EXPERIMENTATION
&
OBSERVATIONS
Since no data are available in the literature regarding physiological requirements of the megagametophyte in sterile culture, it was thought necessary to design the following experiments:

1. Optimal agar concentration in the medium
2. Optimal basal medium
3. Effect of storage on viability of the megaspores as tested in optimal basal medium
4. Effect of sucrose concentrations; 2,4-D and light intensity on germination.
1.1 **Optimal agar concentration in the medium**

To find out the optimal agar concentration, Knop's solution of inorganic salts (full strength) was gelled with 0.5, 0.8, 1.0, 2.0 and 3.0 per cent of Difco-Bacto agar. Data on the time taken for megaspore germination and the percentage germination were recorded as shown in Table I.

<table>
<thead>
<tr>
<th>Agar concentration</th>
<th>Time taken for germination</th>
<th>Number of megaspores</th>
<th>Germination percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5%</td>
<td>72 hrs</td>
<td>44</td>
<td>14</td>
</tr>
<tr>
<td>0.8%</td>
<td>72 hrs</td>
<td>35</td>
<td>12</td>
</tr>
<tr>
<td>1.0%</td>
<td>96 hrs</td>
<td>70</td>
<td>21</td>
</tr>
<tr>
<td>2.0%</td>
<td>96 hrs</td>
<td>47</td>
<td>13</td>
</tr>
<tr>
<td>3.0%</td>
<td>120 hrs</td>
<td>37</td>
<td>6</td>
</tr>
</tbody>
</table>

By the time germination started in higher concentrations of agar, viz., 1.0, 2.0 and 3.0%, fully-developed megagametophytes were observed on medium solidified with 0.5% and 0.8% agar. Subsequent growth was also found to be best supported on 0.8% agar concentration.

1.2 **Optimal basal medium**

Due to well-marked structural simplicity but sharp sensitivity of the fern gametophyte in general, for various
inorganic salts of different concentrations, it was felt desirable to define the medium of inorganic salts which could support optimal growth. The media tried included Knop's (1869, full and half strength), Knudson's (Steeves et al., 1955), Moore's (1903) and Murashige and Skoog's (1962).

All the media were gelled with the optimal concentration (0.8%) of Difco-Bacto agar. Megaspore inoculation was done on the same day and the cultures were maintained under uniform conditions of light and temperature, as mentioned under 'Materials and Methods'. Data on the time taken for germination, and percentage germination, were regularly recorded (Table -II).

**TABLE - II**

<table>
<thead>
<tr>
<th>Media used</th>
<th>Time taken for germination</th>
<th>Number of megaspores</th>
<th>Germination percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knop's (full strength)</td>
<td>96 hrs</td>
<td>44</td>
<td>7</td>
</tr>
<tr>
<td>Knop's (half strength)</td>
<td>72 hrs</td>
<td>60</td>
<td>11</td>
</tr>
<tr>
<td>Knudson's</td>
<td>96 hrs</td>
<td>34</td>
<td>10</td>
</tr>
<tr>
<td>Moore's</td>
<td>72 hrs</td>
<td>52</td>
<td>18</td>
</tr>
<tr>
<td>Murashige and Skoog's</td>
<td>144 hrs</td>
<td>46</td>
<td>2</td>
</tr>
</tbody>
</table>
It is clear from Table II that both, in terms of time taken for germination as well as percentage germination, Moore's medium proved to be most suitable, closely followed by Knudson's medium. Growth on Murashige and Skoog's medium was markedly slower than the rest of the media. The percentage of megaspore germination on Knop's medium (full and half strength) was considerably low. Moreover, the megagametophytes on this medium turned brown soon after their initiation. Thus, only Moore's medium was utilized because of the highest percentage of megaspore germination and better growth of the resultant gametophytes.

1.3 Effect of storage on viability of the megaspores as tested on optimal basal medium

This experiment was designed to see the effect of ageing on megaspore germination. The following three samples of sporocarps collected in different years and localities, were used for this purpose:

a. Sporocarps collected in 1963 from the Botanical Gardens, Lucknow University

b. Sporocarps collected in 1969 from the plants raised in the Department of Botany, Panjab University, Chandigarh

c. Sporocarps collected in 1970 and received from Valparaiso (Indiana), U.S.A.

Megaspores from the above samples were taken and inoculated in Moore's medium gelled with 0.3% agar. Data
on the time taken for germination were regularly recorded (TABLE -III).

<table>
<thead>
<tr>
<th>Sample used</th>
<th>Year of collection</th>
<th>Locality</th>
<th>Time taken for germination</th>
<th>Number of megaspores</th>
<th>Germination percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>1963</td>
<td>Botanical Gardens, Lucknow University</td>
<td>408 hrs</td>
<td>70</td>
<td>6</td>
</tr>
<tr>
<td>(b)</td>
<td>1969</td>
<td>Botany Department, Panjab University, Chandigarh</td>
<td>120 hrs</td>
<td>45</td>
<td>8</td>
</tr>
<tr>
<td>(c)</td>
<td>1970</td>
<td>Indiana (USA)</td>
<td>72 hrs</td>
<td>62</td>
<td>24</td>
</tr>
</tbody>
</table>

It is clear from Table III that the megaspores obtained from sporocarps of sample (c) showed maximum viability as compared to the other two samples. The megaspores of sample (a) were least viable, showing thereby that the period of storage adversely affects the percentage of germination.

1.4 Effect of various sucrose concentrations; 2,4-D and light intensity on germination response

1.4-1 Effect of various sucrose concentrations

To see the effect of various concentrations of sucrose, optimal basal medium was supplemented with 0.5, 1.0, 2.0, 3.0,
4.0, 5.0 and 6.0 per cent of sucrose by weight. Megaspores were inoculated on the medium supplemented with the above sucrose concentrations on the same day. Control cultures on optimal basal medium (without sucrose) were also set along with.

Observations

In control cultures, germination was observed after four days of inoculation. The percentage megaspore germination as well as time taken for germination varied in different concentrations of sucrose (Table IV).

*TABLE - IV

<table>
<thead>
<tr>
<th>Sucrose concentrations</th>
<th>Time taken for germination</th>
<th>Number of megaspores</th>
<th>Germination percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inoculated</td>
<td>Germinated</td>
</tr>
<tr>
<td>Control</td>
<td>96 hrs</td>
<td>51</td>
<td>24</td>
</tr>
<tr>
<td>0.5%</td>
<td>72 hrs</td>
<td>42</td>
<td>26</td>
</tr>
<tr>
<td>1.0%</td>
<td>72 hrs</td>
<td>39</td>
<td>19</td>
</tr>
<tr>
<td>2.0%</td>
<td>96 hrs</td>
<td>74</td>
<td>20</td>
</tr>
<tr>
<td>3.0%</td>
<td>96 hrs</td>
<td>44</td>
<td>7</td>
</tr>
</tbody>
</table>

* In Tables I - IV the figures pertaining to megaspore germination are the mean values of four countings made at random after the first signs of germination.
Table IV clearly shows that 0.5% sucrose is optimal for megaspore germination. Percentage germination progressively falls with an increase in sucrose concentration. Moreover, the germination was delayed in concentrations higher than 0.5% and 1.0%. Sucrose concentrations higher than 3.0% proved supra-optimal for germination because no germination was observed in media incorporated with 4.0, 5.0 and 6.0 per cent.

1.4-2 Effect of sucrose in combination with 2,4-D

In another set of cultures, 0.5% sucrose was incorporated in the optimal basal medium along with different concentrations of 2,4-D (0.5 ppm, 1 ppm-6 ppm). Megaspores were inoculated on the same day.

Observations

Megaspores germinated after three days of inoculation. Maximum percentage (about 90%) germination was observed in the medium supplemented with 0.5% sucrose in combination with 1 ppm 2,4-D. It was of interest to note that the percentage germination was high as compared to the one observed on optimal basal medium supplemented with 0.5% sucrose. The percentage germination decreased with an increase in concentration of 2,4-D in the medium.
INDUCTION OF APOGAMY IN REGNELLIDIUM DIPHYLLUM LINDM.

1. INDUCTION OF SPOROPHYTIC PHASE FROM VEGETATIVE CELLS OF THE FEMALE GAMETOPHYTE (INTACT MEGASPORES) BY SUCROSE.

Female gametophytes raised on BM (sucrose-free)

Transferred to BM + 1-10% sucrose (1S,2S,3S-10S)

2. INDUCTION OF SPOROPHYTIC PHASE FROM VEGETATIVE CELLS OF THE FEMALE GAMETOPHYTE (INTACT MEGASPORES) BY SUCROSE IN COMBINATION WITH 2,4-D.

Female gametophytes raised on BM + 0.5% sucrose

Transferred to BM + 0.5% sucrose + 1 ppm 2,4-D

3. INDUCTION OF SPOROPHYTIC PHASE FROM VEGETATIVE CELLS OF THE EXCISED FEMALE GAMETOPHYTES BY SUCROSE IN COMBINATION WITH 2,4-D.

Female gametophytes raised on BM + 0.5% sucrose + 1 ppm 2,4-D

Transferred to BM + 0.5% sucrose + 1 ppm 2,4-D

BM + 0.5% sucrose + 2,4-D (0.05, 0.1, 0.5, 1-4 ppm)
2. **INDUCTION OF APOGAMY FROM THE MEGAGAMETOPHYTE**

It is important to mention here that no data are available in the literature regarding the induction of sporophytic development directly from gametophytic tissue in heterosporous ferns. Since sucrose and auxins have been demonstrated to affect apogamy in homosporous ferns and organogenesis in angiosperm tissues, the megagametophytes of *Regnellidium diphylloides* were subjected to these chemicals.

The optimal basal medium utilized in this experiment consisted of Moore's solution of mineral salts, ferric citrate (10 mg/l) and Mitscherlich trace elements (1 ml/l). The medium was gelled with 0.8% Difco-Bacto agar. Sterilization and inoculation of megaspores was done as described under 'Materials and Methods'. The experiment was designed in three different ways (Fig. 1) and the results of each are described separately as follows:

1. _Induction of apogamy with intact megaspores on sucrose media._

2. _Induction of apogamy with intact megaspores on sucrose in combination with 2,4-D._

3. _Induction of apogamy from the excised megagametophytes on sucrose in combination with 2,4-D._

2.1 **Induction of apogamy with intact megaspores on sucrose media**

An earlier experiment has demonstrated that sucrose up to a certain level, is helpful in germination. In order
to see whether the effect of sucrose in favour of sporophytic induction begins during 'gametophytic phase' or 'initiative phase', this experiment was performed in two different ways.

2.1-1 **Stock cultures of mega gametophytes raised on optimal basal medium (sucrose-free)**

Megaspores were germinated on optimal basal medium (Moore's media without sucrose). Four-day-old megagametophytes were transferred to the optimal basal medium supplemented with 0.5%, 1.0-10.0% sucrose. These are referred to by the symbols 0.5S, 1S, 2S and so on, the numerals denoting the sucrose concentrations. Subculturing was done after every third week. Cultures in optimal basal medium (without sucrose) were set to act as control.

**Observations**

**Control cultures:** In control cultures, the megagametophyte comprised a few vegetative cells which were polygonal and almost isodiametric with discoid chloroplasts.

**Cultures in different sucrose concentrations:** The megagametophytes transferred to the medium incorporated with different concentrations of sucrose, gradually lost their

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*The terms 'gametophytic', 'initiative' and 'developmental' herein used are after Whittier (1964); though used 'arbitrarily', they undoubtedly facilitate understanding of the apogamous response in terms of time.*
chlorophyll and became brown. After having remained in the same physiological state for six weeks, the brown tissue differentiated cylindrical appendages herein designated as protophylls, first in 3S cultures followed by 2S. The protophylls thus formed (Fig. 2) were erect, cylindrical, green outgrowths which lacked vascular supply and stomata. They failed to develop lamina comparable to that of the normal sexually produced, 2X sporophytes.

Similar response of the megagametophytes was observed on 1S, 4S, and 5S cultures as well, but, as compared to the 3S cultures the response was slower in view of the differentiation of fewer protophylls. On the other hand, the female gametophytes, on cultures with sucrose concentrations ranging from 6S-10S, remained brown throughout the period of experimentation.

2.1-2 Stock cultures of megagametophytes raised on optimal basal medium supplemented with 0.5% sucrose

Megaspores were germinated in optimal basal medium supplemented with 0.5% sucrose. Four-day-old megagametophytes were then transferred to basal medium supplemented with different concentrations of sucrose (1.0-10.0%). Sub-culturing was done after every third week. Control cultures

* The terms protophylls and protocorm are used here because of their striking resemblance with those recorded in some species of *Lycopodium*.
were set on optimal basal medium supplemented with 0.5% sucrose.

