 3 and 5% trehalose were not significant. Growth on 1% trehalose, however, was significantly greater than on any other treatments without B vitamins at the .05 confidence level (Figure 14), and was comparable to the highest average final volumes achieved on sucrose and sorbitol treatments with B vitamins. On both 3 and 5% trehalose growth was essentially level from the time of germination, and on 1% trehalose there was a trend towards leveling off near the end of the 12 wk period. Figure 36 depicts the large volumes achieved by megagametophytes on 1% trehalose, and the similarity with many megagametophytes cultured on sugar treatments with B vitamins. Tissue appeared firm and textured, with slight rhizoid development, and color ranged from white to yellow. Megagametophytes cultured on 3 and 5% trehalose displayed little development of firm tissue (Figure 37).

Growth was greatly enhanced on trehalose with B vitamins for all three concentrations (Figure 38). Average final

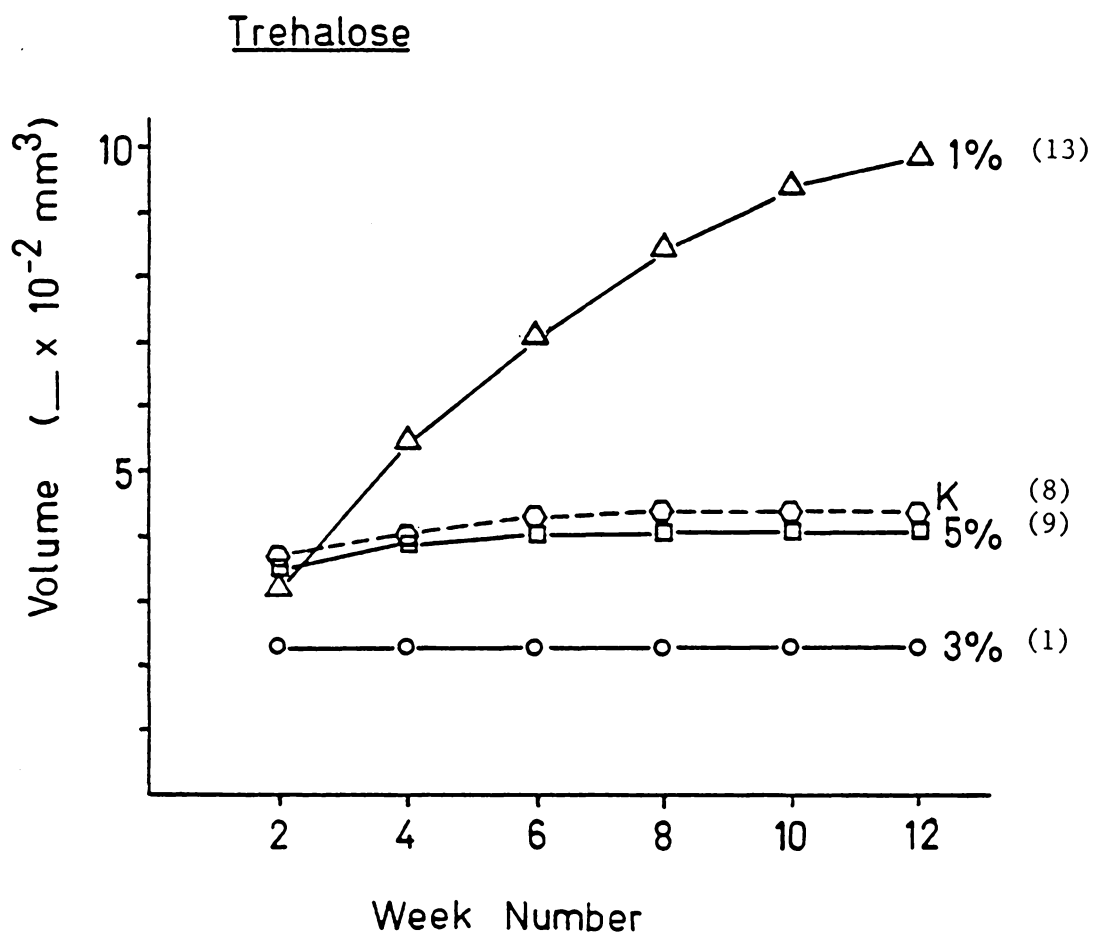


Figure 35: Growth on Trehalose

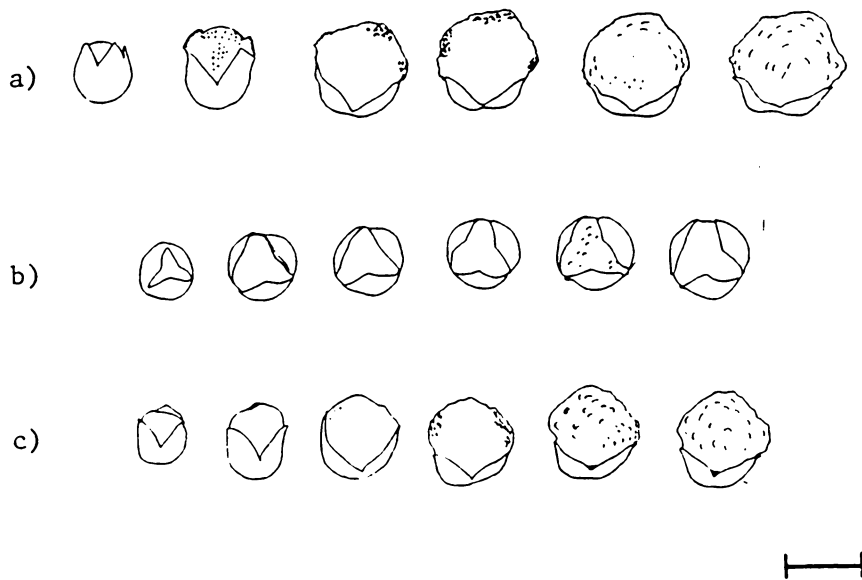


Figure 36: Response of Megagametophytes Grown on 1% Trehalose

a = 1T(1)II, b = 1T(2)III, c = 1T(1)I, scale bar = 0.5 mm.

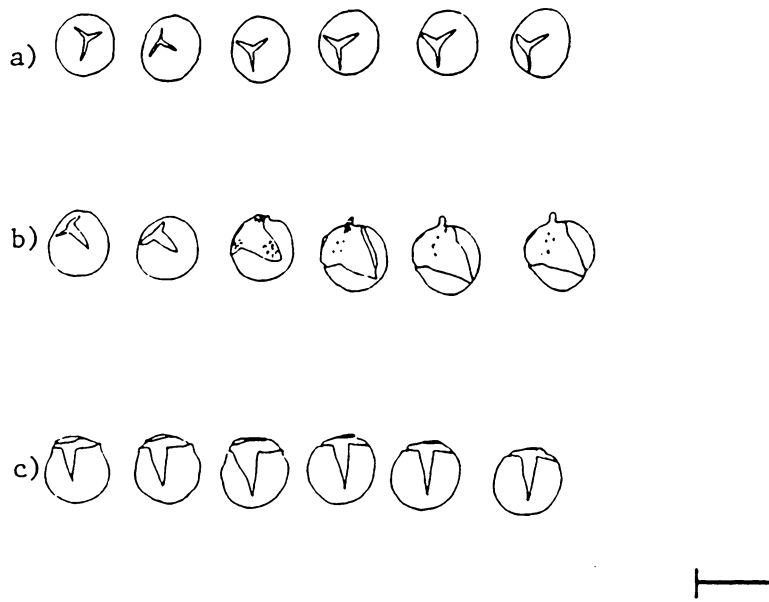


Figure 37: Response of Megagametophytes Grown on 3 and 5% Trehalose

a = 3T(3)IVin, b = 5T(7)Iin, c = 5T(1)III, scale bar = 0.5 mm.

volume on 1% trehalose with B vitamins was comparable to that on 5% glucose with B vitamins, which exhibited the greatest final volume of any treatment (Figure 17). Average final volumes on the three concentrations of trehalose with B vitamins were not significantly different from each other (Figure 15), but 1% and 3% trehalose with B vitamins were significantly higher than K+B at the .05 confidence level. There was no indication that growth was leveling off on any of the three concentrations at the end of the 12 wk period. Figures 39 - 41 depict the response of megagametophytes on the three concentrations of trehalose with B vitamins, many of which rapidly grew out of their megaspore walls. Figure 41 depicts a megagametophyte cultured on 5% trehalose with B vitamins that grew entirely out of its megaspore wall. The tissues of these megagametophytes, as on all sugar with B treatments, appeared firm, textured, and had some rhizoid development. Color of the tissues ranged from white to yellow.

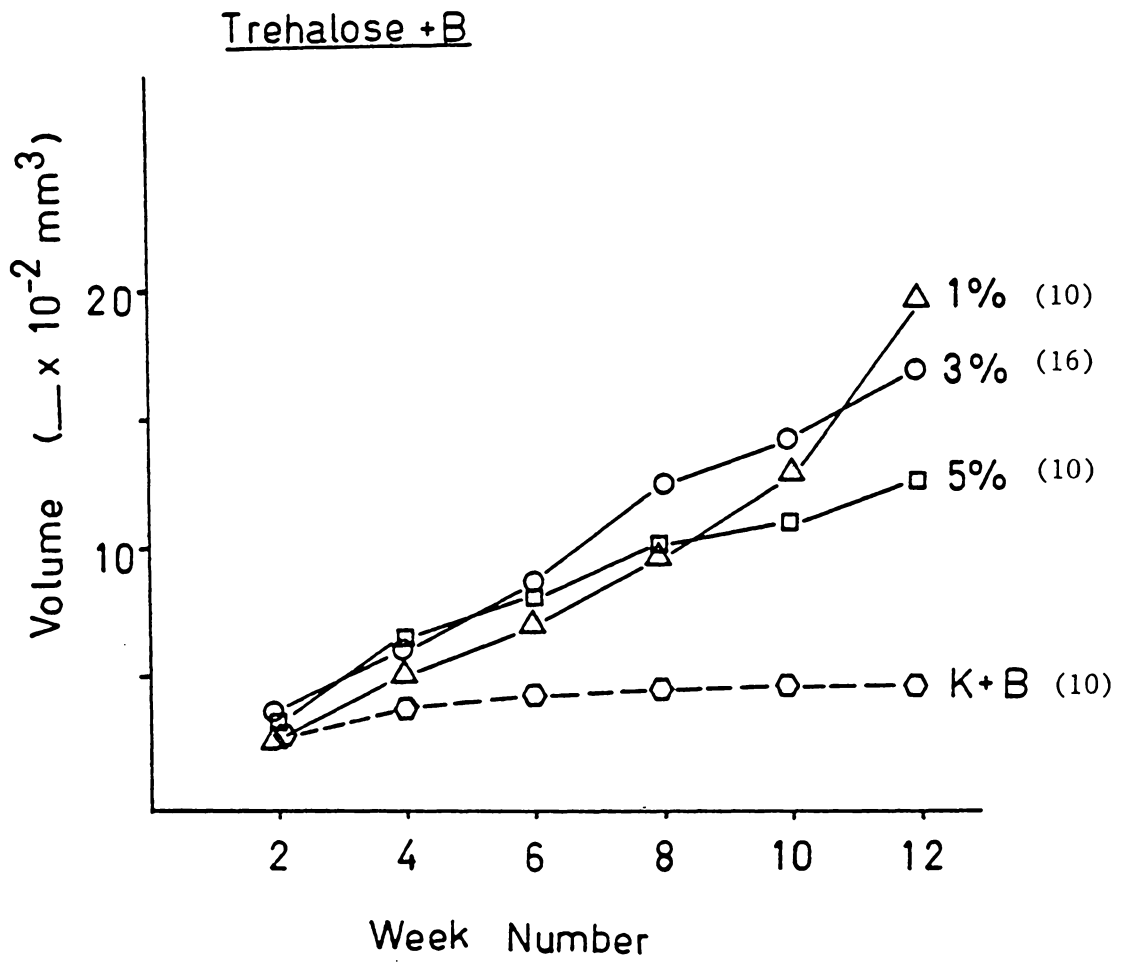


Figure 38: Growth on Trehalose With B Vitamins

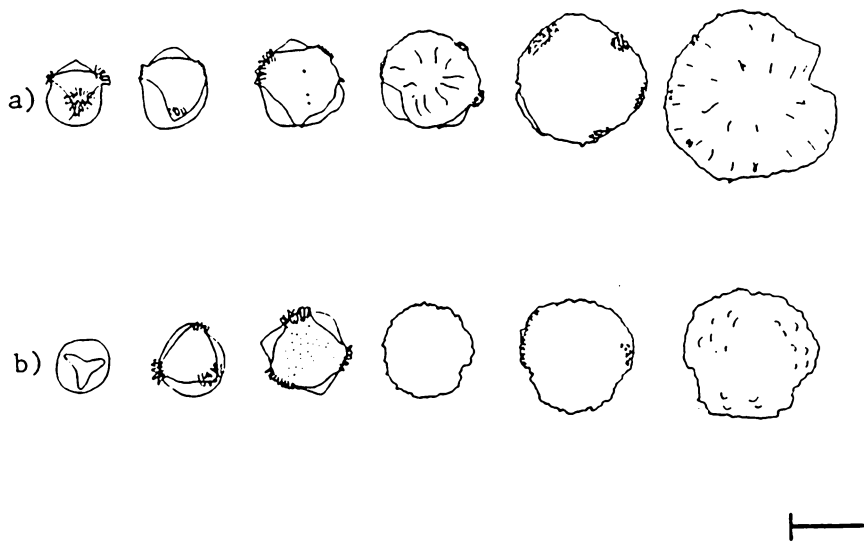


Figure 39: Response of Megagametophytes Grown on 1% Trehalose With B Vitamins

a = 1T+B(2)IIIin, b = 1T+B(5)IIin, scale bar = 0.5 mm.

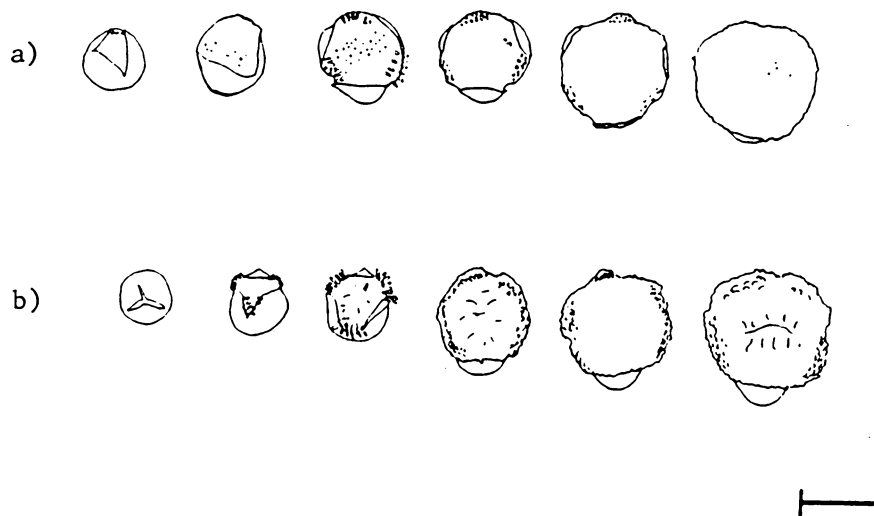


Figure 40: Response of Megagametophytes. Grown on 3% Trehalose With B Vitamins

a = 3T+B(1)IVin, b = 3T+B(2)IVin, scale bar = 0.5 mm.

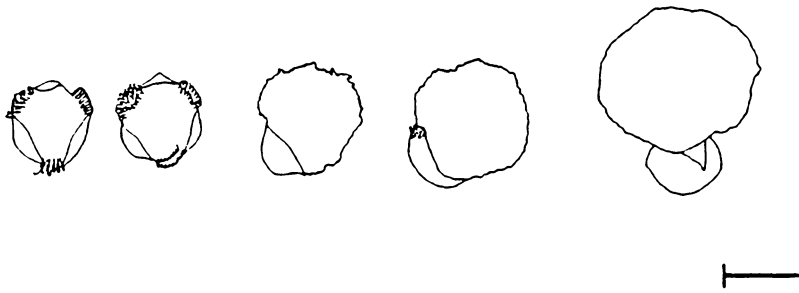


Figure 41: Response of Megagametophytes Grown on 5% Trehalose With B Vitamins

5T+B(5)IIIout, scale bar = 0.5 mm.

### 3.3.5 Growth on Sorbitol With B Vitamins

Final volume achieved on the sorbitol control treatment, 3% glucose with B vitamins, was not significantly different from that on the first of this type (Figure 15). Figure 42 presents the growth of these two treatments. One megagametophyte cultured on the sorbitol control treatment was included in Figure 27. Figures 43 - 46 present growth responses of megagametophytes on sorbitol treatments with B vitamins, which were enhanced on all three concentrations. There was no indication of growth leveling off on either 1 or 3% sorbitol with B vitamins, though growth on the 5% sorbitol treatment with B vitamins did level off. As with sugar treatments with B vitamins, there was rapid growth, resulting in firm and textured tissues. Several megagametophytes grew entirely out of their megaspore walls (Figures 44-a and 46-a). Some rhizoids developed, and color of the tissues ranged from white to yellow.

