INDUCTION OF SPORE FORMATION DURING EARLY DEVELOPMENT IN BLASTOCLADIELLA EMERSONII

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ABSTRACT

INDUCTION OF SPORE FORMATION

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BLASTOCLADIELLA EMERSONII

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The purpose of the research reported in this thesis was to study the induction of spore formation in lag phase plants and its relationship to the internal and external environments of the cell. The developmental age of plants was determined by amount of growth and number of nuclei present prior to induction. The capacity of different nutrient solutions to inhibit formation of papillae was investigated. Changes in cellular morphology, polysaccharide, protein, nucleic acids, and dry weight during induction were followed. The viability of zoospores produced by lag phase plants was measured.

The number of spores released is related to the position of the nuclei in the mitotic cycle. Amino acids and nutrient medium were more effective than glucose in reversing induction of sporogenesis. The morphological and physiological changes occurring during induction of lag phase plants were similar to those observed for log phase plants, with the physiological changes occurring at a slightly accelerated rate in lag phase plants. In terms of viability and general morphology, the zoospores produced by lag phase plants resemble closely those produced by older plants.

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Sally Wood Hennessy

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
MOPS	Morpholinopropane Sulfonic Acid
OC	Ordinary Colorless (Thin-walled Sporangia)
PYG	Peptont-Yeast Extract-Glucose
RNA	Ribonucleic Acid
RS	Resistant Sporangium
TCA	Trichloroacetic Acid

INTRODUCTION

The formation of reproductive structures, whether sexual or asexual, is an important phase in the development of any organism. Sporogenesis and gametogenesis in the fungi and non-vascular plants have frequently been studied as representative systems of differentiation. Research which narrowly approaches spore formation as a static event that merely marks the termination of the development of one organism and the beginning of growth in another obscures the underlying function of the process. It is, rather, a dynamic series of events in which the organism interacts on many levels with its environment. Regardless of the complexity of its development, the cell must retain the basic potential to respond by reproduction, or the species will cease to exist in nature. Mass growth of organisms under "ideal laboratory conditions" often disguises the inherent mechanisms of survival. Through a study of one aspect of sporogenesis, the importance of the potential for spore formation in the aquatic Phycomycete, Blastocladiella emersonii, will be investigated.

The life cycle of Blastocladiella emersonii has been described in detail (Cantino and Lovett, 1964; Cantino, 1966; Cantino, et al., 1968). A swimming zoospore retracts its flagellum and encysts. A small germ tube emerges from the cyst and begins to develop into a rhizoidal system. After a lag period, the nuclei begin to divide mitotically, and the cell increases in volume, developing along one of two main pathways. In the absence of significant amounts of bicarbonate, a thin-walled

sporangium (OC) is formed which can be induced to cleave out zoospores, thus completing the cycle of development. In the presence of bicarbonate, a thick-walled resistant sporangium (RS) is formed. After an incubation period, this cell can also be induced to release zoospores similar to those produced in the OC plant.

In one of the earliest studies of spore formation in Blastocladiella, Cantino and Hyatt (1953) described the germination (in hanging drop mounts) of spores from resistant sporangia. After retraction of the flagellum and encystment, a germ tube emerged and rhizoids developed which attached the tiny plants to the surface of the cover slip from one or more points on the thalli. Often these plants were so reduced in their development that only one to four spores were released through the single papilla formed. Emerson (1941) also found that when Allomyces was grown in conditions of very limited nutrient supply, plants reaching only forty to fifty microns in height could still form reproductive structures. Out of these early works evolved many questions regarding the possible interrelationships of reproduction and development, the focal point of the research to be described herein. A series of these questions is presented below to demonstrate ideas which prompted and guided my work.

If the potential to form spores exists throughout the growth or log phase of the organism, does it also exist during the lag phase of development, before any significant increase in cell size or nuclear number occurs? Lovett (1968) reported zoospore germination in his basal salts medium but did not describe the fate of these young plants. The results of Cantino and Hyatt (1953) mentioned above suggest that lag phase sporogenesis may be possible, although perhaps not equally so for

all spores.

One might also ask whether the number of spores released at any particular time is directly related to the number of nuclei then present or more dependent upon their own cycle of replication and division. After inducing spore formation in Blastocladiella emersonii at the end of 16 hr of growth, Lessie and Lovett (1968) found that a round of nuclear division begins within ½ hr after the medium change. Similarly, gametogenesis in Chlamydomonas could occur only immediately after nuclear division (Kates and Jones, 1964). At least one mitosis must occur prior to fruiting body formation by Physarum (Sauer, et al., 1969), although this mitosis was highly asynchronous and many of the nuclei degenerated (Guttes, et al., 1961). A synchronous round of nuclear division also occurred in Plasmodiophora during the transition from vegetative to reproductive development (Williams and McNabola, 1967). No further mitoses were observed following induction of zoospore formation in Phytophthora (Williams and Webster, 1970), Gilbertella (Bracker, 1966), or Olpidium (Temmink and Campbell, 1968), nor during gametangial formation in Allomyces (Wilson, 1952; Flanagan, 1969). After examining nuclear changes in thin-walled and resistant sporangia, Flanagan (1969) suggested that the degree of nuclear division prior to spore cleavage might be related to existing environmental conditions. This problem as yet remains unsolved for sporulating cells of Blastocladiella emersonii.

Another area in which information is lacking is that of the mechanism of induction of sporogenesis. Murphy and Lovett (1966) suggest that the primary event initiating zoospore production in Blastocladiella emersonii may be "...the dissociation of cellular metabolism from

dependence upon exogenous materials..." (p. 90). The question remains as to whether this dissociation involves one or several metabolic changes. If several biochemical alterations are involved, to what extent is the triggering of spore formation reversible by addition of nutrients? The importance of the single factor nitrogen in gametogenesis in Chlamydomonas reinhardti is demonstrated by the fact that re-addition of nitrogen at any phase in the process, even after gametes are released (both prior to and after gametic copulation), readily causes dedifferentiation to the vegetative state (Jones, 1970). In their review of cellular differentiation, Padilla and Cameron (1968) stress that complex interactions between the nucleo-cytoplasm and the environment govern the degree of reversibility in differentiation. The "point of no return" in fruiting body formation by Physarum polycephalum occurred hours before the initiation of morphological change (Sauer, et al., 1969). In refeeding experiments they also observed that glucose alone merely blocked sporulation; transfer to fresh nutrient medium was necessary for resumption of normal plasmodial growth. After their extensive studies on commitment to resistant sporangial path of Blastocladiella emersonii, Cantino and Goldstein (1962) found that the physiological "point of no return" for this event by no means involved only a single metabolic change, despite the fact that differentiation was triggered by the presence of bicarbonate in the medium. They noted that "the underlying biochemical events, if each of them could be viewed and taken separately, would probably have their points of no return dispersed over a much broader range of time and physiological age, each with its own degree of 'indispensableness,' and each, if rendered inoperative, contributing a certain degree of

somatic 'hybridity' to the protoplast" (p. 646). The possibilty that a similar series of biochemical "points of no return" is involved in the induction of zoospore formation by Blastocladiella emersonii remains to be explored.

Still another facet of the overall problem of induced sporogenesis which deserves attention concerns the possibility that differences in morphological and physiological changes occur during zoospore formation by log as opposed to lag phase plants. Related to this is the question of whether the spores produced after different periods of plant growth have the same potential for development. The early studies described previously, which suggested the ability of very young or poorly nourished plants of aquatic fungi to reproduce, did not speak to these questions. Lovett (1968) noted that salt-germinated spores of Blastocladiella apparently did not increase their RNA content, but he did not analyze them further. Although various amounts of growth have been allowed before inducing zoosporogenesis in Blastocladiella emersonii (12 hr and 20° C - Soll, 1969; 16 hr and 24° C - Murphy and Lovett, 1966; and 18 hr and 22° C - Suberkropp, personal communication), few differences in the zoospores produced have been noted. One possible area of conflict lies in the amount of DNA per spore as reported by Lovett (1963) and Myers and Cantino (1971); this discrepancy has not been linked to variations in growth time or temperature. However, when RS plants of Blastocladiella emersonii are matured for different lengths of time and then induced to form spores, the spores do behave differently (Cantino, 1969).

The mechanism of reproductive structure formation as relates to the interactions between the environment and the morphological and

physiological age of the cell is a fertile area for study where many questions remain unanswered and many are yet to be asked. Zoospore differentiation in Blastocladiella emersonii is an excellent system through which to probe these questions. Many of the key events of the life cycle of this organism have been well defined (Cantino and Lovett, 1964; Cantino, 1966; Cantino, et al., 1968); a good foundation has been laid upon which to build new information and from which to formulate new interpretations. The research to be discussed in this paper deals mainly with the induction of sporogenesis in young, uninucleate plants: its reversibility, morphology, physiology, possible relationship to the nuclear and developmental life cycles, and, to a lesser extent, its final products. This study is not presumed to provide a definitive picture of this aspect of cellular differentiation; its purpose is rather to present a different approach to a familiar problem in order to reveal new aspects of its complexity.

LITERATURE REVIEW

The differentiation of reproductive sturctures and/or cells from vegetative ones has long been an area of great interest to mycologists, as well as other biologists. As early as 1900, Klebs hypothesized that the external conditions which favored these two phases were different, and that environmental conditions favorable to reproduction are more specific. He further suggested that some minimal period of vegetative growth was a necessary prerequisite for reproduction. Thus, long before sophisticated techniques for studying the physiology of differentiation were available, it was clear that the intitiation of reproduction involves an interaction between the internal and external environments of cells.

Induction of Spore Formation

One of the most frequently used methods of inducing formation of asexual reproductive structures, both past and present (Sparrow, 1960; Hawker, 1966), involves removal or limitation of the availability of some external factor, often a nutrient (Morgan, 1967). For the green alga, Chlamydomonas reinhardi, the depletion of exogenous organic nitrogen is the key factor involved in gametogenesis (Sager and Granick, 1954). This was later verified by Kates and Jones (1964) and extended to include inorganic forms of nitrogen. Williams and McNabola (1967) suggested that lack of adequate energy precursors and carbon sources was important in induction of sporogenesis in Plasmodiophora brassicae, while depletion of some component in peptone was indicated in research by Mitchell and Yang (1966) with Aphanomyces euteiches. Any of the nutrient factors which helped to maintain steady-state growth conditions in liquid cultures

of Anabaena cylindrica could prevent spore formation (Fay, 1969).

In many instances, more than simple deprivation of nutrients is necessary for completion of spore formation. The slime mold, Physarum polycephalum, requires exposure to light and nicotinic acid, in addition to more than four days of starvation, to begin the formation of fruiting body structures (Guttes, et al., 1961). Although some studies with the fungi show that spore formation is directly related to replacement of nutrient media with water (Blondel and Turian, 1960; Sparrow, 1960; Renaud and Swift, 1964; Hawker, 1966; Moore, 1968; Flanagan, 1969), the possible involvement of other factors, such as ions, cannot be dismissed. Llanos and Lockwood (1960) may have unknowingly supported this hypothesis by showing that distilled water alone could not induce sporogenesis in Aphanomyces euteiches.

Hypotonic salt solutions have largely replaced water as induction media for sporogenesis, because they give more reproducible results (Machlis, 1953a; Murphy and Lovett, 1966). Water alone gave very asynchronous spore release in Blastocladiella emersonii, whereas calcium specifically induced rapid, synchronous sporulation (Soll, 1970). Mitchell and Yang (1966) found that calcium was also essential for greatest amount of zoospore release in Aphanomyces euteiches. Sensitivity of Anabaena cylindrica to various sporulation ions is dependent upon age; phosphate ions are inhibitory to spore formation by young cultures, while older ones tolerate higher concentrations (Wolk, 1965). In addition to ions, exposure to light (Hohl and Hamamoto, 1967) and cold (Chapman and Vujicic, 1965) induces differentiation of spores in some Phytophthora species. Although there are numerous details available

concerning the role of external factors in initiating or synchronizing spore formation, the exact mechanisms are as yet unclear or unknown.

The Morphology of Differentiation

The internal changes which occur during sporogenesis are better understood than the external changes which initiate the process, primarily because the electron microscope has come into common usage and has allowed detailed studies of internal changes. The morphological differentiation of reproductive structures, especially asexual spores of the lower fungi, includes three distinct yet interrelated areas: vacuolar or vesicular changes, alteration of the plant cell wall, and organellar development (including flagellar formation). Although the order and extent of these changes are not the same for all fungi, their general nature is quite similar.

Vacuolar and Vesicular Changes. In cells which are about to undergo spore production, two distinct types of vacuole-like bodies have been described. The first is a large central or perhaps acentric vacuole described for Allomyces x javanicus, A. arbusculus, and A. anomalus (Ritchie, 1947), Phytophthora parasitica (Hohl, et al., 1966), Phytophthora capsici (Williams and Webster, 1970), and Saprolegnia terrestris (Howard and Moore, 1970), and Saprolegnia ferax (Gay and Greenwood, 1966). The central vesicle in Saprolegnia ferax is distinctive in that it appears to contain many small particles. This central vesicle, the first sign of zoospore production in Phytophthora parasitica, is formed through the coalescing of smaller cytoplasmic vesicles (Hohl and Hamamoto, 1967). In Saprolegnia terrestris, this central vacuole enlarges

prior to cleavage and forms furrows which penetrate the surrounding cytoplasm in much the same manner as in Allomyces. This role seems to be accomplished by other vesicles in the Phytophthora species, although disappearance of this vacuole at the time of cleavage was noted by Williams and Webster (1970).

The role of separating the individual spores, each with its own plasma membrane, is more often credited to smaller bodies called cleavage vacuoles or vesicles (because of stainable material with them). These structures have been reported in several Allomyces species (Blondel and Turian, 1960; Moore, 1964; Renaud and Swift, 1964; Moore, 1968), Phytophthora parasitica (Hohl, et al., 1966), Phytophthora infestans (Elsner, et al., 1967), Albugo candida (Berlin and Bowen, 1964), Olpidium brassicae (Temmink and Cambell, 1966), Plasmodiophora brassicae (Williams and McNabola, 1967), and Gilbertella persicaria (Bracker, 1966), and may have been what Chambers and Willoughby (1964) referred to as the "spongy ground material" separating the spores of Rhizophlyctis rosea. At the time of cleavage, these vesicles, which are often already present in the cytoplasm in large numbers, begin to widen and fuse, forming distinct "lines" along which eventual separation of the spores will occur. The exact source of these vesicles is not known. Hohl and Hamamoto (1967) suggest that those present in Phytophthora may arise from a Golgi-dictyosome complex. In Allomyces, where no Golgi apparatus has been identified, the vesicles may arise from a pinching off of the cell membrane (Renaud and Swift, 1964) or the cisternae of the endoplasmic reticulum (Moore, 1964). The problem of whether the extension of the cleavage plane is furthered by continuous formation and fusing of new vesicles (Blondel and Turian,

1960) or by simple elongation of pre-existing vesicles (Bracker, 1966) has not been resolved. As cleavage is completed, newly-formed spore membranes coalesce with the plasma membrane of the sporangium, leaving the spores completely free from the cell wall.