**Observations**

**Control cultures:** It was interesting to note that in 6-day-old control cultures, some of the vegetative cells of the megagametophyte underwent rapid mitotic divisions and cell differentiation resulting in the formation of typical sporophytic cells. These sporophytic cells could easily be distinguished from the neighbouring gametophytic cells by their shape, and number and size of chloroplasts (Fig. 3).

A study of 10-day-old megagametophytes revealed the presence of vascular elements in the form of tracheids having scalariform thickenings, in the sporophytic cells which in turn are derived from the vegetative cells of the megagametophyte. In the absence of other cell types that usually accompany the xylem elements, only a limited extent of histogenic differentiation could be effected in these cultures. The differentiated tissue, derived from the megagametophyte, then started turning brown and remained in the same state for 3 weeks. Later, only one or two protophylls were seen differentiated from the brown tissue, which turned brown soon after their formation.

**Cultures in different sucrose concentrations:** Some variations in the development of the megagametophytes were
apparent on different sucrose concentrations referred to earlier by 1S-10S symbols. The differentiated tissue derived from the megagametophyte, after transfer to all the above-mentioned sucrose concentrations, lost their normal photosynthetic pigment gradually and turned brown. In 1-7% sucrose concentrations, the cells of the brown tissue resumed mitotic activity and as a consequence thereof, a highly proliferated callus-like mass was formed. Simultaneously, or at a little later stage, protophylls bearing a striking resemblance with those recorded in some species of the genus Lycopodium were differentiated from the above-mentioned proliferated mass of cells.

The response of the brown tissue (derived from the megagametophyte) to various sucrose concentrations, in terms of organogenesis, differed as follows:

In 1S and 2S cultures, the tissue remained brown for 2 weeks, after which the proliferation was observed, followed by differentiation of protophylls along with a large number of rhizoids (Fig. 4). The latter differentiated from the basal portion of the proliferated mass.

In 3S cultures, the tissue remained brown for only a week, after which it started differentiating protophylls similar to those in 1S and 2S cultures (Fig. 5). However, the degree of proliferation as well as the number of
protophylls and rhizoids differentiated per gametophyte are markedly higher than in 1S and 2S cultures. In some individuals in 3S cultures, awl-shaped leaves, as shown in Fig. 6 were observed, comparable at least in external appearance to that produced as the first leaf in sexually produced, 2X sporophytes. They differed, however, from the same in complete absence of vascular supply and stomata. In contrast to the straight vernation of the protophylls these exhibited circinate coiling. The differential behaviour of growth pattern, exhibited by various individuals in 3S cultures, cannot be explained at this stage and needs further experimentation.

The growth response of the proliferated tissue in 4S and 5S cultures was extremely slow as was evidenced by the differentiation of a fewer protophylls and poor rhizoidal growth as compared to 3S cultures.

In 6S and 7S cultures, the brown tissue remained, in the same state for 6 weeks, after which it proliferated into a green mass of cells, which later differentiated a number of green, cylindrical appendages. The rate of differentiation of these organs was, however, markedly slow. A study of 12-week-old cultures on this medium showed the differentiation of the following organs:

1. **Roots**: Positively geotropic roots with profuse growth of root hairs were produced.
ii. **Shoot-like organs**: Radially symmetrical organs with apical parts much like the normal 2X sporophyte, bearing many multicellular, uniseriate hairs on the surface were seen (Fig. 7). No lateral organs were seen on these shoot-like organs.

iii. **Unbranched protophylls**: These protophylls (Fig. 8) comprised typical sporophytic cells with a few stomata but they were devoid of vascular supply. Some other protophylls developed vascular strand in the form of tracheids with spiral thickenings but no stomata were observed.

iv. **Leaves with limited cladification**: Leaves with meagre development of dichotomously branched lamina, were formed (Fig. 9). The differentiation and maturation of tracheids commenced from the basal portion of the petiole and proceeded towards the apex, but no normal lamina was developed. A large number of uniseriate, filamentous, multicellular hairs were also observed on the surface of these appendages.

Some of the undifferentiated cells of the proliferated mass were found to exhibit an interesting behaviour as regards their plane of division. Successive mitotic divisions in transverse plane resulted in the formation of filamentous structures much resembling the uniseriate, multicellular hairs present on the sexually produced 2X sporophytes.
Various parts of the 2X sporophyte possess uniseriate multicellular hairs. The cells of these hairs possess either a few chloroplasts or none. The hairs produced from the haploid tissue, however, were generally comprised of three cells in contrast to 6-12-celled condition in sexually produced, 2X sporophyte. In figure 10 two dermal hairs (differentiated from the proliferated mass) at different stages of their development are depicted. The cells of the young hairs, as seen in figure 10A, are full of chloroplasts and other organelles of the cytoplasm occur in the normal position. During the course of differentiation, the chlorophyll gets degraded and vacuolation results in shifting the nucleus near the cell wall (Fig. 10B). The cell walls of these hairs are loosely joined and get easily separated into single cells (Fig. 11).

The brown tissue in 8S, 9S and 10S cultures remained brown throughout the period of experimentation.

A point of utmost importance that emerges from the experiments described above is that the supply of sucrose during the initial stages of megaspore germination, plays an important role in initiation and development of sporophytic phase. The incorporation of an appropriate concentration of sucrose during megaspore germination in experiment 2.1-2 helped in shifting the gametophytic phase to sporophytic phase. This is indicated by the fact that
the initiation of sporophytic cells and tracheids is attained at a very early stage of development. Furthermore, the differentiation of organs such as protophylls, positively geotropic roots, shoot-like organs, leaves with meagre development of dichotomously branched lamina and uniseriate, multicellular hairs are observed in contrast to the differentiation of protophylls and rhizoids only, in experiment 2.1-1, where no sucrose is incorporated in the medium in the initial stages of megaspore germination.

2.2 Induction of apogamy with intact megaspores on sucrose in combination with 2,4-D

Because of the failure to get a normally-organised sporophyte with root, shoot and leaf in the previous experiment, and also in view of the variable response of the gametophytic tissue to sucrose at different stages of its development, the present experiment was designed in the following three ways:

2.2-1 Stock cultures of megagametophytes raised on optimal basal medium (sucrose-free)

Megaspores were put to germination on optimal basal medium (without sucrose). Four-day-old megagametophytes were then transferred to the basal medium supplemented with 0.5% sucrose in combination with different concentrations of 2,4-D; the concentrations tried include 0.05 ppm, 0.1 ppm, 0.5 ppm and 1-4 ppm. Control cultures were kept alongside
on basal medium.

Observations

Control cultures: In these cultures, the germinating megaspores followed the usual pattern of growth i.e. the megagametophytes comprised of polygonal, almost isodiametric cells with discoid chloroplasts. The tissue of the megagametophyte gradually lost its chlorophyll, became brown and ultimately died.

Cultures on basal medium supplemented with 0.5% sucrose in combination with different concentrations of 2,4-D: To begin with, the megagametophytic tissue, after transference to media supplemented with 0.5% sucrose in combination with different concentrations of 2,4-D, lost its chlorophyll and turned brown. It remained in the same state for six weeks. Subsequently, the brown tissue resumed mitotic activity and developed protocorm (bearing a striking resemblance with similar structures recorded in some species of the genus *Lycopodium*), on the media supplemented with 0.5% sucrose in combination with 1 ppm and 2 ppm 2,4-D. The protocorm differentiated protophylls which were indefinite in shape and position. These were devoid of vascular supply and stomata. It may be recalled here that such protophylls were also obtained earlier from callus-like proliferated mass derived from the megagametophyte in experiment 2.1-1 and 2.1-2.
In the rest of 2,4-D concentrations, the material gave no response.

2.2-2 Stock cultures of megagametophytes raised on optimal basal medium supplemented with 0.5% sucrose

Megasporates were germinated on optimal basal medium supplemented with 0.5% sucrose. Four-day-old megagametophytes were then transferred to the basal medium supplemented with 0.5% sucrose in combination with 0.05 ppm, 0.1 ppm and 1-4 ppm concentrations of 2,4-D. Cultures of megagametophytes on optimal basal medium supplemented with 0.5% sucrose were set to act as control.

Observations

Control cultures: As shown in experiment 2.1-2 in these cultures also, some of the cells of the megagametophyte underwent rapid mitotic divisions and cell differentiation resulting in the formation of typical sporophytic cells full of discoid chloroplasts. Differentiation of vascular elements in the form of tracheids with scalariform thickenings were also noticed. The tissue gradually turned brown and remained in the same state for 3 weeks. After the production of a few protophylls, further growth ceased altogether.

Cultures on medium supplemented with 0.5% sucrose in combination with different concentrations of 2,4-D: The megagametophytic tissue, after transference to media
supplemented with sucrose in combination with different concentrations of 2,4-D, gradually lost its chlorophyll and ultimately turned brown. It remained in the same state for 2 weeks in contrast to 6 weeks in the earlier experiment, after which the protocorm started differentiating protophylls on the media supplemented with 0.5% sucrose in combination with 1 ppm and 2 ppm of 2,4-D.

Optimal response of differentiation, however, was observed on basal medium supplemented with 0.5% sucrose along with 1 ppm 2,4-D. The protophylls differentiated in these cultures (Figs. 12, 13) were mostly unbranched, sometimes forked and rarely trifid. The vascular supply in all the protophylls was absent in the initial stages. At a later stage, in forked protophylls, however, an acropetally developed vascular supply was observed which failed to reach the apices of the protophylls (Figs. 12, 13). In some specimens, two-leaflet condition comparable to that of 2X sporophytes was observed but the leaflets were different in shape.

The formation of such sporophytes with partial morphological differentiation was also observed on optimal basal medium incorporated with 0.5% sucrose and 2 ppm 2,4-D. The rate of growth was, however, comparatively slow in this case. The protophylls were produced in preponderance and very few leaves with flattened lamina were produced.
In terms of overall growth, the haploid sporophytes obtained in the above experiment, were comparatively shorter in size with complete absence of normal shoot system. The whole mass resembled that of a protocorm bearing roots and protophylls without any definite orientation. In contrast, the 2X sporophyte exhibited normal growth with fully differentiated root, shoot and leaf (Fig. 14).

2.2-3 Stock cultures of megagametophytes raised and subcultured on optimal basal medium supplemented with 0.5% sucrose in combination with 1 ppm of 2,4-D

In spite of the fact that the presence of an appropriate sucrose concentration in the initial stages of megaspore germination played a significant role in shifting the gametophytic phase to sporophytic phase in the previous experiments, it failed to induce absolutely normal sporophytic growth with all the three organs, viz. root, shoot and leaf. The present experiment was, therefore, designed to obtain normal looking haploid sporophytes comparable to the sexually-produced 2X ones.

Megaspores in this experiment were directly germinated on optimal basal medium supplemented with 0.5% sucrose in combination with 1 ppm 2,4-D.

Observations

Differentiation of sporophytic cells from the tissue
of the megagametophyte could be observed as early as 2-3 days after megaspore germination. A microscopic study of 4-5 day-old megagametophytes revealed rapid mitotic activity. This was followed by differentiation of cells of different shape and form, some of them being much more elongated with numerous discoid chloroplasts. These sporophytic cells could easily be distinguished from the neighbouring polygonal and almost isodiametric cells of the megagametophytes (Fig. 15). Stomata were also seen in the tissue derived from the megagametophyte (Fig. 16). The stomata were structurally normal with two guard cells around the stomatal aperture.

A study of 6-8-day-old tissue showed the differentiation of vascular elements in the form of tracheids with scalariform thickenings. These were found in groups with in the parenchymatous tissue (Fig. 17). Thus, it is of interest to observe a combination of gametophytic cells, typicall sporophytic cells—tracheids and stomata, derived from the few-celled megagametophyte.