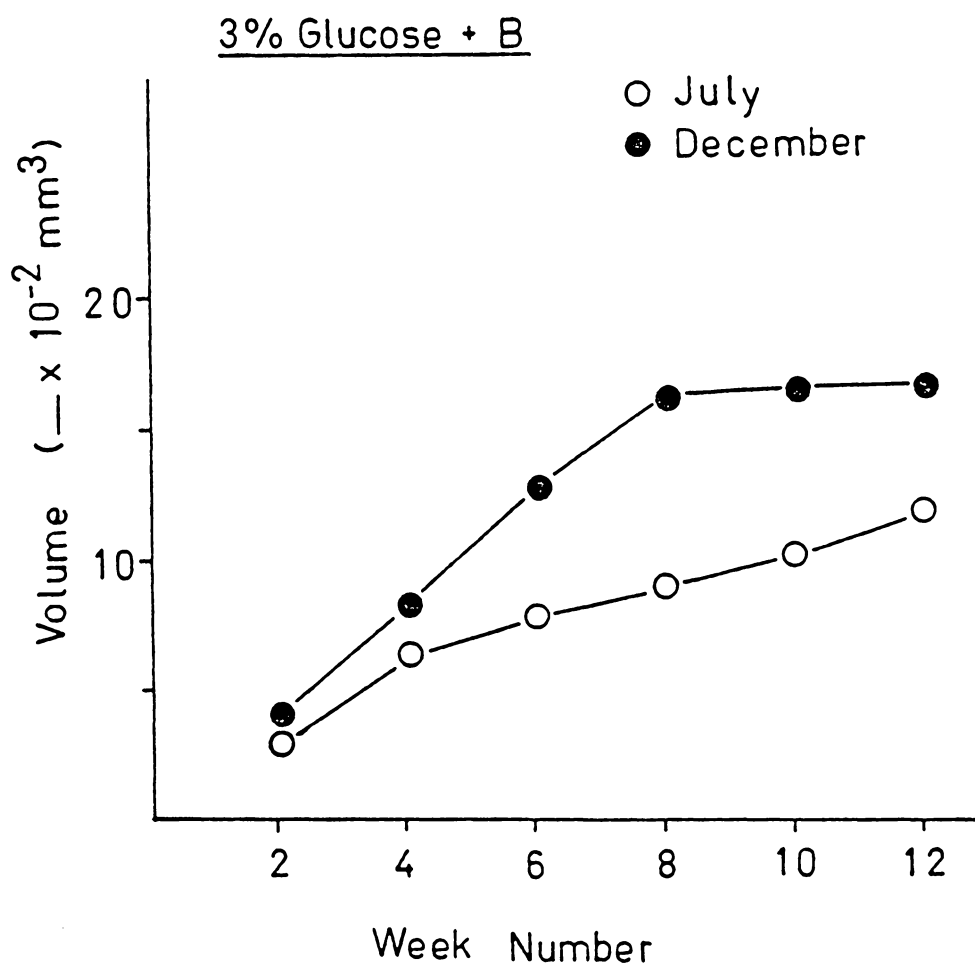


Figure 42: Growth on 3% Glucose With B Vitamins - June and December Experiments

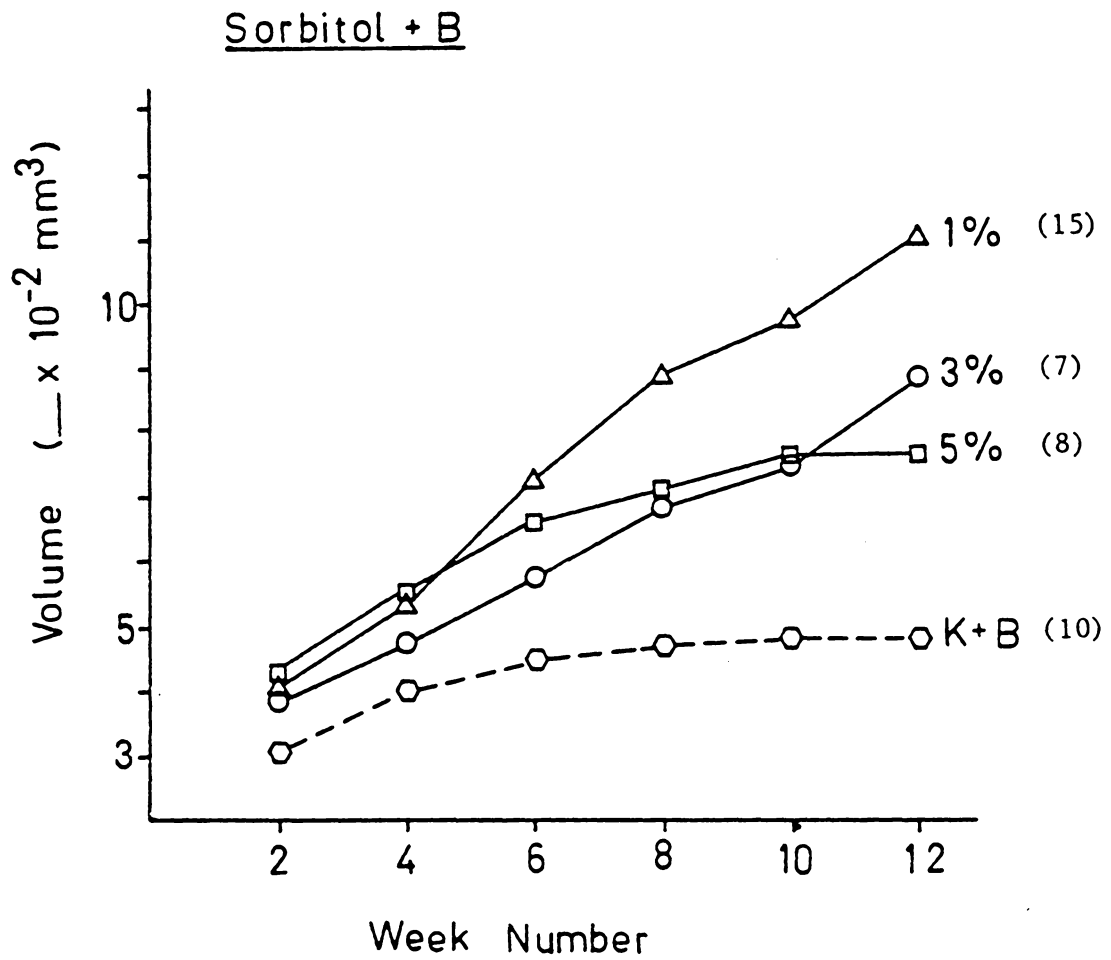


Figure 43: Growth on Sorbitol With B Vitamins

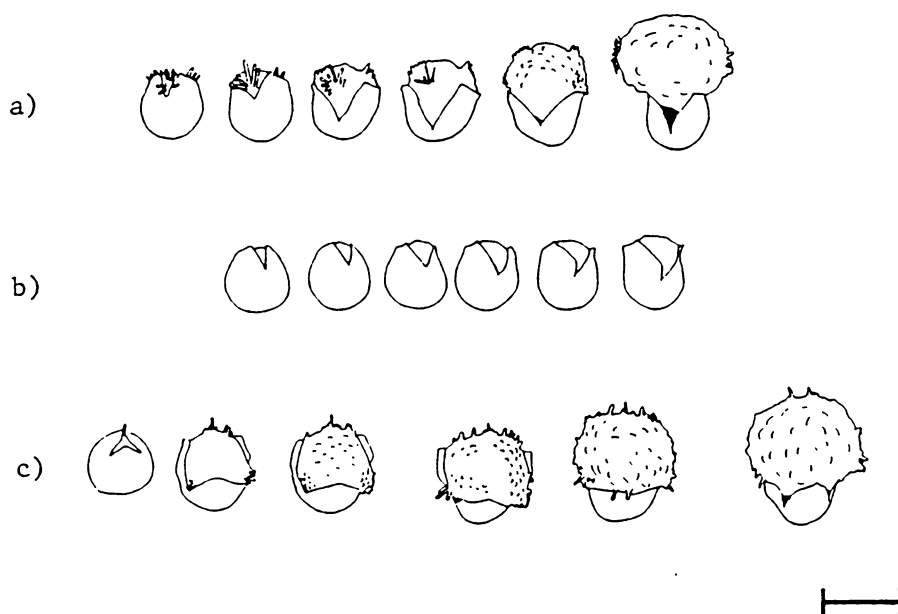


Figure 44: Response of Megagametophytes Grown on 1% Sorbitol With B Vitamins

a = 1Sb+B(4)Iin, b = 1Sb+B(6)IIIout, c = 1Sb+B(3)IIIout, scale bar = 0.5 mm.



Figure 45: Response of Megagametophytes Grown on 3% Sorbitol With B Vitamins

a = 3Sb+B(8)IIIout, b = 3Sb+B(4)IVin, scale bar = 0.5 mm.

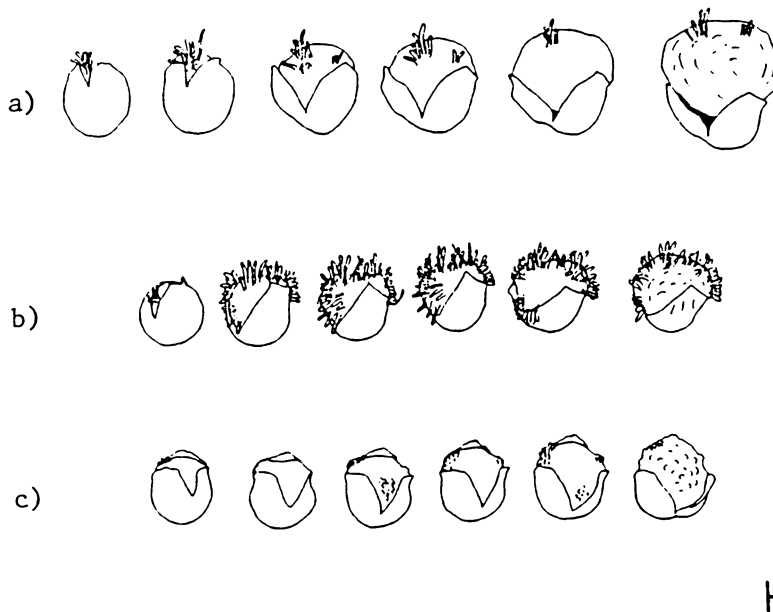


Figure 46: Response of Megagametophytes Grown on 5% Sorbitol With B Vitamins

a = 5Sb+B(5)Iin, b = 5Sb+B(1)Iin, c = 5Sb+B(7)IVin, scale bar = 0.5 mm.

### 3.4 RESPONSE TO THE SECOND TREHALOSE TREATMENTS

Responses to the second set of trehalose treatments were different from the first set. Rates of germination were not different between the 2 sets, but percent germination and final volume exhibited different patterns (Table 4 and Figure 47). Response to the second control treatment (K) was not significantly different from the first K treatment. This last observation suggests that megaspores sown in July and December had a similar potential for response.

Percent germination on 1% trehalose was high in the first treatment, but significantly lower in the second treatment (Table 4). A significantly higher percent germination was exhibited in the second 3% trehalose treatment as compared to the first. Germination percentage on the second 5% trehalose treatment was twice as high as in the first, but this was not a significant difference.

Growth on the two sets of treatments exhibited different patterns. The first set (Figure 35) produced a pattern of growth in which 1% trehalose produced the highest average final volume ( $9.87 \times 10^{-2} \text{ mm}^3$ ), with 3 and 5% trehalose tending to inhibit growth to levels below that on K medium. In the second set of trehalose treatments, however, average final volume increased with increasing concentration of the sugar (Figure 47). One % trehalose still enhanced growth

TABLE 4

Percent Germination on 1st and 2nd Trehalose Treatments  
(July and December)

| <u>Treatment</u> | <u>Percent Germination</u> |            |
|------------------|----------------------------|------------|
|                  | <u>1st</u>                 | <u>2nd</u> |
| K                | 09                         | = * 10     |
| 1T               | 34                         | > 07       |
| 3T               | 02                         | < 19       |
| 5T               | 19                         | = 37       |

\* (=) indicates no significant difference  
at the 0.05 confidence level.  
See Appendix C for all data.

above that on K medium, and achieved almost the same average final volume as before ( $8.76 \times 10^{-2} \text{ mm}^3$ ). Three and 5% concentrations, however, produced larger average final volumes than 1%. In fact, 3 and 5% trehalose produced larger average final volumes than any treatment in either the first or second sets. Growth responses to K medium and 1% trehalose in the second set of treatments were not significantly different from their earlier values (Figure 48).

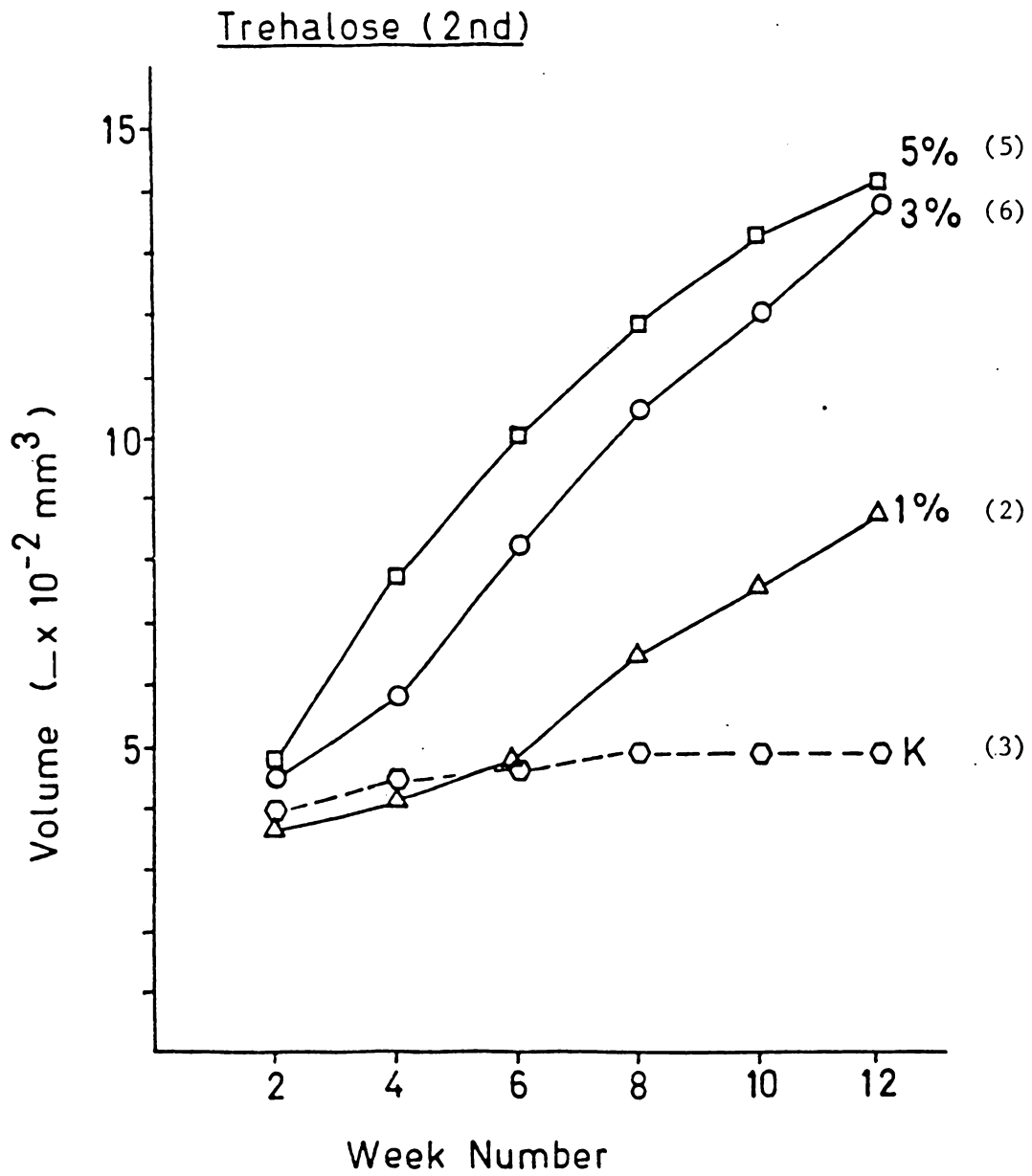


Figure 47: Growth on Trehalose - 2nd Set

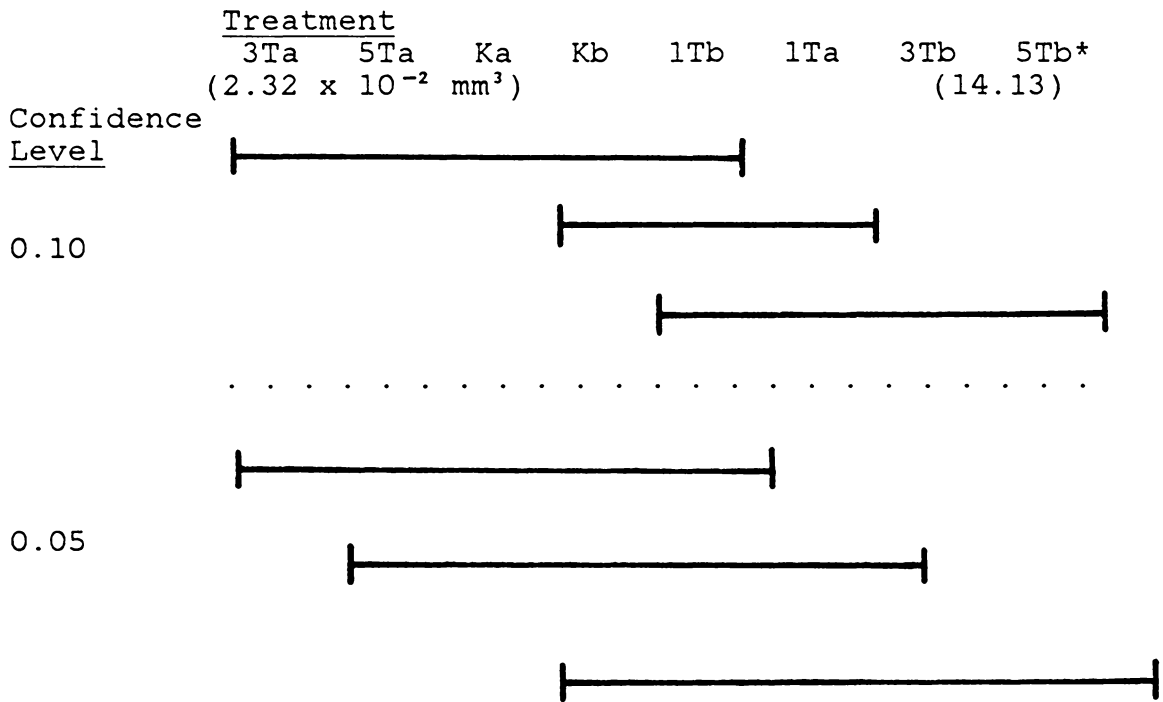


Figure 48: Differences In Final Volume - Trehalose (1st and 2nd Sets)

a = first set of trehalose experiments, b = second set. Treatments sharing a common line are not significantly different at the specified confidence level.

