CHAPTER V

GENERAL DISCUSSION

V.I LIGHT DEPENDENT PYCNIDIAL PRODUCTION

*Botryodiplodia theobromae* has emerged as valuable experimental system for the study of differentiation in eukaryotes as evident from some of the preceding account. Among eukaryotes several fungi offer certain advantages for studying the phenomenon of differentiation (listed in Chapter I). The present experimental system (*B. theobromae*) offers additional advantages which are listed below:

1. Growth and differentiation in *B. theobromae* are mutually exclusive phenomena.
2. A radial colony of about 5.0 cm diameter undergoes synchronous sporulation (differentiation).
3. Pycnidial production (differentiation) in *B. theobromae* is light dependent. The non-sporulating cultures maintained in dark thus provide good controls.

The salient features of the experimental system and the observed changes in nucleic acids and proteins during light induced pycnidial production are discussed at length in this chapter.
Each mycelial zone in a growing mycelial culture of *Botryodiplodia theobromae* (on solid medium or stationary liquid medium) formed over a period of 24 hours, undergoes three distinct phases of development. The first phase is of 24 hours duration, when the vegetative mycelial growth is completed. In the second phase of about 24 hours, mycelia undergo morphogenetic changes leading to the synchronous onset of morphologically differentiated pycnidia in the zone. Pycnidia at this stage contain mostly the sporogenic mass. During the final or maturation phase pycnidia enlarge and the sporogenic mass is differentiated into numerous pycnidiospores.

One minute of light exposure is sufficient to induce sporulation in dark grown cultures. Mycelia can perceive light stimulus anytime during the vegetative phase of growth but their ability to perceive light is completely lost after a certain period of maturation. Thus, mycelia in a dark grown zone form pycnidia only if induced during the 'receptive period'. Only the mycelial growth exposed to light forms pycnidia and the light stimulus is not carried over to the unexposed mycelia.

In the cultures grown under continuous illumination, although the light stimulus is perceived by mycelia during the vegetative phase of growth, yet initiation of events leading to onset of sporulation is held back
until the completion of the vegetative phase. Possibly, light perception results in a stable product(s) which participates in the initiation of sporulation specific events, after the vegetative phase. Production of secondary metabolites is known to be related to differentiation in several fungi (Smith & Galbraith, 1971; Smith & Berry, 1974). In B. theobromae production of a secondary metabolite after vegetative phase of growth could be the key to initiate the changes in the mycelia leading to synchronous development of pycnidia.

A working hypothesis is suggested to explain light induced sporulation in B. theobromae (Fig. 1). The present hypothesis is a modification and extension of the hypothesis put forward by Rakoczy (1962), for photo-induced sporulation in Physarum nudum. According to Rakoczy's hypothesis, a substance A is formed during vegetative growth and is converted to B under the influence of light, leading to sporulation. With ageing, more A accumulates and hence more B is produced, which results in quantitative increase in sporulation. In B. theobromae however, a hypothetical substance A is produced during vegetative phase of growth, both in the light and the dark (Fig. 1). A gets converted into B photochemically, during the vegetative phase in the light grown cultures and in response to light exposure in the dark grown
Fig. 1. A hypothetical scheme to explain photoinduced sporulation in *Botryodiplodia theobromae*.
Fig. 1.

Differentiation phase

Maturation phase

Light/Dark

Onset of sporulation

Mature pycnidia

Formation of secondary metabolite at the end of vegetative phase of growth
cultures. Secondary metabolite $S$ is produced at the end of vegetative phase. $B$ and $S$ combine and the complex $BS$, initiates sporulation specific changes leading to the synchronous development of pycnidia at the end of the differentiation phase. In the absence of light, $A$ is not converted into $B$ and thus metabolite $S$ may combine with $A$ to form $AS$ complex. $AS$ complex would not trigger changes leading to sporulation and thus would not result in sporulation of unexposed cultures or unexposed regions of the cultures. When $A$ complexes with $S$, it may no longer remain sensitive to light, which would explain the loss of light receptiveness by mycelia, on prolonged incubation in the dark. The role of light in morphogenesis is probably only to convert $A$ into $B$, photochemically. Thus, dark grown cultures given short or long exposures, during the 'receptive period', result in similar sporulation responses.