Apart from the above characteristics, in certain portions of the tissue, multicellular, hyaline, cylindrical structures were also observed, the apical part of which appeared to contain the apical meristem. The internal organisation of these apices, however, could not be studied for the paucity of the material.
Further growth of the haploid sporophyte from the above-mentioned tissue was interesting. This tissue developed a calyptra which enclosed the developing tissue at a very early stage, as known to occur in young 2X sporophytes. The growth of the calyptra kept pace with the growth of the sporophyte and was comprised of compactly arranged non-chlorophyllous cells which were much longer than broad, in contrast to the chlorophyllous and bigger cells of the calyptra of 2X sporophytes. A large number of rhizoids originated from all the sides of the megagametophyte as well as the tissue derived from it. This derived tissue turned brown and remained in the same state for 2 weeks. During this period, the growth of the calyptra along with the proliferation of the brown tissue continued to occur (Figs. 18, 19). The tissue then developed protocorm and the initiation and differentiation of sporophytic organs from it was observed after 4 weeks on the same medium. Protophyll was the first externally visible organ developed directly from the tissue of the megagametophyte. It was enclosed in the calyptra (Figs. 20, 21, 22) as the first leaf does in the sexually produced 2X sporophyte. Generally, the first protophyll was straight in vernation (Fig. 23) but rarely it showed circinate coiling (Fig. 24). The rate of differentiation of these protophylls was markedly slow, as only 3-4 protophylls were observed after 5 weeks of culture. Initiation of roots in these haploid sporophytes was much delayed. Only rhizoids were observed in early stages.
of development (Fig. 25), followed by the differentiation of protophylls (Fig. 26). Figure 27 depicts the formation of 2-3 protophylls from the protocorm whereas the root initiation is delayed. On the other hand, in sexually produced 2x sporophytes, the first leaf is the first externally visible organ to be differentiated (Fig. 28). The first root was generally initiated along with the differentiation of the first leaf (Figs. 29, 20, 31).

Although the differentiation of sporophytic organs was maximum in this experiment, no typical shoot system was observed (Fig. 32). A large number of haploid sporophytes thus obtained (Fig. 33) comprised, on the whole, of a green mass bearing a striking resemblance with protocorm, as pointed out earlier. The lateral appendages produced by the protocorm were of variable morphology (Fig. 34), and are described below in details:

1. Unbranched protophylls: These appendages (Fig. 35) were comprised of parenchymatous cells without the differentiation of vascular tissue and stomata.

2. Forked protophylls: A number of such appendages (Fig. 36) were observed which lacked stomata. The vascular supply in the early stages of their development was absent but later on it developed the xylary tissue composed of tracheids which could be traced up to the point of forking but the distal part of the branches was devoid of vascular
differentiation. Some of these protophylls were deeply forked and longer than the others due to limited cladification (cf. Fig. 34).

iii. Leaves with flattened, entire lamina: Very few fronds with distal, flattened lamina were observed (Fig. 36). Even on prolonged culturing, leaves with normal 2-leaflet condition failed to develop. It may be recalled here that similar leaves with flattened lamina were produced in 7S cultures in the previous experiment 2,1-2. The latter, however, were bifid. This similarity of frond form is indicative of the possibility that lower sucrose concentration in this experiment has been compensated by 2,4-D.

iv. Leaves with bilobed lamina: As depicted in Figs. 37, 38, 39, some of the fronds produced bilobed lamina which differed from those of the sexually produced 2X sporophytes (Fig. 40), both in their shape as well as the degree of lobing.

v. Dermal hairs: Multicellular, uniseriate hairs, like those of the 2X sporophyte, were borne on all the sides of the above-mentioned appendages.

vi. Roots: Chlorophyllous, positively geotropic roots (with root hairs) curving upside down, were formed (Fig. 41).

From the overall results of the experiments described
above, the valuable role of an appropriate sucrose concentration in the initial stages of megaspores germination is quite apparent. Best results are obtained in experiment 2,2-3 where appropriate concentrations of both sucrose and 2,4-D are incorporated in the medium during megaspore germination.

It is significant to point out here that bud or cushion-like structure, as are reported in apogamous homosporous ferns, were not observed in the present material. The significance of this differential behaviour of the gametophytic tissue in homo- and heterosporous ferns will be discussed in the later part of the thesis.

2.3 *Induction of apogamy from the excised megagametophytes on sucrose in combination with 2,4-D*

This experiment was designed to see if the excised megagametophytes exhibit growth response different from those with intact megaspores. For this purpose, the megaspores were inoculated in optimal basal medium supplemented with 0.5% sucrose along with 1 ppm of 2,4-D (Fig. 42). Four-day-old megagametophytes (Fig. 43) were excised from the megaspores and transferred to variously modified basal medium. Basal medium supplemented with 0.5%, 1-10% of sucrose; and with low concentration of sucrose (0.5%) in combination with 0.05 ppm, 0.1 ppm, 0.5 ppm, 1 ppm of 2,4-D was utilized for this experiment.
Observations

The excised tissue of the megagametophyte in all the cultures, turned brown 4 days after inoculation and remained in the same state for 10 weeks. However, quantitative increase in mass could be discerned, which probably occurred due to increase in cell number. Subsequently, differentiation of protophylls was observed on media supplemented with 0.5% sucrose in combination with 1 ppm and 1.5 ppm of 2,4-D (Fig. 44). At initial stages, there was no sign of vascular strand within these protophylls. At a later stage, however, a fully developed vascular strand was observed. The growth rate of these plants was extremely slow, as only three protophylls were differentiated within a period of 12 weeks of culture on the same medium (Fig. 45). Subsequently formed protophylls developed lamina which resembled the second leaf of the 2x sporophyte. A few normal roots were also observed arising from the surface of the brown tissue of the megagametophyte, but normal shoot system failed to develop in these cultures.

It was surprising to note that on further subculturing, the sporophytes, instead of attaining adulthood, turned brown and ultimately died. The excised tissue of the megagametophyte in all other media remained brown and failed to differentiate any organ.
3. **EFFECTS OF GLUCOSE, SUCROSE AND COCONUT WATER ON THE SPORELINGS OF REGNELLIDIIUM DIPHYLLUM LINN.**

Sterile cultures of whole plants have been reported in *Marsilea* (Allsopp, 1963), *Regnellidium diphyllum* (Rao, 1966) and *Salvinia* (Gaudet and Huang, 1967; Gaudet and Koh, 1968). Different aspects of morphogenesis in *Marsilea* (Allsopp, 1952, 1953, 1954, 1955, 1963; Allsopp and Rao, 1968) using sterile liquid cultures have been studied in the recent years. The development and morphogenetic control of land and water forms of *Marsilea* have been well described by Allsopp (1963, 1965).

Since the earlier studies on *Marsilea* and *Regnellidium* deal with liquid culture, it was felt desirable to investigate the morphogenetic response of *Regnellidium* on solid media. In order to raise the plants up to the reproductive phase, various supplements were added in the media. Alterations in the morphology of the various organs and their size were regularly recorded. Another purpose of the present study was to determine the optimal nutritional requirements of the plant so as to design further experiments on regeneration, callus induction and differentiation.

To define the optimal basal medium for the growth of the sporophyte, following media gelled with 0.8% agar were tried:
i. Knop's medium (1869, full and half strength)

ii. Knudson's medium (Steeves et al., 1955)

iii. Moore's medium (1903).

A mixture of megaspores and microspores sterilised in 2% calcium hypochlorite solution for 8-10 minutes as already described in 'Materials and Methods' was inoculated on all the above mentioned media.

Observations

Out of the media tried, Knop's medium (full strength) was established as the optimal basal medium for the normal growth of the sporophytes. After fertilization, first leaf was observed within 5-7 days of inoculation.

Effect of glucose: Sporelings transferred on Knop's medium supplemented with different concentrations of glucose (1-5%) showed normal growth sequence i.e. from the first subulate leaf through spatulate and then bifid leaf. Overall growth was, however, much affected when compared to the control. The plants showed stunted growth with small-sized green leaves in all the concentrations. Rhizome part was reduced in all the concentrations. Glucose concentrations higher than 5% proved toxic since the plants turned brown and ultimately collapsed.

Effect of sucrose: Some variations in the development and the rate of growth were apparent on different sucrose
Control cultures: The overall growth and differentiation proceeded at a normal pace in control plants. The adult plants with normal root, shoot and leaf were, however, somewhat frail and in successively older leaves the petioles failed to stay in erect position.

Cultures on different sucrose concentrations: Various concentrations of sucrose incorporated in the medium modified growth response quite considerably, most of them being of quantitative nature. For example, when the plants were cultured in 1% sucrose (Fig. 47), the size of the petiole was reduced as compared to control (Fig. 46), whereas the laminar expansion showed an appreciable increase. In contrast to the control, the leaves grew vertically erect (Fig. 47). In 2% sucrose, the petiole size further decreased, but an appreciable increase in laminar expansion was witnessed. The plants were healthier and larger in size than those in 1% sucrose (Fig. 48). The optimal growth response was observed in 3% sucrose (Fig. 49). These plants were healthier with more expanded lamina. There was an appreciable increase in thickness of rhizome, petiole, and leaf blade; decrease in petiolar length was, however, noticed. In 4% - 5%
sucrose, the plants showed stunted growth with reduced internodal length and laminar area. Figure 50 depicts one such plant in 5% sucrose with overall growth greatly retarded. In sucrose concentrations higher than 5%, the plants were charred and turned brown. Figure 51 shows comparative growth response of 3-week-old sexually produced sporophytes in sucrose concentrations from 1% to 5% (from left to right) whereas figures 52, 53, 54, depict the same after 4 months.

The results of a comparative study encompassing quantitative data of number of leaves, roots, etc., of the plants cultured in different sucrose concentrations, are presented in Table V.

Growth response in relation to foliage: The number of leaves formed 7 days after fertilization was 3 in control and 1-4% sucrose concentrations, while in 5% only two leaves developed. Their number increased after 14 days in all the concentrations, but the maximum increase was observed in 3% sucrose; after 21 days, the number reached 7 and 9 respectively in all the concentrations (Fig. 55).

Length of petiole: It was interesting to note that all the concentrations of sucrose retarded petiolar growth, the effect increased with the increasing concentrations of sucrose(Fig. 56).
Extent of laminar expansion: In 1-3% sucrose the laminar expansion was more than that of the control after 7, 14, 21 and 28 days. In 4% sucrose the expansion remained at the level of the control plants up to 14 days, after which a considerable decrease was observed after 21 and 28 days (as compared to control). A retarding effect on laminar expansion was observed in 5% sucrose (Fig. 57).

Growth response in relation to root formation: Sucrose increased the number of roots as compared to control after 7 days. 1% sucrose was found to be most effective in differentiation of roots after 14, 21 and 28 days. In 5% sucrose, their number exhibited appreciable decrease as compared to the control plants after 14, 21 and 28 days (Fig. 58).

Length of roots: Sucrose concentrations 1-4% increased the root length over control after 7 days. Maximum increase was observed in 2% and 3% sucrose after 28 days, 5% sucrose showed retarding effect on root length (Fig. 59).

It is clear from the foregoing data that Knop's medium supplemented with 3% sucrose supported the optimal vegetative growth of the plants. However, despite the normal growth of the plant in this medium and maintaining cultures up to a period of about one year, none of the plants ever reached maturity to bear the sporocarps (Fig. 60). The
Factors responsible for sporocarp formation are under investigation.

Reversion to juvenility in old cultures

It was striking to observe the production of leaves without lamina in older cultures. These leaves were thick structures with a vascular strand and normal stomata. This condition may arise due to the fall in nutritional level of the medium on ageing or due to the presence of certain toxic metabolic products secreted from the cultured plants.

Effect of coconut water

Sporelings on Knop's and 3% sucrose fortified with 10% coconut water showed poor growth since the plants turned yellow soon after their transfer. New leaves appeared regularly which were small and deficient in chlorophyll. Rhizome length was considerably reduced due to slow growth rate. Sporelings on optimal basal medium supplemented with 5% coconut water, developed new chlorophyllous leaves which were of small size. Rhizome part was much reduced. Plants showed slow growth rate. The overall growth, however, was better than those growing on optimal basal medium fortified with 10% coconut water.
4. REGENERATION OF RHIZOME SEGMENTS

The sporophytes raised in sterile cultures on Knop's medium (full strength) supplemented with 3% sucrose, were used as stock cultures in this experiment. Roots and leaves were removed from the rhizome. Equal-sized rhizome segments, with and without nodal part, were cut and inoculated separately in Knop's medium (full strength) supplemented with 3% sucrose. All the cultures were maintained in uniform conditions of light and temperature as described under 'Materials and Methods'.

Observations

Some variations regarding the activation and growth pattern of rhizome segments with and without nodal part were observed. Segments with nodal part were the first to sprout, while the rhizome segments without nodal part remained quiescent for about two months. Further growth of these two types of segments was interesting. Segments with nodal part, after a few days of inoculation, turned brown. A week later, swelling up of rhizome segments was observed and first sign of growth was evidenced by the production of multi-cellular, uniseriate hairs. Subsequently a normal sporophyte was formed from each of these segments (Figs. 61, 62, 63, 64).
Segments of the internodal parts behaved quite differently. They turned brown and remained in the same state for two months. Their prolonged cultures resulted in the production of small-sized protophylls and chlorophyllous, positively geotropic roots (Figs. 65, 66). These protophylls were comprised of compactly arranged sporophytic cells with discoid chloroplasts without any vascular supply and stomata. A large number of multicellular, uniseriate hairs were seen on the surface of these protophylls.