The Sporangial Wall. The formation of one or more crosswalls separating the sporangium from other non-reproductive cells is a common phenomenon in eucarpic fungi. Ritchie (1947) described the process in Allomyces x javanicus, A. arbusculus, and A. anomalus as beginning almost an hour before the sporangium reaches its completed form. According to him, "micelles" or bundles of wall material were laid down with their axes tangential to the septum in an orderly manner proceeding from the sporangial wall to the center of the cell. In a similar manner, Chapman and Vujicic (1965) described the crosswall of Phytophthora as composed of thickened regions of the inner wall, with aggregates of electron-dense material between. At its completion, the crosswall appeared to bulge into the sporangium. Lessie and Lovett (1968) also recognized crosswall formation as one of the early events in zoospore differentiation in Blastocladiella emersonii, as did Gay and Greenwood (1966) for Saprolegnia ferax.

A major change in the wall of the developing sporangium is formation of one or more papillae. In Blastocladiella emersonii, this involves a localized breakdown of the cell wall and deposition of papillal material by "secretory granules" (Lessie and Lovett, 1968). The papillal material or plug in Allomyces x javanicus is composed largely, if not completely, of pectic substances (Skucas, 1966). Vesicles containing granular material were also found in the region of spore formation in gametangia of Saprolegnia

terrestris, although no plug material is present (Howard and Moore, 1970). The development of the exit tube in Olpidium (Temmink and Campbell, 1968) involves a bulging of the thallus wall, followed by an accumulation of "spongy material" (perhaps related to tiny vesicles found in other fungi) which enlarges and increases the swollen area of the sporangial wall. At the same time as the three layers of wall burst, the material within the bulge expands and forms a solid plug in the mature exit tube. The plasmalemma of the sporangium in Phytophthora capsici becomes undulate and fuses with vesicles which concentrate along its surface (Williams and Webster, 1970). King, et al., (1968) suggested that this fusion could result in the accumulation of enzymes prior to degradation of the papilla. In another species of Phytophthora, Hohl and Hamamoto (1967) noted the presence of fibrous material within the papilla which seemed to develop from a layer of small flat vesicles just beneath. In all cases where plugs are present, they are pushed out or dissolved away immediately prior to zoospore release.

Organization of Organelles and Flagellum Formation. As the differentiation of reproductive structures proceeds, the cytoplasm and organelles of the formerly vegetative cell must be reorganized to form many individual cells, each capable of functioning as an independent unit. Detailed studies of the process have been made in several lower fungi. The single, most central event is the formation of one or more flagella. The process has been described in some detail for Phytophthora capsici by Williams and Webster (1970). The paired centrioles begin to elongate early in zoospore development, even before disappearance of the central vesicles, to form kinetosomes which extended through the cytoplasm

to a length of .66 µ. At the same time, vacuoles and vesicles fuse to form an axonemal vesicle in which the flagellum will be formed. A terminal plate of the kinetid develops from a thickening in the membrane of the axonemal vesicle, resembling a double-concave lens. Near this plate, the kinetsome takes on the 9+2 arrangement of the flagellum, and its inner fibrils progress through the plate to form the axonemal cylinder within the expanding vesicle. Bundles of microtubules are connected to the centriolar base of the kinetosome and extend upward along the outer margins of the nucleus. The entire process is completed soon after cleavage. Studies by Hohl and Hamamoto (1967) with P. capsici and King, et al., (1968) with P. infestans substantiate the above findings, and add the observation that flagella always lie at the periphery of the sporangium, near the sporangial wall, thus suggesting that the cleavage planes form between the vacuoles in which flagella lie. Albugo candida has a method of flagellar development similar to that described for Phytophthora (Berlin and Bowen, 1964). The role of paired centrioles in Saprolegnia ferax has been discussed by Heath and Greenwood (1970).

Renaud and Swift (1964) found a similar mechanism for flagellar formation in the gametes of Allomyces arbuscula. In this organism, however, only one flagellum is produced per gamete, as opposed to the pair of flagella in Phytophthora. In Allomyces gametangia, the two centrioles are not equivalent, for only the large centriole becomes associated with the primary vesicle and is actively involved in flagellar formation. The basal plate, also observed in Phytophthora, develops as the flagellum begins to form. Blastocladiella emersonii, a close relative of Allomyces, shows a similar method of flagellar differentiation (Lessie and Lovett,

1968), with additional presence in the centriolar area of a banded rootlet, the exact role of which is not yet known. A similar organelle in B. britannica is closely connected to the flagellar apparatus (Cantino and Truesdell, 1971). In contrast to the peripheral location of flagella in Phytophthora, the flagellum of Rhizophlyctis rosea (Chambers and Willoughby, 1964) seems to be coiled around the developing zoospore. Thus, despite individual idiosyncrasies among the various genera, the mechanisms of flagellar formation in the spores of lower fungi show many basic similarities.

The formation and reorganization of other internal structures characteristic of spores of the lower fungi have not been studied in the same depth as flagellar formation. Changes in mitochondria have been noted for Phytophthora capsici (Williams and Webster, 1970), Saprolegnia ferax (Gay and Greenwood, 1966), and Blastocladiella emersonii (Lessie and Lovett, 1968). The distribution of the mitochondria in Phytophthora involves a movement of several organelles to a position peripheral to the nuclei of the developing spores during the early stages of cleavage. In Saprolegnia, a fragmentation and rounding up of mitochondria occurs soon after induction of sporangium formation. Alterations in the internal structure of mitochondria were noted at the time of cleavage, the microvilli becoming more straight, unbranched, uniform in length, and peripheral, not extending into the center of the organelle. In contrast, the mitochondria of Blastocladiella fuse after the cleavage of individual zoospores to form a single mitochondrion. Just prior to zoospore release, it becomes positioned around the basal body and rootlet, peripheral to the nuclear apparatus.

Lessie and Lovett (1968) have also described the accumulation

of alpha and beta polysaccharide granules around mitochondria at the time of induction, with some of the smaller beta particles lying inside the organelle. Their role, if any, in zoospore formation is not known. Another structure described in developing sporangia is the lipid body. In Allomyces macrogynus (Blondel and Turian, 1960) and Blastocladiella emersonii (Lessie and Lovett, 1968), several lipid droplets within a limiting membrane (Cantino and Truesdell, 1970) form a ring (or "lipid crown") around each of the nuclei in the early stages of sporangial development. Temmink and Campbell (1968) reported the accumulation of lipid in multivesicular bodies, yielding round lipid droplets, as zoospore formation progressed in Olpidium.

The formation of the nuclear cap is one of the most dramatic changes during zoospore development in Blastocladiella emersonii (Murphy and Lovett, 1966; Lessie and Lovett, 1968) and Allomyces (Blondel and Turian, 1960; Moore, 1968). By the time cleavage is completed, each nucleus is invested with a prominent basophilic cap bound by a double membrane. Soon after induction, the ribosomes, distributed throughout the cytoplasm in growing plants, begin to aggregate. The ribosomes are enclosed by a double membrane formed through the fusion of many small vesicles just prior to the end of zoospore differentiation.

The theme of the balance between basic patterns and distinct specialization is thus aptly demonstrated by morphological changes during the differentiaion of reproductive structures in lower fungi.

The Physiology of Spore Formation

Few intensive studies have been made on the physiological changes

which occur during differentiation of reproductive structures. An alteration in respiration may accompany spore formation. Turian and Chodat (1959) found a rapid rise in O_2 uptake at the time of gametic cleavage in Allomyces macrogynus. In Blastocladiella emersonii, both OC (McCurdy and Cantino, 1960) and RS (Cantino, 1961) spores showed higher levels of oxygen consumption than their respective sporangia. The exact time of the change in respiration is not yet known. Fay (1969) also reported a higher level of endogenous respiration in spores of Anabaena cylindrica and suggested that it might be related to the spore's capacity to mobilize reserve materials more rapidly than vegetative cells.

The increasing emphasis on the role of protein synthesis and breakdown in differentiation is reflected in several studies on the formation of reproductive structures. Jones (1970) found that gametic differentiation in Chlamydomonas reinhardti was accompanied by a 100% increase in protein breakdown during the first four hours after induction. Although a cessation of net protein synthesis and a greatly reduced rate of incorporation of CO_2 into amino acids occurred soon after induction, there was some incorporation of amino acids into protein. Jones thus hypothesized that some degree of protein turnover, used in the formation of specific new protein, was required for gametic differentiation. Through the use of actidione (cycloheximide), an effective inhibitor of protein synthesis in Physarum polycephalum, Sauer, et al., (1969) showed the need for protein synthesis throughout induction until the first morphological signs of fruiting body formation appeared. An increase in soluble protein seems to occur during the formation of spores from RS in Blastocladiella

emersonii (Cantino, 1961). Protein synthesis in OC plants increases for one hour after induction, followed by a sharp decrease prior to spore release (Murphy and Lovett, 1966). Active uptake of arginine begins at about 60% of the germination time and new arginine-rich protein fractions associated with nucleic acids emerge in mature cells preparing to form spores (Dounas and Cantino, 1965b). The possible role of these histone-like proteins in zoospore development and formation of the nuclear cap was discussed.

Changes in nucleic acid synthesis during zoospore differentiation were studied extensively by Murphy and Lovett (1966) for Blastocladiella emersonii. The total DNA and RNA per cell reached its maximum level about one hour after induction and then began to decrease, as reflected in the loss of uracil label. Inhibition studies with Actinomycin D revealed that normal papilla formation requires synthesis of specific RNA at least thirty minutes prior to its appearance and that the RNA directing cleavage and discharge of the spores is probably formed at least one hour before this event. The use of Actinomycin D with cultures of Physarum polycephalum gave similar results, indicating that synthesis of RNA for sporulation occurs several hours prior to fruiting body formation. Nucleic acid analogs lost their effectiveness in stopping differentiation by blocking DNA synthesis at least four hours prior to the time of critical RNA synthesis (Sauer, et al., 1969). DNA synthesis necessary for gametic differentiation in Chlamydomonas was closely related to a requirement for protein synthesis (Jones, 1970).

The process of differentiation of reproductive structures appears to involve a complex system of metabolic changes in which some cell

constituents are broken down and other compounds are formed in response to the specific new needs of the cell.

MATERIALS AND METHODS

In investigating the potential for spore formation in Blastocladiella emersonii and its relationship to the morphological and physiological state of the developing plant, three different approaches were used. Each methodology will be discussed separately, with details given where particular analyses were used. Unnecessary repetition can thus be avoided and a clear understanding of the research technique expedited.

General Procedures

Cultures used to obtain spores for inocula were maintained on PYG agar. Each agar plate (10 cm diameter) was inoculated with 1 ml of spore suspension in PYG broth (Difco) at a density of approximately 1-1.5 x 10^5 spores per ml. Spore density was determined in triplicate with a Model B Coulter Counter with electrolytes NaCl and KCl at 1 x 10^{-2} M each and at threshold settings of 20 (lower) and 100 (upper), and corrected for coincidence. Plates were incubated in the dark approximately 22 hr at 22° C. Spore inocula were obtained by flooding the plates with approximately 5 ml water and pipetting the resulting suspension through sterile filter paper (Whatman No. 4). The spores were chilled for 10-15 min in an ice bath before use.

Nuclear Division Synchrony

One liter of PY½G-P liquid medium (yeast extract - 1.25 g; glucose - 1.5 g; KH_2PO_4 - 0.33 g; K_2HPO_4 - 0.43 g; and water - 1000 ml) in a two liter flask was inoculated with 1.5 x 10^7 spores. Population density was determined after filtration. Prior to and following

inoculation, the culture was aerated at 7.5 liters/min with line air which had been bubbled through water twice to increase humidity and remove impurities. The culture was grown for eight hr under apporximately 350 footcandles of visible light at 22° C. Samples for staining of approximately 2 ml were removed every 15 min, fixed with an equal amount of 10% formaldehyde in 10^{-2} M morpholinopropane sulfonic acid (MOPS) buffer, adjusted pH 6.8, and stored at 7° C.

The plants were concentrated for 3 min (800 g in an ICC Clinical Centrifuge) and washed twice with water. A few drops of each plant suspension were placed on a microscope slide and the water allowed to evaporate. Slides were placed in 1 N HCl for 15 min at 55° C, stained with Azure A (0.5 g/100 ml) for one hr, and excess dye removed by a brief wash in water. Specimens were dehydrated in 95% ethanol, 100% ethanol, and xylene, mounted with glycerin, and observed under oil immersion at 450X with a Leitz microscope. The blue stained nuclei were clearly distinguishable within the unstained cells. Four experiments were run and approximately 100 plants were counted in each to determine the average number of nuclei at each sampling time.

Induction of "Early" Sporogenesis

In order to determine the kinetics of induction and reversal of spore formation in young germlings, approximately 10^7 spores were added to prechilled flasks containing 25 ml of sterile PYG broth. Flasks were swirled gently to assure even distribution of the spores, their contents poured into a large sterile glass Petri dish (14 cm diameter) containing 5 microscope slides, and the plants allowed to grow on the slides for specific amounts of time, as determined by the nature of

the experiment.

Evaluation of the induction of sporogenesis after different periods of growth was made by removing replicate slides after four ½ hr intervals following inoculation and placing them with sterile technique in phosphate buffer (5×10^{-4} M K_2HPO_4 and 5×10^{-4} M KH_2PO_4; pH 6.8) for ten min to remove growth medium. The slides were then transferred to new sterile plates containing 25 ml of the same buffer (hereafter referred to only as phosphate buffer). The completion of the slide transfer is the zero time for all experiments on the kinetics of induction. Triplicate experiments were run for each ½ hr growth interval.

To determine the kinetics of papilla formation after varying growth periods, slides were removed at ½ hr intervals, and 200 randomly selected plants were scored for the presence or absence of papillae under a Wild phase microscope at 600X.