### 3.5 CORRELATION ANALYSIS

The 3 relationships that were analyzed are presented graphically in Figures 49 - 51. Percent germination versus final volume displayed the highest correlation ( $r = 0.81$ ) of the three (Figure 49). Treatments producing high % germination also tended to produce larger megagametophytes. Number of days to 50% germination versus final volume (Figure 50) had a lower correlation ( $r = 0.61$ ) and, therefore, a weaker linear relationship between the two responses. Treatments with rapid germination also tended to produce larger megagametophytes. Figure 50 indicates that no treatments produced both slow germination and large average final size. Number of days to 50% germination versus % germination (Figure 51) exhibited the lowest correlation ( $r = 0.48$ ). There was only a slight tendency for treatments producing rapid germination to also produce higher % germination. Figure 51 indicates, however, that no treatments produced both slow germination and high % germination.

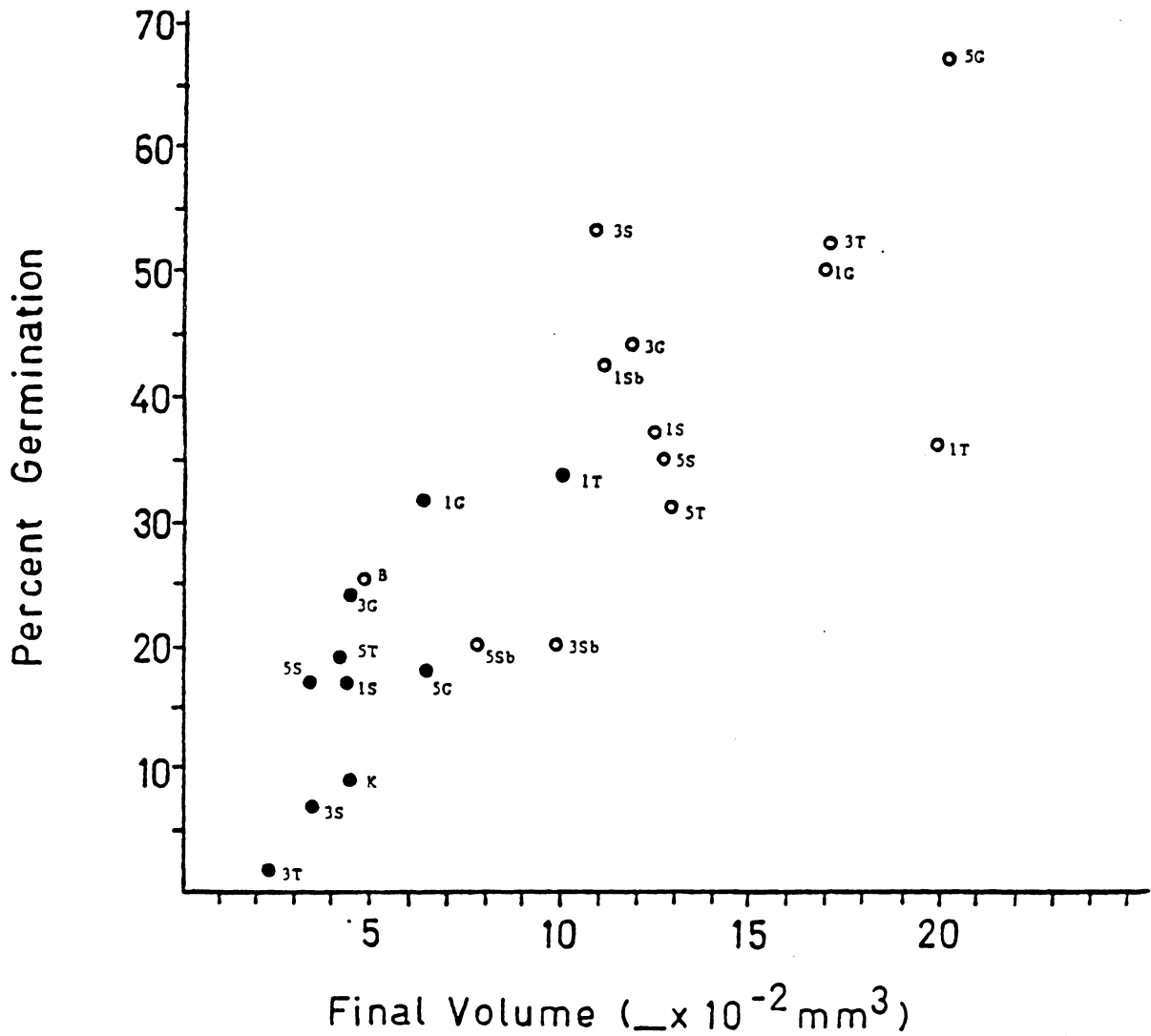


Figure 49: Correlation Between % Germination and Final Volume

● = without B vitamins, ○ = with B vitamins,  $r = 0.81$ .

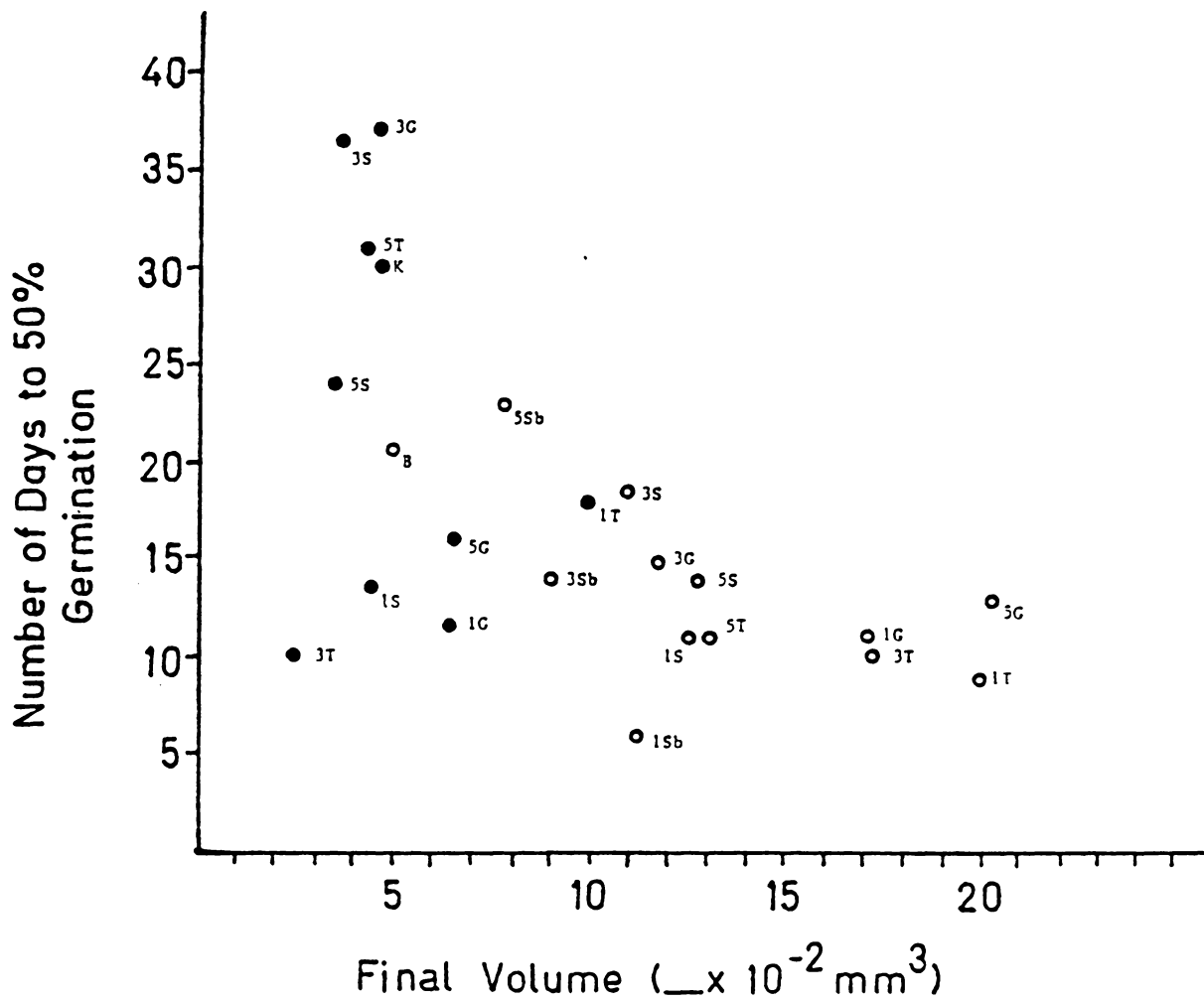


Figure 50: Correlation Between Time to Reach 50% Germination and Final Volume

● = without B vitamins, ○ = with B Vitamins,  
 $r = 0.61$ .

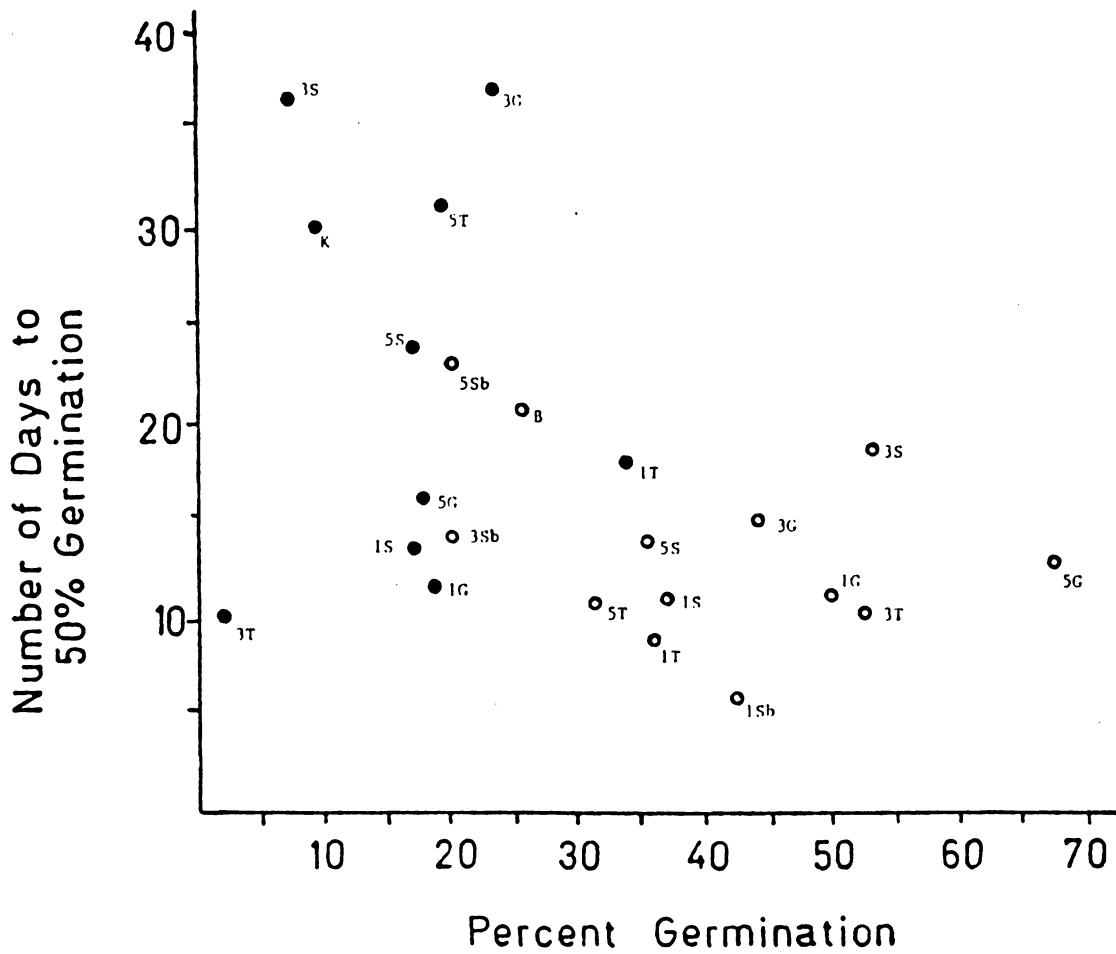


Figure 51: Correlation Between Time to Reach 50% Germination and % Germination

● = without B vitamins, ○ = with B vitamins,  $r = 0.48$ .

### 3.6 RESULTS OF FLUORESCENCE ANALYSIS

Many of the megagametophytes examined contained chlorophyll-a, though the amount was less than in sporophyte tissue (Table 5) (see Appendix D for all data). Whether megagametophytes containing chlorophyll-a were photosynthesizing in culture is unknown at this time. Megagametophytes cultured on most of the carbon treatments had less chlorophyll-a than those cultured on K medium, and those cultured on trehalose and sucrose had less than those cultured glucose and sorbitol. The amount of chlorophyll-a was highest in megagametophytes cultured on 3% Sorbitol with B vitamins, and there was none in those cultured on 1% sucrose. The presence or absence of B vitamins did not appear to determine the amount of chlorophyll-a present, though the amount of chlorophyll-a present in megagametophytes cultured on K medium with B vitamins was less than those cultured on K medium alone. The highest amount of chlorophyll-a in megagametophyte tissue was ca. 25% of that present in an equivalent amount of sporophyte tissue.

TABLE 5

Amount of Chlorophyll-a Present in Megagametophyte and  
Sporophyte Tissue

| Treatment            | <Chlorophyll-a><br>(micrograms/mm <sup>3</sup> ) |
|----------------------|--------------------------------------------------|
| sporophyte<br>(stem) | $1.20 \times 10^{-2}$                            |
| 3%Sb +B              | $4.94 \times 10^{-3}$                            |
| K                    | 2.97 "                                           |
| 1%Sb +B              | 2.40 "                                           |
| 3%G +B               | 1.89 "                                           |
| 5%Sb +B              | 1.74 "                                           |
| 5%G +B               | 1.05 "                                           |
| K+B                  | $9.60 \times 10^{-4}$                            |
| 1, 3, 5% G           | 9.50 "                                           |
| 1, 5% T              | 6.31 "                                           |
| 3%T +B               | 5.00 "                                           |
| 3%S +B               | 4.38 "                                           |
| 5%S +B               | 3.48 "                                           |
| 1%S +B               | 3.27 "                                           |
| 1%S                  | 0.00                                             |

Sb = sorbitol

G = glucose

T = trehalose

S = sucrose

B = B vitamin mixture

### 3.7 CELL SIZE ANALYSIS

Megagametophytes cultured on K medium, and 1, 3, and 5% sorbitol with B vitamins contained cells of approximately the same size (Table 6). Average cross-sectional area of cells in megagametophytes of Selaginella compared favorably with that of fern gametophytes as determined by Whittier (1964a).