It is important to mention that these protophylls resembled the haploid protophylls produced from the megagametophytes in the earlier experiments, with the only difference that the haploid protophylls lacked dermal hairs which are produced on the surface of the protophylls in the present experiment. These dermal hairs resembled the ones produced on the sexually produced 2X sporophytes.
A perusal of the literature shows that very little work has been done on fern callus as compared to the voluminous data available in the case of seed plants. The reports available to date embody callus induction from:

(i) gametophyte generation (Morel and Wetmore, 1951; Steeves and Sussex, 1952; Kato, 1963, 1966; Mehra and Sulklyan, 1969; Partanen, 1972; (ii) leaves (Morel, 1956; Bristow, 1962; Kato, 1965; (iii) rhizome segments (Bristow, 1962) and (iv) excised roots (Mehra and Palta, 1971). The above mentioned studies are important in as much as they illuminate the role of factors which control the differentiation of callus cells into the gametophyte or sporophyte generations. It was felt very desirable to extend work on similar lines to a heterosporous fern, *Regnellidium diphyllum* and later to other materials as well.

The material was obtained from the aseptically growing diploid sporophytes. The rhizome segments, leaf lamina and petiole were excised and inoculated on various callus-inducing media shown in Table VI. In addition to above-mentioned excised plant parts, 7-day-old plantlings were also tried in a few experiments.

5.1 Induction and differentiation of callus from rhizome apex

Rhizome apices excised from the young aseptically
FIG. 67 - SCHEMATIC REPRESENTATION OF EXPERIMENTS ON CALLUS INDUCTION AND DIFFERENTIATION FROM RHIZOME APEX ON MOORE'S BASAL MEDIUM (MB) + SUCROSE + 2,4-D.

Stock cultures
(2 x sporophytes)

Rhizome apex

Excised and inoculated

Callus

MB + 0.5 % sucrose + 1 ppm 2,4-D

MB + 0.5 % sucrose + 2 ppm 2,4-D

Sub-Culturing

MB + 0.5 % sucrose + 1 ppm 2,4-D

MB + 0.5 % sucrose

KB + 3 % sucrose

KB + 4 - 5 % sucrose

DIFFERENTIATION

Cylindrical structures
(radially symmetrical, without vascular system)

Juvenile leaves

(i) Leaf-like appendages
(ii) Shoot apex - Later roots were also differentiated
(iii) Complete sporophyte

Roots

MB = Moore's basal medium, KB = Knop's basal medium
growing sporophytes were inoculated on variously modified callus-inducing media. The results are described below:

5.1-1 Induction of rhizome apex callus on Moore's medium + sucrose + 2,4-D (Fig. 67)

Observations

On Moore's medium supplemented with 0.5% sucrose along with 1-2 ppm 2,4-D, the apex of the rhizome formed green, solid callus after six weeks of inoculation. Since the optimal callus growth was observed on 1 ppm 2,4-D medium, the stock cultures were maintained on this medium.

After six weeks of culture, the callus was excised and subcultured on fresh medium with the same concentrations of sucrose and 2,4-D (Fig. 68). The growth of the callus was exceedingly slow and it turned brown soon after its transfer to fresh medium. The callus was heterogenous with a wide spectrum of variation in size and form of the constituent cells e.g. broadly rectangular (Figs. 69, 70) or more elongated than broad ones (Figs. 71, 72, 73). Figure 74 depicts a callus cell of highly irregular shape and densely clustered chloroplasts. In yet other cells, it was interesting to note repeated mitotic divisions in transverse plane resulting in filamentous structures (Fig. 75).
Differentiation

Differentiation of cylindrical structures (radially symmetrical without vascular system): On regular subculturing of the callus on the medium of its induction for 8 weeks, differentiation of cylindrical structures was observed from all over the surface of the callus. These were comprised of compactly arranged, elongated cells without a vascular strand and stomata as seen in cleared specimens.

Differentiation of juvenile leaves: After subculturing the callus for 8 weeks on basal medium containing 0.5\% sucrose, differentiation of vascularized cylindrical structures, broadly comparable to the cotyledonary leaf, was observed (Fig. 76). At seven weeks, the differentiation of leaf-like appendages (Figs. 77, 78, 79) was observed when the callus taken from the medium of its induction was transferred to Knop's medium incorporated with 3\% sucrose. Some of these appendages bore multicellular, uniseriate hairs structurally comparable to those on the diploid sporophyte (Fig. 77).

Differentiation of shoot apex and complete sporophyte: After 8 weeks of culturing on Knop's medium supplemented with 3\% sucrose, the callus differentiated shoot apex (Fig. 80) which in turn differentiated juvenile leaves on the upper surface and root on the lower as in a normal sporophyte.
FIG. 85. SCHEMATIC REPRESENTATION OF EXPERIMENTS ON CALLUS INDUCTION AND DIFFERENTIATION FROM RHIZOME APEX ON MOORE'S BASAL MEDIUM (MB) + SUCROSE + KINETIN (K) + 2,4-D

Stock Cultures → 7-day-old sporelings

↓

Inoculated

↓

Callus

MB + 0.5% sucrose + 1 ppm K + 1 ppm 2,4-D (in 75% cultures after 8 weeks)

Callus

MB + 0.5% sucrose + 1 ppm K + 2 - 4 ppm 2,4-D (in 50% cultures after 8 weeks)

Callus

MB + 0.5% sucrose + 1 ppm K + 5 ppm 2,4-D (in 100% cultures after 8 weeks)

Callus

MB + 0.5% sucrose + 1 ppm K + 0.25 ppm 2,4-D

↓

D I F F E R E N T I A T I O N

↓

(i) Juvenile leaves

No differentiation

→

↓

(i) Juvenile leaves of first, second and third order

No differentiation

↓

(ii) Adult leaves

(i) Complete sporophyte with adult leaves and roots
Fig. 100: Schematic representation of experiments on callus induction and differentiation from rhizome apex on Moore's basal medium (MB) + sucrose + kinetin (K).

Stock cultures (2 x sporophytes) → 7-day-old sporelings

Inoculated

Callus
MB + 0.5% sucrose + 1 ppm K → Sub-culturing → Callus
MB + 0.5% sucrose + 0.5 ppm K → Callus
MB + 0.5% sucrose + 0.1 ppm K → Callus
MB + 0.5% sucrose + 1 ppm K → Callus
MB + 0.5% sucrose + 2 ppm K → Callus
MB + 0.5% sucrose + 3 ppm K → Callus
MB + 0.5% sucrose + 4 ppm K → Callus
MB + 0.5% sucrose + 5 ppm K

Differentiation

No differentiation → Juvenile leaves of first order → Complete sporophyte → Roots only

Juvenile leaves of first order → Complete sporophyte

Juvenile leaves of first and second order → Complete sporophyte

Complete sporophyte
After 10 weeks of culturing on the same medium the differentiation of complete sporophyte with normal roots and leaves was observed, the first few leaves being spatulate (Figs. 81, 82). After 12 weeks of culturing, a large number of plants with normal leaves and roots was observed from the callus, which by itself had completely obliterated by this time (Figs. 83, 84).

**Differentiation of roots:** Callus from the medium of its induction when transferred to Knop's medium supplemented with 4-5% sucrose, differentiated roots in 20% of the cultures after 10 weeks. Roots were densely covered with yellow hairs.

5.1-2 Rhizome apex callus on Moore's medium + sucrose + kinetin 2,4-D (Fig. 83)

Callus was induced in 7-day-old sporelings inoculated on Moore's medium + 0.5% sucrose + 1 ppm kinetin + 1-6 ppm 2,4-D. After six weeks of culture, the callus was excised and subcultured on the fresh medium of the same composition.

Callus was induced in 50% of the cultures on medium containing 1 ppm 2,4-D, after four weeks. Among the remainders, in 25% cultures the callus was initiated 8 weeks after inoculation. On medium containing 2-4 ppm 2,4-D 25% of the cultures formed callus after four weeks while 25% of the cultures gave response after 8 weeks raising the total number of callused plants to 50 per cent.
On medium containing 5 ppm 2,4-D, callusing was 100%. This medium, was therefore, established as the optimal one. Figures 36, 37, 38, 39 depict callus growth on 2-5 ppm 2,4-D media respectively, 6 weeks after subculturing.

With a further increase of 1% of 2,4-D i.e. 6 ppm, 75% cultures callused. This callus was green, friable being composed of ovoid or elongated cells. As depicted in Fig.90, a few cells developed papillate projections.

**Differentiation**

It can be seen from the preceding experiment that Moore's medium + 0.5% sucrose + 1 ppm kinetin + 5 ppm 2,4-D was optimal for callus induction, and calluses raised on this medium were used for differentiation.

The callus on transference to Moore's medium + 0.5% sucrose + 1 ppm kinetin + 0.25 ppm 2,4-D, differentiated juvenile leaves after 7 weeks.

When 2,4-D was completely omitted from the medium, no differentiation occurred. Transference of callus from the medium of induction to basal medium + 0.5% sucrose + 1-3 ppm kinetin did not show any organogenesis or differentiation.

After 10 weeks of culturing on Moore's basal medium + 0.5% sucrose, the differentiation of juvenile and subsequently the normal adult leaves were observed (Figs.91,92).
The differentiation began at different loci on a given callus. After 12 weeks the differentiation was observed all over the surface of the callus (Fig. 93).

On Moore's medium containing 1-4% sucrose, differentiation of juvenile leaves from all over the surface of the callus was observed after 8 weeks. On regular subculturing on basal medium supplemented with 1 and 2% sucrose, the initiation of the juvenile leaves was observed after 5 weeks (Figs. 94, 95). After 8 weeks of planting on the same medium, the differentiation was observed from all over the surface of the callus.

On 3 and 4% sucrose, callus growth was retarded. On regular subculturing, however, normal, second order juvenile leaves and roots were differentiated on the same medium (Fig. 96).

On 5-7% sucrose concentrations, none of the organs differentiated but callus growth was enhanced on these media. Figures 97, 98, 99 depict the callus growth after 12 weeks on 5-7% sucrose respectively.

5.1-3 Rhizome apex callus on Moore's medium + sucrose + kinetin (Fig. 100)

Green friable callus was induced after 5 weeks when 7-day-old sporelings were inoculated on medium supplemented with 1-4 ppm kinetin and 0.5% sucrose. After six weeks,
the callus was excised and inoculated on fresh medium (Figs. 101, 102).

Since on 1 ppm kinetin medium, fast growing callus was obtained, therefore, the stock cultures were maintained on this medium.

Organogenesis was not observed when the callus was subcultured on the same medium.

When the concentration of kinetin in the medium of callus induction was reduced to 0.5 ppm, differentiation of juvenile leaves, broadly comparable to that of the first cotyledonary leaf was observed (Figs. 103, 104). On 0.1 ppm kinetin, juvenile leaves resembling the first two leaves of the normal sporophyte differentiated (Figs. 105, 106).

On basal medium supplemented with 0.5% sucrose, plantlings with normal roots and leaves, with usual heteroblastic changes, were formed after 8 weeks (Figs. 107, 108).

On 1-4% sucrose media, normal plants with well developed roots and leaves differentiated (Figs. 109, 110). As can be seen in the preceding experiment, in 5-6% sucrose the root formation was observed at 8 weeks on the same medium.

5.2 *Induction and differentiation of callus from rhizome segments*

Rhizome segments completely devoid of roots and leaves
FIG. 111 - SCHEMATIC REPRESENTATION OF EXPERIMENTS ON CALLUS INDUCTION AND DIFFERENTIATION FROM RHIZOME SEGMENTS ON MOORE'S BASAL MEDIUM (MB) + SUCROSE + 2,4-D

Stock Cultures (2 x sporophytes) → Rhizome segments → Excised and inoculated

Callus
MB + 0.5% sucrose + 1 ppm 2,4-D

Callus
MB + 0.5% sucrose + 2 ppm 2,4-D

Callus
MB + 0.5% sucrose + 3 ppm 2,4-D

Sub-Culturing

MB + 0.5% sucrose + 2 ppm 2,4-D

MB + 0.5% sucrose

MB + 1% sucrose

MB + 2% sucrose

MB + 3.5% sucrose

DIFFERENTIATION

Cylindrical structures (radially symmetrical without vascular system)

Juvenile leaves of first order

Juvenile leaves of first and second order

Juvenile leaves of first order

Roots only (leaf formation absent)
were cut 1 cm in length and inoculated on variously supplemented basal medium. The results are presented below:

5.2.1 Callus from rhizome segments on Moore's basal medium + sucrose + 2,4-D (Fig. 111).

Callus was induced on Moore's basal medium supplemented with 0.5% sucrose and 1-3 ppm of 2,4-D. Basal medium with 0.5% sucrose and 2 ppm 2,4-D was found to be optimal for callus induction. Callus on this medium was kept as control.