In experiments involving the reversibility of sporogenesis, slides were prepared as described above and placed in the induction solution (phosphate buffer) after one hr of growth. After ½ hr intervals (up to 2 hr) after induction, pairs of slides were transferred to a Petri dish containing 25 ml of either glucose (3 g/l; pH 7.0), Casamino Acids (0.5 g/l; ph 6.7), or PYG broth (ph 6.8). A separate dish was used for each pair of slides to prevent any interaction between plants in different stages of spore formation. Four hr after induction, when normal papilla formation has reached 90% or above, one of each pair of slides was placed in 5% formaldehyde in 5×10^{-3} M MOPS for later counting. The other slide was immediately scored for papilla formation in the manner described for induction kinetics. The Coplin jars containing the fixed slides

were sealed and stored overnight at 7° C to be counted the next day. Duplicate experiments were conducted for each type of nutrient solution.

Physiological Analyses

The physiology of spore formation by lag phase plants was studied for comparison with data obtained for plants which had completed the exponential phase of growth (Murphy and Lovett, 1966). Large, sterile, glass Petri dishes (without slides) were inoculated with spore suspension as described for the induction studies. After one hr, PYG broth was decanted, and the plants adhering to the glass surface (Turian and Cantino, 1960) were gently washed twice with 12 ml of sterile phosphate buffer. Twenty-five ml of phosphate buffer were then added to each plate. Samples for analyses were collected after four ½ hr intervals after induction by pipetting off approximately 23 ml of the buffer solution, gently scraping the plates with a rubber policeman to lossen cells from the glass surface, and pipetting the resulting plant suspension into centrifuge tubes prechilled to 2-4° C. The suspension was centrifuged at 2-4° C at 1600 g for five min, the plants resuspended in 3 ml of fresh buffer, and the cells then gently sonicated in an ice bath (10 sec. at setting three on a Model W140-D Sonifier Cell Disruptor) to separate clumps. A 0.5 ml sample was immediately removed and fixed with 0.5 ml of 10% formaldehyde in 10^{-2} M MOPS for determination of plant density by hemocytometer. The remaining plant suspension was sonicated in the ice bath at setting 5 for three min and a small sample was examined to insure total breakage. The sonicate was extracted with cold trichloroacetic acid (TCA) (final concentration, 5%) for one hr, centrifuged for 5 min;

the TCA soluble extract was removed, the pellet washed three times with 3 ml samples of 5% TCA, and centrifuged. All supernatant solutions were combined and mixed with an equal volume of 95% ethanol. The resulting precipitate was dissolved in 3 ml of 0.6 N HCl, and the solution was heated for 2 hr at 95° C, placed in small Petri dishes, and dried in a vacuum descicator (with a dish of KOH pellets). After three days, the residue in the dishes was dissolved in distilled water and analyzed for glucose by the Glucostat Method (Washko and Rice, 1961). The cold TCA insoluble fraction was resuspended in 1 ml of 1N NaOH and heated for 10 minutes at 90° C. The solution was analyzed for protein with the Folin phenol reagent (Lowry, et al., 1951). Bovine serum albumin (0.5 mg/ml) was used as a standard. In assays for nucleic acids, the cold TCA insoluble pellet was heated at 90-95° C for 20 min in 2 ml of 5% TCA. The extract was centrifuged, the supernatant solution collected, and the pellet re-extracted with 5% TCA. The two supernatant solutions were combined and analyzed with the orcinol reagent (Dische, 1955). Purified yeast nucleic acid (0.1 mg/ml) was used as a standard.

Dry weights were obtained by filtering the non-disrupted plant suspension through pre-washed filter paper discs (Whatman No. 1), and drying the discs at 80° C in a vacuum oven to constant weight (within 0.1 mg at three successive weighings). Triplicate experiments were made for each time period and each type of analysis.

Viability of Spores

Cultures were prepared and induced as described for the physiological anlayses. The plants remained five hr in the phosphate buffer. The plates

were then swirled gently, the spore suspension pipetted off, filtered through sterile paper (Whatman No. 4), and chilled at 2-4° C for 15 min. A sample was fixed to determine spore density, and 0.1 ml diluted with 25 ml of PYG broth for inoculation of PYG agar plates. The plates were stored overnight at 22° C and scored for total plants. Triplicate tests were made.

Spores used for electron microscopic studies of morphology were obtained as described above, concentrated by centrifugation (1600 g), and fixed with 2% gluteraldehyde and 1% OsO_4 in 10^{-3} M sodium cacodylate and 5×10^{-3} $CaCl_2$. Cells were then dehydrated in an alcohol series, treated with propylene oxide, and embedded in Epon. Sections were cut on a Porter - Blum MT-2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Phillips 100 Electron Microscope. Concentration, fixation, and dehydration were carried out at 2-4° C.

RESULTS

Nuclear Division Synchrony

The synchrony of the first two nuclear divisions in young plants of Blastocladiella emersonii growing in liquid culture was analyzed in terms of average number of nuclei per cell. The results in Figure 1 indicate a decrease in synchrony in the second division with respect to the first division, the division of two nuclei into four taking approximately ½ hr longer to complete. The length of the initial lag period before mitosis was consistently 2½ hr. One possible cause of the decrease in synchrony at the second nuclear division was the asynchronous manner in which the two nuclei divide, the presence of odd numbers of nuclei being repeatedly noted at several sampling intervals (Figure 2).

Induction of "Early" Sporogenesis

Through variation of the germination solutions, the sporulation solutions, and the growth periods of germlings (See Appendix B), young plants could be induced to form small sporangia from which either one or two spores would be released.

Morphology. The morphological changes accompanying zoospore formation by young plants are essentially like those described by Lessie and Lovett (1968) for log phase plants which had been induced to sporulate. For the first 30-60 min after replacement of the nutrient medium with the phosphate buffer, there is little change in the appearance of the plant, although some increase in size is observed (Figure 3). Soon afterward, the cytoplasm takes on a darker, more granular appearance, and a large

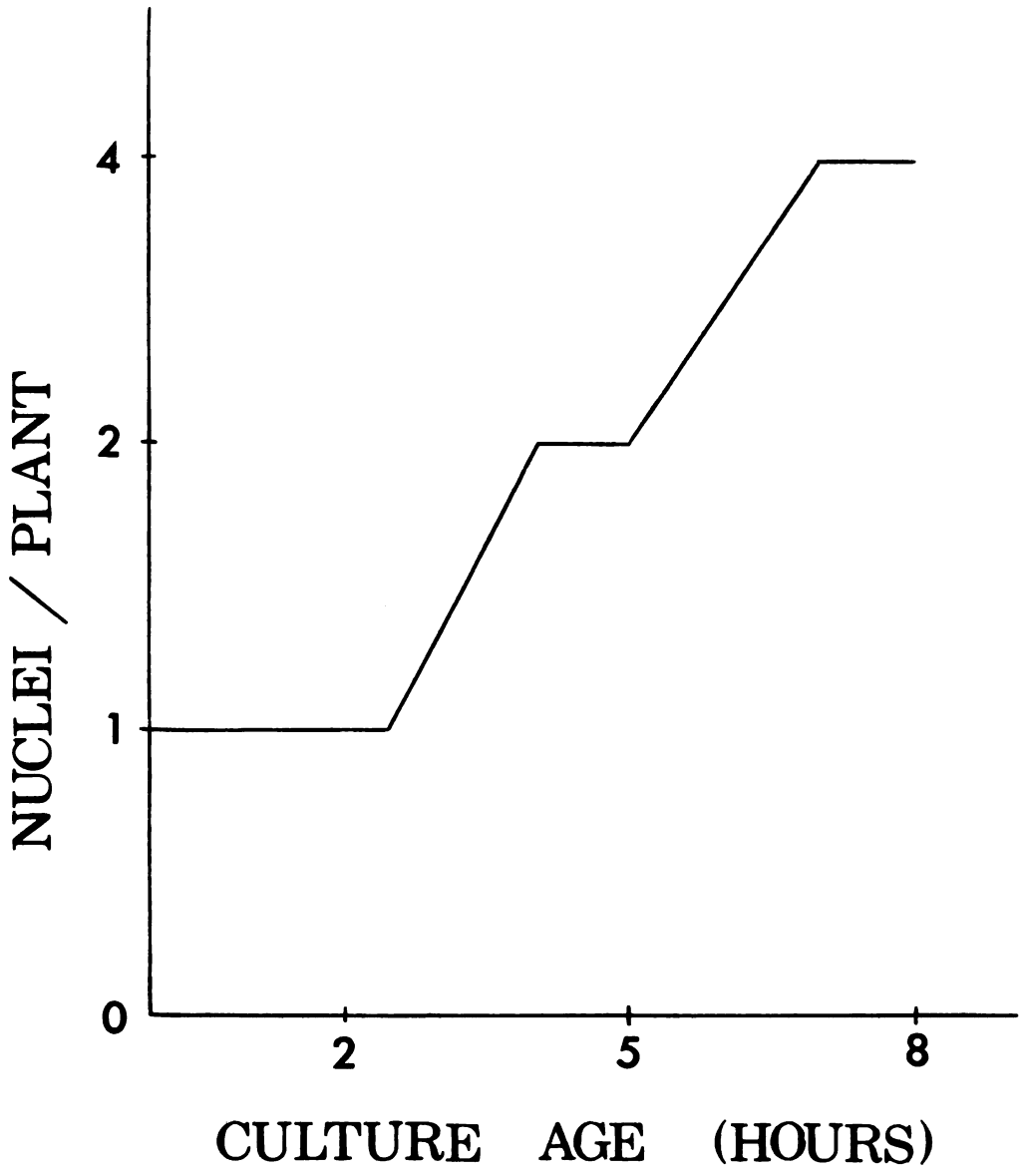


Figure 1. Nuclear Division Synchrony

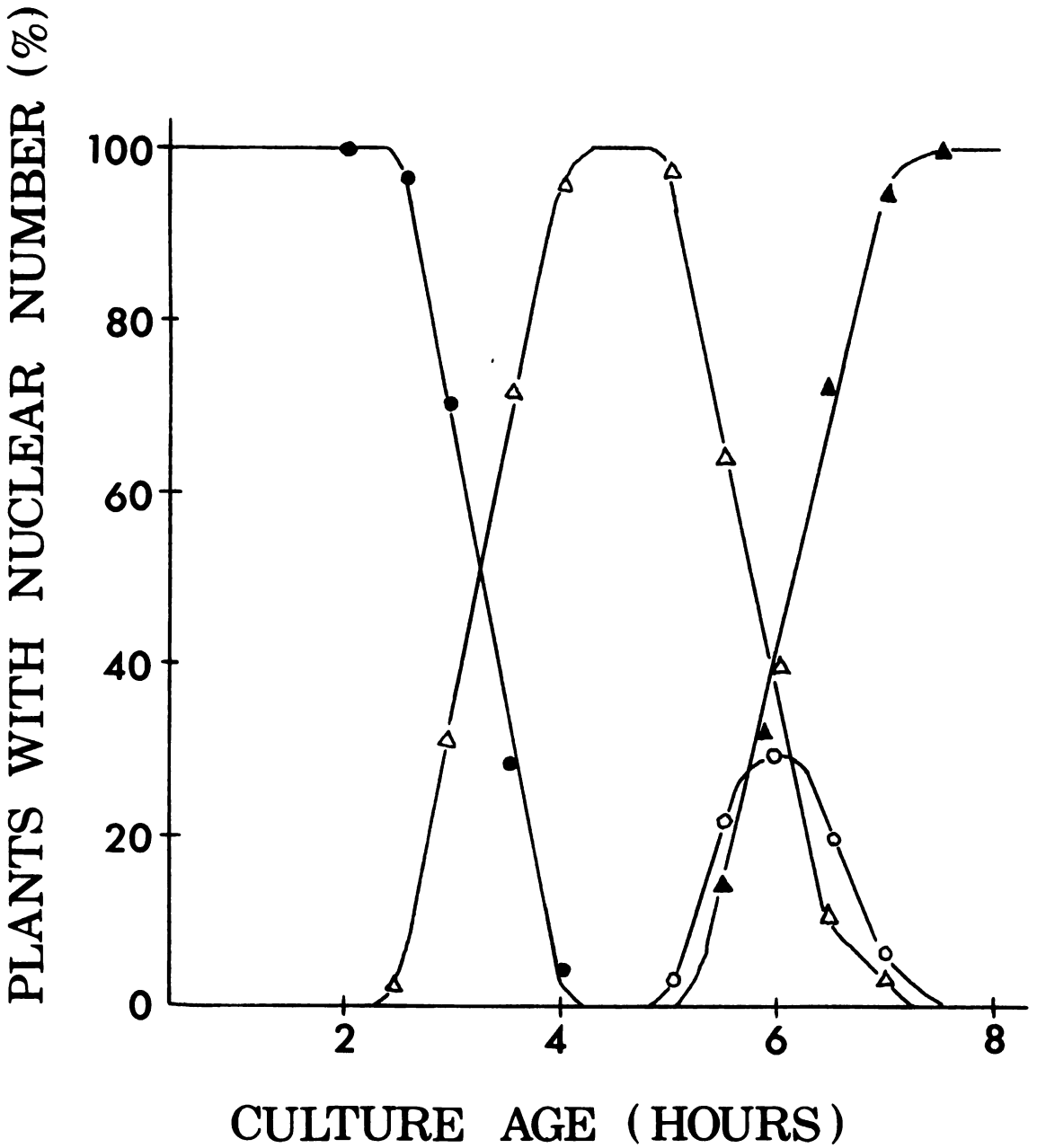
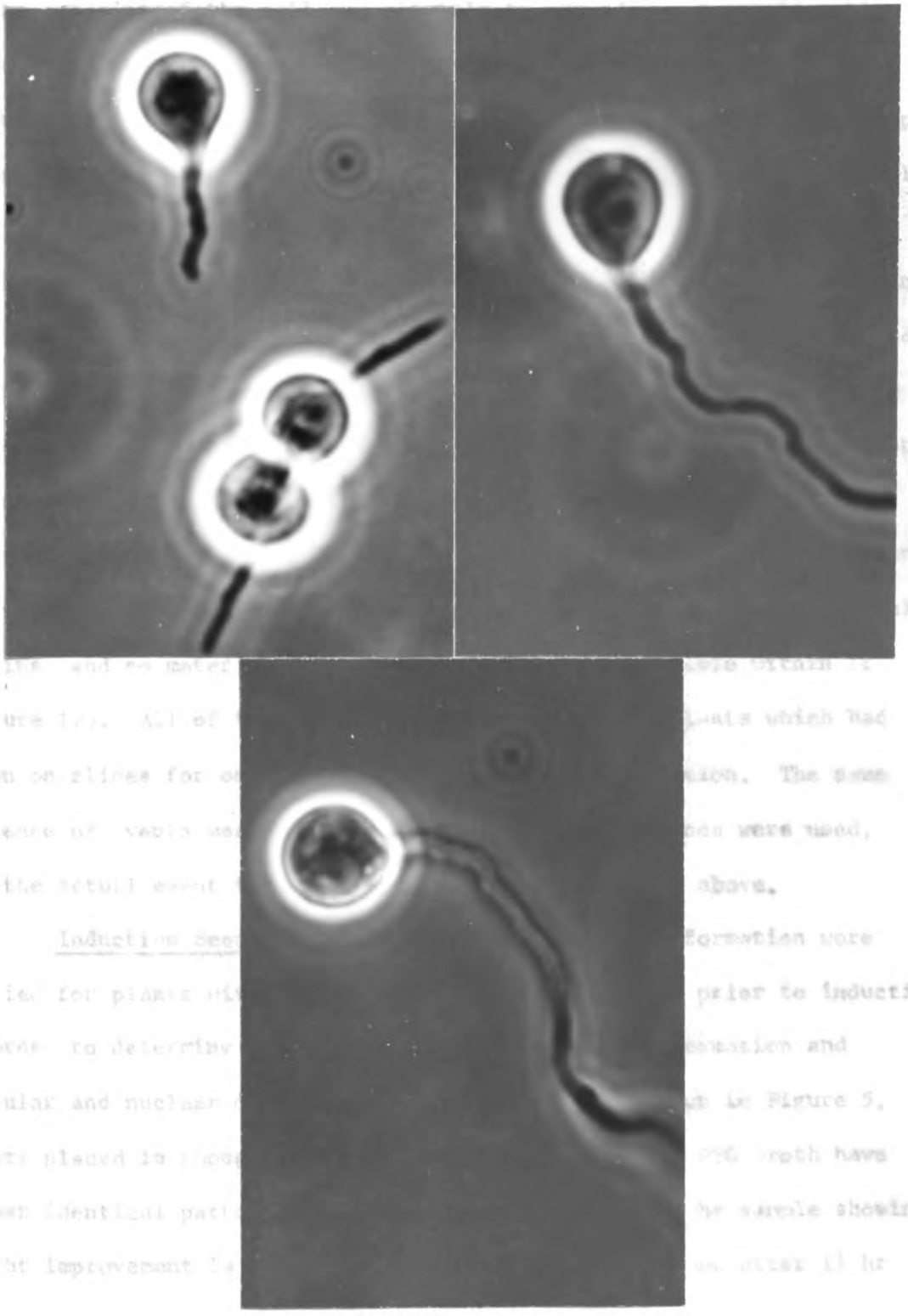


