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Blue-green algal nitrogen fixation associated with deepwater rice in Bangladesh

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BLUE-GREEN ALGAL NITROGEN FIXATION ASSOCIATED WITH

DEEPWATER RICE IN BANGLADESH

by

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This thesis is entirely my own work and has not
previously been submitted for any other degree.

(Abdul Aziz)

May 1985

to my parents

M. A. Karim and Hasina Begum

ABSTRACT

The morphology and nitrogenase activity (ARA) of Gloeotrichia pisum Thur. growing on deepwater rice were studied in situ at Sonargaon, Bangladesh, and compared with results from laboratory studies. The alga showed obvious similarities and dissimilarities.

Hairs, gas vacuoles and akinetes, which were found in the field, were not found in the laboratory, even though the influences of quantity and quality of light flux and nutrient deficiency were tested.

The response of nitrogenase to changes in light flux was rapid. A reduction in flux brought about similar results in the field and in the laboratory. Diel changes in nitrogenase activity showed a maximum at mid-day in the presence of maximum light flux, but considerable activity (3.7% of daily total) took place at night. The activity in a medium free of combined carbon by bacteria free G. pisum indicates that the rate and duration of nitrogenase activity in the dark depend on the preceding light conditions.

In batch culture, higher nitrogenase activity was observed when dark-grown alga was reilluminated, than the maximum activity ever found under continuous illumination.

The variation of nitrogenase activity in batch culture was studied in relation to the growth characteristics and developmental stages of the alga. Maximum activity (1.4 nmol C_2H_4 µg chl a^{-1} min^{-1}), was observed after about one day of growth under continuous light. During this period, juvenile filaments were abundant (hence maximum heterocyst frequency), cyanophycin granules lacking and cultures had the lowest chl a : d. wt ratio. Heterocyst differentiation occurred between 14 and 24 h of growth and cell division was observed only after heterocyst differentiation. It appears that the juvenile filament is the most active nitrogen fixer during the life cycle of the alga. Reasons for low nitrogenase activity of about 0.352 nmol C_2H_4 µg chl a^{-1} min^{-1} in the field have been discussed.

It is estimated from diel changes in N_2-fixing activity and based on ARA of 77.4 nmol C_2H_4 $tiller^{-1}$ min^{-1} and using some approximations, that G. pisum contributed about 4 kg N ha^{-1} $season^{-1}$ in deepwater rice fields at Sonargaon in 1983.

A brief morphological study was also included. In the field, rapid colonization was observed on freshly submerged rice culms. Colonies contained intersheath spaces on their periphery and exhibited zonation of filaments, in the larger ones. The mechanism of the formation of a radiating colony in the laboratory has been described and discussed. Differentiation of a hormogonium into a filament was studied in batch culture. The basal youngest cell differentiated into a heterocyst, whilst the rest of cells divided repeatedly, forming several groups of cells in a row. Subsequently most of these groups of cells were liberated successively as hormogonia.

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It is with pleasure that I acknowledge all the people who helped me in the completion of this work. I am especially grateful to Dr B.A. Whitton, my supervisor, for his guidance and encouragement and for helpful discussion throughout the work.

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LIST OF ABBREVIATIONS

ARA	Acetylene Reduction Assay (Acetylene Reducing Activity)
s	second
min	minute
h	hour
d	day
nm	nannometre
μm	micrometre
mm	millimetre
cm	centimetre
m	metre
km	kilometre
ha	hectare
ml	millilitre
l	litre
μg	microgram
mg	milligram
chl <u>a</u>	chlorophyll <u>a</u>
d. wt	dry weight
M	molar
μmol	micromole
nmol	nannomole
N	normal solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
EDTA	ethylenediaminetetra-acetic acid
P	phosphorus
FRP	filtrable reactive phosphorus
FTP	filtrable total phosphorus
$^{\circ}$ C	degree Celsius
PAR	Photosynthetically Active Radiation (400-700 nm)
K Pa	kilo Pascal
$\bar{x}$	mean
$\pm$	standard deviation
n	number of replicates

1 INTRODUCTION

1.1 Introductory remarks

In Bangladesh deepwater rice is the only crop grown in low-lying areas during the monsoon period (Section 1.2.3). Its importance is evident from the fact that there is no other crop which can be grown in these areas during this season and produce a stable yield year after year with little or no addition of fertilizer. Brammer (1976, 1983) attributed an important part of this fertility to the presence of blue-green algae.