Differentiation

Callus in control cultures remained brown for 4 weeks after which it regenerated cylindrical structures devoid of vascular strand and stomata (Fig. 112).

When 2,4-D was omitted from the medium of callus induction, juvenile leaf resembling the first cotyledonary leaf of embryonic sporophyte, differentiated after 6 weeks (Figs. 113, 114).

On raising the sucrose concentration from 1-5% in the basal medium, variable results were obtained. On 1% sucrose, the juvenile leaves of the first and second order differentiated whereas on 2% sucrose, only the juvenile leaves of the first order were observed.

Callus on 3-5% sucrose could differentiate normal roots with root hairs (Fig. 115).
FIG. 116 SCHEMATIC REPRESENTATION OF EXPERIMENTS ON CALLUS INDUCTION AND DIFFERENTIATION FROM RHIZOME SEGMENTS ON KNOP'S BASAL MEDIUM (KB) + SUCROSE + IAA + KINETIN (K)

Stock Cultures (2 x sporophytes) → Rhizome segments (Excised and inoculated) → Callus

- KB + 0.5% sucrose + 0.5 ppm IAA + 0.5 ppm K

Sub-Culturing

- KB + 0.5% sucrose + 0.5 ppm IAA + 0.5 ppm K
- KB + 0.5% sucrose + 0.5 ppm IAA + 0.25 – 0.1 ppm K
- KB + 0.5% sucrose + 0.5 ppm K + 0.25 – 0.1 ppm IAA

DIFFERENTIATION

- Juvenile leaves of first order
- Juvenile leaves
- Juvenile leaves

(i) Juvenile leaves
(ii) Adult leaves

(i) Juvenile leaves
(ii) Adult leaves

Juvenile leaves; Adult leaves (absent)
Adult leaves with two leaflets, however, did not differentiate on any of these media even though the cultures were kept under observations for five months.

5.2-2 Callus from rhizome segments on Knop's medium + sucrose + IAA + kinetin (Fig. 116)

Callus was induced on Knop's medium incorporated with 0.5% sucrose, 0.5 ppm IAA and 0.5 ppm kinetin. With a view to enhance the growth of the callus, coconut water, casein hydrolysate and yeast extract were also tried in different concentrations with no success.

For differentiation, the callus was transferred to various media and the results are as follows:

Callus on the medium of its induction i.e. Knop's medium + 0.5% sucrose + 0.5 ppm IAA + 0.5 ppm kinetin soon turned brown and remained in this state for five weeks. Subsequently, the callus became green and differentiated awl-shaped juvenile leaves after 7 weeks (Fig. 117).

Juvenile leaves were found to differentiate on basal medium + 0.5% sucrose + 0.5 ppm IAA + kinetin (0.25 and 0.1 ppm) after 8 weeks of culturing. Similar results were obtained on basal medium + 0.5% sucrose + IAA (0.25 and 0.1 ppm) + 0.5 ppm kinetin.

When auxin and kinetin were omitted from the medium of callus induction, the callus differentiated juvenile
FIG 121 - SCHEMATIC REPRESENTATION OF EXPERIMENTS ON CALLUS INDUCTION AND DIFFERENTIATION FROM RHIZOME SEGMENTS ON KNOP'S BASAL MEDIUM (KB) + SUCROSE + 2,4-D + KINETIN (K)

Stock Cultures (2 x sporophytes) → Rhizome segments

Excised and inoculated

Callus
KB + 0.5% sucrose + 0.1 ppm 2,4-D + 0.1 ppm K

Sub-Culturing
KB + 0.5% sucrose + 0.1 ppm 2,4-D + 0.1 ppm K

Callus
KB + 0.5% sucrose + 0.1 ppm 2,4-D + 0.5 ppm K

Callus
KB + 0.5% sucrose + 0.1 ppm 2,4-D + 1.0 ppm K

Callus
KB + 0.5% sucrose + 1 ppm 2,4-D + 1.0 ppm K

Sub-Culturing
KB + 0.5% sucrose + 1.0 ppm 2,4-D + 1.0 ppm K

DIFFERENTIATION

(i) Juvenile leaves of first and second order
(ii) Adult leaves
leaves after 4 weeks of culturing (Fig. 118). These leaves possessed normal vascular system. On regular subculturings of the callus on the same medium, adult leaves with two-leaflet condition differentiated after 6 weeks.

Similar results were obtained on basal medium supplemented with 1% sucrose. The differentiation, however, was much delayed because the juvenile leaves (Fig. 119) differentiated after 8 weeks. As shown in figure 120, the leaves possessed normal vasculature. In 2-5% sucrose only the juvenile leaves were formed, the adult ones were not observed on these media.

5.2-3 Callus from rhizome segments on Knop's medium + sucrose + 2,4-D + kinetin (Fig. 121)

Rhizome segments callused after four weeks on basal medium + 0.5% sucrose + 2,4-D (0.1 ppm) + kinetin (0.1, 0.5 and 1 ppm). Optimal callus growth was observed on medium supplemented with sucrose (0.5%), 0.1 ppm 2,4-D and 0.1 ppm kinetin (Fig. 122). Callus was light green in colour with exceedingly slow rate of growth.

The juvenile leaves which differentiated on this medium, possessed a distinct vascular strand (Fig. 123). Similar results were obtained when the callus was transferred to basal medium containing 1-4% sucrose. No other organ differentiated from the callus during five months of experimentation.
FIG. 124 - SCHEMATIC REPRESENTATION OF EXPERIMENTS ON CALLUS INDUCTION AND DIFFERENTIATION
FROM PETIOLE SEGMENTS ON KNOP'S BASAL MEDIUM (KB) + SUCROSE + NAA + KINETIN (K)

Stock Cultures

(2 x sporophytes)

Petioles of first and second leaves

Excised and inoculated

Callus

KB+ 0.5 % sucrose + 0.1 ppm
NAA + 0.1 ppm K

Callus

KB+ 0.5 % sucrose + 0.5 ppm
NAA + 0.5 ppm K

Sub-Culturing

KB + 0.5 % sucrose + 0.1 ppm NAA + 0.1 ppm K

KB + 1 % sucrose

KB + 2 % sucrose

KB + 3-4 % sucrose

KB+ 0.5 % sucrose + 0.5 ppm NAA + 0.5 ppm K

Cylindrical structures

(i) Shoot apex

(ii) Sporophyte with roots and juvenile leaves; adult leaves absent

(ii) Complete sporophyte with roots and leaves; complete heteroblastic stages recorded

Juvenile leaves

Cylindrical structures
On Knop's medium supplemented with 0.5% sucrose, 1.0 ppm 2,4-D and 1 ppm kinetin, the rhizome segments became swollen after 4 weeks of culture followed by the initiation of callus. Further subculturings on the same medium resulted in sequential differentiation of juvenile (first and second order) and adult leaves.

5.3 **Induction and differentiation of callus from petiole segments**

Petiole segments, approximately 1 cm in length, were excised from the aseptically growing plants and cultured on variously supplemented media. Only the petioles of the first and second juvenile leaves were inoculated since the older ones were not responsive. The results of this experiment are presented below:

5.3-1 **Callus from petiole segments on Knop's medium + sucrose + NAA + kinetin (Fig. 124)**

In the medium supplemented with 0.5% sucrose + 0.1 ppm NAA + 0.1 ppm kinetin, initiation of callus was observed after 4 weeks (Fig. 125). The callus was composed of heterogenous cells. Some of the cells were of exceptionally large size. The chloroplasts were uniformly distributed throughout the cells but in some cases these were grouped together.

**Differentiation**

The callus from the medium of induction i.e. Knop's
medium + 0.5% sucrose + 0.1 ppm NAA + 0.1 ppm kinetin when transferred to the same medium, differentiated cylindrical structures whereas on medium without sucrose, it showed differentiation of roots 3-4 weeks after subculturing; no other sporophytic organ differentiated on this medium.

The callus when transferred to Knop's medium incorporated with 1-2% sucrose, proliferated into dark green nodular masses composed of rapidly dividing cells. Shoot apex was the first organ to be differentiated from this callus (Fig. 126). Later, the normal roots and fronds were formed (Fig. 127). In 2% sucrose the heteroblastic changes leading to normal two-leaflet condition was observed. In 1% sucrose, the sequence of sporophytic organogenesis was similar to that in 2% sucrose, the only difference being that the differentiation of two-leaflet condition was not noticed throughout the period of experimentation (Fig. 128).

The callus taken from the medium of its induction when transferred to Knop's medium supplemented with 3% and 4% sucrose, differentiated vascularized juvenile leaves. The formation of first juvenile leaf was observed one month after subculturing on this medium (Fig. 129). This was followed by the differentiation of second juvenile leaf as shown in Fig. 130.

Induction of petiole callus was also observed on basal medium supplemented with 0.5% sucrose + 0.5 ppm NAA +
0.5 ppm kinetin. However, as compared to the preceding experiment, the callus was light green, nonfriable with a rather slow rate of growth. The callus on transference to the medium of its induction, differentiated cylindrical structures.

5.3-2 **Callus from petiole segments on Knop's medium + sucrose + IAA + kinetin (Fig. 131)**

Callus was induced on knop's medium supplemented with 0.5% sucrose, 0.1 ppm IAA and 0.1 ppm kinetin (Fig. 132). For differentiation, the callus was subcultured on the following media:

1. The medium of induction i.e. Knop's medium + 0.5% sucrose + 0.1 ppm IAA + 0.1 ppm kinetin.
2. Basal medium
3. Basal medium with 1-4% sucrose.

**Observations**

Growth of the callus on medium (1) was very slow. Callus was solid, nonfriable and green in colour. After 4-5 weeks of culturing, numerous roots differentiated from the callus; no other organ differentiated on this medium.

No differentiation occurred on media (2) and (3).
FIG. 131 - SCHEMATIC REPRESENTATION OF EXPERIMENTS ON CALLUS INDUCTION AND DIFFERENTIATION FROM PETIOLE SEGMENTS ON KNOP'S BASAL MEDIUM (KB) + SUCROSE + IAA + KINETIN (K)

- Stock Cultures (2 x sporophytes)
- Petioles of first and second leaves
- Excised and inoculated
- Callus
- Sub-Culturing
- KB + 0.5% sucrose + 0.1 ppm IAA + 0.1 ppm K
- KB + 0.5% sucrose + 0.1 ppm IAA + 0.1 ppm K
- KB + 4% sucrose
- KB

No differentiation
No differentiation

D I F F E R E N T I A T I O N

Roots
FIG. 133 SCHEMATIC REPRESENTATION OF EXPERIMENTS ON CALLUS INDUCTION AND DIFFERENTIATION FROM PETIOLE SEGMENTS ON KNOP'S BASAL MEDIUM (KB) + SUCROSE + 2,4-D + KINETIN (K)

Stock Cultures (2 x sporophytes) → Petioles of first and second leaves
  ↓
Excised and inoculated
  ↓

- Callus
  KB + 0.5% sucrose + 0.1 ppm 2,4-D + 0.1 ppm K
- Callus
  KB + 0.5% sucrose + 0.5 ppm 2,4-D + 0.5 ppm K
- Callus
  KB + 0.5% sucrose + 1.0 ppm 2,4-D + 1.0 ppm K

Sub-Culturing

- KB + 0.5% sucrose + 0.1 ppm 2,4-D + 0.1 ppm K → Juvenile leaves of first order
- KB + 0.5% sucrose → Juvenile leaves of first and second order
- KB + 1-2% sucrose
- KB + 0.5% sucrose + 0.5 ppm 2,4-D + 0.5 ppm K
- KB + 0.5% sucrose + 1.0 ppm 2,4-D + 1.0 ppm K

DIFFERENTIATION

- (i) Shoot apex
- (ii) Complete sporophyte with adult leaves and roots

- (i) Juvenile leaves
- (ii) Adult leaves

Adult leaves
5,3-3 Callus from petiole segments on Knop's medium + sucrose 2,4-D + kinetin (Fig. 133)

Induction and differentiation of callus was observed on the following media:

1. Knop's medium + 0.5% sucrose + 0.1 ppm 2,4-D + 0.1 ppm kinetin
2. Knop's medium + 0.5% sucrose + 0.5 ppm 2,4-D + 0.5 ppm kinetin
3. Knop's medium + 0.5% sucrose + 1.0 ppm 2,4-D + 1.0 ppm kinetin.

On medium (1), solid green callus was initiated after 4 weeks (Fig. 134). After five weeks of culturing on the same medium, the juvenile leaves of first order differentiated (Fig. 135). Juvenile leaves of first and second order developed on basal medium supplemented with 0.5% sucrose. On medium containing 1 and 2% sucrose, differentiation of shoot apex and subsequently complete sporophyte with normal leaves and roots, was observed.