Figure 2. Changes in Nuclear Number
(●---● - one nucleus; △---△ - two nuclei;
○---○ - three nuclei; ▲---▲ - four nuclei)

Figure 3. Induction of Young Germlings
(Germlings at Zero Time, upper left; Germlings After ½ hr in Buffer, upper right; Germlings After 1 hr in Buffer, lower center.
All light micrographs in this thesis were taken with Kodak High Contrast Copy Panchromatic Film, HC 135-36, on a Wild Phase Microscope. All germlings are approximately 8-10 μ in diameter.)



vacuole can be seen, sometimes centrally located, but more often occurring at the anterior of the cell and slightly to one side. A relationship between the location of the vacuole and the area of papilla initiation was not observed. After 30-45 min the vacuole disappears and the cytoplasm becomes clear; the nucleus is again visible as a dark disc (the nucleolus) within a lighter one (the nucleus). By this time a small refractile "crosswall," separating the branched rhizoid from the developing sporangium, is visible. Within 30 min, the first papillar initials are evident, and the papillae (one per cell) expand until they reach a size of one third to one half the diameter of the tiny plants. Also, the ring of lipid droplets around the nucleus can be seen. The zoospore rounds up and becomes separated from the plant cell wall (Figure 4). By 4 hr after induction, the release of a single zoospore per plant is initiated. The plant wall remains, and no material from the original cell is visible within it (Figure 12). All of these observations were made on plants which had grown on slides for one hr in PYG broth prior to induction. The same sequence of events was observed when other growth periods were used, but the actual event times varied from those described above.

Induction Sequences. The kinetics of papilla formation were studied for plants with varying exposures to PYG broth prior to induction in order to determine the relationship between spore formation and cellular and nuclear development. The results are shown in Figure 5. Plants placed in phosphate buffer after 1 and 1½ hr in PYG broth have almost identical patterns of papilla formation, the 1½ hr sample showing slight improvement in synchrony. However, plants induced after 1⅔ hr occasionally released two spores rather than only one. Induction of spore

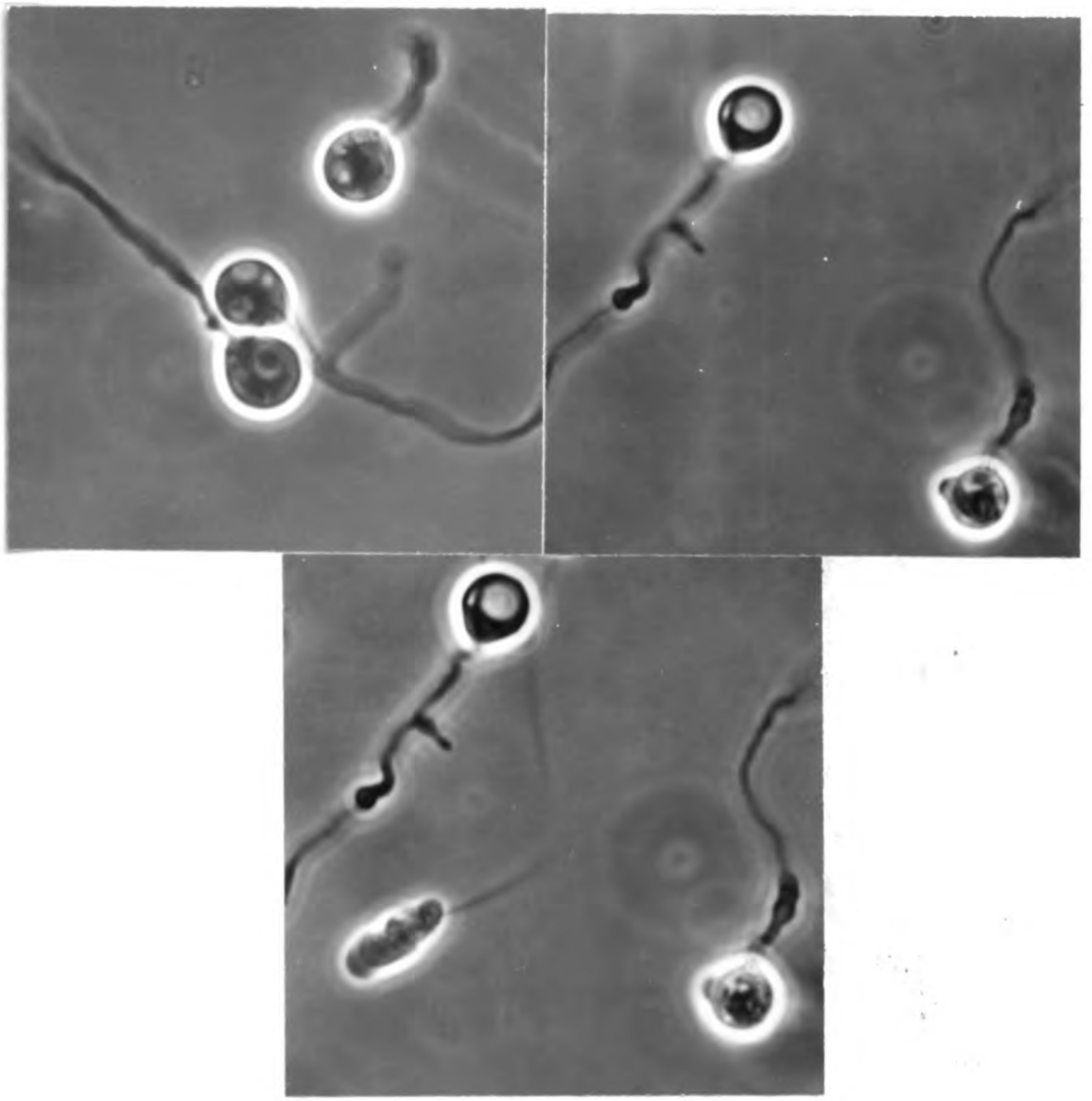
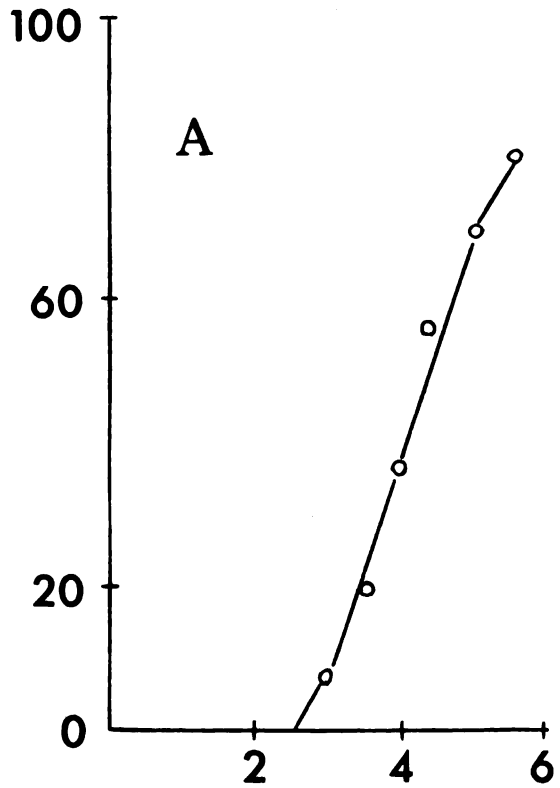


Figure 4. Morphology of Spore Formation
(Germlings After 1½ hr in Buffer, upper left;
Germlings at the Time of Initiation of Zoospore
Release, upper right and lower center)

Figure 5. Kinetics of Papilla Formation
(Veritical Axes - Papilla %; Horizontal Axes - Time in Buffer - Hours; A. - After ½ hr in PYG broth; B. - After 1 hr; C. - After 1½ hr; D. - After 2 hr)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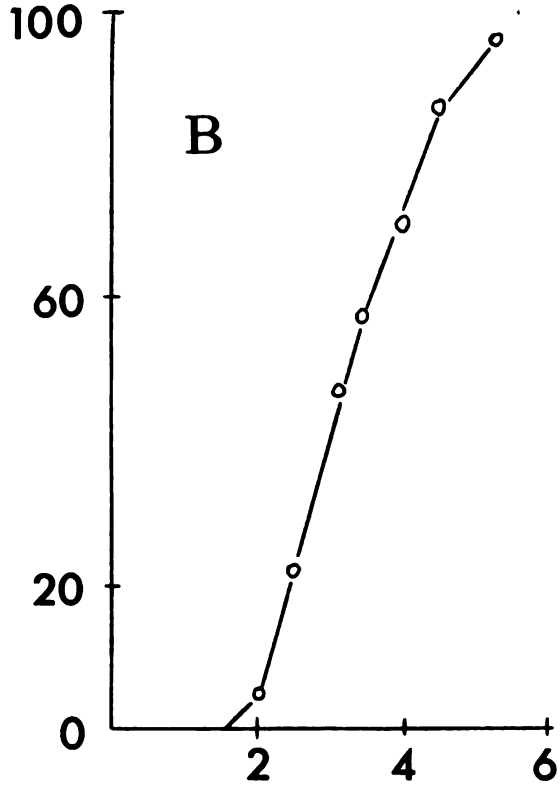
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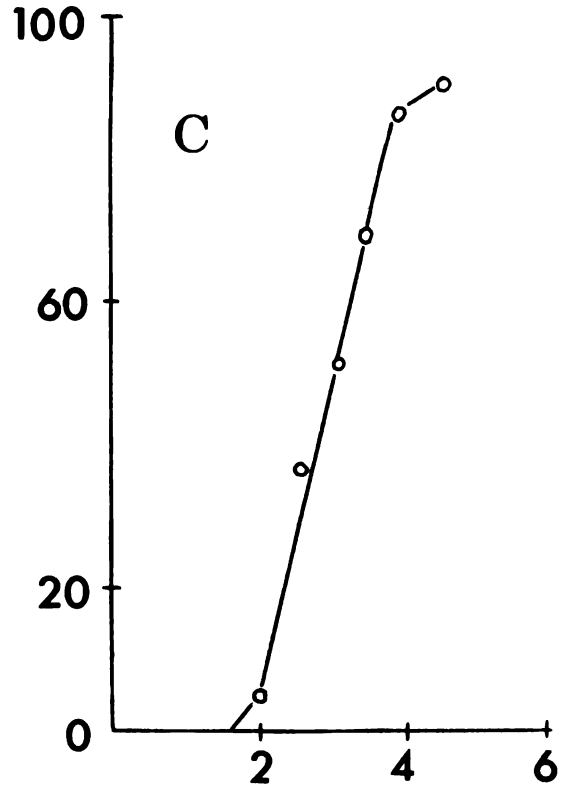
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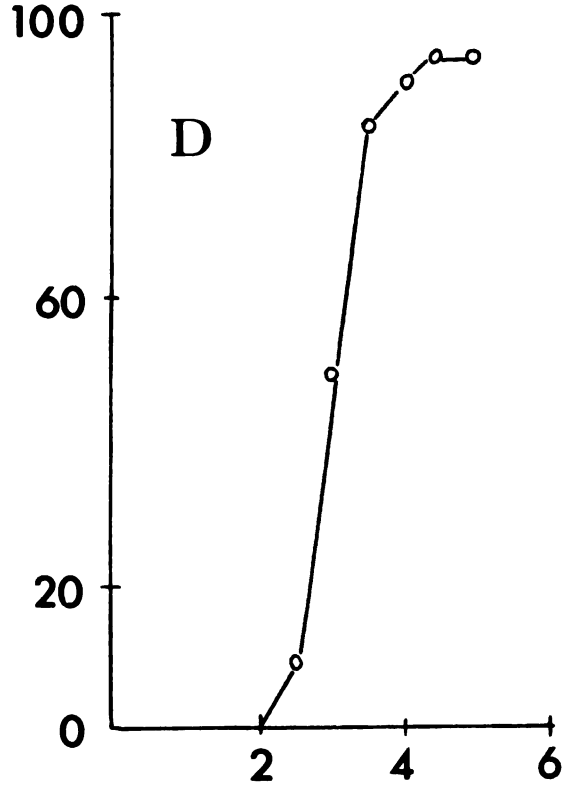
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formation after only ½ hr in liquid medium resulted in an extended lag before initiation of papillae formation. These plants eventually released spores in a normal manner. Plants removed from the nutrient solution after 2 hr showed a ½ hr increased lag phase and production of both one and two spores. In experiments where zoospore discharge was also followed, initiation of release from uni-spored sporangia occurred ½ hr before that from bi-spored sporangia. Most of the plants examined discharged two spores.

