SIROGONIUM STICTICUM: ITS MORPHOLOGY, CYTOLOGY, AND SEXUAL CYCLE

by

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ABSTRACT

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Clonal cultures of Sirogonium sticticum (Engl. Bot.) Kuetzing were established and maintained in Pringsheim's soil-water medium in a controlled environment. Filaments from mature cultures were examined; morphological data and measurements were obtained and recorded. Microchemical reagents and nucleophilic stains were applied to the mature cells to denote the presence and location of specific organelles and metabolites.

The sexual cycle of S. sticticum was studied intensively. Numerous recordings were made beginning with genuflexions through zygote formation; photographs were taken of stages of conjugation. Attention was given to gametic formation with emphasis on the chloroplastic disorganization. The occurrence of protogyny was noted on two occasions. Morphological data were obtained on the sexual structures; the frequency and distribution of the sterile cells were recorded.

Zygospores from a natural collection were germinated and the pattern of growth of the germlings was analyzed. Primary interest

centered on the reorganization of the chloroplastic material in the germling. Orange carotenoid granules were observed in cells of young germlings, becoming more diffuse as the cells increased in number. The reorganization of the ribbon-like chloroplasts was generally accomplished by the 3-cell stage.

INTRODUCTION

Sirogonium sticticum (Engl. Bot.) Kuetzing is an unbranched, filamentous, chlorophytan alga in the family Zygnemataceae, which is one of the most widely distributed groups of fresh-water algae. Members of this family are commonly found as "pond scums" on the surface of ponds, lakes, and slow-moving streams. The genus Sirogonium is far less common in occurrence than Spirogyra, Zygnema, and Mougeotia, the more familiar members of the family.

Several characters are employed in separating the genera Sirogonium and Spirogyra: The orientation of chloroplasts, presence or absence of a mucilaginous sheath, and the type of conjugation. In most species of Spirogyra the chloroplasts are definitely spiraled while in Sirogonium the plastids are relatively straight. A feature often characteristic of the Zygnemataceae is the slimy feeling caused by the pectose mucilaginous sheath externally apposed to the cell wall. The sheath is absent in Sirogonium, resulting in a filament that is coarse to the touch. Sexual reproduction in the Zygnemataceae is commonly of the type known as scalariform conjugation, although lateral conjugation and the formation of parthenospores and akinete-like bodies also occur (Smith, 1950). In Spirogyra a conspicuous tube forms between the two conjugating filaments; the male gamete passes

through the tube into the female gametangium. In Sirogonium a tube linking the cells is absent, resulting in direct conjugation (Transeau, 1951; Tiffany and Britton, 1952; Randhawa, 1959). In addition, the genus Sirogonium possesses both morphological and physiological anisogamy (Fritsch, 1935); Spirogyra demonstrates physiological anisogamy only.

The discussions found in the literature for Sirogonium or any of its species are exclusively of a descriptive and taxonomic nature. In the early literature the present writer has found no indication that investigations have been made on this organism in the laboratory under controlled conditions. However, studies on S. melanosporum have been reported and are in progress (Hoshaw, 1963).

In the present investigation of S. sticticum it was necessary to isolate small fragments of filaments into test tubes of soil-water media to establish clonal cultures. The cultures were maintained in an environmentally controlled chamber. The major objectives of this research, using clonal cultures, include a detailed examination of the vegetative material for morphological and cytological data; the application of methods to induce conjugation; and the critical observations of the sexual stages. In addition, zygospores and germlings were studied from the original collection.

REVIEW OF LITERATURE

Taxonomy and Morphology. The genus Sirogonium was established by Kuetzing (1843) and in his Latin diagnosis S. sticticum was the only species mentioned, thereby instituting this species as the type for the genus. Prior to the establishment of the genus, this alga was known as Conferva stictica Engl. Bot. (Smith, 1813). Historically, all algae were designated as Fucus, Corallina, Ulva, or Conferva. As microscopy developed as a tool for biology, algae were studied in detail by European scientists. The members of the genus Conferva were gradually removed and classified into taxa based on similar characteristics, thus deleting the genus Conferva from the present system of classification (Randhawa, 1959).

Six years after establishing the genus Sirogonium, Kuetzing (1849) validly published the descriptions of S. sticticum (Engl. Bot.) Kuetzing and three other species: S. breviarticulatum Kuetzing, S. Braunii Kuetzing, and S. notabile Kuetzing. DeBary (1858) elaborated on the observations of Kuetzing and stated that S. Braunii and S. breviarticulatum were identical with S. sticticum. Among the many synonyms recorded in the early literature of S. sticticum are Mougeotia stictica Kuetzing, Conferva stictica Engl. Bot. and Spirogyra trispira Meneghini (Kuetzing, 1849). Wolle (1887) also stated that the

names S. breviarticulatum Kuetzing, S. Braunii Kuetzing, Zygnema curvatum Ag. and Choaspes serpentina Gray were once applied to this alga. The discussion of the vegetative and sexual processes given by Gray in 1821 was extremely lucid and quite similar to that of Kuetzing 22 years later (West, 1904). For years after the recognition of this genus, many of its species were included under Spirogyra; recently Prescott (1951) listed Spirogyra pseudofloridana Prescott and Spirogyra stictica Wille from collections in the Western Great Lakes area. One year earlier, however, Smith (1950) included Sirogonium in his text and listed six species. Sirogonium was recognized again as a separate genus by Transeau (1951) who monographed the Zygnemataceae with taxonomic keys and descriptions of each of the 12 species. A more recent monograph of the family was published by Randhawa (1959) in which 15 species were described. Although this alga is distributed over a wide geographic range especially in warm climates (Randhawa, 1959), it is not found in the large quantities as is Spirogyra (Smith, 1950).

Vegetatively, the cells of Sirogonium are cylindrical, united in unbranched filaments, and lack pores (Smith, 1950). Such cells greatly resemble vegetative cells of Spirogyra in that both possess several narrow chloroplasts (Transeau, 1951) and plane septa (Randhawa, 1959). The chloroplasts, usually three to six in S. sticticum (Transeau, 1951; Randhawa, 1959), are comparatively straight, each

bearing numerous pyrenoids, structures possessing a proteinaceous core surrounded by minute starch plates (Randhawa, 1959). This Indian phycologist also stated that the absence of a layer of mucilaginous pectin causes the filament to be coarse to the touch and enables it to be the host for numerous aquatic epiphytic organisms. The length of the average vegetative cell is from 2-4 times its diameter although it may attain a size of 7 or more diameters (Transeau, 1951). The protoplast is uninucleate under normal conditions (Smith, 1950).

Growth and Culture Techniques. Johansen (1940) stated "It is . . . difficult to keep members of the Zygnematales in culture vegetatively and is scarcely worth attempting." Fortunately, other botanists attempted the culturing of the Zygnematales, and more specifically the Zygnemataceae, with good growth being obtained. Bold (1942) in an extensive paper discussed the growth of numerous algae under laboratory conditions and in his lengthy bibliography cited work on such genera as Spirogyra and Zygnema. More recently Allen (1958) and Rickert (1963) have grown and maintained Spirogyra in a controlled environment and Thomas (1963) has also effected excellent growth in the laboratory with Zygnema. Axenic cultures are difficult to obtain in these filamentous algae. Bold (1942) stated,"The filamentous Zygnematales are among the most difficult organisms to obtain in bacteria-free condition because of their gelatinous surface layers and

the absence of motile cells." However, pure cultures have been acquired in Zygnema by treating the alga with weak concentrations of penicillin G (Thomas, 1963).