It is well established that some blue-green algae are capable of fixing atmospheric nitrogen and that part of this nitrogen can eventually be incorporated into the rice plant. Apart from floristic reports, there are no eco-physiological studies of blue-green algae in rice fields of Bangladesh. Studies carried out elsewhere indicate that N_2-fixing activity by blue-green algae in rice fields is affected markedly by light intensity. The present study was therefore planned mainly to evaluate the effect of light intensity on N_2-fixing activity by Gloeotrichia pisum in the field as well as in the laboratory. (G. pisum is a widespread alga in Bangladesh.) Some morphogenetic studies were also planned in order to understand the developmental patterns of the alga and to see if there is a relationship between nitrogen-fixing activity and developmental stages.

1.2 Bangladesh

1.2.1 Physical environments

Bangladesh, a land of 144000 km^2 and situated between 20° 35' to 26° 75' N and 88° 03' to 92° 75' E, stretches from near the foot-hills of the Himalayas to the Bay of Bengal (Fig. 1.1).

The landmass has been formed throughout the Pleistocene and up to the present by sediments washed down from the Himalaya Mountains through the Ganges, Jamuna (Brahmaputra) and Meghna rivers and their numerous tributaries and distributaries (Morgan & McIntire 1959). In terms of the relative age of the landmass, the region may be divided into four parts: hilly lands of the Tertiary (and older) in the south-east; terrace lands of the Pleistocene in the Barind and Madhupur Jungle; tippera surface of the early Recent in the median eastern part; the extensive floodplains of the Recent in the rest of the country.

Fig. 1.1   Bangladesh, showing the major floodplains and
            other physiographic regions (1a, Barind ; 1b,
            Madhupur tract ; 2, Meghna depression ; 3,
            Chittagong Hill tracts); inset shows origin
            of rivers.

Legend:
- Meghna floodplain
- Jamuna "
- Ganges "