Reversal of Induction. The capacity of glucose, Casamino Acids, and fresh PYG broth to reverse spore formation after varying amounts of time in phosphate buffer was tested. All three had some effect on the on the number of papillae formed, but different results were obtained with each solution. Studies made on reversal by glucose showed that even when plants were transferred immediately into glucose from PYG broth, some papilla formation was observed after 3½ hr. The number of papillae steadily increased with time in the buffer prior to transfer to glucose, until the percentages reached values comparable to those of buffer alone (Figure 6). The Casamino Acids inhibited papilla formation until 1½ hr following placement in buffer; and PYG broth was effective until plants had remained 2 hr in phosphate buffer before re-exposure to the nutrient medium (Figure 7). In these experiments, plants were left in the buffer for a maximum of 2½ hr, because at this point the rapid rise in papilla formation normally occurs (Figure 5B).

Physiological Analyses

Polysaccharide Content. The polysaccharide per cell during the first 2 hr post-induction increased during the first ½ hr, then declined

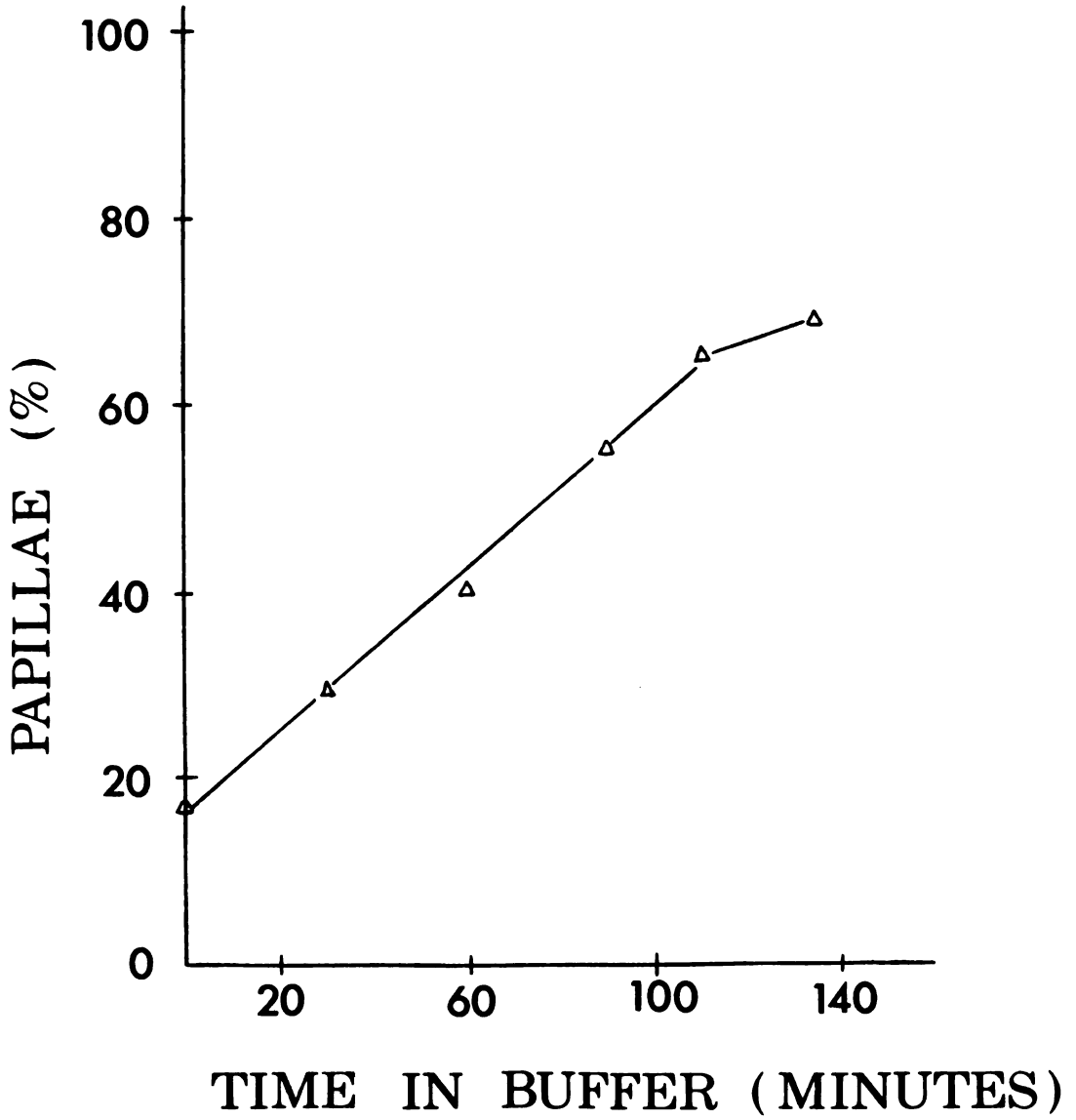

Figure 6. Reversal of Induction by Glucose

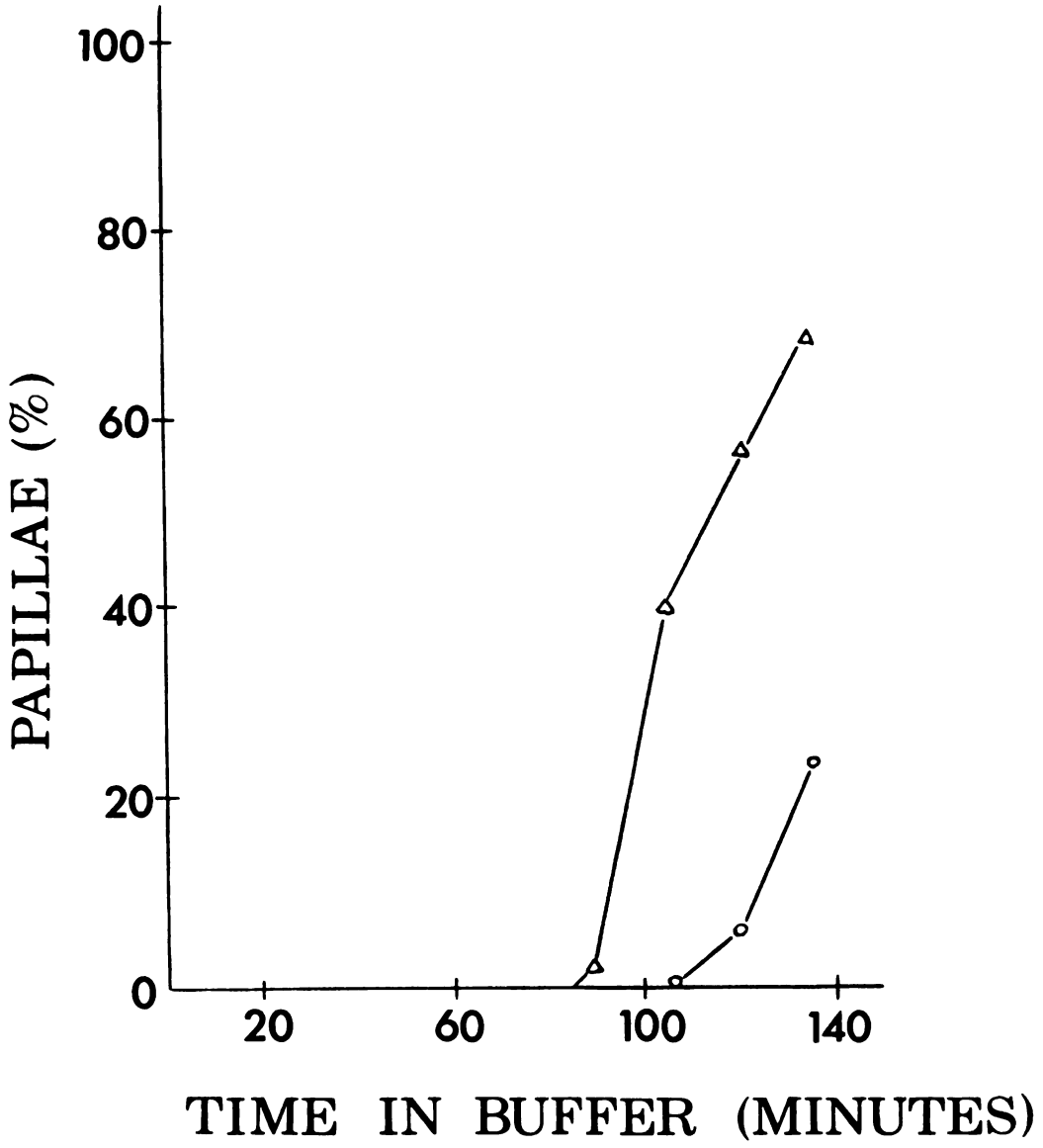

Figure 7. Reversal of Induction by Casamino Acids and PYG broth (Δ---Δ - Casamino Acids; o---o - PYG broth)

during the next 1½ hr to its lowest level by 2 hr (Figure 8).

Protein Content. During the first ½ hr after induction, the protein per cell increased, remained constant for another hr, then dropped to a new low level (Figure 9).

Nucleic Acid Content. The nucleic acid per cell remained fairly constant during the first hr following induction and then decreased slightly (Figure 9).

Dry Weight. The dry weight per cell rose during the first hr after induction, remained constant over the next ½ hr, and then decreased to its original level at the time of induction (Figure 10).

Zoospore Viability

The percentage viability for spores produced by lag phase plants was 89% (Table 1).

Table 1. Viability of Zoospores from Uni-spored Sporangia.

Spore Inoculum	Plants Produced*	Viability
4.2×10^3	3.7×10^3	88
3.95×10^3	3.5×10^3	89
3.04×10^3	2.76×10^3	91

*After incubation on PYG agar for 17 hours in the dark at 22° C.