The initial step in the maintenance of laboratory cultures involves the isolation of the alga from a natural collection. Filamentous forms can be broken into fragments mechanically with the aid of a needle or scalpel (Lewin, 1959); such segments are then transferred to a soil-water medium (Pringsheim, 1946) for growth and development. The use of inorganic media has been employed extensively and described by Bold (1942), Allen (1958), Starr (1960), Hoffman (1961), Machlis (1962), Thomas (1963), and others.

Conjugation. Sexual reproduction in the Zygnemataceae is by conjugation in which amoeboid gametes are involved (Randhawa, 1959). Such union may occur between two different filaments, as in scalariform conjugation, or between adjoining cells of the same filament, thus resulting in lateral conjugation (Smith, 1950). The scalariform type is found in 400 of the 533 species of the family which were described by Transeau (1951). According to Smith (1950) there are many factors conditioning the induction of sexuality in the filaments in nature. These include seasonal periodicity of environmental factors and the ratio between the surface and volume of the cell, with those having the greatest ratio becoming sexual first. Changes in temperature,

illumination and mineral content of the water apparently are of little benefit in promoting sexuality.

In the genus Sirogonium an evolutionary advance in sexual reproduction is seen over that of Spirogyra; the former demonstrates both physiological and morphological anisogamy while the latter shows only physiological anisogamy (Fritsch, 1935; Randhawa, 1959). According to Fritsch (1935) the conjugating cells meet by geniculation. This phenomenon involves a bending or flexing of the fertile cells, thus resulting in a tangled net (Transeau, 1951). During progametangial formation there is usually one division occurring in the female, forming a sterile cell and the gametangium; two divisions occur in the male, thus producing two sterile cells and the gametangium (Transeau, 1951; Randhawa, 1959). Fritsch (1935) stated that these preparatory divisions occur "as a general rule" and Transeau (1951) indicated that "the development of gametangia is highly variable in some collections."

Once the gametangia have developed, small projections arise from the female cell and come in contact with the male cell (Randhawa, 1959). At the junction of the filaments a pectic or mucilaginous ring develops (Fritsch, 1935; Smith, 1950; Transeau, 1951; Randhawa, 1959) causing adherence of the cells. Apposed cells do not develop a conjugation tube (Smith, 1950; Transeau, 1951; Randhawa, 1959). The zygospores, usually ellipsoidal in shape (DeBary, 1858; Transeau, 1951; Randhawa, 1959), possess a median spore wall that may vary

in color from yellow to black and is variously ornamented (Randhawa, 1959).

Zygospore Germination. Various techniques to promote the germination of zygospores or oospores in the laboratory have been explored. Starr (1955) stated that successive drying and wetting proves beneficial in promoting germination for some algae. Aging is often a necessary factor before germination can occur (Starr, 1955). Starr has also included, among his techniques, transfer to fresh media and freezing followed by transfer to fresh media.

In his work on Chlamydomonas, Lewin (1949) found that starvation of the zygotes during their development reduced the thickness of the walls and thereby shortened dormancy. According to Cook (1962), Pringsheim obtained the germination of oospores of Bulbochaete intermedia after a three-month dormancy without special treatment. The germination of the oospores of Bulbochaete hiloensis, however, required that the material be stored in tightly closed containers for several weeks at relatively high temperatures. The action of bacteria and fungi on the vegetative material and oospore walls promoted the liberation of the mature oospores. Transfer of oospores to fresh media from darkness to light resulted in germination (Cook, 1962).

In her work with Spirogyra, Allen (1958) placed zygotes in the dark a few days after they were formed. Mature zygospores were

obtained about ten days after the initiation of the dark treatment. These structures were cut out, dried for a minimum of three days, covered with soil-water supernatant, and returned to a lighted area. Germination occurred in 72 hours on an alternating light-dark cycle or less time was necessary under continuous light to effect germination. Rickert (1963) followed a similar technique, but placed the zygospores on desmid agar (Starr, 1960). Germination occurred in about two weeks.

Fritsch (1935) stated that zygospores of the Zygnemoideae possess thick walls which are three-layered; the outer layer is cuticularized; the middle one bears distinctive markings and is colored; and the inner layer is transparent.

In Sirogonium germination is unusual because the first cross wall of the germling is formed some distance from the point where the filament emerges from the zygospore (Smith, 1950).

METHODS AND MATERIALS

Collection and Isolation. A natural collection of Sirogonium sticticum (Engl. Bot.) Kuetzing was obtained from Dr. G. W. Prescott, Professor of Botany at Michigan State University, in August, 1963. The material was collected from "a small ox-bow type of lagoon, only about 100 x 200 ft., 3 miles south of the town of Kalispell, Montana on U. S. Highway No. 93. The plant formed a yellowish-green mat and was entangled about Elodea" (Prescott, personal communication).

As the collection was examined using a dissecting microscope, filaments which appeared healthy and uniform in structure were selected for isolation. Various methods were used to isolate this material. One method that proved successful was the use of a glass hook for picking up small filaments. This hook was prepared by drawing out a glass pipette to a fine point and then sealing the end by quickly passing the glass through the flame of a Bunsen burner. Another technique applied in isolating fragments of this alga was the use of stainless steel surgical needles to break up the filaments into sections of a few cells. Such pieces were then recovered with a glass pipette or with the end of a surgical needle. Scissors were also employed to cut the net into small sections for pieces to transfer. After fragments were removed from the petri dish containing the natural collection,

they were cleansed by a series of washings in distilled water. The filaments were then placed in test tubes of Pringsheim's soil-water medium (Starr, 1960). When sufficient growth was obtained, the nets were transferred to milk bottles containing the soil-water medium.

Clonal cultures were obtained from germlings as well as from the isolation of small pieces of filaments. Aged zygospores from the Prescott collection were dried for a 24-hr period, then rewet with soil-water supernatant. Germlings resulting from the germination of the zygospores were transferred into soil-water medium.

Growth and Maintenance.

Media. Cultures of S. sticticum were grown and maintained in steamed soil-water medium in test tubes and milk bottles. The preparation of this medium entailed placing a small amount of garden soil, approximately 1/4 teaspoon for test tubes and 1/2 teaspoon for half-pint milk bottles, on the bottom of the vessel. The soil was then covered with distilled-demineralized water, so that the vessels held about 3/4 of their capacity. The tubes and bottles were covered and placed in a steam chamber and steamed for 2 hours on each of two successive days. The soil used exclusively in the growth of S. sticticum was labelled I-3, obtained from the L. B. Lantz farm near Monticello, Indiana. A variation in the soil-water medium was made by adding a small amount of $CaCO_3$ to the test tube or milk bottle

and then placing the soil in the bottom of the glass container.