In medium (2), induction of callus was observed after 8 weeks of planting (Fig. 136). Callus was nonfriable and light green in colour. Differentiation of leaves (juvenile and adult) was observed after 3 weeks.

On medium (3), callus was induced after five months of culturing on the same medium. It was friable, fast
growing and light green in colour. Optimal callus growth (Fig. 137) was recorded on this medium as compared to the preceding media. Callus comprised of irregularly shaped elongated cells containing numerous chloroplasts (Figs. 138, 139). The first organ differentiated was a leaf which was structurally similar to the two leaflet condition of the sexually-produced sporophyte.
II. *CERATOPTERIS PTERIDOIDES* (HOOK.) Hieron.

Considerable amount of work has earlier been done on the gametophyte morphology of *Ceratopteris thalictroides* but the reports are somewhat contradictory and confusing. For instance, according to Kny (1875) and Javalgekar (1960), during early ontogeny an apical cell was established. Later, the apical cell was replaced by a marginal meristem in the lateral position resulting in an asymmetric structure. In sharp contrast, Pal and Pal (1963), and Nayar and Kaur (1969) did not observe a single apical cell and the development and asymmetric form of the adult gametophyte in culture was attributed to a marginal multicellular meristem in variable position.

Another controversial point in the earlier description is the sex expression in relation to the morphology of the gametophyte. Mahabale (1948) could observe only one type of gametophytes in nature i.e. bisexual. Kny (1875), Javalgekar (1960) and Nayar and Kaur (1969) have described two types of prothalli—cordate, bisexual and the ameiotic spatulate; the latter bearing antheridia only. According to Pal and Pal (1963), most of the gametophytes in their cultures were bisexual but some were small, spatulate bearing antheridia only. Besides these two types, a few archegoniate prothalli were also noted by them.
In a recent critical study, Klekowski (1970) has observed two kinds of ontogenetic patterns under uniform culture conditions. The amemistic prothalli developed only antheridia and remained antheridiate throughout their life while those with relatively faster growth, initiated a notch meristem in the lateral position and became cordate and bisexual. The ratio between the spatulate and cordate prothalli in a given spore sample varied in different populations.

In view of the aforesaid points of disagreement regarding the morphology of the gametophyte of *Ceratopteris thalictroides*, it was felt desirable to investigate in detail the morphology of the prothallus of *Ceratopteris pteridoides*.

It is pertinent to mention at this point that *Ceratopteris pteridoides* which was earlier known to be a neotropical species (Copeland, 1947), has recently been reported to occur in China and South-east Asia (De Vol, 1957). Spores used in the present study were obtained from plants growing in nature at Calcutta (West Bengal).

1. **IN VITRO SPOROE GERMINATION AND DEVELOPMENT OF THE PROTHALLUS**

Spores were surface sterilized in 1% aqueous calcium hypochlorite for 8-10 minutes and sown on Knop's medium (full strength) solidified with 0.8% agar. Germination occurred five days after inoculation. First division of
the spore cell resulted in a large protonemal initial and a small rhizoidal initial. Protonemal initial emerged after rupturing the sporodeim and differentiated chloroplasts, and the smaller rhizoidal initial developed into a hyaline thread-like rhizoid. Protonemal cell underwent successive transverse divisions and formed 3-5 celled filamentous protonema which further expanded by both transverse and longitudinal divisions resulting in spatulate form (Nayar and Kaur, 1969). For a while, the increase in size of the gametophyte was due to expansion of the individual cells. Further development of the prothallus was effected by a group of marginal cells. Contrary to earlier reports in *Ceratopteris thalictroides* (Javalgekar, 1960), no apical cell was observed (Figs. 140, 141). As reported by Nayar and Kaur (1969), the cells on either side of the meristematic area expanded more than the meristematic cells resulting in a distinct notch (Figs. 142, 143).

Three distinct types of gametophytes were observed (1) cordate with two equally developed symmetrical lobes (Figs. 144, 145), (2) cordate, asymmetrical with two or more unequally developed lobes (Fig. 146), (3) amertistic spatulate, with or without branches (Figs. 147, 148, 149). As regards the sex expression, in the case of type (1), the prothalli either bore archegonia only and remained archegoniate throughout, as also recorded by Pal and Pal (1963),
(Fig. 144), or developed antheridia first and archegonia later and became hermaphrodite (Fig. 145). In type (ii), the prothalli were all hermaphrodite; antheridia developed first in 30-35 days and archegonia a week later. In ameristic spatulate gametophytes, further growth was inhibited and antheridial formation was observed two weeks after germination (Fig. 147). Some of the ameristic prothalli developed a large number of filamentous branches (Figs. 148, 149). The above mentioned differences in development, morphology and sex expression of the prothalli in a given culture could be detected in 15-day old cultures.

The fact that all the three types of gametophytes were observed persistently under uniform culture conditions make it certain that the differences observed in their adult phenotype and sex expression are not due to the external conditions but indeed genetically controlled. In this respect they resemble with certain strains of C partenopteris thalictroides (Klekowski, 1970).

2. IN VITRO COMPLETION OF LIFE CYCLE AND REGENERATION OF SPOROPHYTES

Fertilization was achieved in the presence of free water and young sporophytes were observed two weeks after fertilization. During the vegetative phase which lasted for about two months, the earlier formed leaves were deltoid with entire lamina (Fig. 150) and the subsequent ones were
leaves for 8-9 weeks in culture; thereafter the fertile leaves devoid of expanded lamina were differentiated; linear, undivided to begin with and 2-3 lobed later (Figs. 152, 153). After the initiation of the fertile leaves, the vegetative leaves showed signs of senescence. As recorded in other species of the genus (Copeland, 1947), the sporangia were borne on the underside of the fertile leaves protected by their reflexed margins. Interestingly, in some 7 month old plants the factor for dimorphism of fronds was lost because the sporangia were also developed on the expanded lamina of the vegetative leaves (Fig. 154). Meiosis (Fig. 155) and other stages of sporogenesis were perfectly normal resulting in 32 spores per sporangium. A comparison of the spores obtained from the sporophytes growing in nature and in vitro however, revealed marked difference in size (Figs. 156, 157); structurally they were identical.

Regeneration: Vegetative buds are known to occur on petiole, leaf blade and roots of a number of unrelated fern genera (MoWeigh, 1937). White (1969) has recently carried out a detailed study on the ontogeny and histogenesis of sporophytic buds in a number of ferns notably Amphoradenium haolicanum, Amphoradenium sarmentosum and Athyrium macraei. The formation of epiphytic buds in Ceratopteris thalictroides and Ceratopteris cornuta in nature has earlier been reported
by Goebel (1887, 1930); Engler and Prantle (1902), Bally (1909), Haberlandt (1914), Holscher and Lingelshein (1915), Holm (1925), Beyerle (1932), Pal and Pal (1962).

The present in vitro study deals with (a) the bud formation on excised and intact sterile leaves of *Ceratopteris pteridoides* and (b) the possible factors controlling the vegetative and fertile phases of the sporophytes developed from the buds.

**Experimentation**

Leaves of different hierarchical status were excised from the sporophytes raised and maintained on basal medium. Sporophytes with intact leaves were also cultured for comparison.

**Results**

Vegetative leaves, both intact and excised, showed competence for bud induction. The number of buds differentiated per leaf as well as the sites of response were predictable. In most cases examined, only one bud appeared on the leaf apex (Fig. 158), but occasionally their number varied from 1-3, randomly distributed on the margins of a given leaf. As to the sites of bud inception in excised as well as intact leaves, a group of embryonic cells which served as the bud initials, appeared near the apices or on
the sinus in the case of lobed leaves which preceded the fertile ones on the shoot apex.

The buds differentiated on the juvenile leaves continued to produce only the vegetative leaves while intact with the parent leaf (Figs. 159, 160, 161). However, when the plantlings were excised and inoculated on the fresh medium, fertile fronds appeared after two weeks. It can be inferred from the foregoing that some unknown factor present in the medium triggered the differentiation of the fertile fronds. In sharp contrast, the buds regenerated on the sinus of the lobed leaves showed ability both for the vegetative as well as the fertile leaves, the latter differentiated after the formation of 2-3 vegetative leaves (Fig. 162). Thus the adult sporophytes bearing fertile fronds could be obtained within one month of culturing of the excised leaves. Figure 163 shows the regeneration of the sporophyte from vegetative leaves intact with the sporophyte, but the fertile ones did not show such response. Interestingly in some cases sporophytic bud initiation and its subsequent development into normal sporophyte was accompanied by rhizoidal differentiation; root initiation, however, remained inhibited (Fig. 164).

It is clear from the above observations that the hierarchical status of the subtending leaf is an important factor that controls the vegetative and fertile phases of the daughter plants.
3. INDUCTION OF APOSPORY

The present study was undertaken to throw light on three aspects of in vitro induction of apospory: (i) to assess the competence of excised vs intact organs of the sporophyte for aposporous response, (ii) the role of carbohydrate metabolism and (iii) response under normal vs starved conditions of the sporophytes.

Experimentation

The sporophytes used in the present experiment were raised on Knudson's medium supplemented with 2% sucrose and the induction of apospory was tried by the following methods:

1. By culturing the detached leaves on suitable nutrient media,
2. By subculturing the juvenile sporophytes to the stage of starvation.

Results

Induction of apospory in the excised leaves: Both the juvenile and adult leaves were detached from the shoot and cultured on the basal medium. Subculturing was done at regular intervals on the fresh medium. The excised leaves turned brown soon after their transfer to the medium and remained in this state for four weeks. Later, the differentiation of gametophytic cells was observed on the juvenile leaves of the first, second and third hierarchical order; the
adult leaves failed to show any response. Thus, in accord with the earlier reports on terrestrial ferns (Bell and Richards, 1958; Takahashi, 1962, 1969), it seems certain that an important requirement for the aposporous response is the juvenile nature of the leaves.

**Site of response:** Both the leaf lamina and stipe gave rise to the aposporous gametophytes (Figs. 165, 166, 167), in the former case, these regenerated on both the dorsal and ventral surface. As regards the degree of response, several gametophytes developed on one leaf, generally near the apex and the basal part of the lamina.

Although most of the aposporous gametophytes remained spatulate, a few cordate ones were also observed bearing antheridia and archegonia. It may be pointed out here that in the case of aposporous gametophytes of *Ampelopteris prolifera*, the archegonia were formed only when the gametophytes were detached from the subtending leaves (Mehra and Sulklyan, 1969).

**Comparison of the haploid and aposporous diploid gametophytes:** The prothallial shapes such as filamentous, spatulate and cordate seen in cultures raised from spores were also observed among the aposporous gametophytes. In overall dimensions, however, these were smaller than the haploid gametophytes. Symmetrical, cordate, archegoniate prothalli, as recorded in cultures raised from spores were
It was quite interesting to observe some intermediate structures combining the features of the gametophyte and the sporophyte. Such outgrowths have earlier been reported by Mehra and Sulklyan (1969), Takahashi (1969). Figure 168 shows one such elongate cylindrical structure arising from leaf lamina. Their microscopic examination revealed antheridia on the margins and isolated tracheids in the gametophytic cells of the expanded portion (Fig. 168). It was further noted that some of the marginal cells of these intermediate structures regenerated filamentous gametophytes (Fig. 169, arrowed). As shown in figure 168 intermediate outgrowths as well as gametophytic structures could arise from the same leaf lamina. Figure 170 depicts the differentiation of another such structure originating from the margin of the petiole. Its cells with wavy walls (Fig. 171) bore striking resemblance with the epidermal cells of the leaf, but it was devoid of vascular supply and stomata.

The excised roots of 1-3 hierarchial sequence failed to show any response. These results are in agreement with the previous reports on Pteris vittata and Lygodium japonicum (Takahashi, 1969), but differ from those recorded earlier by Mehra (1975).

Aposporous response of starved sporophytes: Young sporophytes established on 2% sucrose medium were transferred
and maintained on the basal medium. No subculturing was done on fresh medium.

At four weeks, the leaves showed signs of senescence, and at ten weeks, the aposporous gametophytes were induced on intact leaves and the roots (Figs. 172, 173). The sites and the growth of aposporous gametophytes was similar to that recorded in the preceding experiment. These gametophytes were induced on both the surfaces of the lamina. Interestingly, the roots intact with the parent plant, in sharp contrast to the excised ones used in the preceding experiment, also induced aposporous gametophytes (Fig. 173). In gross morphological features as well as the presence of tracheids and sex organs, these gametophytes were indistinguishable from those obtained from the leaves.