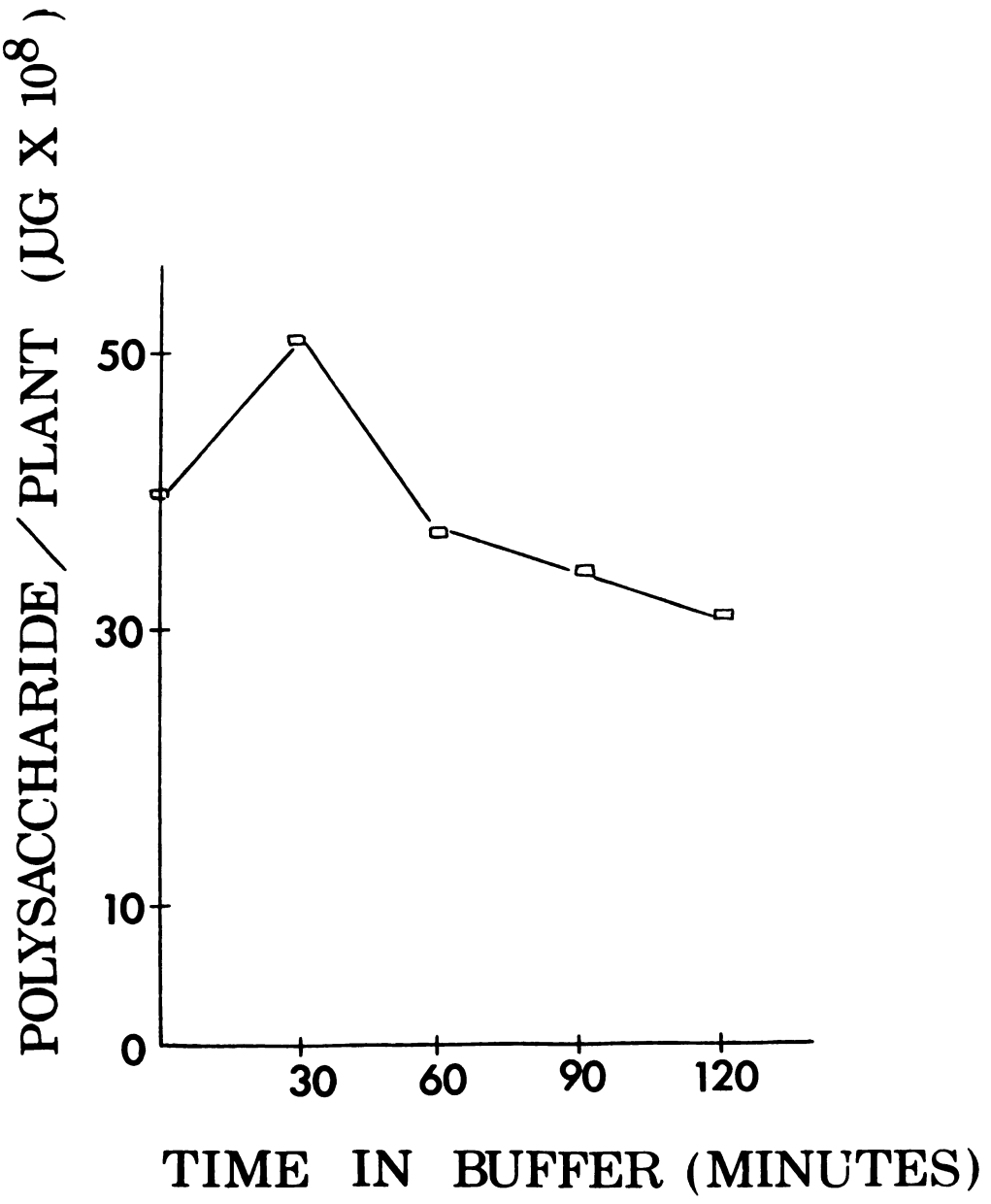

Figure 8. Post-Induction Polysaccharide Change

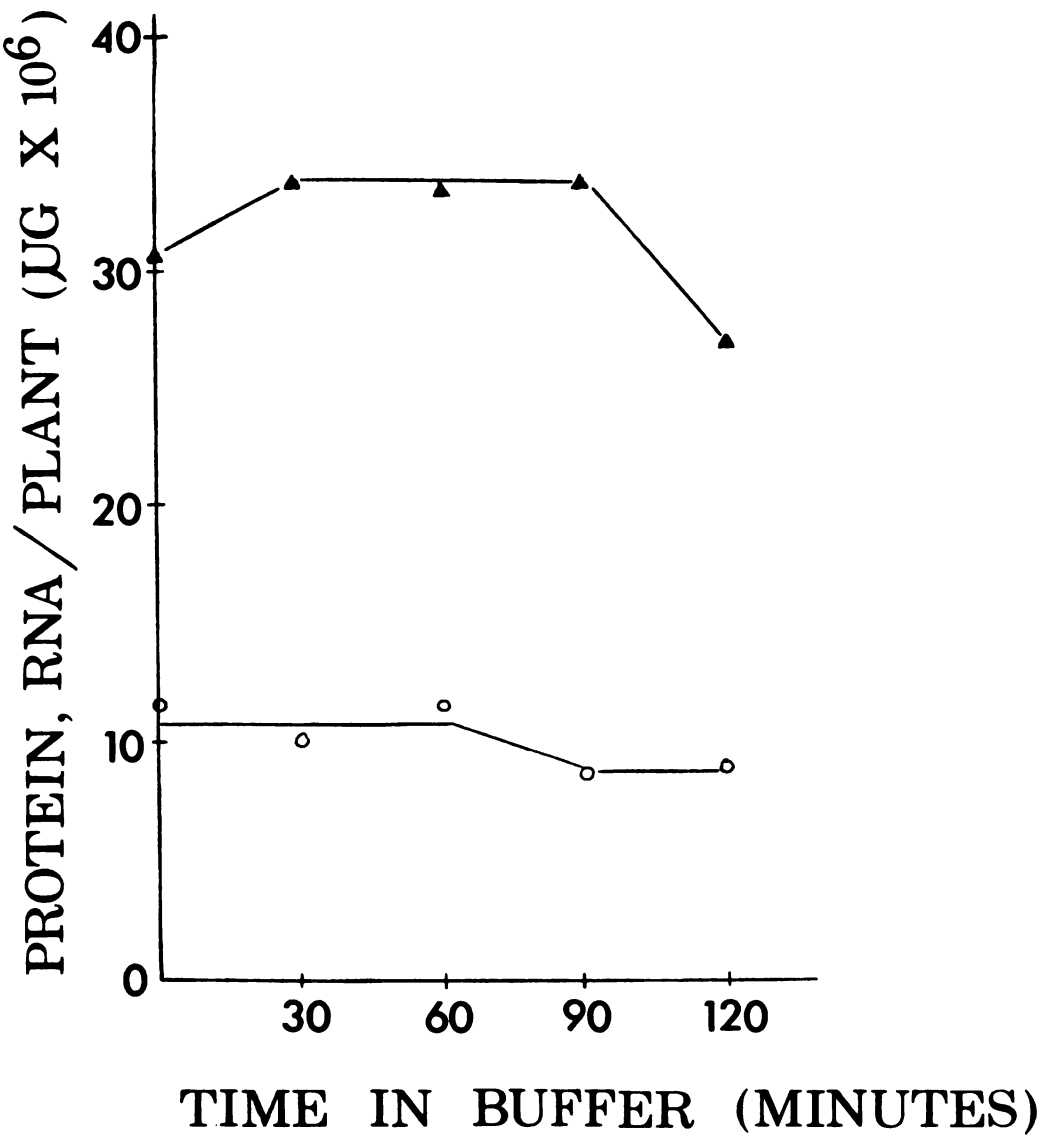

Figure 9. **Post-Induction Protein and RNA Changes**
(▲---▲ - protein; o---o - RNA)

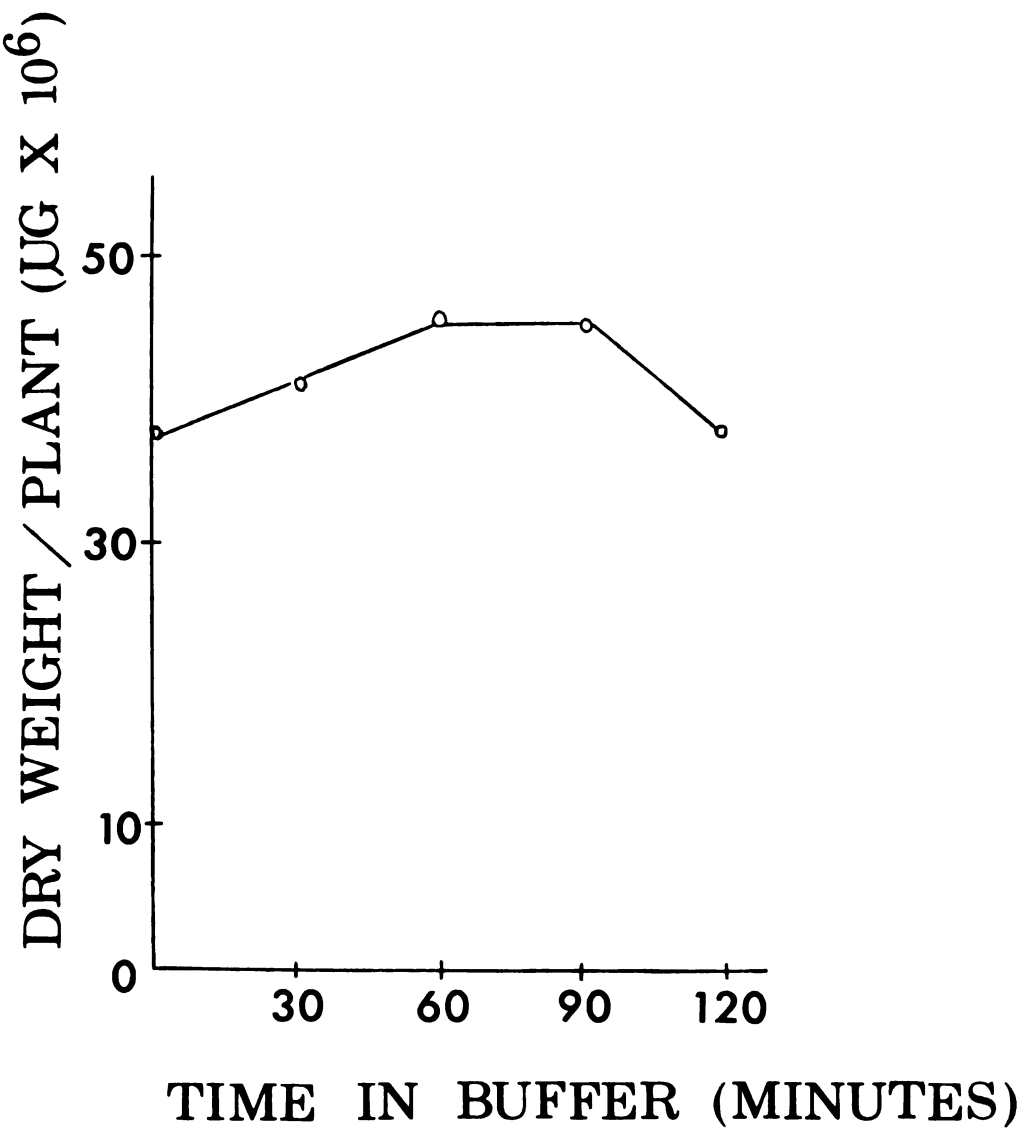

Figure 10. Post-Induction Dry Weight Change

DISCUSSION

Nuclear Division Synchrony

The results of these experiments support related work of others and suggest that there is a relationship between nuclear behavior and the potential of the plant to form spores. In comparing these results with those of Turian and Cantino (1959), the difference in the rate of nuclear division at 22° and 24° C is evident. At the higher temperature, the first division begins prior to 2 hr rather than 2½ hr. The doubling times were also different: at 22° C, 2 hr were required for the cell to go from the two to the four nucleate stage, while only slightly more than 1½ hr were required at 24° C. The 22° C incubation was used in order to parallel studies with Petri dish cultures grown at room temperature (22° C).

Studies on the sensitivity of Blastocladiella emersonii to radiation also show that nuclear divisions during early growth are synchronous (Deering, 1968). Although different culture conditions (agar plates and 23° C) and different sample sizes (50 cells) were used, his results compare favorably with mine. Deering also observed that resistance to radiation reached its maximum at about 1½ hr after germination. Following this, the young plants became sensitive to radiation, sensitivity increasing to a maximum 3 hr after germination. Sensitivity to radiation is an often used indicator of initiation of DNA synthesis. In many systems, cells are sensitive prior to DNA replication. Lovett (1968) followed development of young plants of B. emersonii microscopically and found that the first nuclear division began 140-200 min after inoculation (in cells grown in

synthetic medium in Bellco spinner flasks at 24° C). The first division was not complete until approximately 4 hr after inoculation. These results are almost identical with mine, despite the temperature difference. This similarity is probably due to the fact that in most synthetic, liquid media described to date, B. emersonii grows at a reduced rate compared to that obtained with PYG. Lysine enriched liquid and agar media are effective in growing cultures of B. emersonii, albeit less so than PYG broth and agar (Cantino and Goldstein, 1971). On synthetic media, the first measureable increase in DNA content of developing plants of B. emersonii occurs after 2 hr of growth (Lovett, 1968). This increase continued for almost 2 hr, then the DNA per cell levelled off. In the light of the work by Deering and Lovett, the nature of the nuclear cycle in B. emersonii begins to become evident. Unlike the mitospores of Allomyces, which reportedly begin nuclear division as soon as they are released (Flanagan, 1969), B. emersonii zoospores undergo a lag period of more than 1 hr. Some time later, probably near 1½ hr post-germination, the nucleus changes in preparation for the approaching replication of DNA. By 2 hr, replication of DNA starts; soon afterward nuclei begin mitosis. Some DNA replication continues throughout most of mitosis, indicating that there is no conventional G_2 phase detectable between replication and division.

My induction studies substantiate this description of nuclear development in young plants of B. emersonii and strongly favor the existence of a direct relationship between spore formation and the nuclear cycle. The capacity to produce consistently single-spored sporangia for at least one hr, possibly even 1½ hr, after germination confirms the existence of

a true lag phase, similar to that described by Machlis (1957) for Allomyces. The fact that a few of the cells induced to form spores after only 1½ hr of growth proceed to form bi-spored sporangia suggests that changes are occurring within the nuclei to which the process of spore formation is sensitive. The decrease in synchrony toward the end of papilla formation by such plants may be the result of a "catch-up" process in which those cells already in the phase of "preparation for replication" must then move through the complete replication and division cycle before spore formation and discharge occur. This hypothesis of the priority of nuclear commitment over spore formation is also in agreement with results obtained for plants induced after 2 hr in nutrient medium. The ½ hr extension of the lag before papilla formation begins, and the observation that zoospores are always released from single-spored sporangia ½ hr before those from bi-spored ones, suggest that the few cells which have not yet moved into the changing nuclear condition are able to reproduce more quickly, whereas those already committed to nuclear division must finish the prescribed events before zoospore release. These results do not explain the prolonged lag before papilla formation for plants placed in phosphate buffer after only ½ hr in PYG broth; this phenomenon may be related more closely to other aspects of development discussed later.

This possible relationship between the nuclear cycle and spore formation may help to explain the disagreement as to the need for, or existence of, a round of mitosis prior to spore formation, and the variation in synchrony of papilla formation after different periods of growth. The occurrence of mitosis prior to sporogenesis may be a function of the phase of the nuclear cycle. Given the apparent increase in nuclear

division asynchrony with increasing nuclear number, the range of possible "nuclear ages" within a population of cells, each with several hundred nuclei, becomes very large, and the likelihood of obtaining identical results with varying growth periods becomes minuscule.

This suggestion does not eliminate the possibility that some organisms may actually require a round of nuclear division or some other method of bringing about an equalization of "nuclear ages" before the formation of their reproductive structures. In gameteformation, where a reduction division is involved, synchrony in all nuclei is common. If cells of Chlamydomonas are removed from nitrogenous media prior to this division, they will not form gametes. Reduction is necessary before differentiation of new chloroplasts, flagella, eyespots, etc. can proceed (Kates and Jones, 1964). In yeasts, the change from equational to reductional division is a key factor in the transition from the growing to the sporulating state (Pontefact and Miller, 1962). The nuclei of the resistant sporangia of Euallomyces appear to require development to advanced prophase I before germination can be induced (Wilson, 1952). The synchrony of nuclear divisions in Physarum (Guttes, et al., 1961) could be necessary for the aggregation of numerous individual cells of the plasmodium to form an organized fruiting body. The results of my work imply that for Blastocladiella emersonii, the extent to which mitosis occurs prior to sporogenesis depends upon the physiological development of nuclei at the time of induction. The possible relationship of other cellular constituents to this nuclear cycle and to the triggering mechanisms for sporogenesis will be examined in the last section of this discussion.

Induction of "Early" Sporogenesis

In evaluating the usefulness of any system of cellular differentiation for morphological or biochemical analyses, a key factor which must be considered is the synchrony with which the transition from one state to another occurs (Baldwin and Rusch, 1965). Lessie and Lovett (1968) stated that sporogenesis in Blastocladiella emersonii involves two distinct differentiative events: "...the presporulation modification of the plant to form a multinucleate zoosporangium and the subsequent formation of the individually organized uninucleate zoospores..." (p. 221). The distinction between these two phases is based on the time sequence and morphological changes following induction rather than biochemical reorganization since the latter criterion involves a complex, ongoing process from which only select events have been separated out and studied. According to these authors, the end of the first phase and beginning of the second is the point at which papillae are fully formed and lipid droplets are visible around the nucleus. In keeping with the purpose of my research, only the first phase of this differentiation sequence will be evaluated in terms of synchrony.

One definition of synchrony in sporogenesis in B. emersonii states that "...the entry of all the plants into a single stage during 5% of the total generation time was taken to indicate that the cultures were 95% synchronized..." (p. 75; Murphy and Lovett, 1966). The use of a similar definition for my system would be far less meaningful, since the generation time is deliberately abbreviated. The time required for 100% papilla formation is longer than in that of Murphy and Lovett (1966), while the times between induction and initiation of papilla formation

are the same. The degree of synchrony possible in the induction of spore formation during the early stages of development may depend upon the synchrony of germination. Improvement of synchrony requires further experimentation with germination solutions.

The potential to form spores is present even in the very early stages of development (see p.42). The ability to induce papilla formation and release of a single spore in 90-95% of the plants examined indicates that this potential is, at a given time, the same for each cell in the population. The observation by Cantino and Hyatt (1953) that varying numbers of spores were released by tiny sporangia was probably a summary of many experiments with varying conditions, especially the amount of dissolved nutrients in their spore suspensions, rather than a case of different spore potentials.