The present investigation has indicated that the information imparted by light stimulus is not movable from one part of the colony to the other. As a result, in a growing colony each zone produces pycnidia synchronously, 24 hours after the pycnidial formation in the preceding zone. Fig. 2 summarizes the morphological events as they occur in two adjacent zones in light and dark grown cultures, represented on a time scale.
Fig. 2. Schematic diagram indicating events in zones B and C of light and dark grown cultures during incubation, represented on a time scale.
Fig. 2.
Exposure of the dark reared mycelial inoculum to light for 1 minute, during the process of inoculation, resulted in the production of pycnidia in dark. About 70% of the mycelial growth formed pycnidia in the absence of light and the peripheral area remained unsporulated. The sporulation observed in the dark grown cultures, is therefore, a result of a carry over of stimulus from the light induced inoculum to dark grown mycelia. However, it has been indicated earlier that in a growing culture, the light stimulus is not transferred from exposed to unexposed mycelia. The mycelial inoculum used in this experiment is similar to the inoculum used in other experiments except that the mycelia in the inoculum, in this experiment, received light stimulus for one minute, during inoculations.

It is difficult to visualize the presence of two kinds of stimuli in the same organism. A probable hypothetical explanation however, could be provided, for the 'carry over effect' observed in dark grown cultures, originating from light induced inoculum. During the process of inoculation, it is possible that a large percentage of mycelia get injured on the periphery of the inoculum. Light may affect the physiology of the injured mycelia in such a way that light influence is substituted by a biochemical change(s)
Also further mycelial growth from the exposed inoculum might exhibit similar altered physiology. However, the growing culture reverts to normal physiology, after a certain period of time, so that only 70% of the colony formed pycnidia without light. In intact mycelia, on the contrary, light causes specific localized change(s) and therefore stimulus is not passed on to any unexposed part of the culture. Earlier Mehta et. al. (1972) reported that both light and injury are necessary for abundant pycnidial production of _B. theobromae_ on the host plant.

The 'carry over effect' observed during present investigation can also be explained based on present hypothesis. A change in the physiology of mycelia, originating from exposed inoculum, may lead to the conversion of _A_ into _B_, by a biochemical pathway. Alternatively, the mycelia could produce _B_ directly instead of _A_ being converted into _B_. When _S_ is produced at the end of the vegetative phase, it would combine with _B_ and the complex _BS_ would result in the sporulation of the culture in complete darkness.

V.2 CHANGES IN NUCLEIC ACIDS DURING DIFFERENTIATION

In the cultures maintained in light an increase in the nucleic acids was observed after 60 hours of incubation
whereas there was a sharp decrease in the nucleic acid levels (mg/g dry wt. of mycelium) in non-differentiating cultures. Sudden discontinuation of mycelial growth at 60 hours (due to limited surface available for growth) might have caused degradation of nucleic acids to minimum basic level in the dark grown cultures, which was then maintained during subsequent incubation in dark.

In the light grown cultures increase in the nucleic acids coincided with the onset of pycnidial production in the zone B. Extensive hyphal branching is known to be involved in the pycnidial morphogenesis. According to Nishi et. al. (1968) growing apices of fungal hyphae have higher amounts of nucleic acids, compared to the non-growing hyphae. Thus, extensive hyphal branching such as seen in differentiating cultures here may be responsible for the observed increase in the nucleic acid levels. The completion of pycnidial morphogenesis would then be responsible for the sudden degradation of nucleic acids observed after 72 hours. The sharp changes in the nucleic acid levels in differentiating cultures would thus support the observed synchronous nature of sporulation at a biochemical level. The gradual increase in the DNA, observed after 76 hours of incubation in the differentiating cultures coincided with the production of pycnidiospores inside pycnidia. Since the spores are
rich in DNA but not so in RNA, no simultaneous rise in RNA content was observed during the pycnidiospore production. Thus there is a close association between nucleic acid content and the various events during differentiation and maturation of pycnidia in the Zone B.