TABLE 6  
Cell Size Analysis

| <u>Treatment</u> | <u>n</u> | <u>Cross-Sectional Area</u> ( <u>mm<sup>2</sup></u> ) |
|------------------|----------|-------------------------------------------------------|
|------------------|----------|-------------------------------------------------------|

Selaginella megagametophytes

|      |   |              |
|------|---|--------------|
| K    | 2 | .0032 ±.0008 |
| B1Sb | 4 | .0026 ±.0008 |
| B3Sb | 3 | .0028 ±.0007 |
| B5Sb | 4 | .0025 ±.0011 |

Fern gametophytes (from Whittier, 1964a)

|            |    |       |
|------------|----|-------|
| 0% Sucrose | 20 | .0021 |
| 0.5 "      | "  | .0021 |
| 2.5 "      | "  | .0028 |
| 6.0 "      | "  | .0025 |

### 3.8 CELLULAR ORGANIZATION OF MEGAGAMETOPHYTES

Sections of several megagametophytes that exhibited normal or enhanced growth are shown by Figure 52 f-h. The megagametophyte grown on K medium (Figure 52-f) was structurally similar to other Selaginella megagametophytes (Robert, 1971; Foster & Gifford, 1974). There was a basal zone of lipid reserve tissue with little cellularization in the distal end of the megagametophyte, and the upper end (proximal end of megaspore) consisted of highly cellularized tissue. Unlike the descriptions of Robert (1971), however, there was no indication of a membranous structure, the diaphragm, which divides the megagametophyte of Selaginella kraussiana into upper and lower zones. At this stage of development there were also no rhizoids nor archegonia present. The megaspore wall was not removed from this megagametophyte, and can be seen in section.

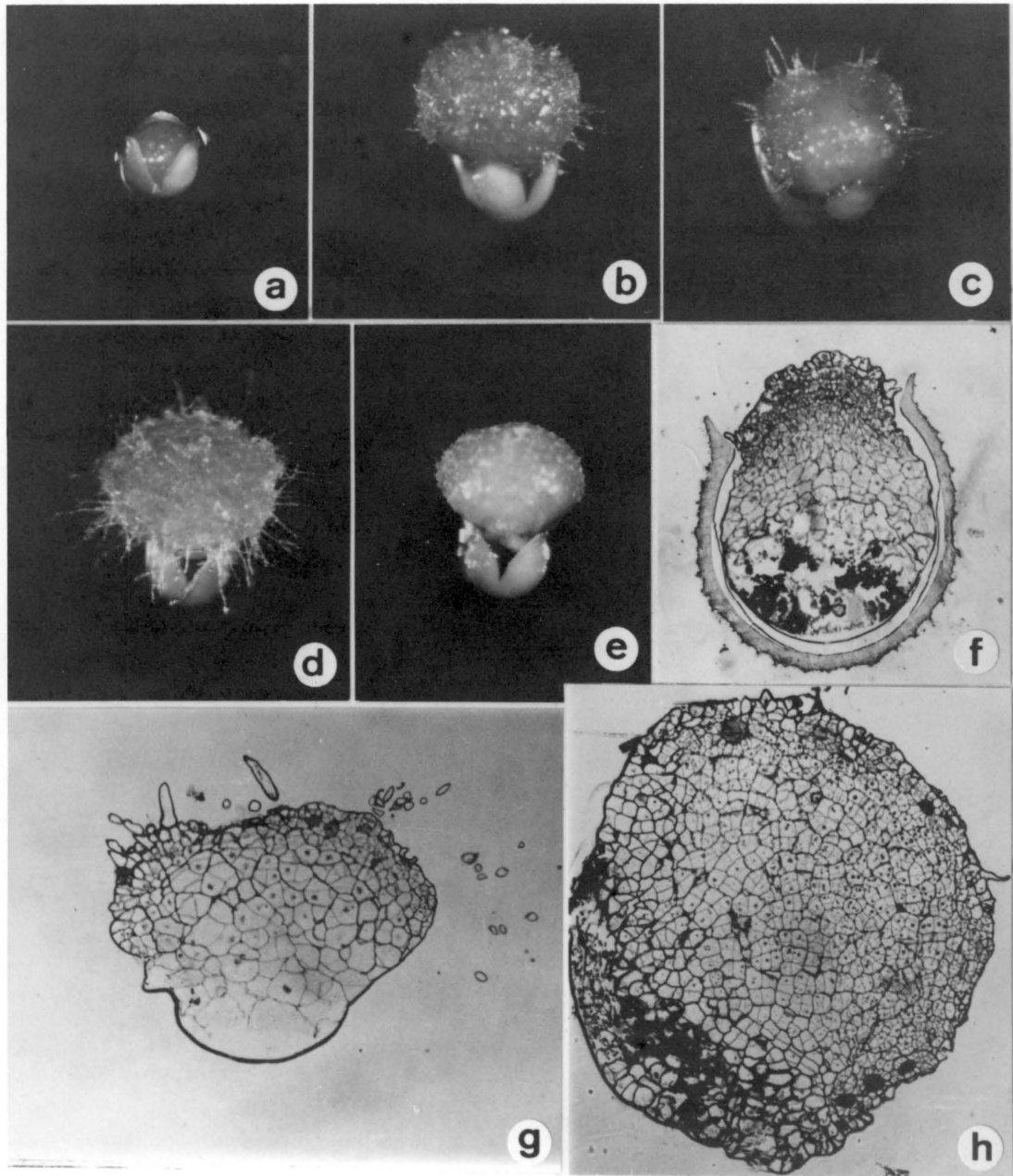
Several megagametophytes grown on sorbitol with B vitamins provided the first observations of the internal anatomy resulting from enhanced growth. One of these megagametophytes, cultured on the 1% sorbitol treatment, that was alive when selected for fixation and imbedding, displayed a highly cellularized internal anatomy, with some remnant of the lipid reserve tissue present at the basal end (Figure 52-h). Notably, enhanced growth did not occur at

the expense of the total depletion of the lipid reserve. Locations of recent cell divisions, determined by the presence of thin cell walls, indicated that cell divisions were not located in specific regions, but were dispersed internally throughout the tissue. One megagametophyte grown on 3% sorbitol with B vitamins exhibited a smaller, less cellularized, tissue (Figure 52-g). Both of these megagametophytes (Figure 52 g,h) had archegonia, with egg cells, located immediately below their proximal surfaces.

Figure 52: Normal and Enhanced Growth of Megagametophytes -  
External and Internal Observations

a. Growth on K medium. b. on 3% trehalose (2nd set).  
c. on 5% trehalose (2nd set). d. on 3% glucose with  
B vitamins. e. on 1% sorbitol with B vitamins. a-e. x 29.

f. Normal internal anatomy (on K medium). g. on 5%  
sorbitol with B vitamins. h. on 1% sorbitol with B  
vitamins. f-h. x 110.



## Chapter IV

### DISCUSSION

#### 4.1 THE EFFECTS OF B VITAMINS

The known metabolic roles played by the B vitamins are important to an understanding of these experiments. The B vitamin mixture used by Wetmore and Morel (1951) to enhance growth in megagametophytes of Selaginella was also used by them (Morel & Wetmore, 1951) to enhance growth in culture of the gametophyte of the fern, Osmunda cinnamomea. This vitamin mixture consisted of thiamin, niacin, pantothenate, pyridoxine, biotin, and inositol. Inositol is not considered to be a vitamin, but its growth enhancing properties place it in the category of a growth factor (Lehninger, 1975). A brief discussion of the known roles of each of these components now follows.

Thiamin serves as a prosthetic group in the enzyme cocarboxylase (thiamin pyrophosphate). This enzyme is involved in the mainstream of carbohydrate metabolism by allowing pyruvate formed in glycolysis to enter the Krebs cycle through decarboxylation to form acetaldehyde, which then reacts with coenzyme-A to form acetyl-coA (Salisbury and Ross, 1978). Therefore, thiamin is extremely important

in allowing available carbohydrates to be utilized for cellular metabolism.

Niacin is a major component of the pyridine nucleotides, NAD and NADP, which function as coenzymes in a large number of oxidation-reduction reactions in respiration, photosynthesis, nitrogen metabolism (nitrate reductase), and lipid degradation.

Pantothenate (coenzyme A) serves as a carrier of acyl groups at the beginning of the Krebs cycle, and in lipid synthesis and degradation.

Pyridoxine, in the form of pyridoxal phosphate, forms an important part of amino transferase enzymes, through which the bulk of amino acids are synthesized. The enzyme transfers amino groups of amino acids to the alpha carbon of keto acids.

Biotin is a coenzyme used in condensation reactions of acetate units (from acetyl-coA) to form new fatty acids in lipid synthesis.

Inositol, a cyclic sugar alcohol, is probably synthesized in plant and animal tissues by the cyclization of D-glucose (Cosgrave, 1980). It is not a building-block of any known coenzyme, but is a component of inositol phosphoglyceride, a lipid molecule found in membranes. Robert (1971) determined that granules in the lipid reserve of Selaginella

kraussiana, which he likened to aleurone grains, consisted of inositol hexaphosphate (phytic acid), though the connection here may be illusory.

#### 4.2 UTILIZATION OF B VITAMINS AND SUGARS

Treatments with B vitamins alone speeded germination and increased % germination, but did not effect growth. This suggests that, by themselves, B vitamins had no effect on the utilization of native reserve foods stored in the megaspores.

Some sugar treatments alone affected certain aspects of germination and growth of megagametophytes. This suggests that, under specific circumstances, the sugars in the media were used by some megagametophytes.

Sugars with B vitamins in every case enhanced germination and growth of the megagametophytes. From the above controls, one can conclude that these effects are due to the enhanced utilization of sugars in the media when stimulated by B vitamins. The effects observed are probably not due to enhanced utilization of the native reserves within the megaspores.

Specific analyses of the responses of megagametophytes to the various treatments follows.

#### 4.3 GERMINATION TIMING AND NUTRITION

Speed of Germination may have been linked to the stage of development of the megagametophyte at the time the megaspore was shed, and to the length of the ensuing time period during which the megaspore remained dormant before inoculation onto a medium favorable for germination (i.e. with continuous water availability). Prolonged dormancy may have diminished viability in megaspores due to increased dehydration and depletion of the nutritional reserve tissue. Megaspores with diminished viability, when inoculated onto agar, may have recovered to germinate, though the time to do so may have varied according to the extent of degradation within the megaspore, and the particular nutritional treatment on which the megaspore had been inoculated. For example, if megaspores were placed on a nutritional treatment consisting of B vitamins with sugar, perhaps less viable ones recovered quickly to germinate. Fully viable megaspores may have germinated more quickly than normal, and younger megaspores may have developed rapidly too. A pattern of response such as this may explain the more rapid and tightly clustered germinations that occurred on B vitamin treatments (Figure 3). The B vitamin mixture may have played an important role in this enhancement of germination due to facilitation of any of the following:

lipid reserve utilization, membrane synthesis, carbohydrate utilization, energy production, cell wall synthesis, protein synthesis.

Variation in number of days to germinate may have been introduced in three ways. The first was through forced drying, dehiscence, and dispersal of some megaspores before they had reached normal maturity. These might require additional time before they could be anatomically and physiologically capable of germinating. The second possible source of variation was the collection and inoculation of megaspores that matured long before collection, and that had lain dormant within strobili. Inspection of numerous strobili has shown that many megaspores are too large to be dispersed, and remain trapped for varying lengths of time. Some older megaspores were probably utilized in this experiment, since larger megaspores were preferentially used. Many of the megaspores which did not germinate might have been too old. Much of the variation in germination rate on treatments without B vitamins might possibly be so explained. A third cause of variation may have involved genetic differences amongst megaspores as a result of segregation and assortment of chromosomes at meiosis. None of the four megaspores in any tetrad was likely genetically identical. Support for genetic variation comes from the

differing colors and production of rhizoids in megagametophytes on a single treatment. This genetic variation may have extended to other, less visible aspects of metabolism.

Sugar treatments without B vitamins did not produce significant differences in the average number of days to germinate (Figure 1). However, a sugar that could be metabolized by megagametophytes might increase the speed of germination as a result of enhanced metabolism. A nutritionally inert sugar could be expected to slow germination since it would confront megaspores only as an osmotic agent and make water less available. The increase in average final size on glucose treatments (Figure 21) over that on K medium is an indication that megagametophytes can metabolize this sugar. Further support comes from the observations that % germination was significantly higher on 3 and 5% glucose than on K medium (Table 2-c), and all 3 concentrations tended to exhibit more rapid germination than on K medium. One % trehalose and 1% sucrose also tended to increase the rate of germination, but they appear to have been inhibitory at 3 and 5%. This may indicate an ability to use these sugars only in low concentrations. The differences in average number of days to germinate between treatments without B vitamins were not significant though

average final volumes suggest that some treatments may have been more favorable than others. The B vitamins may have allowed more rapid utilization of supplemental carbohydrates, possibly explaining why total treatments with B vitamins germinated significantly faster than total treatments without B vitamins. Ideally, study of the effects of different sugars on germination response should use megaspores that are developmental age-mates, though the difficulties inherent in selecting them are obvious.

#### 4.4 PERCENT GERMINATION AND NUTRITION

Many of the factors influencing the speed of germination may also have helped determine % germination. The age and condition of each megaspore undoubtedly influenced germination. A premature megaspore might develop and germinate, but a post-mature one might not recover depending upon the sugar treatment. Table 2-c indicates that among treatments without B vitamins only 1 and 3% glucose, and 1% trehalose, significantly increased % germination. This was in general accord with the ability of these sugar treatments to enhance growth. This provides further evidence that these sugars may have been utilized by megagametophytes. Unlike germination speed, which lacked significant differences among treatments without B vitamins, %

germination showed significant differences among these treatments. This may be because germination is an all-or-nothing phenomenon, and variation as a result of different ages of megaspores was not so strong a factor.

The significant enhancement in % germination among treatments with B vitamins, even on K medium (Table 2-d), might be explained in much the same way as for germination rate. The B vitamins, which play important roles in carbohydrate, lipid, and protein metabolism, may have stimulated recovery of lost viability in older megaspores, which might not have germinated otherwise. Perhaps the addition of B vitamins to K medium facilitated utilization of the lipid reserve, resulting in an increase in available nutrition and viability. This is contradicted, however, by the lack of any enhancement of growth by B vitamins on K medium. Work is needed to determine the actual status of the lipid reserve through time to provide a clearer picture of the effect of B vitamins on its utilization.

The addition of B vitamins might have allowed utilization of all concentrations of sugars which were not previously usable. There were no significant differences between the highest % germinations occurring on each of the three sugars in the presence of B-vitamins (Table 2-h). There is strong

evidence that sucrose, which seems not to have been used at any of the 3 concentrations without B vitamins, was metabolized readily with the addition of B vitamins. This is judged by the increase in observed growth. Determining the biochemical pathways through which sucrose is utilized only in the presence of B vitamins may provide valuable future research. Whether these pathways are normally active in low levels in the megagametophyte, or are completely absent, will provide an interesting direction for future research.

In treatments without B vitamins there were suggestions that higher concentrations of sugars inhibited growth responses that lower concentrations could induce. On glucose, for example, 1% enhanced % germination significantly, as did 3% at a lower level. Five % glucose, however, did not increase % germination significantly. On trehalose as well there was great enhancement of % germination on 1% concentration. On 3 and 5%, however, significant enhancement did not occur and growth responses appeared inhibited. Perhaps megagametophytes have the ability to metabolize these sugars, but only at low rates due to production of only small amounts of the necessary enzymes. Higher concentrations may then pose problems due to osmotic effects. This reasoning is speculative, and

further testing is needed. The addition of B vitamins may possibly have alleviated the deleterious effects of effects of high concentration by allowing higher rates of utilization through increased enzyme production. This may have resulted in enhanced metabolism and higher % germination on all concentrations.

Despite the apparent inhibition of growth below that on K medium (Figure 29), megagametophytes might be capable of utilizing some sucrose when grown on much lower concentrations. The sporophyte contains higher amounts of trehalose than sucrose (White and Towers). There may, therefore, be more trehalase available than invertase in the sporophyte. Based on this observation one can speculate that there may be a corresponding lower level of invertase available or inducible in the megagametophyte. If this were the case then 1% sucrose may have already been too much for the system to metabolize, and the observed lack of growth on this treatment may be the result of osmotic difficulty. One should not yet conclude that megagametophytes are incapable of metabolizing sucrose. The ability to utilize trehalose, on the other hand, may have been greater. One % could possibly be used to enhance metabolism, whereas higher concentrations might still create problems osmotically for the tissue. Interesting responses might be observed by

culturing megagametophytes on much lower concentrations of sucrose and trehalose. Quantitatively assaying the invertase and trehalase present in megagametophytes cultured on a range of concentrations of each sugar would be interesting. Future research should focus on whether trehalase is present in megagametophyte tissue normally, or if growth on trehalose induces its synthesis.

#### 4.5 GROWTH AND NUTRITION

The enhancement of growth possibly involved the same metabolic factors that increased speed of germination and % germination. Glucose enhanced growth at all concentrations. Trehalose greatly enhanced growth at 1% concentration, and sucrose concentrations may have been too high to effect any enhancement. One % trehalose may have been near the correct concentration for the postulated amount of trehalase present, or inducible, in the tissue. Therefore, the megagametophyte may have had the supplemental nutrition necessary to support enhanced growth. This undoubtedly entailed higher rates of membrane, cell wall, and protein synthesis. Additional coenzymes may be necessary to have enhanced and sustained growth, since growth on most sugar treatments with B vitamins did not level off within the 12 wk period. The production of additional coenzymes on 1%

trehalose may have been stimulated since these were not supplied in the medium, and growth did not level off on this treatment. Except for 1% trehalose, growth on all other non-B treatments leveled off within the 12 wk period.