The extended lag period for plants induced after only ½ hr of "growth" in PYG broth is probably more the result of germination of spores than of the physiological stage of development of the germling. The morphological changes involved in the transformation of the swimming zoospore of Blastocladiella emersonii into a cyst, and then a young germling, were discussed by Soll, et al., (1969) and reviewed by Truesdell and Cantino (1971). Soll and Sonneborn (1969) found that germination in PYG broth tended to be highly asynchronous, especially encystment. The results of work (p.43) with other induction times show that the number of spores produced by a cell is not dependent on the number of nuclei at the time of induction, since only one was present up to 2½ hr. Despite the similarity in lag periods for plants induced at 1 and 1½ hr, the role of germination synchrony in the kinetics of subsequent papilla formation

has not been eliminated. The degree to which the kinetics of zoospore germination may or may not be "smoothed out" by growth and nuclear division remains to be learned.

Through reversal of induction by various nutrient solutions, a clearer picture of the metabolic processes involved in commitment to sporulation can be obtained. Studies by Sauer, et al., (1969) with Physarum polycephalum showed that glucose was only partially inhibitory to fruiting body maturation, whereas a semi-defined citrate-hematin medium was competely inhibitory (Daniel and Baldwin, 1964). Decreased inhibition of gametogenesis by re-exposure to nitrogen (ammonium chloride or potassium nitrate) was also noted by Kates and Jones (1964) for Chlamydomonas. With Blastocladiella emersonii induction of sporogenesis can be only partially blocked during the first 1½ hr by glucose at a concentration similar to that in PYG broth. Evidence that glucose loses its inhibitory effect after plants have been in the buffer for 1½ hr suggests that the early phase of induction may be sensitive to depletion of glucose. However, because glucose alone cannot completely reverse even early stages of induction, involvement of other factors is certain.

Exposure of plants to Casamino Acids inhibited formation of papillae for the first 1-1½ hr of induction and allowed resumption of growth (in terms of an increase in cell size and nuclear number). This indicates that at approximately 1½ hr after induction depletion of exogenous nitrogen probably causes changes in metabolism which result eventually in spore production. It is important to note here that this time interval coincides with the beginning of the cell's sensitivity to radiation. Perhaps this depletion influences the preparations for nuclear division.

The fact that Casamino Acids can inhibit papilla formation to a greater extent than glucose suggests that amino acids may replace glucose during early induction and thus counteract the effects of depletion of the cell's energy reserves.

The reversal of induction by PYG broth until the final stages before initiation of papilla formation (2 hr) indicates that more than depletion of glucose or nitrogen is involved in triggering sporogenesis. The complex nature of peptone and yeast extract makes selection of a single factor impossible. Machlis (1953b) observed that yeast extract "...supplies the metabolic machinery through many pathways and byways, makes unnecessary numerous steps in the synthesis of the organism, and reduces the overall energy requirements for growth and maintainance..." (p. 450). This may explain why PYG can inhibit papilla formation when Casamino Acids cannot. The capacity of Casamino Acids to support growth before the critical time in induction substantiates this hypothesis, as does the observation that the kinetics of papilla formation beyond the inhibitory phase for each solution show similarities.

In summary, the results of the reversal experiments suggest that the hypothesis put forward by Cantino and Goldstein (1962) about the differentiation from OC to RS pathway is applicable to induction of zoospore formation as well. Biochemical differentiation necessary for sporogenesis may involve many interrelated changes in metabolism, each with its own point of no return. Further studies using defined media, and experiments on the inhibitory capacity of its components, are necessary before a fuller understanding of the biochemical differentiations involved in induction can be gained.

Physiological Analyses

Polysaccharide Content. The changes in polysaccharide per cell were analyzed during the first two hr following induction. During this time, before the formation of papillae, some of the reactions between the nutrient depleted external environment and the internal metabolism of the cell become evident. The initial rise in the level shows that internal reserves are still being converted into polysaccharide during the first 30 min after replacement of the medium with buffer. Polysaccharide may be used in the completion of the plant wall, or set aside in the form of storage material such as the granules described by Lessie and Lovett (1968). In studies on the distribution of ^{14}C - glucose during sporogenesis, Cantino (1965) recorded an initially rapid uptake of glucose between inoculation with swimming spores and the single rhizoid phase some three hr later. In my system, however, the time between inocualtion and initiation of rhizoidal branching was only $1\frac{1}{2}$ hr (Figure 11). Lovett (1968) reported similar results. The interval between Cantino's two readings makes detailed comparison between my system and his difficult. Further studies with labelled glucose using shorter sampling intervals in the early phases of germination and growth would be necessary to pinpoint the time of uptake more exactly. In the same study, Cantino found that by the time sporogenesis was initiated, ^{14}C from the labelled glucose had been incorporated into polysaccharide, chitin, protein, DNA, RNA, lipid, and a pool of small soluble molecules, thus demonstrating the many ways in which the cells use glucose. My attempts to analyze the changes in free glucose per cell during the induction process proved

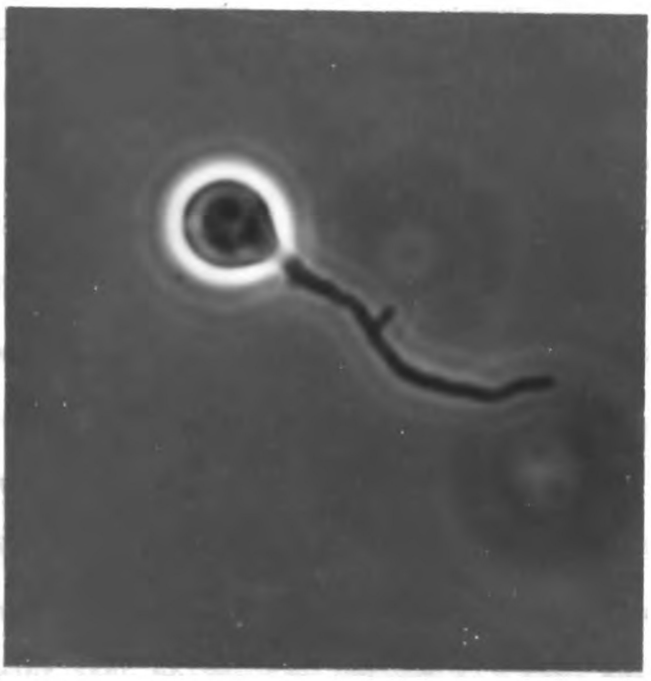

Figure 11. Initiation of Rhizoidal Branching (Germling After ½ hr in Buffer, Showing the Development of a Rhizoidal Branch)

unsuccessful.

The rapid decline in polysaccharide during the next ½ hr interval suggests that one of the earliest events in starvation-induced sporogenesis is the rapid conversion of stored polysaccharide into forms in which it can be used by the cell. This hypothesis is supported by data obtained for log phase plants (Cantino and Goldstein, 1961). The fact that glucose alone could not completely inhibit formation of papillae in any reversal experiments adds new meaning to this hypothesis. If glucose is used initially to provide reserves and wall structure, and only secondarily as an energy source, its effectiveness in maintaining a growth-oriented over a starvation metabolism would be short-lived. The finding (Cantino, 1965) that only 19% of the ^{14}C from the labelled glucose found its way into protein, the largest cell component at sporogenesis, suggests that glucose is used preferentially in polysaccharide and wall metabolism (28% of the label went into polysaccharide and 23% into chitin). Physiological changes in cells undergoing partial reversal by glucose were not followed, so that the role of glucose in inhibiting spore formation remains unknown. Studies on lag phase sporogenesis (Murphy and Lovett, 1966) and the physiology of early germination (Lovett, 1968) did not deal with the changes in polysaccharide in differentiating cells.

Protein Content. The protein in lag phase cells, like polysaccharide, continues to increase during the first ½ hr after induction. Soll and Sonneborn (1971) showed that the early stages of differentiation in Blastocladiella emersonii, involving the transition from the swimming spore to the "round cell" with a germ tube initial, required no protein synthesis. These authors did note a breakdown of prelabelled protein

during germination, but only at about the time that protein synthesis is first detected. Lovett (1968) found similar results; the initial rise in protein per cell does not begin until 70-80 min after inoculation. If this is the case, then plants induced to form spores after only 1 hr in PYG broth should show no increase in total protein over that in the spore itself. This does not mean that no protein synthesis occurs during the first hour, since both Lovett (1968) and Soll and Sonneborn (1971) noted uptake of labelled leucine 20-30 min after inoculation, probably balanced out by the breakdown of pre-existing spore proteins. At the end of this first hr of development, Lovett reported a total protein content of 2.5×10^{-5} µg per cell, while I found a larger amount of 3.1×10^{-5} µg per cell. The use of a defined medium as opposed to that of PYG broth may account for the difference in results.

Log phase plants of Blastocladiella emersonii during sporogenesis (Murphy and Lovett, 1966) continued to synthesize protein following nutrient replacement for at least 2 hr after induction, albeit at a reduced rate from that before induction. Although protein synthesis in my system does continue after induction, net protein ceases to increase after the first ½ hr and remains at this level for another hr. One source of difference between the results of log phase studies and mine might be that larger internal pools of amino acids build up during the log phase of development. After studies using the incorporation of labelled amino acids, a more definite statement can be made as to whether this levelling off results only from a cessation of all protein synthesis or from a turnover that causes no detectable increase. Gametic differentiation in Chlamydomonas (Jones, 1970) shows a 100% increase in proteolytic

activity during the 4 hr immediately following the initiation of carbon and nitrogen starvation, thus suggesting that protein breakdown should not be ignored for Blastocladiella emersonii.

The results of the reversal of induction by nutrient solutions adds a different perspective to this question of cessation of protein synthesis versus protein turnover. The capacity of Casamino Acids to reverse induction for 1½ hr, and the continued incorporation of labelled leucine noted by Murphy and Lovett (1966), suggest that pathways of protein synthesis have not been shut down but rather diverted elsewhere. Domnas and Cantino (1965a) found a decrease in total soluble amino acids during the last 5% of the generation time in B. emersonii. An increase in the size of amino acid pools following induction was also noted by Murphy and Lovett (1966), and Domnas and Cantino (1965a) noted a decrease in soluble protein at the end of the generation time, suggesting that some protein breakdown may be involved in the levelling off of the protein content of lag phase cells.

The decreased level of protein per cell after 1½ hr in the induction medium implies that protein degradation exceeds protein synthesis. In log phase plants, Murphy and Lovett (1966) found a sharp decrease in the incorporation of labelled leucine at 1½ hr post-induction, indicating that protein synthesis was tapering off. The fact that Casamino Acids no longer inhibited papilla formation beyond this point further supports the conclusion that the metabolic machinery has reached a point of no return with respect to amino acids, after which it is committed to proceed toward spore formation. Whether the products of this protein breakdown are incorporated into new proteins required by the spore for membranes,

flagella, etc. or are used for energy following the decrease in polysaccharide reserves is not yet known. The system described in this paper, in which the exact number of spore products from each plant is known, seems to offer an excellent means to explore this problem further.

Nucleic Acid Content. Because of the manner in which cells were grown, the analyses of nucleic acids did not involve a separation of DNA and RNA. Lovett (1968) noted that the DNA per germling did not increase until 2 hr after inoculation. In log phase cultures induced to undergo sporogenesis, no synthesis of DNA was noted beyond the first hr after induction (Murphy and Lovett, 1966). If synthesis of new DNA is blocked during the first hr of induction, the plants grown for only one hr in nutrient solution would have no opportunity to increase their DNA content. Thus the DNA content in lag phase cells is assumed to remain fairly constant during induction. Although some loss in DNA was noted for log phase plants (Murphy and Lovett, 1966), the decrease occurred after papilla formation, whereas all the analyses of lag phase cells were made prior to initiation of papilla formation. Turian and Cantino (1959) found the RNA content in ungerminated spores to be approximately 15 times that of DNA. Since RNA increases during the first hr while DNA does not, the DNA/RNA ratio is probably even smaller for one hr germlings. Thus the loss in nucleic acid can probably be accounted for mainly by degradation of cellular RNA.

The amount of nucleic acids per cell after one hr of growth in PYG broth was approximately 1.1×10^{-5} µg. This is in good agreement with the combined DNA and RNA of young germlings analyzed by Lovett (1968) - approximately $.92 \times 10^{-5}$ µg per cell. As was the case for protein, the

the slight variation in the two results may be related to the different growth media used. In a normally developing germling, RNA begins to accumulate in measurable amounts after 40-45 min and increases at a constant rate through the first 4 hr of development (Lovett, 1968). However, in lag phase plants removed from nutrient after 1 hr of growth, the RNA per cell remains fairly constant over the first hr after induction and then decreases sharply. The sensitivity of the DNA and RNA in young plants to nutrient depletion was suggested by Lovett's (1968) observation that spores germinated in a salt medium "...produce rhizoids which elongate and branch normally, although the young plants fail to reach the stage of nuclear division. They also apparently fail to increase their RNA content..." (p. 967).

Sporogenesis in log phase plants of Blastocladiella emersonii (Murphy and Lovett, 1966) shows an increase in RNA content per cell during the first ½ hr after induction at the same rate as that found for plants prior to induction. During the next hr, the rate of increase tapers off, with a decrease occurring at the initiation of papilla formation. One possible explanation for these events occurring earlier for lag phase plants than for log phase plants is the larger amount of reserve materials which can be accumulated during extended growth in PYG broth. These reserves may enable plants to withstand the metabolic alterations caused by starvation for an increased amount of time. The rate of incorporation of labelled uracil into log phase plants after induction increased during the first ½ hr, followed by a rapid decline to zero over the next hr, with loss of uracil label therafter. A similar analysis would be necessary for lag phase plants to explain the lack of change in total RNA during

the first hr following induction. The suggestion that some RNA synthesis may be taking place despite the net decrease in RNA per cell stems from the fact that the RNA required for papilla formation is formed at least ½ hr before this event, and the RNA specific to zoospore cleavage was present at least 1 hr before it took place (Murphy and Lovett, 1966). A similar study of induction of fruiting body formation in Physarum showed blockage of RNA synthesis beyond a critical point prior to spore production was ineffective in stopping the event (Sauer, et al., 1969). The possible relationship of the changes in RNA to the reversal experiments is not clear. If the mRNA needed to direct formation of papillae is already present ½ hr before the process begins, Casamino Acids must not be able to initiate or complete rapidly enough the formation of new RNA. The capacity of PYG broth to act at this point may be related to the ease with which it can by-pass metabolic pathways to produce needed molecules more immediately. Experiments with precursors of nucleic acids are necessary to clarify the degree of commitment to spore formation reached during synthesis of RNA.

Dry Weight Analysis. The changes in dry weight of young plants during induction were similar to those obtained by Murphy and Lovett (1966). Dry weight continued to increase after induction. The levelling off period was shorter than that for log phase plants, probably due to smaller amounts of reserves. In both cases, a decrease in dry weight occurred at the time of initiation of papilla formation.