Attempts to grow this organism in a chemically defined medium were made during the course of this research. The media employed and their methods of preparation were as follows:

Bristol's Solution (Starr, 1960)

Stock solutions of the following salts were employed:

$NaNO_3$	10.0g in 400 ml water
$MgSO_4 \cdot 7H_2O$	3.0g in 400 ml water
KH_2PO_4	7.0g in 400 ml water
$CaCl_2$	1.0g in 400 ml water
K_2HPO_4	3.0g in 400 ml water
NaCl	1.0g in 400 ml water

Ten ml of each of these stocks and a drop of 1% $FeCl_3$ were added to 940 ml of distilled water. The pH was varied by changing the amounts of monobasic and dibasic potassium phosphate; a pH of 6.8 was used.

Modified Bristol's Solution

and Agar (Starr, 1960)

The same procedures were followed as above, but 15g of agar were added. In addition, 40 ml of soil-water supernatant were substituted for 40 ml of distilled water. Minute amounts of biotin, vitamin B-12, and

Stein's trace element solution (Stein, 1958) were added in some instances.

Desmid Agar Medium (Starr, 1960)

This medium was employed without the addition of agar. To 1000 ml of pyrex-distilled water, 10 ml of each of the following were added:

0.1% solution of $MgSO_4 \cdot 7H_2O$

0.1% solution of K_2HPO_4

1.0% solution of KNO_3

Minute amounts of biotin, vitamin B-12, and Stein's trace element solution (Stein, 1958) were added in some instances.

Czurda's Medium (Allen, 1958)

To 995 ml of pyrex-distilled water, one ml of the following solutions was added:

10.0% KNO_3

1.0% K_2HPO_4

1.0% $MgSO_4$

0.2% $FeSO_4$

0.5% $CaSO_4$

The pH was adjusted to 6.8 using H_2SO_4 and KOH. Again, biotin, vitamin B-12, and Stein's trace element

solution (Stein, 1958) were added in small amounts.

Trace Element Solution (Stein, 1958)

H_3BO_3	2.86g
$MnSO_4 \cdot H_2O$	2.05g
$ZnCl_2$	0.10g
$CuSO_4 \cdot 5H_2O$	0.08g
$H_2MoO_4 \cdot H_2O$	0.02g
Double distilled water	1.0 liter

Controlled Environment. The cultures of S. sticticum were grown in a chamber on a 12:12 hr light-dark cycle. The temperature remained 20 $\pm$ 2° C with illumination of 2150-3250 lux from 110-watt cool-white fluorescent light bulbs. Unsuccessful attempts were made to grow the alga at a temperature of 26° C on a 16:8 hr light-dark cycle at 3250-4300 lux furnished by 40-watt cool-white fluorescent bulbs.

Cytology and Morphology.

Measurements. Morphological data were obtained by placing filaments on clean slides and covered with #2 cover slips. The preparations were examined using a Bausch and Lomb zoom compound microscope having 10X, 43X, and 97X (oil immersion) objectives. An American Optical phase-light microscope with objectives of 10X, 20X,

43X, and 97X was also employed. Both optical instruments were fitted with eyepiece micrometers which had been calibrated by using a stage micrometer to obtain measurement in microns. Units indicating the length and width of the cell, the length and width of the chloroplasts, the proportions of the nucleus as well as the number and relative size of the nucleoli, and the dimensions of the zygospore were recorded. In addition, the number of pyrenoids per plastid were counted and recorded.

Staining. Vital stains were applied to the cells to determine the presence of specific organelles or metabolites. To establish the presence of mitochondria, a solution of Janus Green B in water (1: 10, 000) was used in mounting the filaments on the slide. To denote the presence and location of pectin, an aqueous solution of ruthenium red (1:5, 000) was applied to the filaments and allowed to react for approximately one hour. To determine the distribution of fats and oils within the cell, Chrysoidin Y was used in a 1:10, 000 solution.

Other stains that were used to demonstrate the presence of metabolites included Sudan III to locate oil, fat, and cutin (Jensen, 1962). Iodine-potassium iodide (I-KI) (Johansen, 1940) was applied to denote the cellular areas important in starch synthesis and storage.

The structure of the nucleus was studied intensively and attempts to observe somatic divisions were made. Material for

observations of mitosis was preserved in the following fixative: 0.5g of iodine, 1g of potassium iodide, 4 ml of glacial acetic acid, 24 ml of commercial formalin, and 400 ml of distilled water (Cave and Pocock, 1951). Once the alga was placed in the fixing solution, it could be retained without cytological alteration up to 72 hours. The fixed material was placed on a slide in acetocarmine, heated, and observed. A mordant of 0.4% ferric hydroxide in propionic acid and two parts absolute alcohol (Cave and Pocock, 1951) was sometimes added before the acetocarmine treatment. Propiocarmine (Harris, personal communication) was substituted for the more commonly used aceto-carmine resulting in a better defined stain.

Induction of Conjugation. Various techniques were applied to induce conjugation in S. sticticum. The simplest attempt was that of removing the net from the milk bottle by means of a sterile wire loop and placing the net in a deep petri dish (20mm x 100mm) containing fresh soil-water supernatant. This dish was then placed under a light bank at 3250-4300 lux at 26 $\pm$ 2° C or returned to the culture chamber. Periodic observations of the nets were made and sexual stages examined and recorded photographically. Modifications of this basic method included placing the dish in lower light intensity (ca. 1100 lux) in the chamber and using a 1:1 mixture of distilled water-soil-water supernatant for the algae.

The method described by Hoshaw (1961) for Cosmarium and Oedogonium was attempted in S. sticticum. Mating dishes were prepared with deep petri dishes fitted with a watch glass on a glass triangle. Soil-water supernatant was put in the watch glass; filaments of S. sticticum were transferred to the liquid. The bottom of the petri dish was filled with 20 ml of a freshly prepared 5% solution of $NaHCO_3$. The mating dishes were covered and placed under a light of 3250-4300 lux at 26 $\pm$ 2° C. Observations were made in 24 and 48 hours.

Filaments indicating early stages of sexuality were removed from the net and placed on the surface of 1.5% water agar--15g of agar; 1000 ml distilled water (Allen, 1958)--and on Bristol's agar (Starr, 1960) for study. These plates were returned to the growth chamber and were observed daily.

Zygospore Germination. Filaments containing zygospores from the original collection were kept at 6° C in a refrigerator until needed. To promote germination, a few drops of the liquid containing zygospores were placed in the bottom of a small petri dish. The lid remained off the dish for 24 hours to allow the material to dry out. Soil-water supernatant was added and the dish was placed under a fluorescent light at an intensity of 3250-4300 lux and a temperature of 26 $\pm$ 2° C.

Attempts were made to remove the zygospores from the old female gametangial walls using stainless steel dissecting scissors. Zygospores liberated in this manner should show a greater percentage of germination as the gametangial walls apparently inhibit their development.

Due to the scarcity of the material, no attempts at zygospore germination were made from those zygotes formed in culture.

Microscopic and Photographic Equipment. The compound microscopes used during the course of this study have been described previously. A Bausch and Lomb Stereozoom dissecting microscope was used for isolating and scanning plates to locate sexual stages. Photographs used in this thesis were taken with an American Optical phase-light microscope equipped with a Zeiss adapter and camera. All of the pictures were taken on Kodachrome II Type A film from which color prints were produced.

OBSERVATIONS

The material used for these studies was obtained from isolations made from a natural collection supplied by Dr. G. W. Prescott. Of the several clones resulting from the isolation of small pieces of filament, two were used as the basis for maintaining the population. Zygospores from the natural collection were germinated and detailed examinations of the germlings were made.