100 km
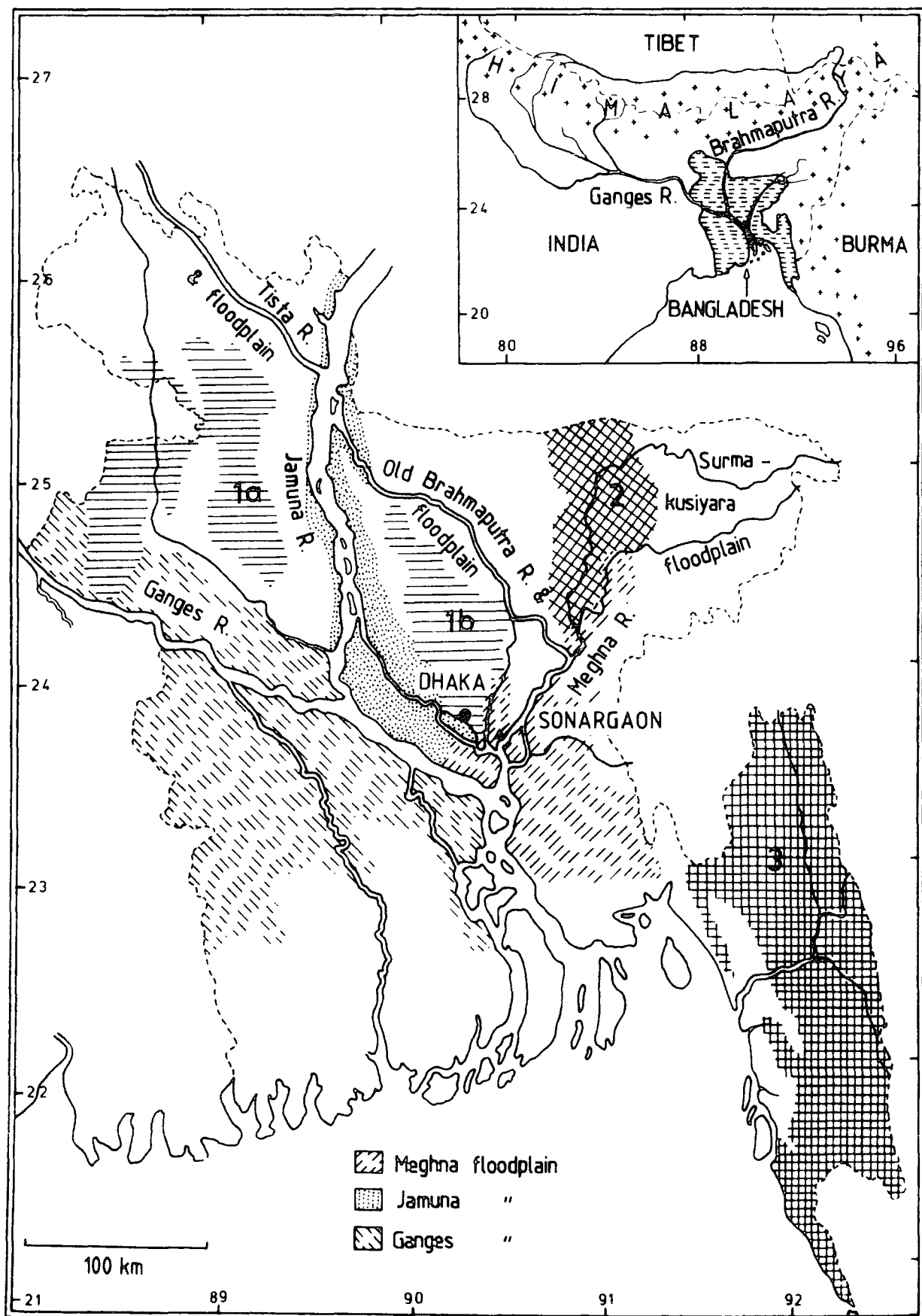
The extensive floodplains of Bangladesh lie almost at the sea-level in the south and rise gradually towards the north, with a maximum altitude of only about 46m (except hilly areas). A number of mighty rivers with their tributaries and distributaries and numerous bils (low-lying areas, some holding water throughout the year; as distinct from a chak which has no area of standing water in winter) all over the country, are the most significant physiographic characteristics. There are 5 river systems as shown in Fig. 1.1 (Ahmad 1968):

(1) Ganges or Padma and its deltaic streams;

(2) Jamuna's affluents and channels;

(3) Meghna and Surma-Kusiyara system;

(4) North Bengal rivers; the Tista is the most important;

(5) Rivers of the Chittagong Hill Tracts and adjoining plains;
the Karnaphuli is the most important.

Both the Ganges and Jamuna river systems originate from the vicinity of the crest of the Himalayas. The Surma and Kusiyara rivers unite forming the Meghna, which grows rapidly after confluence with the old Brahmaputra. All these rivers flow generally from the north to the south. However, out of 15.5 million km^2 catchment area of the river system, about 7.5% lies within Bangladesh. The activity and behaviour of these rivers is of utmost importance in determining the economic conditions of the people (Ahmad 1968). The country suffers from twin problems of flood and drought. From May to October the combined discharge of rivers totals about $142 \times 10^3 \ m^3 \ s^{-1}$ but this dwindles to only $7 \times 10^3 \ m^3 \ s^{-1}$ during the dry season (November to April). About 2.4 billion tons of sediment is discharged annually (Allison 1975, Rashid 1977).

Flooding is a part of the normal cycle of the seasons. Snow melt in the upper Himalayas, rainfall, silting up of river beds, topography and configuration of the country are the major factors which contribute to the annual flooding (Ahmad 1968, Allison 1975, Rashid 1977). The three major floodplains are the Ganges, Jamuna and Meghna (Fig.1.1). Flooding occurs first in the eastern part of the country followed by middle and western parts. Rate of water rise is normally 5-8 cm per day and usually two flood peaks occur, first in July and the second higher peak in early September (Allison 1975). Less than half of the country can be regarded as flood-free and this includes the hilly areas, Barind and Madhupur uplands. It is estimated that nearly 75% of

the floodplains is flooded deeper than 30 cm for 3-5 months during the rainy season (Brammer 1983). Floods normally affect about one-third of the country's cultivable land.