**Induction of apospory by raising stock culture on sucrose-free basal medium**

In order to test whether the aposporous response was due to the presence of sucrose in the stock cultures in preceding experiment, the sporophytes were raised on sucrose-free basal medium and were used as stock cultures. First five leaves and roots of the juvenile sporophytes were excised and 10 of each type were cultured separately on culture media for one month. The results are recorded in Table VII.
<table>
<thead>
<tr>
<th>Plant part</th>
<th>Leaf No. in hierarchical sequence</th>
<th>Total number of explants used</th>
<th>Number of explants regenerated</th>
<th>Total No. of explant regenerated</th>
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<tr>
<td></td>
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<td>Gametophytes</td>
<td>Sporophytes</td>
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<td>Lamina</td>
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<td>Stipe</td>
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It is evident from the results shown in Table VII that the leaf lamina and stipe showed regeneration of both the sporophyte as well as the gametophyte, irrespective of the presence or absence of sucrose in the medium.

Stipes of the first three leaves differentiated only the aposporous gametophyte and those of the leaves of higher order did not differentiate any of the structures. Aposporous response, was thus restricted to the first three leaves.

4. INDUCTION OF APOGAMY

The spores were directly germinated on 0.5% sucrose and the meristic prothalli were selected for the induction of apogamy. These were transferred to 1-3% sucrose media. Control cultures on 0.5S media were also set along with.

The development of gametophytes on 0.5S and 1S (Figs. 174, 175) media was similar to the control cultures but the growth was faster on 0.5S than on 1S. On higher concentrations, i.e. 2S-5S, the two-dimensional growth was markedly reduced as was evidenced by an overall reduction in size of the gametophytes (Figs. 176, 177, 178). On 6S-8S media, the gametophytic cells became necrotic and further growth was inhibited. Figure 179 shows comparative growth response of gametophytes in sucrose concentrations ranging from 1% to 5% (from left to right).

The meristic prothalli raised on 3S-5S media exhibited normal expansion of wings. On 4S medium an apogamously
developed leaf was observed after 4 months of culture (Fig. 177) and another leaf was observed a week later on 3S medium. No apogamous response was recorded in 1S, 2S and 5S media during the corresponding period. On 5S medium, the response was observed after 6 months of regular subculturing on the fresh medium. Lower concentrations of sucrose, viz., 1% and 2% proved to be suboptimal for the induction of apogamy.

The apogamously developed leaf was more dissected (Figs. 180, 181) than the cotyledonary leaf of the zygotic sporophytes. The differentiation of the apogamous leaf was followed by shoot apex and root in that sequence. In the zygotic embryos, on the other hand, the cotyledonary leaf was simple, deltoid (Fig. 182) and the first root developed synchronously (Fig. 183). The apogamously-formed haploid sporophytes showed slow growth and were smaller in size than the zygotic sporophytes, and as is known in all the earlier cases of induced apogamy, the apogamous plantlings failed to enter into the reproductive phase for as long as 6 months (Figs. 184, 185). Figure 186 shows comparative growth response of haploid and diploid sporophytes raised in vitro.
After the first publication of morphological description of the gametophyte of *Anogramma leptophylla* by Goebel (1877), no detailed information is available regarding its development, formation of tubers and sex organs (Nayar and Kaur, 1971). It may be recalled at the outset that the gametophyte being perennial, it bears a striking resemblance with that of a bryophyte plant rather than a short-lived gametophyte of other homosporous ferns (Proskauer, 1963). Secondly, the growth is predominantly lop-sided; and the axiate growth pattern, which characterizes the fern gametophyte in general (Albaum, 1938), is deranged. Thirdly, the three-dimensional growth is restricted to the formation of tuber, and thus the main body of the thallus is constructed as a result of two-dimensional growth only. The present series of experiments were undertaken to see how far the above-mentioned deviations affect the apogamous response under controlled culture conditions and the known exogenous chemical factors.

The basal medium for the optimal growth of the prothallus consisted of Knop's medium (full strength) of mineral salts, 10 mg/l ferric citrate and 1 ml/l Nitsch trace elements. The
medium was gelled with 0.8% agar. Spores were sterilized in 2% calcium hypochlorite solution for 8-10 minutes.

Observations

The spores germinated five days after inoculation. The papillate germ cell emerged after rupturing the spore wall (Figs. 187a,b), and underwent first mitotic division resulting in two highly unequal cells, the smaller rhizoidal initial and the larger prothallial initial; the axis of growth in the former being generally at right angle to the latter. The rhizoidal initial developed into a rhizoid (Figs. 187c,d) and the prothallial initial underwent successive transverse divisions to give rise to a uniseriate filament up to five cells long (Fig. 187e). But sometimes in 2-celled filaments, a vertical division was laid in its terminal cell and the two resultant cells underwent further transverse divisions to give rise to V-shaped, twin protonemata (Fig. 187f,g). In yet other cases, the spore cells divided by a vertical wall and gave rise to two almost equal-sized cells; the two resultant cells gave rise to a filament each. In such cases the first rhizoidal cell was delayed since it differentiated after a transverse division in one of the two cells of V-shaped protonemata (Fig. 187h). The two branches of the V-shaped protonema sometimes grew equally (Fig. 187g) or in other cases, one of the branches over-topped the other (Fig. 187i). Thus unlike most of the
homosporous fern gametophytes, the pattern of protonemal development was variable.

Initiation of spatulate stage and branching pattern: A vertical division in the terminal cell of the filamentous protonema initiated two-dimensional growth. After a short period of transverse and vertical divisions, a spatulate prothallus was formed (Figs. 188, 189). No apical meristem was involved in the formation of the spatulate prothallus. In some cases the growth was very irregular and such gametophytes differentiated more than one marginal meristems (Figs. 190, 191). As a result of meristematic activity of marginal meristems at different loci, a large number of gametophytes became lobed structures before the onset of tuber formation (Figs. 187k, 1). As shown in figure 192, the mature gametophyte was not cordate, there being no distinct apical meristem. Three-dimensional growth was restricted to the formation of tubers; thus unlike the Pteridium prothallus, no central cushion was formed within the expanded portion of the gametophytes.

Formation of tubers and sex organs: Tubers were consistently observed in all the cultures and served three purposes: (i) perennation, (ii) vegetative reproduction and (iii) production of sex organs. One or more tubers could develop anywhere from the marginal cells of the gametophyte; the site of inception was demarcated by the differentiation of a large number of rhizoids around it (Fig. 193). The marginal cells
near the rhizoids divided in different planes (Figs. 193, 194) and gave rise to radially symmetrical tubers (Fig. 195). The tubers, developed in the proximal portion of the gametophyte, were generally longer than those present on the distal portion and were able to penetrate the substratum; those on the distal portion generally accumulated starch.

Tubers also served as a means of vegetative propagation, since they gave rise to a number of new lobes with two-dimensional growth. These new lobes behaved like the mother prothallus when detached along with the subtending tuber.

As regards the sites of sex organs, the antheridial formation was restricted to the proximal region of the tubers (Fig. 196). Occasionally they were observed on the surface of the gametophyte (Fig. 197) as well in close proximity of the tuber. Archegonia were present in the distal region of the tuber (Fig. 198). Such archegonia-bearing tuber has earlier been described under the term "archegoniophore" by Goebel (1877). Figure 199 depicts the regeneration of young gametophytes from the older prothallus when cultured on fresh basal medium.

2. **INDUCTION OF APOGAMY**

Knop's medium (full strength), supplemented with ferric citrate (10 mg/l) and Nitsch's trace elements (1 ml/l), was established as the optimal basal medium for the development of the gametophytes.
Gametophytes of two different age-groups: (i) 2-week-old filamentous and (ii) 6-week-old, spatulate, were transferred to media containing 0.5, 1-8% of sucrose and glucose. Control cultures on basal medium were also set for comparison.

Variation in the rate of prothallial growth was apparent on different sugar concentrations. The growth on 0.5S medium was optimal followed by 1S and 2S. Gametophytes proliferated profusely resulting in numerous lobed structures. Tuber formation was regularly observed in all the above-mentioned sucrose concentrations. Similarly sex differentiation was noticed in all the cultures as described in the preceding experiment.

As expected, the extent of proliferation was markedly reduced corresponding with the increase in concentration of sucrose in the medium e.g. 3S-8S. Figures 200 - 209 show comparative growth response on basal medium, 0.5S and 1S-8S media. As is clear from figures 204-209, in cultures ranging from 3S-8S, the growth was markedly reduced. While the tubers and antheridia were developed normally in the aforesaid cultures, the formation of archegonia was not observed in prothalli as old as one year. The gametophytes continued to proliferate in all the cultures and no apogamous sporophytic structures were differentiated on any of the media (Figs. 210-214).
Apogamous response on sucrose-free medium: After maintaining the cultures for one and a half year on the basal medium, in 2% gametophytes cultured, the first leaf differentiated apogamously from the expanded, lobed part of the gametophyte (Fig. 215). Thus, the site of origin of the first cotyledonary leaf served as an indication of its being apogamous because, as pointed out earlier, the archegonia, and later the embryo sporophytes develop on or in the close proximity of the tubers. These apogamous juvenile leaves were isolated together with their subtending prothallial tissue and subcultured on fresh medium in flasks. Root and shoot formation remained suppressed, as was observed three months after the initiation of the cotyledonary leaf.

To the writer's knowledge, the only comparable report of induced apogamy in the absence of sucrose has recently been published by Rigby (1973) in Pellaea glabella.

Growth response on glucose-containing media: The pattern of development on glucose-containing media was similar to that on sucrose. However, the proliferation of the gametophytes in different concentrations of glucose was considerably reduced as compared to the corresponding sucrose concentrations. After one year of culturing on the same medium, archegonia were developed in 0.5% and 1% glucose. Subculturing was regularly done on fresh media and the cultures were maintained for about two years. No apogamous sporophyte was observed on any of the glucose-enriched media throughout the period of experimentation.
FIG. 216: SCHEMATIC REPRESENTATION OF EXPERIMENTS ON THE INDUCTION AND DIFFERENTIATION OF GAMETOPHYTIC CALLUS ON MOORE'S BASAL MEDIUM (MB) + SUCROSE (S) + 2,4-D

Germinating spores (Stock Cultures)

MB+2%S+0 ppm 2,4-D
(yellow, nodular)

Sub-Culturing

MB+2%S+1 ppm 2,4-D
(yellow, brown, solid)

Callus

MB+2%S+2 ppm 2,4-D
(brown, solid)

MB+2%S+3 ppm 2,4-D
(yellow, solid)

MB+2%S+4 ppm 2,4-D
(brown, solid)

MB+2%S+5 ppm 2,4-D
(yellow, friable)

MB+2%S+6 ppm 2,4-D
(brown, solid)

MB+2%S+7 ppm 2,4-D
(yellow, friable)

MB+2%S+8 ppm 2,4-D
(brown, solid)

MB+2%S+9 ppm 2,4-D
(brown, solid)

Sub-Culturing

MB+2%S+0.5 ppm 2,4-D
MB+2%S+1 ppm 2,4-D
MB+2%S+2 ppm 2,4-D
MB+2%S+3 ppm 2,4-D
MB+2%S+4 ppm 2,4-D
MB+2%S+5 ppm 2,4-D
MB+2%S+6 ppm 2,4-D
MB+2%S+7 ppm 2,4-D
MB+2%S+8 ppm 2,4-D
MB+2%S+9 ppm 2,4-D

(i) Rhizoids
(a) Gametophytes

(ii) Rhizoids
(a) Gametophytes

(iii) Rhizoids
(a) Gametophytes

(iv) Rhizoids
(a) Gametophytes

(v) Rhizoids
(a) Gametophytes

(vi) Rhizoids
(a) Gametophytes

(vii) Rhizoids
(a) Gametophytes

(viii) Rhizoids
(a) Gametophytes

(ix) Rhizoids
(a) Gametophytes

(x) Rhizoids
(a) Gametophytes

(xi) Rhizoids
(a) Gametophytes

(xii) Rhizoids
(a) Gametophytes
3. **INDUCTION AND DIFFERENTIATION OF CALLUS FROM THE GAMETOPHYES OF ANOGRAMMA LEPTOPHYLLA**

These experiments were designed to induce callus from the gametophyte generation of this fern and to determine the role of various exogenous factors in the differentiation of gametophyte and sporophyte (apogamously) from the gametophytic callus.

**Results**

Induction of gametophytic callus was tried both from the germinating spores as well as the aseptically-raised adult prothallii; positive results were obtained only in the first case.