Differentiation apparently involves continuous degradation and synthesis of RNA and DNA. Whether changes in nucleic acids is merely a reflection of morphogenetic phenomenon or has a specific role to play during differentiation is not yet clear. The ratio of RNase activity at pH 6.2 and 5.2 varied between 0.59 to 0.95, suggesting the participation of both RNase A (cytoplasmic enzyme) and RNase B (particulate enzyme), (Ingle and Hageman, 1965; Hadziyev et. al., 1969), at all the stages investigated. The anti parallel pattern of nucleic acid contents observed in the sporulating and non-sporulating cultures, was also true for RNase activities assayed at pH 5.8. Activity of RNase at pH 5.8 represents the sum of activities of both RNases, A and B (Ingle and Hageman, 1965; Hadziyev et. al., 1969). Although it is not necessarily correct to assume that the level of nucleic acids be entirely regulated by RNase activity (Gustafson and Wright, 1972), the reverse patterns of both nucleic acid contents and RNase activity in sporulating and non-sporulating cultures, suggests the involvement of nucleic acids.
and a possible regulatory role for RNase during differentiation. In *Physarum polycephalum*, replication of DNA, prior to light induction is obligatory for sporulation (Sauer et al., 1969a). Synthesis of DNA is also essential for differentiation of appresorium and vesicle of germinating bean rust uredospores (Staples, 1975).

In the present experimental system, the quantitative changes are accompanied by some qualitative changes in the nucleic acids during differentiation. In the dark grown cultures, DNA eluted as one peak. DNA was also eluted as single peak, in the light grown cultures, after 68 hours in light. However, unlike in the dark grown cultures, the radioactivity in the DNA region of light grown cultures, was confined only to the first half of the UV peak. Assuming that the incorporation in the DNA region is due to RNA-DNA complex (Hemleben-Vielhaben, 1966; Cherry, 1964; Richter and Senger, 1965; Sebesta et al., 1965; Hayashi and Spiegelman, 1961; Schulman and Bonner, 1962; Mead, 1964; Mandel and Borkowska, 1964), the displaced radioactive peak in the DNA region would indicate preferential transcription of certain parts of genome which eluted with lower salt concentration. This DNA associated with RNA is known to be rich in G-C content (Hemleben-Vielhaben, 1966) and thus might represent preferential transcription
of nucleolar DNA. Later, after 72 or 76 hours of incubation in light, DNA eluted as double peaks. The main nuclear DNA peak was accompanied by a heavy satellite DNA peak. Elution of DNA as a double peak, by MAK column, has been reported earlier (Hadziyev et. al., 1969; Wolf, 1967; Comb et. al., 1964). Since satellite DNA eluted with lower salt concentration and DNA rich in high G-C content is known to elute with low concentration of salt (Sueoka and Cheng, 1962), we have tentatively attributed the heavy satellite DNA as nucleolar DNA (Wallace and Birnstiel, 1966; Gall, 1968). There is a possibility that this heavy satellite DNA might have originated as a result of several fold amplification of G-C rich DNA segment(s), being preferentially transcribed in 68 hour old cultures in light. The satellite DNA thus appeared simultaneously with the increase in DNA contents between 68 to 72 hours of incubation in light. Satellite DNA rich in G-C content, assigned to nucleolar origin has been reported from other fungi such as Physarum polycephalum (Braun and Evans, 1969) and Blastocladia emersonii (Comb et. al., 1964), although there is some discrepancy about the satellite DNA in the later (Myers and Cantino, 1971).

In the MAK profiles of nucleic acids from the light grown cultures, the radioactivity profiles extended beyond
the OD 260 nm curves. This high specific activity heavy fraction has also been observed during differentiation in *Blastocladiella* (Murphy and Lovett, 1966) and *Achlya* (Griffin and Breuker, 1969), but the precise characterization of the heavy fraction has not been followed.

The fractionation profile of total nucleic acids from non-differentiating cultures, showed neither two peaks of DNA, nor the radioactivity appeared in the heavy RNA. This might mean that differentiation involves addition of r-RNA cistrons. The extra chromosomal amplification of ribosomal genes as distinct satellites has been described by Birnstiel et. al. (1968), Brown and Weber (1968) and Gall (1968). Perhaps new ribosomal RNA population is required for the synthesis of sporulation specific proteins (Hsu and Weiss, 1969; Lodish, 1970; Dube and Rudland, 1970; Steitz et. al., 1970).