The determination of dry weight of cells which have been scraped from Petri dish cultures has an inherent problem: small fragments of broken cells remain in the supernatant solution after low speed centrifugation

and are lost when it is pipetted off. Some plant breakage may also occur during the brief sonication to disperse clumps, and small fragments will then pass through the filter paper. The amount of plant loss detected in fixed preparations used for hemocytometer counts was slight, but may not have been completely accurate. These sources of error probably resulted in the difference between my values and those obtained by Lovett (1968). Since plants were handled identically at each sampling interval, it is likely that the changes in dry weight are valid, but not necessarily the absolute values.

Zoospore Viability

The spores produced by lag phase plants do not differ significantly in viability from those formed by log phase plants. The zoospores escape from their uni-spored sporangia in a very consistent manner. The end of the cell opposite to the flagellum moves out first (Figure 12, upper left). Each spore appears to push itself with the now active flagellum through a narrow opening (exit pore), assuming a dumbell shape at the halfway point of escape (Figure 12, upper right). Once the cell is outside the sporangium, it remains quiesent momentarily and then vibrates rapidly, as if to pull out the flagellum which remains inside (Figure 12, lower right). As soon as the flagellum is jerked out, the spore swims away normally. The spores elongate and swell slightly after release (Figure 12, lower left). The apparent difficulty involved in the escape of spores, more so than seen in log phase plants, brings up the possibility of disorganization or damage to some of the cells' organelles. Electron micrographs of zoospores released from uni-spored sporangia indicate

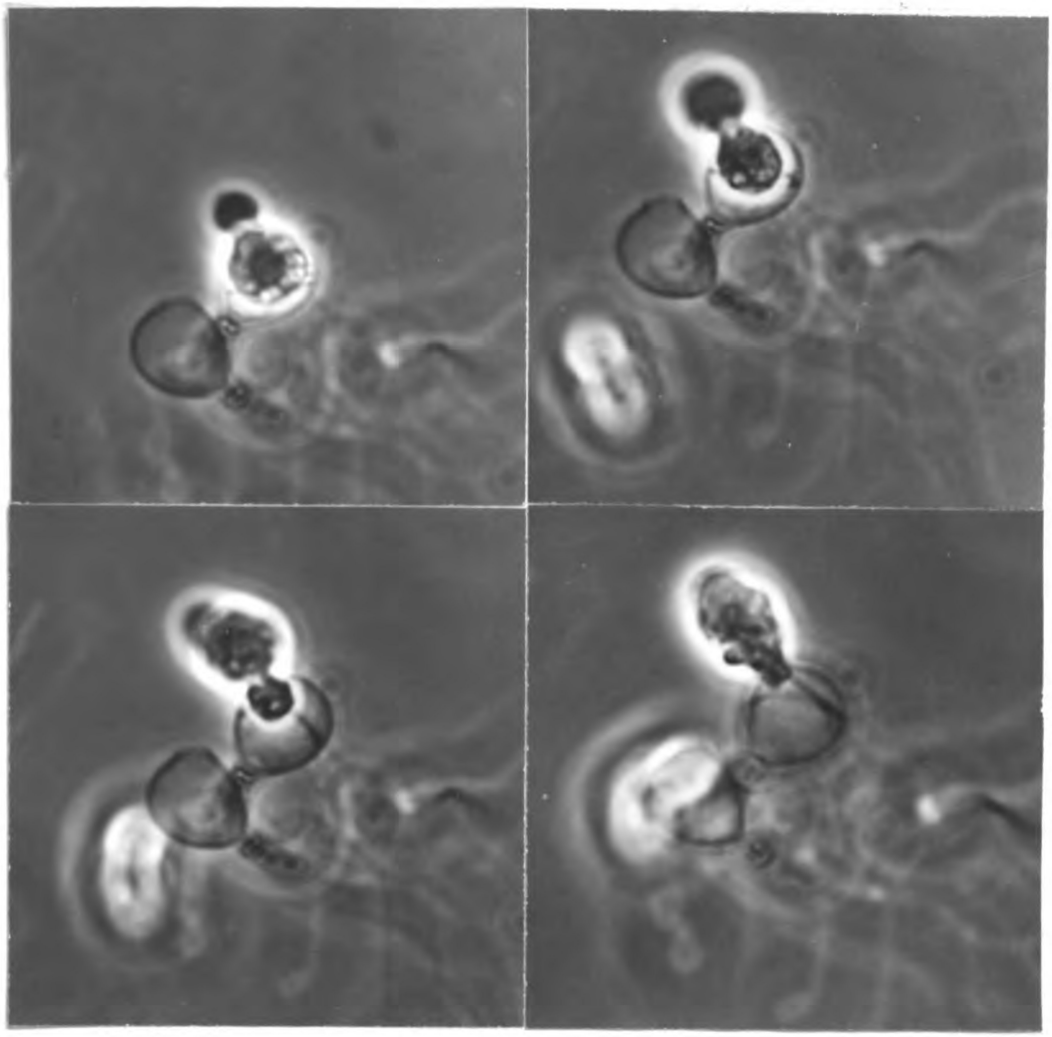

Figure 12. Zoospore Release from Uni-spored Sporangium

normal cellular organization, although a slight distortion of the mitochondrion and the nuclear cap may occur (Figure 13). The results are too preliminary to reach a firm conclusion at the present time. The data from viability studies show that any cellular disorganization which might occur during discharge does not radically impair the potential for germination and plant development.

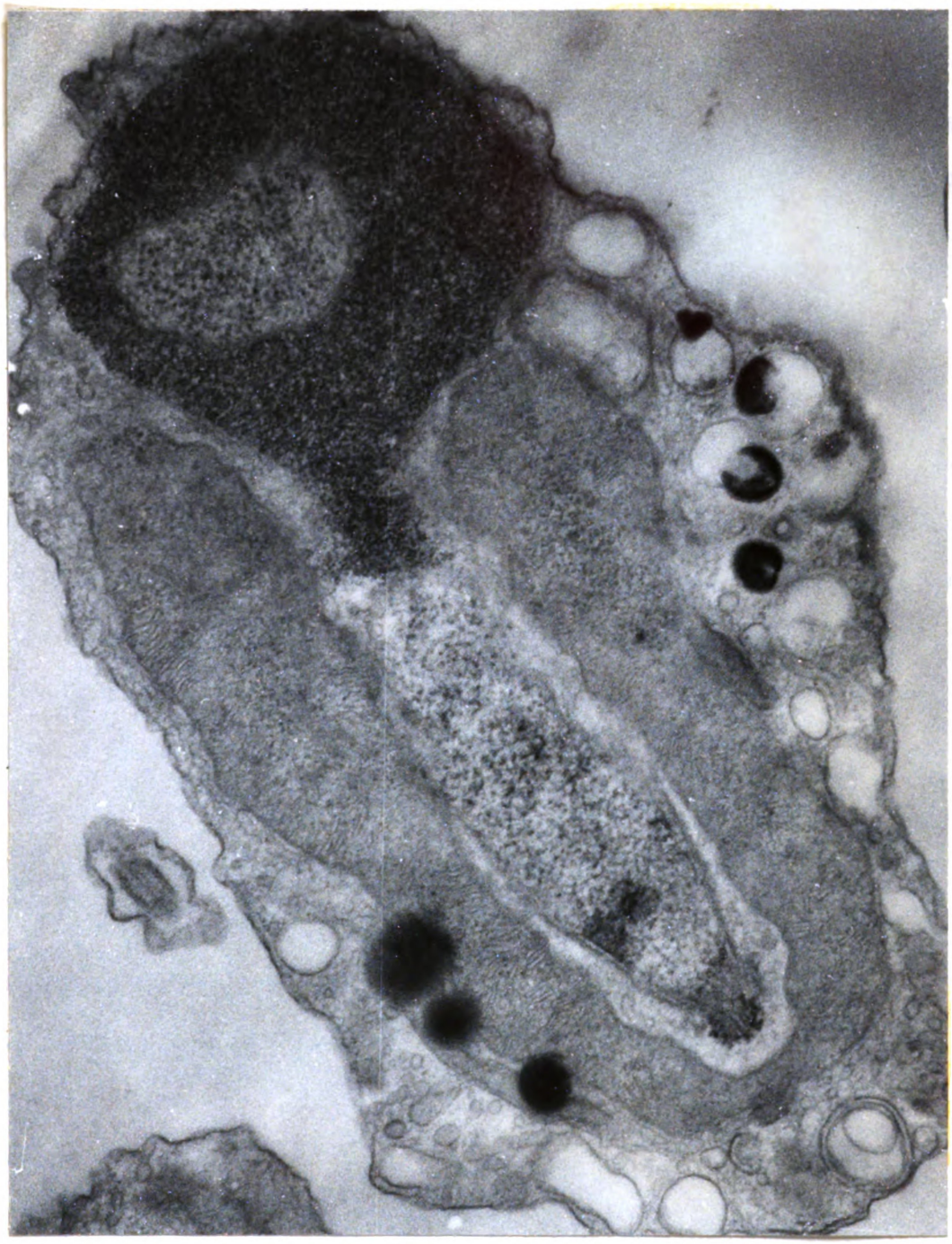

Figure 13. Morphology of Zoospore from Uni-spored Sporangium (X 32,400)

SUMMARY AND CONCLUSIONS

The research described in this thesis deals with the potential of a cell of Blastocladiella emersonii to form spores at the beginning of its life cycle, and the factors which relate to the induction of spore formation. The results answer many of the questions posed in the introduction, but, as is often the case with new information, leave many new questions in their wake.

Despite many years of laboratory culture, the plants of Blastocladiella emersonii have maintained their capacity to form spores at any phase of their OC life cycle, including the lag phase before any significant growth has occurred. At particular time intervals, probably growing shorter with each additional nuclear division, all the cells in a population have the potential to produce the same number of spores. The actual number of spores released for any specific induction time is more closely related to the "nuclear age" of each nucleus, and any further development of the nuclear cycle which might be allowed by internal reserves, than to the actual number of nuclei present at the time of induction. The round of nuclear division described by Lessie and Lovett (1968) is not an essential prerequisite to spore formation at every phase of the life cycle, since synchronous spore formation can occur before any nuclear division takes place. The suggestion by Flanagan (1969) that the degree of nuclear division prior to cleavage of mitospores by Allomyces might be related somehow to environmental conditions certainly holds true for spore production in the early stages of development of Blastocladiella emersonii, and probably for zoosporogenesis in the later stages as well.

The results of experiments involving re-addition of nutrients to plants which have been induced to sporulate, in conjunction with studies on the physiological changes during induction, support the hypothesis that several important shifts in metabolism are occurring in response to the depletion or removal of external nutrients. The first decrease detected is in the young plant's polysaccharide reserves. This step is only partially inhibited by addition of glucose, but appears to be completely reversed by exposure of the plants to Casamino Acids, since vegetative growth is thereby resumed. These results also indicate that depletion of nitrogen may be the most important single factor in the induction of spore formation by Blastocladiella emersonii. Some breakdown of RNA occurs after one hr in buffer; its effect on the commitment of the plant to sporulate can be reversed up until 1½ hr by Casamino Acids and 2 hr by fresh PYG broth. The decrease in the total protein of the cell begins at 1½ hr, and the addition of amino acids does not reverse the effect of this decline on sporogenesis. Only fresh PYG broth can return the cell at this point to normal growth. The final point of no return, probably a sum of all these individual events, seems to coincide with the initiation of papilla formation.

The morphological changes which occur during induction of spore formation are essentially the same as those described for log phase plants (Lessie and Lovett, 1968) with the exception of the observance here of the large vacuole. The source of this vacuole and the reason for its presence in young plants only are not known. The physiological changes occurring after induction of spore formation in young plants show many similarities to those described for log phase plants, but also

distinct differences. The period of continued increase in some cell components is generally shorter (dry weight and protein) or non-existent (RNA) in lag phase plants. Periods of levelling off, where synthesis appears to balance breakdown, are found more frequently in lag phase plants. Decreases in particular components in germlings likewise begin earlier (though in much the same sequence) than reported for older plants. Each of these factors may be influenced by the different pre-existing pools within the cells after various exposures to nutrient (15½ hr for log phase and 1 hr for lag phase). The morphological and physiological changes which occur during the induction process appear, like nuclear changes, to be influenced by previous environmental conditions. The spores released by lag phase plants had the same organelles and also had the same potential for growth as those previously described for log phase spores.

Questions remain to be answered involving the mechanism of reversal and the specific proteins involved in the formation of spores. Research into the relative inhibitory power of other precursors and use of labelled nutrients should help answer many of these questions. This research has shown that the induction of spore formation in Blastocladiella emersonii involves a dynamic series of events in which the external environment, the nucleus, and the developmental state of the cell each play an integral role.

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APPENDICES

APPENDIX A

CHEMICALS USED

ORGANIC:

Azure A	- Matheson Coleman and Bell - Norwood, Ohio.
Bacto-Peptone	- Difco Laboratories - Detroit, Michigan.
Bovine Serum Albumin	- Nutritional Biochemicals Corporation - Cleveland, Ohio.
Casamino Acids	- Difco.
Formaldehyde	- Mallinckrodt Chemical Works - St. Louis, Missouri.
D-Glucose	- Eastman Organic Chemicals - Rochester, New York.
Glucostat Reagent	- Worthington Biochemical Laboratories - Freehold, New Jersey.
L-Glutamic Acid	- Sigma Chemical Company - St. Louis, Missouri.
Glycine	- Sigma.
MOPS	- Calbiochem - Los Angeles, California.
Orcinol	- Sigma.
PYG (agar and broth)	- Difco.
TCA	- J. T. Baker Chemical Company - Phillipsburg, New Jersey.
Yeast Extract	- Difco.
Yeast Nucleic Acid	- Calbiochem.

INORGANIC:

All inorganic chemicals used were "analytical reagent" grade.

APPENDIX B

INDUCTION SOLUTIONS

Several preliminary experiments were made to determine the relative synchrony of papilla formation in various possible induction solutions. Plants were grown on slides for one hr as described previously (p.20) and induced with the different solutions. The results are shown below in Table 2.

Table 2. Synchrony in Various Induction Solutions

Number	Solution	Concentration	T_{50}* (hours)
1	K_2HPO_4 KH_2PO_4	5×10^{-4}M 5×10^{-4}M	3.25
2	KCl	10^{-3}M	3.25
3	$CaCl_2$	10^{-3}M	3.5
4	$CaCl_2$ MOPS	10^{-3}M 10^{-3}M	4.0
5	NaH_2PO_4 Na_2HPO_4	5×10^{-4}M 5×10^{-4}M	4.0

*Time (after induction) at which 50% of the plants had papilla.