Growth. Of the media employed, S. sticticum grew best in soil-water medium. The growth response of the alga to defined inorganic solutions and agar is summarized in Table 1. It is quite probable that other organisms in the unialgal cultures greatly influence the pattern of development in liquid or agar media.

The growth of the alga was influenced by temperature, light, and photoperiod. During the course of this study, it was found that a temperature of 20 $\pm$ 2° C and a 12:12 hr light-dark cycle resulted in healthy and vigorous growth. The application of a 16:8 hr light-dark cycle at 26° C resulted in rapid degeneration of the cultures.

Morphology. Critical observations and measurements of vegetative cells were made from clonal cultures. The diameter and

Table 1. Growth response of S. sticticum in various media.

Medium	Results
Soil-Water Extract Agar	The filaments died within 48 hours; the action of bacteria was most influential on the death of the algae.
Bristol's Solution	A small amount of growth occurred after 6 days; the cells were chlorotic and heavily contaminated with fungus.
Bristol's Solution + Biotin	After 6 days growth, a few healthy cells had been formed, but most of the growth was chlorotic and fungal contaminated.
Bristol's Solution + Vitamin B-12	After 6 days, a large number of healthy cells were visible; however, the fungus was beginning to inhibit further development.
Bristol's Solution + Trace Elements	After 6 days, a small amount of new growth had occurred, but most filaments were black.
Bristol's Solution + Biotin, B-12, and Trace Elements	After 4 days, no new growth had occurred and most of the original inoculum was dead.
Czurda's Medium + Biotin, B-12, and Trace Elements	After 5 days, the original inoculum was dead and had turned black, with a burned appearance.
Desmid Solution	Most of the vegetative cells were very healthy; the fungus apparently inhibited further growth. Algae forms a ball instead of a net.
Desmid Solution + Vitamin B-12	A slow rate of growth was seen in this culture but the cells were very healthy. There was some fungal contamination.

Table 1. (Continued)

Medium	Results
Desmid Solution + Biotin	A rapid growth resulted and healthy cells were observed. Fungal contamination apparently inhibited more noticeable growth.
Water Agar	After 48 hours the filaments were dead, probably because of the bacterial growth rate.
Soil-Water Medium	Extremely vigorous growth was found in this medium. Fungal contamination was present, but the growth rate of the alga surpassed that of the fungus.
Soil-Water Medium + $CaCO_3$	Vigorous growth resulted in this medium, but cultures could not be sustained for as long a period of time as in the soil-water medium.

length of the cell, the width and length of the chloroplasts, the width and length of the nucleus, and the width and length of both cultured and naturally formed zygospores were determined. The results of these determinations, as well as the dimensions listed in Transeau (1951) and Randhawa (1959), are shown in Table 2. In a typical vegetative cell, the plastids, pyrenoids, and nucleus are easily discernible (Fig. 1).

In addition to morphological characters such as the width and length of the cell and the number of chloroplasts in each cell, the size, shape and distinctive markings of the zygospore are important taxonomically. The taxonomic key devised by Transeau (1951) uses the condition of the median spore wall--smooth or ornamented--as the initial character leading to specific nomenclature. Randhawa (1959) employs the shape of the zygospore as the primary character for identifying the species. When a mature zygospore of S. sticticum is squashed, the smooth yellow median spore wall is visible (Fig. 2).

Cytology. Filaments of S. sticticum were subjected to a variety of stains to determine the presence of specific organelles or metabolites. Janus Green B in aqueous solution, specific for location of mitochondria, was applied as a vital stain. The preparations were examined carefully but the activity and position of mitochondria could not be definitely ascertained. Examination with a phase-light

Table 2. Vegetative morphology of S. sticticum.

Structure	Range	Average	Transeau (1951)	Randhawa (1959)
Diameter of cell[1]	46.8 - 57.6μ	48.0μ	38 - 56μ	38 - 56μ
Length of cell[1]	108.0 - 413.6μ	199.6μ	80 - 300μ	80 - 300μ
Length of chloroplast[2]	64.8 - 254.0μ	158.2μ	--	--
Width of chloroplast[2]	3.6 - 14.4μ	8.1μ	--	--
Length of nucleus[2]	10.8 - 18.0μ	14.2μ	--	--
Width of nucleus[2]	7.2 - 10.8μ	8.6μ	--	--
Number of chloroplasts/cell[1]	2 - 4	2.8	3 - 6	3 - 6
Number of pyrenoids/plastid[1]	4 - 17	9.4	--	--
Width of cultured zygospores[3]	37.6 - 56.4μ	47.7μ	--	--
Length of cultured zygospores[3]	65.8 - 84.6μ	72.5μ	--	--
Width of natural zygospores[3]	57.0 - 64.6μ	60.8μ	41 - 67μ	40 - 67μ
Length of natural zygospores[3]	91.2 - 117.8μ	100.6μ	68 - 127μ	68 - 127μ

[1]Based on 100 measurements
[2]Based on 20 measurements
[3]Based on 15 measurements

microscope also failed to disclose the position of the chondriosome. Rod-shaped structures, stained dark blue, were observed with the aid of phase-light microscopy. It was not possible to state definitely that these bodies were mitochondria, due partially to the presence of numerous bacillus-type bacteria on the outer cell wall.

To ascertain the location of pectin, ruthenium red was employed as a vital stain. Filaments were placed in the stain for approximately one hour before critical observations were made. This metabolite was located by the pink coloring imparted to the structures composed of pectin. The septa indicated a pectinaceous composition. In addition, a ring of pectin was noted at the point of adherence of the two papillae (Fig. 3).

The application of Chrysoidin Y in aqueous solution indicated the presence of oil and fat substances in the zygospore. However, a more definitive stain was obtained using Sudan III. When this microchemical reagent was applied to young germlings, several large red droplets, denoting the presence of lipid compounds, were observed. These droplets were scattered throughout the cells of the germling. When zygospores were squashed and stained with Sudan III, a large quantity of fat and oil was noted (Fig. 4).

To locate the centers of starch synthesis and storage within the cell, iodine-potassium iodide (I-KI) was employed. Filaments placed in this solution were critically observed. The pyrenoids

quickly turned a blue-black color and became clearly visible, indicating the location of starchy substances (Fig. 5).

Numerous attempts to observe mitotic divisions in the filaments were made. Due to their rapid growth rate, germlings proved the most suitable material for the study of cell division. Smears were prepared, using propiocarmine as the nucleophilic stain. S. sticticum has a relatively narrow nucleus usually containing a single nucleolus, although two nucleoli have been noted occasionally. Heterochromatin is present in the interphase nucleus in large proportions, thus often giving the appearance of numerous minute chromosomes. In the investigation of the nuclear behavior of S. sticticum one instance of probable mitosis in a metaphase stage was observed. The nuclear membrane and nucleolus were not visible. No estimate of the chromosomal number was made.

Conjugation. S. sticticum is a homothallic organism in which conjugation frequently occurs in the culture vessel. As the filaments elongate, they become attached to each other in various places, thus forming a net. The attachment initiates the first stage in conjugation. Frequently zygospores will be formed in material on which no effort to induce conjugation has been made.