The elution pattern in the soluble region (t-RNA region) of the MAK profile was significantly different between sporulating and non-sporulating cultures, which might mean that specific population of t-RNA was being preferentially transcribed during differentiation. Specific activity (incorporation/OD unit) of RNA and that associated with DNA was much higher in the light grown cultures than in the dark. This suggests that heavy
transcription is specifically required for differentiation which is not needed in the cultures, not undergoing differentiation. \(^3\)H Uridine incorporation associated with satellite DNA (25% of total DNA), present in differentiating cultures was twice as much as main nuclear DNA. Role of new ribosomal RNA (Sauer, et. al., 1969b) and soluble RNA (Chet and Rusch, 1970) synthesis has been emphasized earlier during morphogenesis in Physarum polycephalum. The well known case is that of amphibian oocyte where a thousand fold amplification of ribosomal genes during differentiation has been clearly shown (Miller and Beatty, 1969).

DNA has earlier been reported to undergo similar modification during differentiation in both prokaryotes and eukaryotes. In the dimorphic bacterium, Caulobacter crescentus, the conversion of Swarmer cell to stalked vegetative cell is accompanied by the appearance of a satellite DNA (Shapiro et. al., 1971). The dormant spores of Bacillus cereus T contain a heavy satellite DNA which disappears during germination (Douthit and Halvorson, 1966). Germination of wheat embryos involves a deletion of r-RNA cistrons and G-C rich regions of the chromosomal DNA (Chen and Osborne, 1970), suggesting that amplification of genes must occur in wheat embryos before maturation.
V.3 CHANGES IN SOLUBLE PROTEINS DURING DIFFERENTIATION

According to the central dogma of gene action, changes at transcriptional level observed during differentiation should be reflected in the production of new proteins. Soluble protein composition was therefore analyzed, electrophoretically in order to assess changes in the soluble proteins at various stages.

Soluble protein composition of mycelial cultures varied significantly between 48-96 hours of incubation in light as well as in dark (after 48 hours of preincubation in dark). Several new protein bands appeared during differentiation, while few of them disappeared and some of the protein bands remained unchanged. Further incubation subsequent to 96 hours did not alter the basic soluble protein pattern of cultures in light or dark.

Many of the changes observed between 48-96 hours of incubation were common to both light and dark grown cultures and thus were not related to light induced pycnidial differentiation. Changes in soluble protein pattern and intensity of bands, not related to differentiation, has been demonstrated in few species of *Penicillium* (Bent, 1967).

The protein pattern of differentiating cultures were distinguished by the presence of a prominent pair of
protein bands in the center of the gels and an extra protein band in the low mobility region of the gel. On the other hand, a pair of protein bands present approximately in the center of the gels of dark grown cultures was absent from the protein pattern of cultures grown in light.

No significant changes in the total contents of soluble proteins was observed at any stage. However relative proportion of some proteins increased considerably over the period of incubation suggesting that some proteins were being preferentially synthesized over the others.

Earlier work in other fungi also indicated changes in the soluble protein pattern associated with differentiation processes. Zeldin and Ward (1963) have demonstrated differences in the soluble protein pattern of the differentiated presporangial stage and the plasmodial state of *Physarum polycephalum*. Different morphogenetic forms of *Blastocladiella emersonii* have been characterized by specific protein patterns (Cantino & Goldstein, 1962). Changes in the protein pattern and intensity of bands has been shown earlier in *Penicillium griseofulvum* and *Sclerotium rolfsii* (Bent, 1967; Chet, et. al., 1972).
In *Trichoderma viride* where conidiation is similarly induced by light, no quantitative or qualitative differences in the proteins and nucleic acids could be detected between the induced colonies and the control colonies maintained in dark (Stavy et. al., 1970, 1972).

Unlike in *B. theobromae* where a 5.0 cm diameter colony undergoes synchronous sporulation, in *T. viride* only the extreme peripheral zone of mycelial cultures (about 2-10mm, in width) responds to light stimulus (Galun, 1971). Changes occurring in relatively few hyphae in *T. viride* thus might be masked by massive vegetative mycelial contribution.