The climate is characterized by heavy rainfall, high humidity and temperature, and fairly marked seasonal variations. The mean maximum temperature during summer is in the range of 32-34° C. Throughout the monsoon more or less similar temperature prevails having a maximum of 30-32°C by day and a minimum of 24-26° C at night time. Mean minimum temperature is 9-13° C during January (Manalo 1975, Johnson 1982).

Rainfall has vital significance for agriculture (Ahmad 1968, Manalo 1975, Rashid 1977, Johnson 1982). Most parts of the country during November to February are almost completely rainless. April and May are considered as the season of "little rain" and a very important time for sowing, seedling establishment etc.(Section 1.2.2). In this period and including March, drought is the normal expectancy. June to September is the monsoon period and the months of maximum rainfall (80% of total precipitation: Allison 1975). During this period the maximum rainfall occurs in the north-east and south-east is 3000-5000 mm; around 2000 mm occurs in the central part and 1200 - 1500 mm in the west part of the country. Timing of the arrival of the heavy monsoon rain is very important, as it is linked with the fate of two major crops, the aus and aman. Heavy early monsoon rains could destroy aus (harvested before flooding) by early flooding, while late heavy rains could inundate and destroy the deepwater rice (Section 1.2.3). By October rainfall drops rapidly.

Humidity is high throughout the year, reaching over 80% everywhere during the monsoon.

For most of the monsoon period cloudy conditions prevail in marked contrast to the rest of the year. Johnson (1982) mentioned almost unremitting cloudiness during monsoon but more than ten cloudless days monthly from December to March. The hours of bright sunshine are at a maximum in January.

Ahmad (1968) classified soils into eight categories which correspond broadly to local names, while Brammer (1971) identified as many as 15 soil types throughout the country, based on geological origins and properties. In all floodplains, there is a characteristic pattern of permeable, usually loamy, soils on the highest parts and

impermeable, usually clay, soils on the lower parts (Brammer 1983).
However, about 25% of the country is non-calcareous floodplain
(alluvium) soil while about 25% of the total area is occupied by
calcareous floodplain soil (Johnson 1982).

1.2.2 Agriculture

The country's economy is predominantly agricultural and about 75%
of the total population of over 90 million is employed in it. The total
cropped area is about 129000 km^2 (12.9 million ha). A wide variety of
crops are grown and are broadly classified as:

(a) Bhadoi crops: correspond to the rainy season, grown usually in
the months of March to May and harvested in July to mid August
or November to January,

(b) Rabi crops: correspond to the dry season, grown in October to
December and harvested in mid January to mid April.

Aus and aman (transplanted and deepwater rice) paddy and jute are the
main bhadoi crops, while boro paddy, tobacco, pulses, vegetables etc.
are the main rabi crops. However, about 80% of the total cropped area
is occupied by the paddy and this amounts to an area of about 103000 km^2
(10.3 million ha) (Agricultural Yearbook of Bangladesh 1982).   During
monsoon aman paddy is the only growing crop found in the field (Section
1.2.3). Details of cropping patterns, crop estimates and land
utilization have been published recently in the Agricultural Yearbook of
Bangladesh(1982).

Land preparation, sowing, harvesting etc. are mostly done by
traditional methods using indigenous agricultural tools and appliances
(Indigenous Agricultural tools and equipment of Bangladesh 1982, Catling
et al. 1983). Irrigation facilities are limited and farmers mainly
depend on rain water. Only about 15700 km^2 (1.57 million ha) of land
(under different crops) were under irrigation in 1979-80. Urea and TSP
(triple super phosphate) are the major fertilizers used by the farmers.
Pesticides of different groups are sometimes used.

Average rice yield (unmilled) is low: 135 tons/km^2 (1.35 tons
ha^{-1}). However some high yielding rice varieties have been introduced.
Jute is the second most important crop, which together with tea, is the
leading export crop.