Of the techniques attempted to induce conjugation, only one was successful. Healthy nets were placed in deep petri dishes and

covered with fresh soil-water supernatant. Some stages of sexuality were noted in several cultures, but two dishes, each showing an appreciable amount of conjugation, were selected for detailed study.

The first step in the sexual cycle of S. sticticum is the genuflexing or meeting of two filaments. As these filaments touch, they adhere to each other, and small projections arise from a cell of each until the filaments are united. Such projections are termed papillae (Fig. 6). The cells that produce papillae are referred to as progametangia. The progametangial cells of each filament are responsible for the formation of the gametangia. A small gametangium is formed from one filament; due to the size, it is termed the male gametangium. The opposite filament forms the female gametangium which is larger than the male and often quite inflated.

To initiate the formation of the gametangium, the progametangium usually undergoes somatic division resulting in the gametangium and a small cell. This cell is considered a sterile cell and is usually 70μ or less in length, but may attain a length of 100μ, as opposed to a minimum length of 108.0μ for vegetative cells in the laboratory cultures that were studied. The dimensions of this cell and the adjacent gametangia are shown in Table 3. The division of the progametangial cells is variable in its occurrence and distribution in the area of the gametangia (Table 4).

Table 3. Dimensions of gametangia and adjacent sterile cells of S. sticticum.

Sterile cell adjacent to gametangium			Gametangium			
	Average length	Range	Average Size Length	Width	Range Length	Width
Male	52.0 μ	19.0-103.0 μ	40.0 μ	43.0 μ	28.0 μ	66.0 μ
Female	61.3 μ	28.0-103.0 μ	89.0 μ	61.0 μ	169.0 μ	56.0 μ

Table 4. Frequency and distribution of 25 sterile cells in S. sticticum.

Location	Number	Per Cent
Adjacent to male gametangium only	10	40%
Adjacent to female gametangium only	3	12%
Adjacent to both gametangia	12	48%
Total	25	100%

After gametangia are formed, the vegetative structures of these reproductive cells begin to change. The ribbon-like chloroplasts disorganize, forming detached sections of chloroplasts within the gametangium. As the plastids disorganize, the nucleus becomes obscured and is not receptive to nucleophilic stains (Fig. 7). As the process of conjugation advances, the entire protoplast within each gametangium masses, forming an oval-shaped body which at this point is termed a gamete. Morphological anisogamy is evident in the gametic formation; the male gamete is considerably smaller in volume than the female (Figs. 8 and 9).

When the gametes are ready to fuse, the male gamete disorganizes and flows through the connecting tube formed by the papillae into the female gametangium. Fusion occurs and a zygote is formed. The newly formed zygote remains green for a period of time (Fig. 10). The shape of the zygote may vary, depending principally on the shape of the female gametangium (Fig. 11) although it usually is ellipsoid to ovoid. Eventually, each zygote develops a three-layered wall and during maturation turns a dark brown (Fig. 12) and is now termed a zygospore.

Usually the male gamete forms first (Fig. 13), while protoplastic disorganization is still occurring in the female gametangium. However, in two instances it has been observed that the female gamete is the first to form (Fig. 14). Since attempts to follow this pair of

gametangia through the formation of a zygote were unsuccessful, it is questionable as to the eventual fate of the male gamete. It is possible that a physiological inhibitor prevented the male gamete from forming. It is also possible that protogyny occurs in a greater number of the conjugating filaments than has been observed by the present writer.

Double conjugation--two pairs of gametangia adjacent to each other in both filaments--was not observed in S. sticticum. This phenomenon has been noted in other species, notably in S. melanosporum (Randhawa) Transeau and in S. phacosporum Skuja (Hoshaw, personal communication).

Zygospore Germination. Zygospores from a natural collection were germinated after a 24-hr drying period. Germination occurred within 72-96 hrs after rewetting the material with soil-water supernatant. In S. sticticum the zygospore swells and turns green prior to the emergence of the germling (Fig. 15). One-celled germlings (Fig. 16) appear as the zygospores break open, showing a definite line of dehiscence. This line of dehiscence has not been observed before the zygospore ruptures. In the one-celled germling the chloroplastic material is extremely dense, occupying most of the area within the cell wall. The tip of the apical cell contains colorless cytoplasm and is usually relatively free of plastid material (Fig. 17). As this first cell elongates prior to somatic division resulting in a 2-celled germling,

elongation also begins in the chloroplastic substance with the formation of ribbon-like strands being evident toward the apical and basal ends. When the cell has undergone mitotic division, the daughter cells usually demonstrate considerable differentiation in the chloroplasts (Fig. 18). By the time the germling is 3 or more cells in length, all of the chloroplasts have reorganized and the typical ribbon-like structures have reformed (Fig. 19).

A small dense aggregation of orange material has been observed in the cells of the young germlings. In the one-celled stage this body is located in the region of the undifferentiated chloroplastic material. As the germling increases in length, small orange structures have been observed in several cells, scattered throughout the chloroplastic material. This body is primarily localized in the apical cell, but as the germling undergoes somatic divisions, the orange granules appear to be randomly distributed in several cells. It is hypothesized that this body is composed of carotenoid pigment.

Germlings of S. sticticum are generally uniform in size. However, two instances of large germlings have been observed. The size of one of the large germlings suggested the possibility of ploidy. The usual zygospore averages 60.8μ wide and 100.6μ long; the zygospore of the large germling measured 98.8μ x 152.0μ. The diameter of the cells of this germling was 98.8μ as opposed to the usual 48.0μ. Of the two germlings in which this abnormality was noted, neither

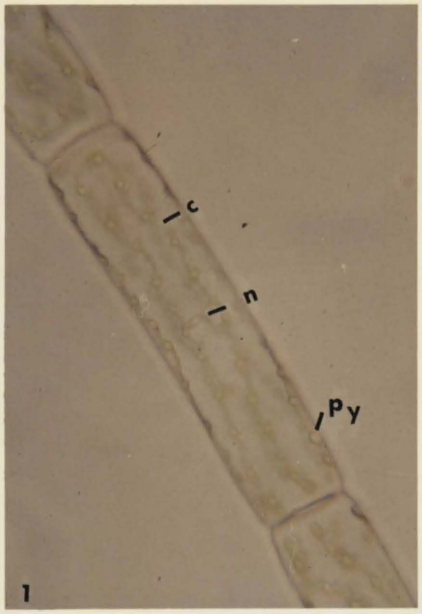
attained a length of more than two cells. If this phenomenon is a result of ploidy as is postulated, it is quite probable that the chromosomal complement hindered further mitotic divisions (Fig. 20).

Fig. 1. A typical vegetative cell of Sirogonium sticticum from a laboratory culture. Note the nucleus (n), chloroplasts (c), and pyrenoids (py). Approximately X 225.

Fig. 2. A zygospore of S. sticticum squashed to show the principal taxonomic characteristic, a smooth yellow median spore wall. Approximately X 465.

Fig. 3. A pair of gametangia of S. sticticum denoting the pectic ring (arrow) in response to staining with ruthenium red. Approximately X 225.

Fig. 4. A zygospore of S. sticticum, smeared and stained with Sudan III to demonstrate lipid content as indicated by the red droplets. Approximately X 225.