Leveling off of growth might at first be interpreted to indicate a general depletion of water and/or sugar from the agar. However, growth on all sugar treatments with B vitamins, and on 1% trehalose, did not level off. This suggests that other factors were responsible for the slowing of growth. In treatments with B vitamins additional enzyme cofactors may have provided the means for utilization of available nutrition, with a resultant continuous increase in size. This suggests the possibility that growth on treatments lacking B vitamins was restricted by a depletion of cofactors in the tissue. The relatively continuous growth on 1% trehalose was possibly due to the greater ability of the tissue to metabolize this sugar. If correct, this would have resulted in a higher level of metabolism, and possibly a production of the cofactors needed to sustain enhanced growth. One % trehalose, the sugar found in substantial quantity in normal sporophytes, may have induced a certain potential for indeterminate growth in the megagametophyte, a pattern of growth commonly found in portions of the sporophyte.

Notably, growth on K medium with B vitamins was not enhanced, and leveled off as rapidly as growth on K medium without B vitamins (Figure 18). In this case nutrition was possibly in short supply, resulting in the cessation of growth. Future observations should include a determination of whether megagametophytes cultured on K medium with B vitamins have depleted their lipid reserves as a result of facilitation by additional enzyme cofactors.

Morel and Wetmore (1951) observed greatly enhanced growth, and induction of callus tissue, in gametophytes of the fern, Osmunda cinnamomea, cultured on the identical B vitamin mixture. Perhaps gametophyte morphology results, not only from normally limited energy supplies, as hypothesized by DeMaggio (1963) and Whittier (1978), but also from the inability of this tissue to produce sufficient quantities of enzyme cofactors needed to sustain enhanced metabolism.

The lack of apogamy in the present series of experiments suggests that neither of these interpretations may be fully correct. Results from Morel and Wetmore's experiment (1951), and from the present work, may indicate that providing these gametophytes with additional enzyme cofactors to utilize supplemental sugars leads only to maintenance and growth of the gametophyte morphology. The

induction of apogamy with high concentrations of sugars, shown to occur in many homosporous plants may, therefore, depend upon more than just the availability of greater amounts of energy.

The ability of megagametophytes, when in the presence of B vitamins to utilize all 3 sugars at any of the three concentrations, to enhance metabolism and growth may indicate that the megagametophyte of Selaginella is deficient in enzyme cofactors. This may result in its normally restricted growth and small size. This would not be a totally correct interpretation, though, as the addition of B vitamins alone did not increase growth. Both B vitamins and carbon sources (sugars and sorbitol) were necessary to produce growth enhancement. Instead, consideration should be given to the possibility that the amount of both cofactors and endogenous nutritional sources normally strike a balance that allows the organism to achieve its purpose of producing archegonia and supporting early development of the sporophyte.

#### 4.6 METABOLISM OF SORBITOL

Enhanced growth on sorbitol treatments with B vitamins suggests that megagametophytes can utilize this polyalcohol in metabolism. Determining the response of megagametophytes to simple osmotic differences was not achieved, therefore, since the selected osmoticum was apparently inappropriate. Future research concerning the effects of osmotic differences on megagametophyte growth must include an osmoticum that can be proven to be nutritionally inert, but otherwise harmless, to the living tissue. The enhanced growth of megagametophytes observed on all treatments with B vitamins (except K medium) may actually result from some osmotic factors in conjunction with B vitamins. This alternative explanation, which implies that the various sugars were not utilized metabolically, seems unlikely.

Since megagametophytes grow to large sizes on sorbitol and B vitamins, this probably indicates some ability to utilize this polyalcohol. Since sorbitol is chemically very similar to mannitol, the use of mannitol by Whittier (1975), and interpretation of his results as a response to osmotic factors may be incorrect. That a basal level of carbohydrate was necessary before increasing concentrations of mannitol could induce greater amounts of apogamy suggests that enhanced metabolism, derived from supplemental

nutrition, allowed for the metabolic utilization of mannitol. The induction of apogamy seen by Whittier (1975) may not have been due to osmotic differences at all. This remains speculative, however, until the nutritional inertness of mannitol is examined further.

The sorbitol control treatment, 3% glucose with B vitamins, exhibited significantly faster rates of germination than the first such treatment (Figure 2). This may indicate that there were no longer any premature megaspores among those inoculated, since these were collected six months later. Perhaps all megaspores were either ready to germinate, were less viable due to older age, or were totally non-viable. The variation in response possibly introduced through the use of potentially premature megaspores in the July sowing may have been removed. The possible reduction may result from the distinctly fewer cones, and therefore megaspores, that were produced in the winter months. The megaspores collected in December were probably somewhat older on the average than those collected in June, when production of megaspores was higher.

Total % germination on the sorbitol and B vitamin treatments was significantly lower than that on any other sugar with B vitamin treatments treatment (Table 2). Average final volumes were also lower (Figure 16). Perhaps

the decrease in percent germination resulted from fewer viable megaspores in a population of older megaspores. Another possible factor may have involved a less efficient utilization of sorbitol as a carbon source in comparison to the sugars. The lower final volume achieved on the sorbitol treatments may corroborate the interpretation that it could not be utilized so easily as the sugars.

#### 4.7 CORRELATION BETWEEN RESPONSES

The high correlation ( $r = 0.81$ ) between percent germination and final volume provides evidence that both responses may have resulted from the ability of a particular nutritional treatment to enhance general metabolism. The abilities to germinate successfully and to grow to large sizes may both depend upon enhancement of synthesis and degradation reactions within the tissues, thus producing a high correlation between the two. Successful germination possibly also depends to a degree on the ability of the megagametophyte tissue to imbibe water, and therefore may not relate strictly to enhanced metabolism. The lower correlations between germination speed and final volume ( $r = 0.61$ ), and germination speed and % germination ( $r = 0.48$ ), perhaps can be explained by the amount of variation occurring in germination speed as a result of using

megaspores of several developmental stages and levels of viability. Another possible reason is that the final volume achieved results from the ability of a treatment to enhance cell divisions and general metabolism, while germination speed may simply be a function of the ability of the tissues to imbibe water and expand. Though both may respond to supplemental nutrition in a positive manner they are not directly linked to each other and correlation is lower between the two. The low correlation between germination speed and percent germination may also have similar causes.

#### 4.8 REPEATED TREHALOSE TREATMENTS

Initial observations indicated some peculiar patterns of growth and % germination on intermediate concentrations of trehalose and sucrose. This prompted the repetition of the trehalose treatments. Final data analyses, however, failed to confirm the apparent patterns. None of the concentrations of sucrose without B vitamins were significantly different from each other in either % germination or average final volume. Three and 5% trehalose, as well, did not produce significantly different results from each other. There was no basis, therefore, for considering these responses to be peculiar. The intermediate concentrations of sucrose and trehalose were

not significantly depressed below the 1 and 5% concentrations. However, the very different responses obtained in the second trehalose treatments still require discussion and a possible explanation.

Though highly speculative, the difference in results between the first and second trehalose treatments may possibly be explained in terms of light availability. In the higher light environment of the growth chamber the megagametophytes may possibly have been light enhanced (see Discussion - Fluorescence Analysis). In the lower light environment of the laboratory benchtop metabolism may have been altered. The presence of a light-absorbing pigment (chlorophyll-a) perhaps indicates that light might be sensed and utilized in some way. Dr. William Ruf of Indiana University (personal communication - August, 1981) has observed that of 100 megaspores of Selaginella pallescens cultured in the dark, only one germinated. Percent germination in the light was much higher. This suggests that megagametophytes have some requirement for light. Differences in growth of Selaginella megagametophytes seen in cultures that differed in light quality, as well as intensity (and in many other factors as well), may be similar to the response of many types of algae which will photosynthesize in high light environments, but when placed

under light-limiting conditions will revert to photoheterotrophy (Droop, 1974; Neilson and Lewin, 1974). Under these conditions algae will use available light to provide the energy necessary to absorb organic materials from the environment. Though this explanation for the difference in results on the two trehalose treatments is highly speculative, directions for further experimentation are indicated.

#### 4.9 THE PRESENCE OF CHLOROPHYLL-A IN MEGAGAMETOPHYTES

Fluorescence analysis has confirmed the presence of chlorophyll-a in the tissue of the megagametophyte, and also that different nutritional treatments affect the amount present. Whether the megagametophyte of Selaginella is a photosynthetically competent organism, capable of producing carbohydrates, is an important question that must be answered before the true nutritional status of this organism is understood. If, in fact, the megagametophyte is producing carbohydrates, then the initial premise of the tissue's reliance on lipids, rather than on carbohydrates, may be partially incorrect. Work is necessary to determine if carbon dioxide is being taken up and incorporated into organic compounds. Though no megagametophytes turned green in this experiment, many became bright yellow. This was

probably due to carotenoid pigments which may have masked the chlorophyll. The bright yellow color of many megagametophytes indicated that these tissues were absorbing blue light. Whether this light was used for photosynthetic reactions is unknown at present.

The amount of chlorophyll-a present in the tissue was greatest on 3% sorbitol with B vitamins (Table 5). Inhibition of chlorophyll synthesis appeared to increase from the monosaccharides to the disaccharides. The inhibition of chlorophyll synthesis on sucrose media has been observed in other plants. Edelman and Hanson (1971) observed that sucrose suppresses chlorophyll formation in carrot callus cultures, and El Hinnawy (1974) determined both that sucrose suppresses, and that glucose has little effect, on chlorophyll synthesis in callus cultures of Melilotus alba. In this experiment trehalose also inhibited chlorophyll synthesis. The reason for the greater inhibition of chlorophyll synthesis on disaccharides is unknown. Perhaps there is some type of metabolic feedback inhibition occurring more on disaccharides than on monosaccharides.

Culture on sorbitol with B vitamins produced the highest amounts of chlorophyll-a. This may be due to the nature of the molecule, which is a polyalcohol. Sorbitol may not be

metabolized along the same biochemical pathways as sugars. There may not be a similar type of hypothetical feedback occurring, and chlorophyll synthesis may be enhanced rather than inhibited. Further work on the presence of chlorophyll-a in the megagametophyte of Selaginella, and its implications, is needed.

#### 4.10 CELL SIZE ANALYSIS

The production of cells of the same cross-sectional area on 4 different substrate concentrations (0, 1, 3, and 5% sorbitol) in megagametophytes of Selaginella indicates that cell size here is under some internal control, and cannot be altered simply by adjusting the osmotic concentration in the environment. Therefore, the sizes of these cells result from normal internal metabolism rather than from osmotic conditions of the external environment. Whittier (1964a) observed the same result when he cultured fern gametophytes on a wide range of sucrose concentrations.

The observation that cells were the same sizes on a variety of treatments and in megagametophytes exhibiting normal or enhanced growth, provides direct evidence that the enhancement of growth results from the production of more, but not bigger, cells.

#### 4.11 CELLULAR ORGANIZATION OF THE TISSUES

Megagametophytes exhibiting normal growth appeared to be organized as is generally recognized for Selaginella (Bierhorst, 1971 Robert, 1971).

If the lipid reserve is the source of nutrition for young sporophytes, then the complete cellularization of one normal megagametophyte was curious. Apparently this megagametophyte had utilized the lipid reserves, but growth was not otherwise very different from normal. Foster and Gifford (1974) state that the basal portion of the megagametophyte does eventually become totally cellularized if fertilization does not first occur, so this may not be so unusual an observation at all.

Enhanced megagametophytes were highly cellularized. From the patterns of thin-walled cells, divisions occurred throughout the tissue. There were no indications of mitoses, which suggests that the rate of cell division may be low. The tissue could be described as a callus, in agreement with the description by Wetmore and Morel (1951). However this should not imply that the tissue was without any organization. In fact, archegonia were restricted to the proximal surface of the megagametophyte. The cause of this polarity is unknown, but research involving the

determinants of archegonial initiation and location could prove to be very interesting from a morphogenetic point of view.

#### 4.12 THE ORIGINAL HYPOTHESIS VERSUS THE RESULTS

The original intent of this experiment was to determine if manipulation of nutrition could alter development of the gametophyte of a heterosporous plant in a manner that would lend support to Lang's hypothesis. Culturing the megagametophytes of Selaginella on supplemental carbohydrates, with and without the enhancement provided by B vitamins, has produced results that would seem, at first, to fail to support Lang's hypothesis. Supplying megagametophytes with carbohydrates, and possibly enhancing their ability to use them, produced larger megagametophytes with no indication of sporophytic development.

The possible explanations for the failure of apogamy to be induced in these series of experiments, using only Selaginella martensii, may be several. The first one involves a misinterpretation of the actual nutritional conditions that may exist during the inceptions of the 2 generations. The presence of a large amount of lipid material in the megagametophyte led to my presumption that lipid-based nutrition may have helped determine gametophyte

morphology, and the presence of carbohydrates in the sporophyte were presumed to determine this morphology. Perhaps the actual situation is different. Future work should involve culture of megaspores on lipids. The actual source of nutrition for megagametophytes, whether from the lipid reserve, any starch grains in the cells, or possibly even some amount of photosynthesis, needs to be determined.

Normally the most basal areas of the lipid reserve do not cellularize until fertilization and the beginning of sporophyte development (Foster and Gifford, 1974). The occurrence of cellularization at this point may indicate that the nutrition stored in the reserve is being made available to the developing sporophyte for early growth and development. If the megagametophyte can be shown to derive nourishment from starch grains, or photosynthesis, then perhaps the real function of the lipid reserve is to give the embryo the resources necessary to reorganize sporophyte morphology. Whether the maintenance of gametophyte morphology is a result of a loss of organization due to some type of nutritional starvation of the tissue is unknown. The culture of sporophyte tissue on very low levels of carbohydrates in the dark might provide further observations concerning the ability of nutritional level to determine morphology.

Another possible explanation of why apogamy was not induced in megagametophytes of Selaginella martensii could derive from the reason why apogamy does occur in other plants. Gametophytes of some ferns, and of some species of Lycopodium, can produce sporophytes without fertilization. In these instances, both gametophytes and sporophytes will contain the same amount of genetic material. These plants also tend to have high ploidy levels (Manton, 1950). In some species of Lycopodium the haploid number of chromosomes is 104, 110, or 136. There is evidence that these plants are polyploid. Halving and doubling the chromosome number can not be considered to produce truly haploid and diploid organisms. If a gametophyte is diploid and the sporophyte tetraploid, for example, a reversion to sporophytic growth might be a relatively simple event in the gametophyte tissue. In these cases the alternation of dissimilar generations may occur as a result of the normal environmental differences described by Lang (1909). Perhaps the control of ploidy level over morphology may have been weakened because the gametophyte is no longer really haploid, and the sporophyte is no longer really diploid. Cultural manipulation of gametophytes on high sugar concentrations may easily disrupt the normal alternation of generations. Ploidy level may determine gametophyte versus

sporophyte morphology, but only when the two generations are truly haploid and diploid. Selaginella, on the other hand, has low numbers of chromosomes, and there is little indication of polyploidy in any of the species examined (Jermy, et. al., 1967). In most species of Selaginella the haploid number of chromosomes is 8, 9, 10, or 11. The megagametophyte, as well as having half the number of chromosomes as the sporophyte, may actually be haploid. Manipulation of nutrition in this case may not be enough to convert growth to the sporophytic mode. One way of testing Lang's hypothesis more effectively may involve obtaining diploid gametophyte tissue by culturing the sporophyte under conditions of low light and low nutrient availability. If this occurs the possible control of the ploidy level over morphology may be loosened, and cultural manipulations of the type carried out in the present series of experiments may prove more fruitful in determining the validity of Lang's hypothesis.

One xerically-adapted species of Selaginella (S. rupestris) has been described, in which microspores are apparently never produced (Webster and Steeves, 1974). Sporelings are produced, however, through either parthenogenesis or apogamy. This indicates that under conditions of extreme stress some species of Selaginella can

adapt to survive in a similar manner as homosporous plants. Determining the ploidy level of megagametophytes of these xeric species would be of great interest, as would growing them under various nutritional regimes.

Finally, despite all the possibilities mentioned to explain why apogamy did not occur in Selaginella, a re-evaluation of Lang's hypothesis may be in order. The amount of supplemental nutrition most likely made available to megagametophytes cultured on sugars with B vitamins must have been very great. Even with this great increase of available energy, megagametophyte morphology was still maintained. Though Lang's hypothesis seems to fit well the experimental and empirical data from homosporous plants, it may be too simplistic for the heterosporous plants. One consistent distinguishing feature between homosporous and heterosporous plants is that homosporous gametophytes are exosporic in their development, and heterosporous gametophytes are endosporic. These two different developmental paths diverged hundreds of millions of years ago (e.g. Selaginella has existed since Middle Pennsylvanian time, about 300 million years ago), and heterosporous gametophytes have probably been selected for remaining reduced in size. Although many homosporous groups have produced heterosporous derivatives, the reverse has

never happened. The heterosporous plants benefited from separation of the sexes, allowing outcrossing to occur more frequently. Heterosporous gametophytes are reproductively mature when shed, and are capable of producing sporophyte offspring almost immediately after germination. They are thus well adapted both for growth in low light, and for short, dry growing seasons. The megagametophyte remained within the megaspore wall, which provided a protective barrier for the highly nutritional lipid reserve tissue within. Constant selective pressure would favor those that remained small and endosporic. Thus, after millions of years of natural selection, the morphology of the megagametophyte generation may be too deeply entrenched to allow easy disruption of the normal alternation of generations.