1.2.3 Deepwater rice

Deepwater rice is variously called broadcast aman, low-land aman
and floating rice. There are hundreds of cultivars and these are

perhaps physiological races of <u>Oryza</u> <u>sativa</u> L. The distinguishing character of this group of rice is that the internode, leaf sheath and blade can elongate by increase in cell length with rising water, can withstand short periods of total submergence (1-10 days), produce nodal roots and tillers, form knees, and when uprooted, float on the water surface.

Deepwater rice is grown in nearly 15700 km^2 (1.57 million ha) of land which is about 12.2% of the total cropped area (Agricultural Yearbook of Bangladesh 1982). The bulk of the deepwater rice is grown in the intermediate 0.91 to 1.83 m normally flooded zone (Allison 1975). Total elongation of the stem could be 2.5 to 25 cm day^{-1} (Choudhury & Zaman 1970). Zaman <u>et</u> <u>al</u>. (1975) showed linear increase of culm length despite fluctuation of flood levels occurring.

Pure strands of deepwater rice are grown in about 49-67% of fields, while in the rest the plant is mostly grown mixed with <u>Aus</u> and rarely with other crops (Catling <u>et</u> <u>al</u>. 1983). Land preparation, sowing, weeding and harvesting are usually done during March and April, late March and April, May and June, and end of October and November respectively. Land preparation and sowing thus could be affected by possible drought (Section 1.2.1). Overall mean yield is about 225 tons/km^2 (2.25 tons ha^{-1}). Catling <u>et</u> <u>al</u>. (1983) identified four factors associated with higher yields of deepwater rice :

(1) the Meghna floodplain having higher early rainfall and a milder flooding pattern;

(2) high yielding varieties, e.g. <u>Khama,</u> <u>Pankaish,</u> and <u>Kartik</u> <u>Sail</u>;

(3) maximum water depths of 1.5 to 1.8 m;

(4) pure strands of deepwater rice.

It is believed that the stable yield of deepwater rice is maintained by an annual deposit of alluvium from the seasonal flood and certainly by biological activity in the floodwater itself, specially that of blue-green algae (Brammer 1976, 1983, Martinez & Catling 1982).

Catling <u>et</u> <u>al</u>. (1983) reported that fertilizer is applied in about 21% of all deepwater rice fields. No significant difference in yield was observed between fertilized and non-fertilized fields. In less than 1% deepwater rice fields, insecticides are used before (for rice-borer) or after flood recession (for ear-cutting caterpiller).

1.2.4 <u>Aquatic vegetation and algal flora</u>

Biswas (1927) made a detailed study of the aquatic macrophytes of

what is now Bangladesh and West Bengal in India. He recorded 60 different taxa including pteridophytes, discussed ecological aspects and classified the water body into four zones: bottom zone (blue-green algae, resting spores etc); zone of phanerogams (rooted plants forming an aquatic meadow); intermediate zone (algae and submerged floating phanerogams); surface zone of micro- and macro-plankton. Recently Islam & Paul (1978) and Islam et al. (1980) surveyed the aquatic vegetation of a haor at Sylhet and a bil at Rajshahi district respectively. A total of 52 macrophytes were recorded from Sylhet during the monsoon period, with the following six taxa as dominant : Eichhornia crassipes, Hydrilla verticillata, Ipomoea aquatica, Pistia stratiotes, Nymphoides indicum and N. cristatum. Islam and co-workers have published lists of aquatic algae covering almost all the districts of Bangladesh and recently Catling et al. (1981) surveyed algae associated with deepwater rice. Algae which occur commonly as epiphytes on rice and other hydrophytes are Gloeotrichia pisum, G. natans, species of Anabaena, Nostoc, Microchaete, Oedogonium, Bulbochaete, Coleochaete, Chaetophora, Gomphonema etc. ; among the free-living forms, commonly occurring taxa are Gloeotrichia natans, Anabaena spp., Aulosira fertilissima, species of Spirogyra, Rhopalodia, Fragilaria etc., and among benthic forms are species of Chara and Nitella (Islam 1972, 1973, Islam & Sarma D. 1968, 1976, Islam & Sarma P. 1976, Islam et al. 1980, Islam & Begum 1981, Catling et al. 1981). On the deepwater rice culms, Anabaena occurred most commonly followed by Gloeotrichia, Oscillatoria, Chroococcus, Nostoc, Lyngbya and Microchaete in decreasing order (Martinez & Catling 1982). They also observed periodicity of blue-green algae in deepwater rice fields, being abundant during the early part of flooding and decreasing with the receding of floodwater.