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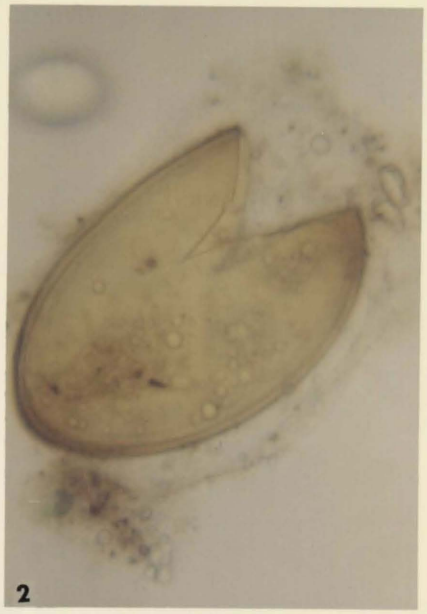

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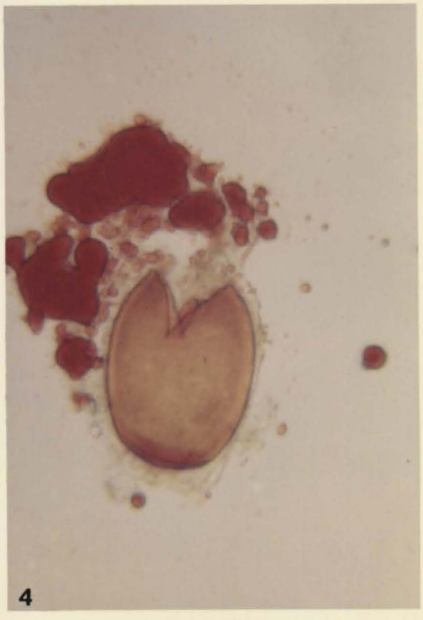

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Fig. 5. A vegetative filament of S. sticticum showing response to treatment with I-KI to locate areas of starch metabolism which are stained blue-black in color. Approximately X 225.

Fig. 6. Early genuflexion in S. sticticum showing the attachment of the papillae (p) and progametangia (pr). Approximately X 225.

Fig. 7. A pair of conjugants of S. sticticum illustrating initial stage of chloroplastic disorganization; note the male gametangium (mg), female gametangium (fg), sterile cell (st) and oil globules (o). Approximately X 225.

Fig. 8. Mature gametes of S. sticticum and the associated sterile cells; note the male gamete (m), female gamete (f), and sterile cell (st). Approximately X 225.

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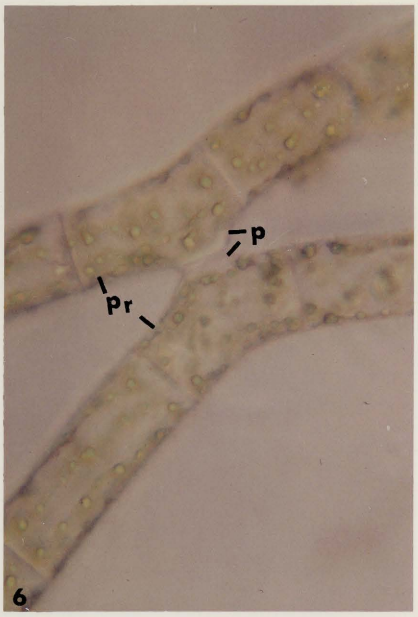

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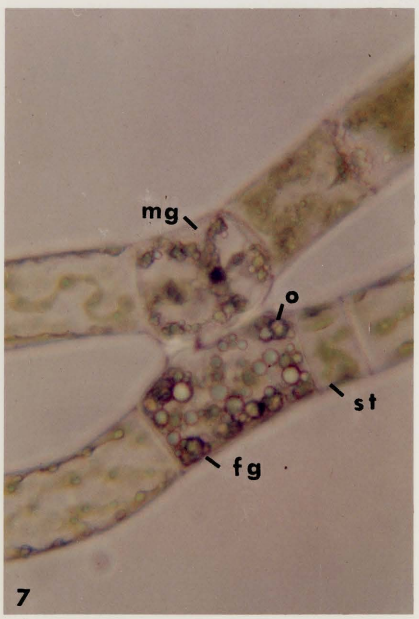

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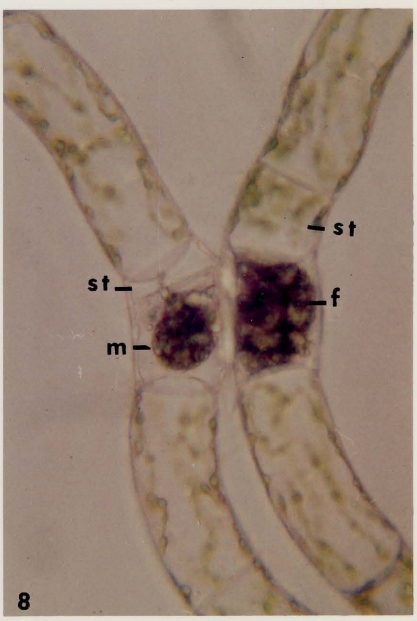

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Fig. 9. A pair of gametes of S. sticticum illustrating morphological and physiological anisogamy; note the female gamete (f), male gamete (m), and sterile cells (st). Approximately X 225.

Fig. 10. Newly formed green zygote of S. sticticum in the female gametangium; note male gametangium (mg), female gametangium (fg), zygote (z), and sterile cells (st). Approximately X 225.

Fig. 11. Recently formed green zygote (arrow) of S. sticticum that differs from the typical shape as seen in Fig. 10. Approximately X 225.

Fig. 12. A mature zygospore (arrow) of S. sticticum formed in clonal culture. Approximately X 225.