## Chapter V

### CONCLUSIONS

Megagametophytes of Selaginella martensii are capable of enhanced growth on several types of carbon sources. Glucose and trehalose without B vitamins increase % germination of megaspores, and final volumes of tissue, over that on simple mineral salts medium (K medium). Sucrose appeared to lack any growth enhancing properties. With B vitamins, the 3 sugars and sorbitol enhance all aspects of growth over K medium. Whether this indicates that B vitamins facilitate metabolism of these carbon sources is unclear, but suggestive.

Some megagametophytes of Selaginella martensii contain chlorophyll-a, and the amount present varies according to the type of carbon source on which the tissue is cultured. Sorbitol treatments produced the most chlorophyll-a, and sucrose treatments produced the least. Whether megagametophytes are capable of photosynthesis is presently unknown. A detailed analysis is necessary in order to more fully understand the nutritional status of this organism.

Response to culture on particular sugars may vary with changes in the cultures conditions. Different light levels may have been responsible for observed differences in

response to trehalose. Determination of the light sensitive reactions that may exist in megagametophytes is necessary to provide important information on their growth and development.

Comparisons of megagametophytes exhibiting normal and enhanced growth indicated that, since cell sizes were the same, larger megagametophytes are produced through the production of more cells.

Megagametophytes cultured on K medium exhibited an internal anatomy that agreed with past descriptions for Selaginella. Megagametophytes exhibiting enhanced growth were highly cellularized organisms, contained archegonia with eggs, and displayed a pattern of cell divisions throughout the tissue rather than at localized sites.

Apogamy did not occur with culture on supplemental nutrition. This may have been due to either a misinterpretation of the conditions necessary to induce apogamy, or Lang's hypothesis is too simplistic to explain alternation of generations in this heterosporous plant, or perhaps the species of Selaginella chosen was less susceptible to manipulation than others might have been.

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Appendix A  
MEDIA COMPONENTS

I. Knudson's Mineral Salts Medium (Caponetti, 1977)

A. Macroelement stock solution (4x strength)

| Ingredient                                               | Amount    |
|----------------------------------------------------------|-----------|
| (1) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ | 2.000 gm  |
| (2) $(\text{NH}_4)_2\text{SO}_4$                         | 1.000 gm  |
| (3) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$            | 0.500 gm  |
| (4) Water to make                                        | 1000.0 ml |

B. Phosphate Stock Solution

| Ingredient                   | Amount    |
|------------------------------|-----------|
| (1) $\text{K}_2\text{HPO}_4$ | 25.000 gm |
| (2) Water to make            | 100.0 ml  |

C. Microelement Stock Solution

| Ingredient                                              | Amount    |
|---------------------------------------------------------|-----------|
| (1) $\text{H}_2\text{SO}_4$ (conc.)                     | 0.500 ml  |
| (2) $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$           | 2.500 gm  |
| (3) $\text{H}_3\text{BO}_3$                             | 2.000 gm  |
| (4) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$           | 0.050 gm  |
| (5) $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$           | 0.030 gm  |
| (6) $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$           | 0.015 gm  |
| (7) $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ | 0.025 gm  |
| (8) Water to make                                       | 1000.0 ml |

D. Ferric Citrate Stock Solution

| Ingredient                                                       | Amount   |
|------------------------------------------------------------------|----------|
| (1) $\text{FeC}_6\text{H}_5\text{O}_7 \cdot 5\text{H}_2\text{O}$ | 2.500 gm |
| (2) Water to make                                                | 100.0 ml |

## E. Final Medium

| Ingredient                   | Amount    |
|------------------------------|-----------|
| (1) Macroelement solution x4 | 250.0 ml  |
| (2) Water                    | 700.0 ml  |
| (3) Phosphate solution       | 0.5 ml    |
| (4) Microelement solution    | 0.5 ml    |
| (5) Ferric citrate solution  | 0.4 ml    |
| (6) Water to make            | 1000.0 ml |

Adjust pH to 5.5.  
Solidify with 0.9% Agar.

II.B Vitamin Mixture (Wetmore and Morel, 1951)

| Ingredient       | Amount              |
|------------------|---------------------|
| (1) Thiamine     | $10^{-6}$ gm/liter* |
| (2) Niacin       | $10^{-6}$ "         |
| (3) Pantothenate | $10^{-6}$ "         |
| (4) Pyridoxine   | $10^{-6}$ "         |
| (5) Biotin       | $10^{-8}$ "         |
| (6) Inositol     | $10^{-4}$ "         |

\* DeMaggio, personal communication.

# Appendix B

## GERMINATION RATE AND VOLUME MEASUREMENTS - ALL DATA

| Treatment     | Germination<br>Rate (Days) | Volume( $\frac{\text{---}}{\text{Y}}$ x $10^{-2}$ mm <sup>3</sup> ) |       |       |       |       |       |
|---------------|----------------------------|---------------------------------------------------------------------|-------|-------|-------|-------|-------|
|               |                            | Y1                                                                  | Y2    | Y3    | Y4    | Y5    | Y6*   |
| -----         | -----                      | -----                                                               | ----- | ----- | ----- | ----- | ----- |
| <u>K</u>      |                            |                                                                     |       |       |       |       |       |
| (3)IIin**     | 53                         | 2.04                                                                | 2.04  | 2.36  | 2.54  | 2.73  | 2.91  |
| (4)Iout       | 20                         | 2.65                                                                | 3.25  | 3.60  | 3.94  | 3.94  | 3.94  |
| (5)IVout      | 37                         | 6.09                                                                | 6.09  | 6.09  | 6.09  | 6.09  | 6.09  |
| (6)IIin       | 37                         | 5.65                                                                | 6.63  | 6.63  | 6.63  | 6.63  | 6.63  |
| (7)IIIout     | 25                         | 3.54                                                                | 3.83  | 3.83  | 4.16  | 4.16  | 4.16  |
| (10)Iin       | 25                         | 3.72                                                                | 4.22  | 5.10  | 5.10  | 5.10  | 5.10  |
| (9)Iin        | 35                         | 2.91                                                                | 2.91  | 3.45  | 3.45  | 3.45  | 3.45  |
| (11)IIIout    | 04                         | 3.25                                                                | 3.25  | 3.25  | 3.25  | 3.25  | 3.25  |
|               | -----                      | -----                                                               | ----- | ----- | ----- | ----- | ----- |
| mean=         | 29.5                       | 3.73                                                                | 4.03  | 4.29  | 4.40  | 4.42  | 4.44  |
| $\pm 1$ S.D.= | 14.5                       | 1.43                                                                | 1.58  | 1.49  | 1.43  | 1.39  | 1.36  |

(\* = Y1 through Y6 represent volume measurements taken at two-week periods from the day of germination through the twelfth week.)

(\*\* =this notation is used to keep track of each germinated megaspore. For example, K(3)IIin means the megaspore growing on plain mineral salts medium, dish number 3, the second quarter of the dish, the inner position on the agar.)

K+B

|          |      |       |       |       |       |       |       |
|----------|------|-------|-------|-------|-------|-------|-------|
| (1)I     | 31   | 2.13  | 2.65  | 2.65  | 3.25  | 3.25  | 3.25  |
| (2)I     | 31   | 3.25  | 3.25  | 4.70  | 4.70  | 5.37  | 5.37  |
| (3)IIin  | 32   | 4.70  | 4.70  | 4.70  | 4.70  | 4.70  | 4.70  |
| (3)IIout | 11   | 3.60  | 5.16  | 6.77  | 7.44  | 7.44  | 7.44  |
| (4)Iin   | 24   | 2.65  | 3.25  | 3.94  | 3.94  | 3.94  | 3.94  |
| (4)IVin  | 39   | 2.65  | 2.65  | 2.65  | 2.65  | 2.65  | 2.65  |
| (5)IIout | 10   | 2.81  | 4.16  | 4.34  | 4.34  | 4.34  | 4.34  |
| (5)IVin  | 09   | 3.88  | 5.16  | 5.16  | 5.37  | 5.58  | 5.58  |
| (7)Iin   | 05   | 2.51  | 4.58  | 4.70  | 4.70  | 4.70  | 4.70  |
| (7)IIIin | 13   | 3.25  | 4.97  | 5.94  | 6.63  | 6.63  | 6.63  |
|          |      | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=    | 20.5 | 3.14  | 4.05  | 4.56  | 4.77  | 4.86  | 4.86  |
|          |      |       |       |       |       |       |       |
| ±1 S.D.= | 12.2 | 0.76  | 1.01  | 1.29  | 1.44  | 1.46  | 1.46  |

1G

|           |      |       |       |       |       |       |       |
|-----------|------|-------|-------|-------|-------|-------|-------|
| (1)Iin    | 47   | 2.65  | 2.65  | 2.65  | 2.65  | 2.65  | 2.65  |
| (2)Iout   | 51   | 3.60  | 3.60  | 3.60  | 3.60  | 3.60  | 3.60  |
| (2)IVout  | 05   | 4.40  | 7.03  | 12.48 | 12.48 | 12.48 | 12.48 |
| (3)Iin    | 17   | 2.69  | 3.25  | 3.60  | 6.09  | 7.11  | 7.11  |
| (3)IIIout | 10   | 2.53  | 3.51  | 4.34  | 4.34  | 4.34  | 4.34  |
| (3)IIout  | 13   | 2.49  | 3.94  | 3.94  | 3.94  | 3.94  | 3.94  |
| (4)IIin   | 10   | 3.39  | 7.11  | 8.07  | 8.07  | 8.07  | 8.80  |
| (4)IVin   | 24   | 3.52  | 3.52  | 3.52  | 3.52  | 3.52  | 3.52  |
| (4)IVout  | 10   | 3.12  | 3.88  | 4.11  | 4.11  | 4.11  | 4.11  |
| (5)IIIin  | 35   | 3.25  | 3.25  | 3.25  | 3.25  | 3.25  | 3.25  |
| (6)IIin   | 08   | 3.05  | 10.22 | 13.35 | 15.06 | 18.90 | 18.90 |
| (6)IIIout | 05   | 3.23  | 3.51  | 3.51  | 3.51  | 3.51  | 3.51  |
|           |      | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=     | 19.6 | 3.16  | 4.62  | 5.54  | 5.89  | 6.29  | 6.35  |
|           |      |       |       |       |       |       |       |
| ±1 S.D.=  | 16.2 | 0.54  | 2.27  | 3.70  | 4.00  | 4.87  | 4.89  |

3G

|            |      |       |       |       |       |       |       |
|------------|------|-------|-------|-------|-------|-------|-------|
| (2) IVout  | 05   | 2.32  | 2.87  | 3.94  | 3.94  | 3.94  | 3.94  |
| (4) IIin   | 08   | 3.34  | 4.16  | 4.16  | 4.16  | 4.16  | 4.16  |
| (4) IIIout | 46   | 3.45  | 3.45  | 3.45  | 3.45  | 3.45  | 3.45  |
| (6) IVout  | 16   | 2.83  | 2.83  | 3.11  | 3.11  | 3.11  | 3.11  |
| (7) Iout   | 42   | 4.34  | 5.51  | 5.51  | 5.51  | 5.51  | 5.51  |
| (7) IIin   | 27   | 3.94  | 4.34  | 4.90  | 4.90  | 4.90  | 4.90  |
| (7) IIIin  | 42   | 4.04  | 4.58  | 4.58  | 4.58  | 4.58  | 4.58  |
| (7) IIIout | 18   | 4.04  | 4.58  | 4.58  | 4.58  | 4.58  | 4.58  |
| (7) IVin   | 37   | 3.88  | 3.88  | 3.88  | 3.88  | 3.88  | 3.88  |
| (7) IVout  | 37   | 3.94  | 5.16  | 5.58  | 5.58  | 5.58  | 5.58  |
| (10) Iout  | 27   | 4.04  | 4.70  | 5.51  | 5.51  | 5.51  | 5.51  |
|            |      | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=      | 27.7 | 3.65  | 4.19  | 4.47  | 4.47  | 4.47  | 4.47  |
| ±1 S.D.=   | 14.3 | 0.61  | 0.87  | 0.85  | 0.85  | 0.85  | 0.85  |

5G

|            |      |       |       |       |       |       |       |
|------------|------|-------|-------|-------|-------|-------|-------|
| (3) Iout   | 13   | 2.73  | 3.66  | 3.66  | 3.66  | 3.66  | 3.66  |
| (3) IIout  | 24   | 3.51  | 4.58  | 4.58  | 4.58  | 4.58  | 4.58  |
| (5) IIIin  | 26   | 3.05  | 3.51  | 4.04  | 4.04  | 4.04  | 4.04  |
| (7) IIIout | 15   | 3.05  | 3.72  | 3.72  | 3.72  | 3.72  | 3.72  |
| (7) IVin   | 16   | 4.32  | 11.09 | 14.11 | 16.04 | 16.04 | 16.04 |
|            |      | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=      | 18.8 | 3.33  | 5.31  | 6.02  | 6.41  | 6.41  | 6.41  |
| ±1 S.D.=   | 5.8  | 0.62  | 3.26  | 4.54  | 5.40  | 5.40  | 5.40  |

1S

|           |      |       |       |       |       |       |       |
|-----------|------|-------|-------|-------|-------|-------|-------|
| (1) IIin  | 26   | 3.25  | 3.94  | 3.94  | 3.94  | 3.94  | 3.94  |
| (2) IIin  | 27   | 3.05  | 3.72  | 4.04  | 4.04  | 4.04  | 4.04  |
| (2) IV    | 39   | 3.51  | 4.04  | 5.16  | 5.41  | 5.65  | 5.65  |
| (4) IVin  | 08   | 2.69  | 3.51  | 3.51  | 3.51  | 4.04  | 4.04  |
| (5) IIout | 15   | 4.70  | 5.65  | 5.65  | 5.65  | 5.65  | 5.65  |
| (5) IVin  | 06   | 3.34  | 4.04  | 4.04  | 4.04  | 4.04  | 4.04  |
| (7) Iin   | 12   | 3.94  | 4.16  | 4.16  | 4.58  | 4.58  | 4.58  |
|           |      | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=     | 17.5 | 3.37  | 3.97  | 4.15  | 4.24  | 4.33  | 4.33  |
| ±1 S.D.=  | 11.9 | 0.70  | 0.82  | 0.91  | 0.96  | 0.96  | 0.96  |

3S

|           |      |       |       |       |       |       |       |
|-----------|------|-------|-------|-------|-------|-------|-------|
| (3)IIin   | 43   | 3.72  | 4.58  | 4.58  | 4.58  | 4.58  | 4.58  |
| (3)IIIout | 43   | 3.05  | 3.35  | 3.35  | 3.35  | 3.35  | 3.35  |
| (5)Iin    | 36   | 2.91  | 2.91  | 2.91  | 2.91  | 2.91  | 2.91  |
| (5)IVout  | 18   | 2.65  | 3.25  | 3.25  | 3.25  | 3.25  | 3.25  |
| (8)IIout  | 27   | 3.40  | 3.40  | 3.40  | 3.40  | 3.40  | 3.40  |
| (8)IVin   | 37   | 3.25  | 3.25  | 3.25  | 3.25  | 3.25  | 3.25  |
|           |      | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=     | 34.0 | 3.16  | 3.46  | 3.46  | 3.47  | 3.47  | 3.48  |
| ±1 S.D.=  | 9.8  | 0.38  | 0.58  | 0.58  | 0.57  | 0.57  | 0.57  |

5S

|           |      |       |       |       |       |       |       |
|-----------|------|-------|-------|-------|-------|-------|-------|
| (2)I      | 22   | 1.89  | 2.13  | 2.13  | 2.13  | 2.13  | 2.13  |
| (3)Iout   | 33   | 2.65  | 2.65  | 2.65  | 2.65  | 2.65  | 2.65  |
| (3)IVin   | 52   | 3.40  | 3.40  | 3.40  | 3.40  | 3.40  | 3.40  |
| (3)IVout  | 52   | 4.04  | 4.04  | 4.58  | 4.58  | 4.58  | 4.58  |
| (4)Iout   | 24   | 3.45  | 3.51  | 3.66  | 3.66  | 3.66  | 3.66  |
| (4)IIIin  | 24   | 2.83  | 2.91  | 2.91  | 3.40  | 3.40  | 3.40  |
| (4)IVin   | 24   | 2.28  | 2.65  | 3.40  | 3.66  | 3.66  | 3.66  |
| (7)IVout  | 10   | 2.28  | 2.91  | 2.91  | 2.91  | 2.91  | 2.91  |
| (8)Iin    | 14   | 2.28  | 2.28  | 2.28  | 2.28  | 2.28  | 2.28  |
| (9)IIIout | 54   | 4.34  | 4.70  | 4.70  | 4.70  | 4.70  | 4.70  |
|           |      | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=     | 30.9 | 2.94  | 3.12  | 3.26  | 3.34  | 3.34  | 3.34  |
| ±1 S.D.=  | 16.2 | 0.83  | 0.80  | 0.87  | 0.87  | 0.87  | 0.87  |