Spores germinated on the basal medium in ten days and the sporelings were subsequently transferred to media supplemented with different concentrations of sucrose and glucose in combination with auxins alone or with kinetin. The results obtained are presented below:

3.1 **Induction of callus on Moore's medium + sucrose + 2,4-D (Fig. 216)**

As shown in figure 216 A-E, the concentration of sucrose was kept constant at 2% in all the media. Callusing of the young gametophytes was observed after six weeks on Moore's medium supplemented with 2% sucrose in combination with 2,4-D (0.5, 1-9 ppm).
On medium containing 0.5 ppm 2,4-D, yellow, nodular callus was induced (Fig. 217). Free hand sections of the nodules revealed actively-dividing parenchyma cells; no tracheids or stomata were found in these nodules. The callus, after regular subculturing on the medium of its induction, regenerated normal gametophytes and rhizoids (Fig. 218). Interestingly, the callus on basal medium supplemented with 2% sucrose (Fig. 216 A), also effected regeneration of gametophytes and rhizoids. No sporophytic organogenesis was observed on this medium.

On medium with 1 ppm 2,4-D (Fig. 216 B), yellowish-brown, solid callus was induced. The colour of the callus was brown at the time of initiation in 2-5 ppm 2,4-D. Abundant rhizoid formation was noticed all over the surface of the callus.

When regular subculturing was done on media containing 1,2 ppm 2,4-D, the callus turned green and later differentiated cylindrical, tuberoid structures and rhizoids (Fig.219). The tubers lacked prothallial outgrowths but possessed numerous rhizoids. On media containing 2% sucrose, the callus differentiated gametophytes, rhizoids and tubers (Fig. 216).

On 3-5 ppm 2,4-D concentrations (Fig. 216C), the callus remained solid, brown in colour with very slow rate of growth. Differentiation of rhizoids was observed from the calli
growing on the medium of their induction as well as on the basal medium containing 2% sucrose (Figs. 220, 221, 222).

In higher concentrations of 2,4-D viz., 6-7 ppm (Fig. 216 D, E) the callus was yellow in colour, its nature being solid in 6 ppm but friable in 7 ppm concentration (Figs. 223, 224); in 6 ppm 2,4-D the callus initiation was soon followed by the differentiation of rhizoids.

Friable callus, induced on 7 ppm 2,4-D medium (Fig. 216 E) could easily be separated into single cells and cell groups (Figs. 225, 226). Figure 227 depicts the differentiation of rhizoids from the callus cells on the medium of its induction. On regular subculturing on the basal medium and on basal medium containing 1-2% sucrose, the callus differentiated rhizoids and gametophytes. In sucrose concentrations ranging from 3-5%, the callus turned brown and differentiated only a few rhizoids. Interestingly, no sporophytic differentiation occurred on any of the sucrose-containing media. This is in contrast to a few recent reports in which a given concentration of sucrose in the medium either triggers the differentiation of gametophyte or sporophytic organogenesis (Kato, 1962, 1965; Bristow, 1962; Mehra and Sulklyan, 1969; Mehra and Palta, 1971).

The solid brown callus induced on media containing 8-9 ppm 2,4-D (Fig. 216 F) showed complete regeneration of gametophytes with rhizoids on the medium of callus induction
Fig. 231: Schematic representation of experiments on the induction and differentiation of gametophytic callus on Moore's basal medium (MB) + sucrose (S) + 2,4-D + kinetin (K)

Germinating spores (Stock Cultures) → Inoculated

Callus

MB + 0.5% S + 1-2 ppm 2,4-D + 0.5 ppm K
(yellowish-green, friable)

Sub-Culturing

MB + 0.5% S + 1-2 ppm 2,4-D + 0.5 ppm K

Callus

MB + 0.5% S + 3 ppm 2,4-D + 0.5 ppm K
(green, friable)

Sub-Culturing

MB + 0.5% S + 3 ppm 2,4-D + 0.5 ppm K

Callus

MB + 0.5% S + 4-6 ppm 2,4-D + 0.5 ppm K
(yellowish-green, nodular)

Sub-Culturing

MB + 0.5% S + 4-6 ppm 2,4-D + 0.5 ppm K

Germinating spores (Stock Cultures)

Rhizoids

Rhizoids

Rhizoids

Differentiation

Differentiation

Differentiation
FIG. 236: SCHEMATIC REPRESENTATION OF EXPERIMENTS ON THE INDUCTION AND DIFFERENTIATION OF GAMETOPHYTIC CALLUS ON KNUDSON'S BASAL MEDIUM (KnB) + GLUCOSE (G) + 2,4-D
Since the callus induction was soon followed by the differentiation of rhizoids in this experiment, the factors responsible for callus induction and its differentiation could not be ascertained. Consequently, no further experiments were performed for differentiation from this callus.

3.3 Induction of callus on Knudson's medium + glucose + 2,4-D (Fig. 236)

Callus was induced from the germinating spores on Knudson's medium supplemented with 2% glucose in combination with 0.5, 1-8 ppm 2,4-D. Callus was solid and brown in colour on medium containing 0.6 ppm and 1 ppm 2,4-D. Except for a few rhizoids, the callus showed complete absence of differentiation on 0.5 ppm 2,4-D medium (Figs. 236 A; 237, 238).

Since optimal callus growth was observed on medium containing 1 ppm 2,4-D, therefore, the stock cultures raised on this medium, were used for differentiation. The callus was heterogeneous, being composed of variable forms of cells containing chloroplasts. It was of unusual interest to observe some callus cells which behaved like spore cells at the time of germination. Prior to the initiation of cell division (Fig. 239) their cytoplasmic components moved towards one side of the cell and the first mitotic division resulted in two highly unequal cells; the larger cell functioned as the germ cell while its sister, smaller one developed into a rhizoid.
FIG. 250 - SCHEMATIC REPRESENTATION OF EXPERIMENTS ON THE INDUCTION AND DIFFERENTIATION OF GAMETOPHYTIC CALLUS ON MOORE'S BASAL MEDIUM (MB) + SUCROSE (S) + KINETIN (K) + AUXINS

Germinating spores

(Stock Cultures)

A

B

C

D

Inoculated

Callus

Callus

Callus

Callus

Sub-Culturing

Sub-Culturing

Sub-Culturing

Sub-Culturing

MB + 0.5% S + 0.1 ppm K + 0.1 ppm NAA

(green, solid)

MB + 0.5% S + 0.1 ppm K + 1-2 ppm NAA

(brown, solid)

MB + 0.5% S + 0.1 ppm K + 1-2 ppm IAA

(brown, solid)

MB + 0.5% S + 0.1 ppm K + 2 ppm 2,4-D

(brown, solid)

Rhizoids

Rhizoids

Rhizoids

Rhizoids

Rhizoids

Rhizoids

Rhizoids

Rhizoids
On regular subculturing of the callus on the medium of its induction (Fig. 236 B), the callus differentiated rhizoids as well as gametophytes on a number of loci (Figs. 240, 241). Cylindrical, tuberoid structures bearing rhizoids were also noticed on this medium (Fig. 242). When the callus was subcultured on basal medium, the differentiation did not proceed beyond the formation of rhizoids (Fig. 243). When 1-2% glucose was added in the basal medium, the callus differentiated rhizoids and gametophytes. On 3-5% glucose media, tubers were also differentiated along with the differentiation of rhizoids and gametophytes. On yet higher concentrations of glucose i.e. 6%, the callus turned brown and differentiated only a few rhizoids.

Yellow, nodular callus was induced on media containing 2-8 ppm 2,4-D (Figs. 244, 245, 246, 247). On maintaining the callus on media containing 2-3 ppm 2,4-D (Fig. 236 C), the callus differentiated rhizoids and gametophytes. On the other hand, on 4-6 ppm 2,4-D (Fig. 236 D) only rhizoid differentiation was noticed. On 7,8 ppm 2,4-D (Fig. 236 E), the callus turned brown and remained in this state for about a year after which it regenerated gametophytes and rhizoids (Figs. 248, 249).

3.4 Induction of callus on Moore's medium + sucrose + kinetin + auxins i.e. NAA, 2,4-D, IAA (Fig. 250)

Different concentrations of auxins in combination
with 0.5% sucrose and 0.1 ppm kinetin were utilized in this experiment.

Dark green callus was induced when basal medium was supplemented with 0.5% sucrose along with 0.1 ppm NAA and 0.1 ppm kinetin. Only a few rhizoids were seen on the callus growing on the medium of its induction (Figs. 250 A, 251).

Callus was brown and solid on Moore's medium supplemented with 0.5% sucrose and 1-2 ppm NAA along with 0.1 ppm kinetin. It differentiated rhizoids from all over the surface of the callus (Figs. 250 B, 252).

Initiation of brown solid callus was observed on the basal medium supplemented with 0.5% sucrose in combination with 0.1 ppm kinetin and 1-2 ppm IAA. Abundant rhizoids were differentiated from the callus (Figs. 250 C, 253).

Callus was also induced on media having the same concentrations of sucrose (0.5%) and kinetin (0.1 ppm) along with 2 ppm 2,4-D. On regular subculturing on the same medium (Fig. 250 D), the callus differentiated rhizoids but the number of rhizoids was much less as compared to those on NAA and IAA (Fig. 254).

All the calli obtained in this experiment were transferred to basal medium supplemented with 0.5% sucrose. The differentiation did not proceed beyond the formation of rhizoids (Fig. 250 A-D).
IN DARK CONDITIONS

- PHYTIC CALLUS ON KNUDSON'S BASAL MEDIUM (KnB) + GLUCOSE (G) + YEAST EXTRACT (YE) + 2,4-D

FIG. 25S - SCHEMATIC REPRESENTATION OF EXPERIMENTS ON THE INDUCTION AND DIFFERENTIATION OF GAMETO-

Phyto Callus on Knudson's Basal Medium (KnB) + Glucose (G) + Yeast Extract (YE) + 2,4-D

- Inoculated Germinating Spores (Stock Cultures)
  - KnB + 2% G + 0.5% YE + 1-2 ppm 2,4-D
  - (Gametophytes turned brown)
  - KnB + 2% G + 0.5% YE + 1-2 ppm 2,4-D
    - Tubers with rhizoids

- Sub-Culturing
  - Spatulate gametophytes
    - KnB + 2% G + 0.5% YE + 1-2 ppm 2,4-D
      - (Gametophytes turned brown)

- KnB + 2% G + 0.5% YE + 1-2 ppm 2,4-D
  - Inoculated (light cultures)
  - Callus
    - KnB + 2% G + 0.5% YE + 2 ppm 2,4-D
      - (yellow, friable)

- CALLUS
  - KnB + 2% G + 0.5% YE + 2 ppm 2,4-D
    - (yellow, friable)

- Sub-Culturing
  - Callus
    - KnB + 2% G + 0.5% YE + 1-2 ppm 2,4-D
      - (yellow, friable)

- IN DARK CONDITIONS
  - Germinating spores (Stock Cultures)
    - KnB + 2% G + 0.5% YE + 1-2 ppm 2,4-D
      - (Gametophytes turned brown)

DIFFERENTIATION

- Rhizoids
  - (i) Rhizoids
  - (ii) Gametophytes
  - (iii) Tubers
  - (iv) Gametophytes

- Tubers with rhizoids
3.5 Induction of callus on Kundson's medium + glucose + 2,4-D + yeast extract in dark (Fig. 255)

The sporelings raised in the light on Kundson's medium were transferred to variously supplemented media and the cultures were placed in dark.

Yellow, friable callus was induced on Kundson's medium supplemented with glucose (2%), yeast extract (0.5%) and 2,4-D (1-2 ppm) (Fig. 256). Callus was heterogeneous and its constituent cells could easily be separated (Figs. 257, 258); the cells were elongated with spirals. Chloroplasts were generally scattered in the cells or were present in groups on one side of the cell.

In order to study the effect of increasing the supply of carbohydrate, the callus was transferred to the basal medium containing 1-6% glucose. Cultures were also maintained on basal medium and on the medium of callus induction.

Three subculturings of the callus on the medium of its induction resulted in differentiation of rhizoids and tubers (Fig. 259). On regular subculturings, after five months, the callus regenerated gametophytes as well.

On the basal medium, the callus cells did not undergo any differentiation although it increased in size. At best on regular subculturings it differentiated rhizoids.
Callus, when transferred to basal medium supplemented with 1-2% glucose, differentiated rhizoids and juvenile gametophytes (Figs. 260, 261). Figure 262 shows the differentiation of juvenile gametophytes from all the sides of the callus on basal medium supplemented with 2% glucose.

On 3-6% glucose media, the callus turned brown and did not differentiate any structure.

The medium used in the present experiment i.e. Knudson's + glucose + 2,4-D + yeast extract, was established as the optimal medium for callus induction.

The cultures placed in light on this medium did not induce callus, instead the spores germinated and formed the spatulate gametophytes. Further growth of the juvenile gametophytes was inhibited; the tissue turned brown and produced elongated tubers which possessed a large number of rhizoids (Fig. 263). On regular subculturing of the gametophytes to fresh medium, no callus was induced.