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m
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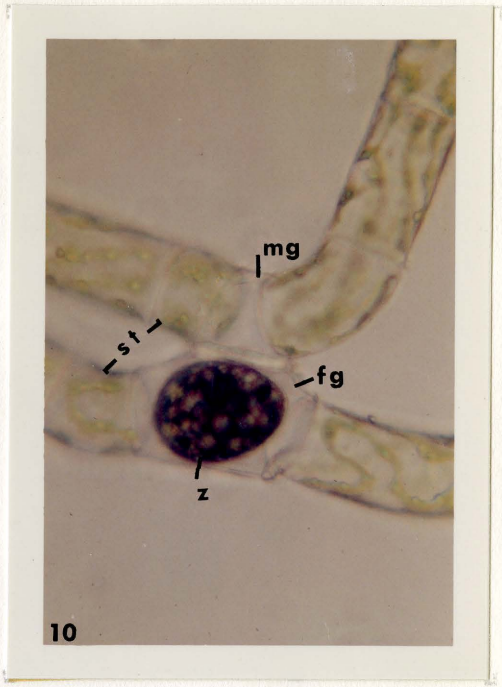
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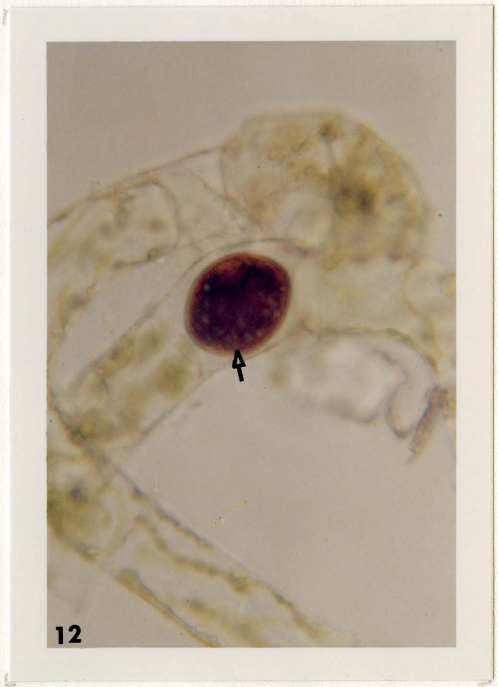
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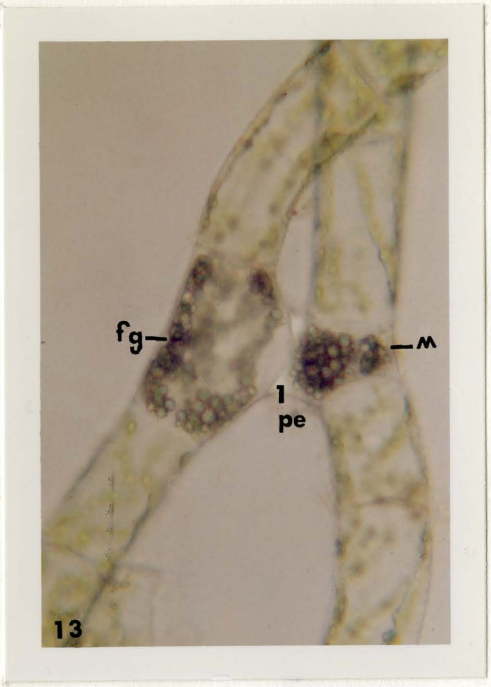
Fig. 13. The common manner of gametic formation of S. sticticum in which the male gamete (m) forms first while the female gametangium (fg) still shows chloroplastic breakdown. Also note the pectic ring (pe). Approximately X 225.

Fig. 14. Example of protogyny in S. sticticum; the male gamete has not yet formed; note the female gamete (f), male gametangium (mg) and sterile cell (st). Approximately X 225.

Fig. 15. A zygospore of S. sticticum from a natural collection in the female gametangium after aging and rewetting to promote germination. Approximately X 225.

Fig. 16. A young germling of S. sticticum emerging from the zygospore. Approximately X 225.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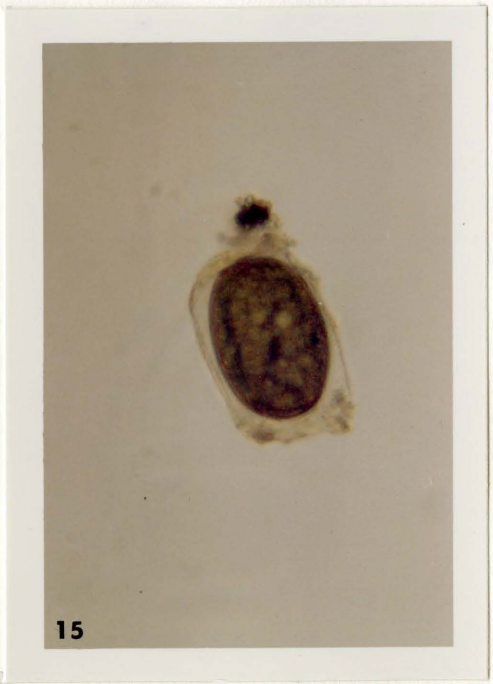
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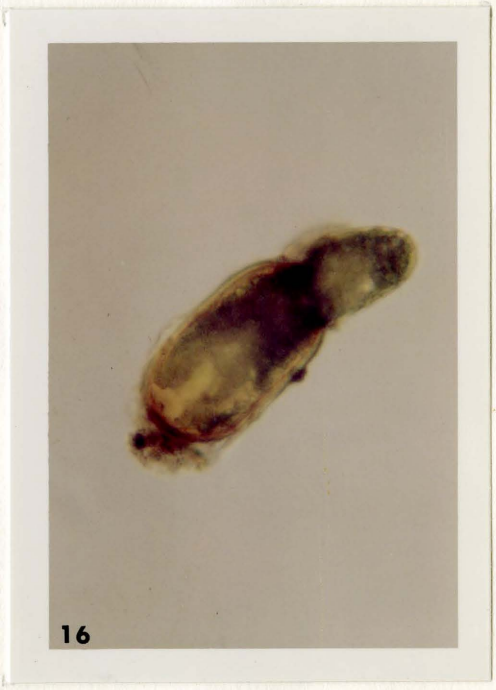
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Fig. 17. A one-celled germling of S. sticticum with disorganized chloroplastic material and clear cytoplasm at the apical end. Approximately X 225.

Fig. 18. A two-celled germling of S. sticticum showing complete reorganization of the chloroplasts. Approximately X 105.

Fig. 19. A portion of a several-celled germling showing the typical vegetative structures of S. sticticum. Approximately X 105.

Fig. 20. An unusually large germling of S. sticticum accounted for, possibly, on the basis of ploidy. Approximately X 225.

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DISCUSSION

Growth and Maintenance. Although difficulty in growing members of the Zygnemataceae is noted in most of the early literature (Johansen, 1940; Bold, 1942), in recent years excellent growth has been obtained in steamed soil-water media by several workers (Allen, 1958; Hoshaw, 1963; Rickert, 1963). In accordance with the findings of Allen (1958) and Rickert (1963) on Spirogyra, it was observed that growth of S. sticticum in defined inorganic media was poor. It is quite probable that the use of other defined media would yield more healthy and vigorous growth than those media employed during the course of this study. A better growth response of the alga in nutrient culture might be obtained by varying the concentration of the salts in the inorganic solutions.

The necessity of obtaining pure cultures of algae has been emphasized by many workers (Pringsheim, 1946; Rickert, 1963; Thomas, 1963). Before more extensive work can be done on S. sticticum, it would be highly desirable to secure and maintain this organism in a pure culture. The absence of the external pectic sheath in this genus should facilitate axenic growth. However, in addition to the presence of bacteria, a parasitic fungus occurred on the clonal

cultures and appeared to have been instrumental in the response of this alga to various media. Techniques to eliminate the fungal contaminant must be employed before bacteria-free cultures can be secured.

Morphology and Cytology. The morphological data obtained on S. sticticum agree with that of Transeau (1951), Tiffany and Britton (1952), and Randhawa (1959). Since all measurements were obtained from clones originating from isolations of a single natural collection, the variability of morphological observations within the species expressed by previous authors is not as great. The necessity of obtaining more collections from which clones can be established in the laboratory is evident for a more thorough knowledge of the organism.

A possible influence on the morphological data recorded in this thesis is the fact that the vegetative material used was obtained exclusively from laboratory-grown cultures. Since neither Transeau (1951) nor Randhawa (1959) mention growth in a controlled environment in the monographs published on the Zygnemataceae, it is assumed that all material surveyed by these investigators was from field collections. It is probable that the morphology of S. sticticum is slightly altered when grown in a controlled environment.