1T

|           |      |       |       |       |       |       |       |
|-----------|------|-------|-------|-------|-------|-------|-------|
| (1)I      | 17   | 2.53  | 4.97  | 7.62  | 9.09  | 10.43 | 11.31 |
| (1)II     | 21   | 4.33  | 13.10 | 13.88 | 14.65 | 16.32 | 18.11 |
| (2)III    | 22   | 3.04  | 3.94  | 3.94  | 3.94  | 3.94  | 3.94  |
| (4)Iout   | 32   | 4.04  | 4.04  | 4.70  | 4.70  | 4.70  | 4.70  |
| (4)Iin    | 10   | 1.92  | 3.94  | 6.71  | 6.71  | 7.44  | 7.44  |
| (4)IIout  | 72   | 2.53  | 2.53  | 2.53  | 2.53  | 2.53  | 2.53  |
| (4)IVout  | 42   | 3.66  | 3.66  | 3.66  | 3.66  | 3.66  | 3.66  |
| (4)IVin   | 71   | 2.73  | 2.73  | 2.73  | 2.73  | 2.73  | 2.73  |
| (5)Iin    | 05   | 2.52  | 4.46  | 7.98  | 11.77 | 17.36 | 20.21 |
| (5)IIout  | 12   | 3.34  | 6.01  | 7.98  | 9.69  | 9.69  | 9.69  |
| (5)IIIin  | 18   | 4.46  | 7.98  | 8.53  | 8.53  | 8.53  | 8.53  |
| (5)IIIout | 05   | 3.25  | 5.65  | 13.10 | 21.22 | 24.47 | 24.47 |
| (5)IVin   | 09   | 3.13  | 7.98  | 9.21  | 10.43 | 10.43 | 10.98 |
|           |      | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=     | 25.8 | 3.19  | 5.46  | 7.12  | 8.43  | 9.40  | 9.87  |
| ±1 S.D.=  | 22.8 | 0.77  | 2.87  | 3.63  | 5.37  | 6.59  | 7.10  |

3T

|          |       |       |       |       |       |       |       |
|----------|-------|-------|-------|-------|-------|-------|-------|
| (3)IVin  | 10    | 2.32  | 2.32  | 2.32  | 2.32  | 2.32  | 2.32  |
|          |       | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=    | 10    | 2.32  | 2.32  | 2.32  | 2.32  | 2.32  | 2.32  |
| ±1 S.D.= | ----- | ----- | ----- | ----- | ----- | ----- | ----- |

5T

|           |      |       |       |       |       |       |       |
|-----------|------|-------|-------|-------|-------|-------|-------|
| (1)III    | 58   | 4.04  | 4.70  | 4.70  | 4.70  | 4.70  | 4.70  |
| (1)IV     | 31   | 3.56  | 3.56  | 3.56  | 3.56  | 3.56  | 3.56  |
| (2)IIIin  | 30   | 3.05  | 4.04  | 4.04  | 4.04  | 4.04  | 4.04  |
| (3)IIIin  | 51   | 3.40  | 3.40  | 4.70  | 4.97  | 5.24  | 5.51  |
| (3)IIIout | 17   | 2.73  | 3.40  | 3.40  | 3.40  | 3.40  | 3.40  |
| (3)IVin   | 32   | 4.40  | 4.40  | 4.40  | 4.40  | 4.40  | 4.40  |
| (4)IIIin  | 15   | 2.49  | 3.40  | 3.40  | 3.40  | 3.40  | 3.40  |
| (6)IVout  | 51   | 4.04  | 4.04  | 4.04  | 4.04  | 4.04  | 4.04  |
| (7)Iin    | 27   | 3.40  | 3.66  | 4.04  | 4.34  | 4.34  | 4.34  |
|           |      | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=     | 34.7 | 3.46  | 3.84  | 4.03  | 4.09  | 4.12  | 4.15  |
| ±1 S.D.=  | 15.3 | 0.63  | 0.48  | 0.51  | 0.56  | 0.62  | 0.68  |

1G+B

|           |    |      |       |       |       |       |       |
|-----------|----|------|-------|-------|-------|-------|-------|
| (2)Iin    | 15 | 2.61 | 3.40  | 3.66  | 4.41  | 5.16  | 5.16  |
| (2)IIout  | 13 | 2.69 | 6.79  | 8.53  | 10.75 | 12.97 | 18.11 |
| (4)IIout  | 29 | 4.70 | 5.51  | 5.51  | 5.76  | 6.01  | 6.01  |
| (4)IIIin  | 05 | 2.73 | 6.31  | 11.66 | 16.32 | 21.22 | 23.17 |
| (4)IIIout | 15 | 3.94 | 5.16  | 5.51  | 5.51  | 5.51  | 5.51  |
| (4)IVin   | 10 | 3.85 | 12.36 | 13.99 | 16.05 | 18.11 | 21.22 |
| (4)IVout  | 05 | 3.25 | 8.53  | 9.09  | 10.98 | 13.99 | 17.36 |
| (6)Iin    | 11 | 3.94 | 9.69  | 9.69  | 15.61 | 20.04 | 24.47 |
| (6)IIin   | 06 | 2.89 | 8.53  | 13.99 | 19.91 | 25.82 | 37.93 |
| (6)IIout  | 06 | 2.74 | 8.53  | 10.43 | 14.27 | 18.11 | 29.30 |
| (6)IIIout | 16 | 2.65 | 4.18  | 5.10  | 6.01  | 6.01  | 6.01  |
| (6)IVin   | 14 | 3.45 | 4.70  | 5.51  | 6.39  | 7.44  | 9.09  |
| (6)IVout  | 14 | 3.45 | 6.39  | 9.09  | 13.10 | 14.65 | 15.61 |
| (7)Iout   | 08 | 3.66 | 3.66  | 3.66  | 3.66  | 3.66  | 3.66  |
| mean=     |    | 11.5 | 3.37  | 7.12  | 9.04  | 11.57 | 13.87 |
| ±1 S.D.=  |    | 6.3  | 0.64  | 2.97  | 4.58  | 6.30  | 8.13  |

3G+B

|           |    |      |       |       |       |       |       |
|-----------|----|------|-------|-------|-------|-------|-------|
| (4)Iout   | 14 | 2.89 | 16.32 | 22.27 | 29.23 | 36.18 | 45.76 |
| (4)IVin   | 15 | 2.33 | 4.34  | 5.94  | 6.17  | 6.39  | 7.44  |
| (4)IIout  | 15 | 2.88 | 4.70  | 5.51  | 7.02  | 8.53  | 11.77 |
| (4)IIIout | 50 | 3.66 | 3.66  | 3.66  | 3.66  | 3.66  | 3.66  |
| (5)Iout   | 11 | 3.34 | 13.10 | 15.61 | 15.61 | 15.61 | 15.61 |
| (5)IIIin  | 45 | 2.73 | 2.73  | 2.73  | 2.73  | 2.73  | 2.73  |
| (5)IVin   | 17 | 1.65 | 2.28  | 2.28  | 2.53  | 2.53  | 2.53  |
| (6)IIIout | 11 | 2.65 | 4.34  | 4.34  | 4.34  | 4.34  | 4.34  |
| mean=     |    | 22.3 | 2.77  | 6.43  | 7.79  | 8.91  | 10.00 |
| ±1 S.D.=  |    | 15.8 | 0.61  | 5.25  | 7.21  | 9.23  | 11.42 |

5G+B

|           |    |      |       |       |       |       |       |
|-----------|----|------|-------|-------|-------|-------|-------|
| (1)III    | 20 | 2.73 | 4.40  | 5.16  | 5.16  | 5.16  | 5.16  |
| (3)Iin    | 11 | 3.25 | 3.94  | 7.98  | 10.43 | 10.43 | 10.43 |
| (4)Iin    | 13 | 2.73 | 4.46  | 19.22 | 24.47 | 39.47 | 48.10 |
| (4)IIout  | 23 | 2.83 | 4.34  | 6.79  | 22.27 | 29.30 | 37.93 |
| (4)IVin   | 39 | 3.05 | 3.05  | 4.34  | 4.34  | 4.34  | 4.34  |
| (5)IIIout | 09 | 5.03 | 11.77 | 17.36 | 21.22 | 21.22 | 21.22 |
| (5)IVin   | 05 | 2.32 | 3.94  | 9.69  | 12.36 | 13.10 | 13.10 |
| mean=     |    | 17.1 | 3.13  | 5.12  | 10.08 | 14.32 | 17.57 |
| ±1 S.D.=  |    | 11.5 | 0.88  | 2.97  | 5.90  | 8.33  | 13.10 |

1S+B

|           |    |      |      |       |       |       |       |
|-----------|----|------|------|-------|-------|-------|-------|
| (3)Iout   | 08 | 6.05 | 7.70 | 19.22 | 23.74 | 28.25 | 32.16 |
| (3)IIout  | 11 | 3.05 | 4.70 | 4.70  | 4.70  | 4.70  | 4.70  |
| (4)Iout   | 06 | 2.69 | 5.51 | 9.09  | 10.43 | 11.77 | 12.36 |
| (4)IIin   | 06 | 2.89 | 4.70 | 6.01  | 8.22  | 10.43 | 15.61 |
| (4)IIout  | 08 | 2.69 | 3.45 | 11.77 | 12.44 | 13.10 | 13.10 |
| (4)IVin   | 08 | 2.83 | 3.45 | 11.80 | 16.01 | 20.21 | 22.30 |
| (5)IIIin  | 17 | 3.25 | 4.04 | 4.19  | 4.34  | 4.70  | 4.70  |
| (5)IIIout | 11 | 3.45 | 3.83 | 7.44  | 8.53  | 9.76  | 10.98 |
| (6)Iout   | 14 | 3.05 | 3.66 | 5.51  | 10.43 | 11.77 | 11.77 |
| (6)IVout  | 27 | 5.16 | 6.01 | 6.79  | 11.77 | 11.77 | 11.77 |
| (7)Iin    | 21 | 3.66 | 4.04 | 6.79  | 8.53  | 8.53  | 8.53  |
| (7)IIin   | 08 | 2.65 | 3.40 | 4.70  | 9.09  | 9.69  | 9.69  |
| (7)IVin   | 13 | 2.73 | 3.40 | 3.40  | 3.40  | 3.40  | 3.40  |
| mean=     |    | 12.2 | 3.40 | 4.45  | 7.80  | 10.13 | 11.39 |
| ±1 S.D.=  |    | 6.3  | 1.04 | 1.28  | 4.34  | 5.37  | 6.67  |

3S+B

|          |    |      |      |       |       |       |       |
|----------|----|------|------|-------|-------|-------|-------|
| (1)Iin   | 20 | 2.73 | 5.65 | 6.79  | 6.79  | 6.79  | 7.44  |
| (1)IIIin | 25 | 2.65 | 4.34 | 6.01  | 6.39  | 6.39  | 6.39  |
| (1)IV    | 25 | 2.29 | 2.73 | 2.73  | 2.73  | 2.73  | 2.73  |
| (3)Iin   | 21 | 3.05 | 3.66 | 4.04  | 4.34  | 4.52  | 4.70  |
| (3)IIout | 19 | 3.33 | 7.98 | 8.53  | 9.11  | 9.69  | 12.36 |
| (3)IIIin | 14 | 2.73 | 3.45 | 6.01  | 7.44  | 7.44  | 7.98  |
| (4)Iin   | 20 | 2.65 | 3.05 | 4.04  | 4.34  | 4.34  | 4.34  |
| (4)Iout  | 18 | 2.49 | 9.09 | 13.99 | 15.16 | 16.32 | 19.22 |
| (4)IVin  | 13 | 1.92 | 4.46 | 9.09  | 12.40 | 18.11 | 23.17 |
| (5)IIout | 17 | 3.94 | 7.98 | 12.36 | 22.27 | 24.05 | 25.82 |
| (6)IIout | 16 | 5.51 | 5.51 | 6.79  | 6.79  | 6.79  | 6.79  |
| (7)Iout  | 14 | 5.51 | 5.51 | 8.53  | 9.11  | 9.69  | 9.69  |
| (7)IVin  | 06 | 6.01 | 7.98 | 8.54  | 9.09  | 10.43 | 10.43 |
| mean=    |    | 17.5 | 3.28 | 5.53  | 7.50  | 8.92  | 9.79  |
| ±1 S.D.= |    | 5.2  | 1.22 | 2.15  | 3.21  | 5.22  | 6.18  |

5S+B

|           |      |       |       |       |       |       |       |
|-----------|------|-------|-------|-------|-------|-------|-------|
| (3)Iout   | 13   | 2.32  | 3.45  | 6.79  | 7.44  | 13.10 | 17.36 |
| (3)IIIin  | 10   | 1.92  | 3.05  | 3.05  | 3.05  | 4.04  | 17.36 |
| (3)IVout  | 18   | 2.89  | 5.16  | 9.69  | 14.46 | 19.22 | 22.27 |
| (4)IIin   | 08   | 2.73  | 5.65  | 7.44  | 7.98  | 8.53  | 9.09  |
| (4)IIout  | 15   | 2.28  | 3.66  | 4.04  | 5.16  | 5.34  | 5.51  |
| (4)IIIin  | 08   | 2.73  | 4.97  | 9.09  | 12.36 | 14.65 | 22.27 |
| (7)IIIout | 56   | 3.40  | 3.66  | 3.66  | 3.66  | 3.66  | 3.66  |
| (8)IIin   | 51   | 3.66  | 3.66  | 3.66  | 3.66  | 3.66  | 3.66  |
| -----     |      | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=     | 22.4 | 2.74  | 4.16  | 5.93  | 7.22  | 9.02  | 12.65 |
| ±1 S.D.=  | 19.6 | 0.58  | 0.95  | 2.65  | 4.25  | 5.96  | 8.06  |

1T+B

|           |     |       |       |       |       |       |       |
|-----------|-----|-------|-------|-------|-------|-------|-------|
| (1)IV     | 12  | 3.83  | 4.97  | 11.20 | 14.65 | 14.65 | 14.65 |
| (2)Iin    | 15  | 1.92  | 2.32  | 3.45  | 5.16  | 6.79  | 8.53  |
| (2)IIIin  | 10  | 2.73  | 5.10  | 8.53  | 13.10 | 30.60 | 70.47 |
| (5)Iin    | 09  | 2.59  | 8.53  | 11.77 | 16.50 | 21.22 | 28.25 |
| (5)IIin   | 09  | 2.89  | 6.95  | 10.43 | 15.32 | 20.21 | 33.31 |
| (6)IIIin  | 07  | 3.01  | 5.65  | 6.01  | 8.53  | 8.53  | 8.53  |
| (6)IVin   | 07  | 2.73  | 6.31  | 8.53  | 10.15 | 11.77 | 15.61 |
| (7)Iin    | 11  | 3.25  | 4.46  | 4.70  | 4.70  | 6.01  | 6.01  |
| (7)IIIout | 06  | 1.74  | 4.46  | 6.01  | 7.44  | 8.57  | 9.69  |
| (7)IVout  | 03  | 2.53  | 3.05  | 3.05  | 3.05  | 3.05  | 3.05  |
| -----     |     | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=     | 8.9 | 2.72  | 5.18  | 7.37  | 9.86  | 13.14 | 19.81 |
| ±1 S.D.=  | 3.4 | 0.60  | 1.82  | 3.18  | 4.83  | 8.56  | 20.26 |

3T+B

|           |    |       |       |       |       |       |       |
|-----------|----|-------|-------|-------|-------|-------|-------|
| (1)IIlin  | 10 | 2.32  | 5.10  | 8.53  | 10.43 | 11.77 | 11.77 |
| (1)IIlout | 21 | 4.40  | 5.23  | 5.51  | 6.39  | 7.74  | 9.09  |
| (1)IVin   | 10 | 2.73  | 5.10  | 9.69  | 18.11 | 24.47 | 24.47 |
| (2)Ilin   | 10 | 1.92  | 3.25  | 6.01  | 7.98  | 9.09  | 9.69  |
| (2)IIlout | 10 | 1.92  | 4.40  | 5.16  | 6.01  | 9.09  | 9.09  |
| (2)IVin   | 10 | 2.73  | 8.71  | 11.77 | 18.11 | 22.27 | 37.80 |
| (3)IIlin  | 05 | 2.32  | 3.60  | 4.34  | 4.70  | 4.70  | 4.70  |
| (3)IIlout | 15 | 11.54 | 18.59 | 20.21 | 20.21 | 20.21 | 20.21 |
| (3)IVout  | 10 | 3.01  | 5.51  | 6.01  | 6.73  | 7.44  | 7.44  |
| (5)IIlin  | 17 | 4.16  | 4.70  | 8.53  | 17.36 | 20.27 | 23.17 |
| (5)IIlout | 06 | 2.32  | 4.65  | 10.43 | 12.54 | 14.65 | 30.60 |
| (5)IIIlin | 14 | 5.79  | 6.95  | 9.69  | 13.10 | 13.10 | 13.10 |
| (6)IIIlin | 11 | 3.25  | 3.25  | 3.25  | 3.25  | 3.25  | 3.25  |
| (7)IIlout | 09 | 4.40  | 6.01  | 10.98 | 25.82 | 31.88 | 37.93 |
| (7)IVin   | 09 | 4.40  | 5.72  | 6.79  | 7.98  | 7.98  | 7.98  |
| (7)IVout  | 09 | 3.83  | 6.95  | 13.99 | 22.63 | 22.63 | 22.63 |
| mean=     |    | 11.0  | 3.82  | 6.11  | 8.81  | 12.58 | 14.41 |
| ±1 S.D.=  |    | 4.0   | 2.34  | 3.62  | 4.24  | 6.98  | 8.26  |