Limited information regarding the application of microchemical reagents to members of the Zygnemataceae has been found in the literature. Although the results of the staining techniques applied to

S. sticticum were relatively good, more work with cytological stains should be done. A comparative study using several species of Sirogonium is suggested by this author to enhance the validity of the reaction of the microchemical stains. The application of different techniques such as polarized light for the detection of the chondriosome, pectinase and the hydroxylamine-ferric chloride reaction (Jensen, 1962) for localization of pectic substances are suggested. The use of a micromanipulator to dissect out specific organelles might also prove extremely beneficial.

Of the metabolites studied in this research, probably the most important one is the lipid substance. In several references in the literature it is stated that large amounts of fats and oils accumulate in the gametangia and zygospores. Although gametangia have been stained, appreciable lipid substances were not denoted. Oil and fat compounds were noted in the zygospores which is in accordance with the findings of Fritsch (1935) who stated that a large amount of lipid material accumulates in the zygospores.

Attempts to observe mitotic division were made on S. sticticum. This author also applied nucleophilic stains to S. melanosporum in efforts to observe somatic division. Although there is no record of chromosomal numbers in the literature for any member of the genus, counts have been obtained for Spirogyra. Doraiswami (1946) in his work on Spirogyra columbiana Czurda noted a phenomenon at both ends

of the nucleus, referring to this formation as a polar cap. Such structures were occasionally seen in cells of S. melanosporum. No such formations were viewed in S. sticticum. The Feulgen reaction (Jensen, 1962) to detect the presence of DNA was applied to S. melanosporum with a negative result. Petter (1933) failed to obtain a positive reaction with the Feulgen treatment on Spirogyra. Godward (1950) in working with Spirogyra found that the nucleoli contain coiled structures resembling chromatic material. Such observations were noted in S. sticticum and S. melanosporum. The stained nucleoli appeared to contain a dense granular material when viewed under high magnification.

The necessity for more cytogenetic work on Sirogonium cannot be over-emphasized. Since ploidy has been reported in Spirogyra (Allen, 1958) and is presumed to occur in other members of the Zygnemataceae (Fritsch, 1935; Godward, 1956) chromosomal studies are of great importance in validating this assumption. According to Godward (1956), the problem of speciation within Spirogyra and other related genera may be resolved by the application of cytotaxonomic characters.

Sexuality. Attempts to induce conjugation in S. sticticum have been largely unsuccessful. Those cultures showing an appreciable number of stages of sexuality were few. Conjugation had apparently begun in the growth vessel rather than as a result of manipulations by the present writer.

A primary problem impeding further study of S. sticticum is the inability to induce sexuality by manipulating environmental or nutritional factors. Methods in addition to those employed by Allen (1958) and Rickert (1963) on Spirogyra are advocated in future studies. A change in the nutritional composition of various media, particularly a lowered amount of nitrogen, may induce sexual stages. The amount of dissolved oxygen in the water and the pH are possible influences on conditioning conjugation in nature. Randhawa (1959), from field observations, noted that S. sticticum attains its dominant vegetative growth at a pH of 9 and oxygen concentration of 0.9 ppm. However, conjugation only occurred under a range of pH 8-8.5. In attempting laboratory experiments with pH as a variable factor, it might be possible to control sexuality in S. sticticum at will.

The detailed observations of more stages of sexuality is definitely necessary before conclusive evidence can be presented in frequency and distribution of sterile cells, gametic formation, and reproductive morphology. One aspect that needs more thorough investigation is that of the protogynous sexual response. It is possible that this phenomenon occurs more regularly than has been noted by this writer. While the initial stages of fusion of the gametes have been observed, the ultimate combination of the two protoplasts to form a zygote has not been viewed. In S. melanosporum once the male gamete has flowed into the female gametangium, it forms a dense rounded

structure, nearly identical to the type formed in the male gametangium prior to the movement of the male gamete. It is exceedingly doubtful that this phenomenon occurs in S. sticticum.

Double conjugation, a process in which two adjacent female gametangia in one filament conjugate with two adjacent male gametangia in another filament, has been observed and illustrated by Transeau (1951). Observations of cross conjugation, a situation in which adjacent conjugants of opposite sexes are found in both filaments, have been noted by Transeau (1951) and Randhawa (1959). Neither of these types of conjugation has been observed in the current investigation of S. sticticum; however, both examples have been noted in S. melanosporum and S. phacosporum (Hoshaw, personal communication).

Zygospore Germination. Although the results of the germination of zygospores of S. sticticum from a natural collection were quite good, more study is needed on this phase of the life cycle of the alga. No percentages comparing viable and nonviable zygospores have been obtained. Zygospores formed in cultured material should be germinated and contrasted with those from a field collection. It is possible that those formed in the laboratory will develop in a slightly different manner.

Another area in which additional research is needed involves an explicit study of the ontogeny of the chloroplasts in the young

germlings. A study of this nature could entail a comparison of the ontogeny of the chloroplasts in several species of Sirogonium. If a method of marking the chloroplasts of each gamete could be devised, it would then be possible to determine the plastids responsible for the reorganization of the chloroplasts in the germling. Allen (1958) stated that the chloroplast of the female gamete reorganizes in the germling, while the plastid of the male gamete nearly disintegrates in the process of gametic fusion.

When the breakdown of the chloroplast of the male gamete of Spirogyra occurred, the only remaining material appeared as orange bodies of carotenoid granules (Allen, 1958). In S. sticticum orange granules have been noted in young germlings. When only one cell has emerged from the zygospore, this orange structure is concentrated near the tip of the apical cell and usually is partially associated with the disorganized chloroplast. As the germling elongates, the cell divides mitotically and at the 2- or 3-celled stage, these granules appear more numerous, smaller, and are located randomly in all cells. These orange or carotenoid structures have been observed in germlings of S. melanosporum (Dennis, personal communication) and S. phacosporum (Levinson, personal communication) as well as in S. sticticum. Since this granule of somewhat questionable origin has been noted in Spirogyra (Allen, 1958), it may be a characteristic of the germlings of

several or all members of the Zygnemataceae. A detailed study of several genera with reference to this structure is proposed to elucidate the occurrence and distribution of these granules.

SUMMARY

1. Clonal cultures of Sirogonium sticticum (Engl. Bot.) Kuetzing were established and maintained in Pringsheim's soil-water medium from a collection supplied by Dr. G. W. Prescott.

2. Vegetative cells from the clonal cultures were examined critically to obtain morphological data. The information compiled from this study was compared with previously cited morphological data.

3. Microchemical reagents were applied to S. sticticum. Starch, lipid substances, and pectin were denoted in the cells. Nucleophilic staining revealed heterochromatin as diffuse particles throughout the nucleus. One or two nucleoli were present.

4. Stages in sexual reproduction were observed and recorded photographically. Detailed morphological observations were made on the conjugating filaments and the results were tabulated.

5. Zygospores were germinated and the progress of their development was recorded. Attention was focused on chloroplastic reorganization and the distribution of the carotenoid granules in the cells of the young germlings.

6. Areas in which more research is needed are discussed. Such fields include the maintenance of S. sticticum in defined media

and in pure culture; the establishment of new clones from other collections to validate morphological and cytological data; the observations of more sexual stages to be able to predict a consistent pattern of conjugation; and the utilization of more intensive cytogenetic techniques to observe chromosomes and their behavior.

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