5T+B

|           |    |      |       |       |       |       |       |
|-----------|----|------|-------|-------|-------|-------|-------|
| (2)IIlout | 18 | 3.45 | 5.10  | 5.16  | 5.16  | 5.16  | 5.16  |
| (2)IVout  | 10 | 2.73 | 3.88  | 4.34  | 4.34  | 4.34  | 4.34  |
| (4)Ilin   | 31 | 3.01 | 5.16  | 6.39  | 6.92  | 7.45  | 7.98  |
| (4)Iout   | 09 | 2.73 | 7.70  | 9.09  | 13.99 | 19.22 | 26.27 |
| (4)IIlin  | 09 | 3.01 | 11.77 | 19.22 | 21.85 | 24.47 | 26.89 |
| (5)IIlin  | 11 | 3.25 | 4.04  | 4.04  | 4.04  | 4.04  | 4.04  |
| (5)IIlout | 19 | 6.39 | 13.99 | 18.58 | 23.17 | 29.13 | 35.09 |
| (6)IIlout | 08 | 3.25 | 4.16  | 4.43  | 4.70  | 6.39  | 7.19  |
| (7)IVin   | 25 | 4.16 | 6.01  | 6.01  | 6.01  | 6.01  | 6.01  |
| (7)IVout  | 11 | 3.25 | 5.10  | 6.01  | 6.39  | 6.39  | 6.39  |
| mean=     |    | 15.1 | 3.52  | 6.69  | 8.33  | 9.66  | 11.26 |
| ±1 S.D.=  |    | 7.8  | 1.09  | 3.48  | 5.76  | 7.35  | 9.33  |

1Sb+B

|           |    |      |      |       |       |       |       |
|-----------|----|------|------|-------|-------|-------|-------|
| (2)Iout   | 09 | 4.16 | 5.51 | 7.28  | 11.77 | 12.36 | 12.36 |
| (3)Iin    | 43 | 4.58 | 5.51 | 7.44  | 7.98  | 8.26  | 8.53  |
| (3)IIin   | 09 | 4.04 | 6.39 | 9.09  | 11.77 | 13.99 | 19.22 |
| (3)IIout  | 06 | 4.04 | 7.44 | 10.98 | 15.61 | 20.21 | 26.82 |
| (3)IIIout | 06 | 4.04 | 4.97 | 6.79  | 7.98  | 8.53  | 8.53  |
| (4)Iin    | 10 | 3.88 | 5.51 | 6.79  | 8.53  | 10.43 | 13.99 |
| (4)IIin   | 05 | 4.04 | 5.37 | 9.09  | 11.77 | 12.36 | 13.10 |
| (4)IIout  | 06 | 5.51 | 6.95 | 9.69  | 10.43 | 10.98 | 11.77 |
| (4)IIIin  | 05 | 4.04 | 5.37 | 7.98  | 10.98 | 11.77 | 13.10 |
| (5)IIout  | 20 | 5.16 | 5.51 | 6.39  | 6.39  | 6.39  | 6.39  |
| (6)IIIin  | 06 | 3.51 | 4.70 | 4.70  | 4.70  | 5.16  | 5.16  |
| (8)IIout  | 10 | 4.04 | 4.04 | 5.51  | 5.51  | 5.51  | 5.51  |
| (8)IVin   | 06 | 3.66 | 6.01 | 7.98  | 9.69  | 9.69  | 9.69  |
| (8)IVout  | 11 | 4.34 | 4.70 | 5.51  | 6.01  | 6.79  | 6.79  |
| (9)Iout   | 05 | 3.05 | 3.66 | 4.04  | 4.34  | 4.34  | 4.34  |
| mean=     |    | 10.5 | 4.14 | 5.44  | 7.28  | 8.90  | 9.78  |
| ±1 S.D.=  |    | 9.8  | 0.60 | 1.00  | 1.92  | 3.20  | 4.13  |

3Sb+B

|           |    |      |      |      |      |       |       |
|-----------|----|------|------|------|------|-------|-------|
| (1)Iin    | 33 | 4.04 | 4.04 | 4.04 | 4.04 | 4.04  | 4.04  |
| (4)IVin   | 15 | 3.40 | 4.40 | 5.16 | 6.01 | 6.79  | 7.44  |
| (5)IVout  | 10 | 3.66 | 6.01 | 6.79 | 9.09 | 10.43 | 14.92 |
| (6)IVout  | 06 | 4.70 | 5.37 | 5.51 | 5.51 | 6.01  | 6.01  |
| (8)IIout  | 10 | 4.04 | 4.70 | 5.16 | 6.39 | 7.98  | 9.09  |
| (8)IIIout | 14 | 4.04 | 4.70 | 7.11 | 9.09 | 9.09  | 12.36 |
| (9)Iout   | 23 | 4.04 | 4.34 | 6.79 | 7.98 | 7.98  | 7.98  |
| mean=     |    | 15.9 | 3.99 | 4.79 | 5.79 | 6.87  | 7.47  |
| ±1 S.D.=  |    | 9.3  | 0.40 | 0.68 | 1.13 | 1.91  | 2.09  |

5Sb+B

|               |      |       |       |       |       |       |       |
|---------------|------|-------|-------|-------|-------|-------|-------|
| (1)IIin       | 06   | 3.66  | 4.16  | 5.16  | 6.01  | 6.01  | 6.39  |
| (4)Iin        | 06   | 3.66  | 4.16  | 5.51  | 6.79  | 6.79  | 6.79  |
| (5)Iin        | 10   | 5.37  | 9.09  | 11.77 | 13.10 | 14.65 | 15.61 |
| (5)IIIin      | 33   | 4.58  | 5.16  | 5.51  | 5.51  | 5.51  | 5.51  |
| (6)IVout      | 33   | 4.34  | 6.79  | 9.09  | 9.09  | 9.09  | 9.09  |
| (7)Iin        | 23   | 4.34  | 4.70  | 5.16  | 5.16  | 5.16  | 5.16  |
| (7)IVin       | 23   | 4.58  | 5.51  | 6.01  | 6.79  | 7.98  | 7.98  |
| (9)Iout       | 23   | 3.66  | 4.04  | 4.70  | 4.70  | 4.70  | 4.70  |
| -----         |      | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=         | 19.6 | 4.27  | 5.45  | 6.61  | 7.14  | 7.49  | 7.65  |
| $\pm 1$ S.D.= | 11.1 | 0.60  | 1.73  | 2.49  | 2.76  | 3.25  | 3.53  |

Sorbitol Control Treatment3G+B

|               |     |       |       |       |       |       |       |
|---------------|-----|-------|-------|-------|-------|-------|-------|
| (3)Iin        | 12  | 4.34  | 6.71  | 7.97  | 7.97  | 7.97  | 7.97  |
| (3)IVin       | 08  | 4.16  | 6.39  | 6.92  | 7.98  | 7.98  | 7.98  |
| (4)IIimid     | 08  | 4.40  | 8.53  | 10.43 | 10.43 | 10.71 | 10.98 |
| (6)Iout       | 27  | 4.04  | 6.39  | 6.39  | 7.98  | 7.98  | 7.98  |
| (6)IIIimid    | 10  | 3.66  | 7.98  | 13.99 | 22.27 | 23.37 | 24.47 |
| (7)Imid       | 10  | 3.20  | 7.98  | 12.85 | 15.61 | 15.61 | 15.61 |
| (9)IIin       | 10  | 4.04  | 11.77 | 25.82 | 33.31 | 33.31 | 33.31 |
| -----         |     | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=         | 9.7 | 3.97  | 8.23  | 12.95 | 16.26 | 16.49 | 16.72 |
| $\pm 1$ S.D.= | 1.5 | 0.46  | 1.92  | 6.90  | 9.99  | 10.09 | 10.22 |

Repeat Trehalose Treatments1T

|           |       |       |       |       |       |       |       |
|-----------|-------|-------|-------|-------|-------|-------|-------|
| (5)Iin    | 21    | 3.66  | 4.70  | 5.51  | 8.53  | 10.43 | 12.36 |
| (5)IIimid | 23    | 3.66  | 3.66  | 4.04  | 4.34  | 4.70  | 5.16  |
|           | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=     | 22    | 3.66  | 4.18  | 4.78  | 6.44  | 7.57  | 8.76  |
| ±1 S.D.=  | 1.41  | 0.00  | 0.74  | 1.04  | 2.95  | 4.04  | 5.09  |

3T

|           |       |       |       |       |       |       |       |
|-----------|-------|-------|-------|-------|-------|-------|-------|
| (1)IVmid  | 13    | 6.01  | 6.79  | 12.36 | 13.10 | 16.32 | 21.22 |
| (2)IIIin  | 14    | 4.34  | 6.01  | 12.36 | 19.22 | 23.17 | 26.82 |
| (2)IIImid | 27    | 4.34  | 5.16  | 5.16  | 5.51  | 5.51  | 5.51  |
| (4)IIimid | 26    | 4.34  | 4.34  | 4.34  | 4.34  | 4.34  | 4.34  |
| (5)Iin    | 19    | 3.36  | 5.51  | 6.01  | 6.39  | 6.39  | 7.44  |
| (6)Imid   | 19    | 4.04  | 6.79  | 9.09  | 14.00 | 16.32 | 17.36 |
|           | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=     | 19.7  | 4.46  | 5.77  | 8.22  | 10.43 | 12.00 | 13.78 |
| ±1 S.D.=  | 5.84  | 0.87  | 0.96  | 3.69  | 5.91  | 7.48  | 9.34  |

5T

|           |       |       |       |       |       |       |       |
|-----------|-------|-------|-------|-------|-------|-------|-------|
| 5T(1)Iin  | 33    | 6.01  | 6.39  | 6.78  | 6.78  | 6.78  | 6.78  |
| (1)IVmid  | 12    | 4.70  | 6.79  | 13.99 | 16.32 | 18.11 | 20.21 |
| (4)IIIout | 22    | 4.34  | 6.79  | 6.79  | 7.44  | 7.44  | 7.44  |
| (5)IVout  | 13    | 4.34  | 6.39  | 8.53  | 9.69  | 11.77 | 11.77 |
| (6)IIIout | 19    | 4.34  | 12.36 | 13.99 | 19.20 | 22.27 | 24.47 |
|           | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=     | 19.8  | 4.75  | 7.74  | 10.02 | 11.89 | 13.27 | 14.13 |
| ±1 S.D.=  | 8.47  | 0.72  | 2.59  | 3.70  | 5.56  | 6.76  | 7.88  |

Trehalose Control TreatmentK

|          |       |       |       |       |       |       |       |
|----------|-------|-------|-------|-------|-------|-------|-------|
| (1)Iout  | 22    | 3.05  | 3.66  | 4.04  | 4.04  | 4.04  | 4.04  |
| (1)IIin  | 23    | 3.66  | 4.70  | 4.70  | 5.51  | 5.51  | 5.51  |
| (6)IVmid | 13    | 5.16  | 5.16  | 5.16  | 5.16  | 5.16  | 5.16  |
|          | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| mean=    | 19.3  | 3.96  | 4.51  | 4.63  | 4.90  | 4.90  | 4.90  |
| ±1 S.D.= | 5.51  | 1.09  | 0.77  | 0.56  | 0.77  | 0.77  | 0.77  |

# Appendix C

## PERCENT GERMINATION

| Treatment | No. of Megaspores<br>Innoculated | No. of Megaspores<br>Germinated | Percent<br>Germination |
|-----------|----------------------------------|---------------------------------|------------------------|
| K         | 90                               | 08                              | 8.9                    |
| K+B       | 59                               | 15                              | 25.1                   |
| 1G        | 38                               | 12                              | 31.5                   |
| 3G        | 70                               | 17                              | 24.2                   |
| 5G        | 57                               | 10                              | 17.5                   |
| 1S        | 47                               | 08                              | 17.0                   |
| 3S        | 83                               | 06                              | 07.0                   |
| 5S        | 58                               | 10                              | 17.2                   |
| 1T        | 45                               | 14                              | 33.5                   |
| 3T        | 44                               | 01                              | 02.2                   |
| 5T        | 48                               | 09                              | 18.7                   |
| 1G + B    | 44                               | 22                              | 50.0                   |
| 3G + B    | 32                               | 14                              | 43.7                   |
| 5G + B    | 24                               | 16                              | 66.7                   |
| 1S + B    | 41                               | 15                              | 36.5                   |
| 3S + B    | 36                               | 19                              | 52.8                   |
| 5S + B    | 34                               | 12                              | 35.2                   |
| 1T + B    | 36                               | 13                              | 36.1                   |
| 3T + B    | 42                               | 22                              | 52.3                   |
| 5T + B    | 45                               | 14                              | 31.1                   |
| 1Sb + B   | 40                               | 17                              | 42.5                   |
| 3Sb + B   | 40                               | 08                              | 20.0                   |
| 5Sb + B   | 40                               | 08                              | 20.0                   |
| 3G + B*   | 40                               | 07                              | 27.5                   |
| All G - B | 165                              | 39                              | 23.6                   |
| All S - B | 188                              | 24                              | 12.8                   |
| All T - B | 137                              | 24                              | 17.5                   |

|           |     |     |      |
|-----------|-----|-----|------|
| All G + B | 100 | 52  | 52.0 |
| All S + B | 111 | 46  | 41.4 |
| All T + B | 123 | 49  | 39.8 |
| All non-B | 580 | 95  | 16.0 |
| All +B    | 393 | 162 | 41.0 |

Repeat Trehalose Treatments

|    |    |    |      |
|----|----|----|------|
| K  | 39 | 4  | 10.3 |
| 1T | 45 | 3  | 6.7  |
| 3T | 37 | 7  | 18.9 |
| 5T | 27 | 10 | 37.0 |

(\* = Sorbitol Control Treatment)

(K = simple mineral salts medium, G = glucose, S = sucrose,  
T = trehalose, Sb = sorbitol, + B = addition of  
B vitamin mixture)

Appendix D  
FLUORESCENCE DATA

| <u>Treatment</u> | <u>No. Megagam-<br/>etophytes<br/>Extracted</u> | <u>Vol. of<br/>Tissue<br/>Extracted<br/>(<math>\times 10^{-2}</math> mm<sup>3</sup>)</u> | <u>Fluorescence<br/>due to<br/>Chlorophyll<br/>(corrected)*</u> |
|------------------|-------------------------------------------------|------------------------------------------------------------------------------------------|-----------------------------------------------------------------|
| K                | 1                                               | 9.5                                                                                      | .1943                                                           |
| K+B              | 8                                               | 47.6                                                                                     | .7772                                                           |
| 1,3,5% G         | 13                                              | 78.6                                                                                     | 1.7487                                                          |
| 1% S             | 5                                               | 32.3                                                                                     | 0.0000                                                          |
| 1,5% T           | 7                                               | 53.9                                                                                     | 0.3886                                                          |
| 3%G + B          | 2                                               | 21.1                                                                                     | 0.5829                                                          |
| 5%G + B          | 2                                               | 37.8                                                                                     | 0.5829                                                          |
| 1%S + B          | 10                                              | 192.6                                                                                    | 1.3601                                                          |
| 3%S + B          | 10                                              | 144.1                                                                                    | 1.3601                                                          |
| 5%S + B          | 9                                               | 131.0                                                                                    | 0.7772                                                          |
| 3%T + B          | 10                                              | 137.7                                                                                    | 1.5544                                                          |
| 1%Sb + B         | 2                                               | 21.4                                                                                     | 0.9715                                                          |
| 3%Sb + B         | 2                                               | 11.6                                                                                     | 1.1658                                                          |
| 5%Sb + B         | 2                                               | 26.2                                                                                     | 0.7772                                                          |
| Stem Tissue      |                                                 | 4930.0                                                                                   | 1975.3                                                          |

\* - The corrected measurements of fluorescence were derived by multiplying actual fluorescence reading by a pre-determined correction factor = 1.943.

Corrected fluorescence readings were divided by the amount of tissue extracted to derive fluorescence per mm<sup>3</sup>.

The linear equation derived by measuring fluorescence of serial dilutions of chlorophyll-A was:

$$\text{Corrected} \\ [\text{Chlorophyll-A}] = \text{Fluorescence} \times .05982773 + .04476125 \\ (\text{micrograms/liter extract})$$

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GROWTH AND DEVELOPMENT OF THE MEGAGAMETOPHYTE OF THE  
VASCULAR PLANT  
SELAGINELLA (LYCOPSIDA) ON DEFINED MEDIA

by

Alan Leonard Koller

(ABSTRACT)

Megagametophytes of the heterosporous lower vascular plant, Selaginella, were cultured on a variety of types and concentrations of carbon sources (glucose, sucrose, trehalose, and sorbitol), with and without B vitamins, in an attempt to induce apogamy. Without B vitamins growth was enhanced on glucose and trehalose, but not on sucrose. With B vitamins growth was enhanced on all types and concentrations of carbon sources. Enhanced growth involved the production of greater numbers of cells in the tissue. Chlorophyll-a was present in megagametophytes cultured on many of the treatments, including control treatments without supplemental carbon. Apogamy was not induced.