1.3 N_2-fixation by blue-green algae in rice fields

N_2-fixing blue-green algae grow abundantly in tropical and subtropical regions and are particularly common in rice fields (Watanabe & Yamamoto 1971). Since De (1939) provided evidence, while working on blue-green algae of (present day) Bangladesh, that these organisms are the main agents for N_2-fixation in the rice field soil, an enormous volume of material on this subject has been published from various countries (Singh 1961, Venkataraman 1972, Roger & Kulasooriya 1980, Roger & Watanabe 1982). However, so far, no study has been reported in Bangladesh on the N_2-fixing activity of blue-green algae in rice fields.

The probable importance of blue-green algae in the nitrogen economy of Bangladesh deepwater rice fields has been stressed by several authors (Brammer 1976, 1983, Catling et al. 1981, Martinez & Catling 1982). Compared to lowland or other rice fields, very little work has been done on N_2-fixing activity by blue-green algae in deepwater rice fields (Kulasooriya et al.1980, 1981a, 1981b, Watanabe & Ventura 1982). Maintenance of natural nitrogen fertility of rice fields has been explained by the blue-green algal N_2-fixation, its slow mineralization and accumulation in soil, and that the rice plant influences increased N_2-fixation and reduces loss (De & Sulaiman 1950, Hirano 1958, Subrahmanyan et al. 1965, Watanabe 1965, App et al. 1980, Tirol et al. 1982).

Under a relatively low light intensity (800 lux) a N_2-fixation rate of 10-20 Kg N ha^{-1} crop^{-1} (extrapolating nitrogen fixing activity of 5.1 µmol N plant^{-1} h^{-1} based on two determinations at heading and maturing stages of plant, by ARA technique) has been observed in deepwater rice fields mainly due to epiphytic Nostoc, Anabaena, Calothrix and Gloeotrichia (Kulasooriya et al. 1981a) and this value corresponds closely to that found by App et al. (1980). The blue-green algae attached to submerged weeds also play a positive role in nitrogen cycling by fixing 2 Kg N ha^{-1} crop^{-1} under rice cultivation and 4 Kg N ha^{-1} crop^{-1} under fallow land (Kulasooriya et al. 1981b). In lowland rice fields, variable N_2-fixation rates have been found and a contribution in the range of 20-30 Kg N ha^{-1} crop^{-1} appears to be typical (Agarwal 1979, Venkataraman 1981, Roger & Watanabe 1982). The presence of blue-green algae can lead to a 10-15% increase in grain yield in the total absence of chemical fertilizer (Agarwal 1979, Roger & Watanabe 1982). Recently, Watanabe & Ventura (1982) found by ^{15}N study in a deepwater rice plot that about 15% of the total nitrogen in deepwater rice had been supplied by blue-green algal N_2-fixation, though they suggested that elsewhere this value was likely to be higher.

N_2-fixation in rice-fields can be influenced by a variety of factors, the cumulative effect of which determines the ultimate nitrogen gain (Sethunathan et al. 1981). The most important are likely to be light intensity and nitrogenous compounds in the surroundings. The rate of N_2-fixation is usually light dependent (Fogg 1974, Roger & Reynaud 1979). Deficiency of light limits N_2-fixation, e.g. highest activity was recorded when the plant canopy gave the least cover (Watanabe, Lee &

De Guzman 1978, Boddey & Ahmad 1981) and during cloudy weather, higher
activity in unplanted fields than in planted fields (Yoshida & Ancajas
1973). On the other hand, high light intensity may inhibit N_2-fixation
(Reynaud & Roger 1978). Asymmetric curves of ARA with a maximum either
in the morning (with a low decreasing activity in the afternoon) or in
the afternoon, recorded by Alimagno & Yoshida (1977), have been
explained by Roger & Reynaud (1979) as the inhibitory effect of high
light intensity and optimal light intensity in the afternoon
respectively. However, no study has so far been made on N_2-fixation by
blue-green algae in deepwater rice fields down the water column or with
respect to diel and seasonal variation.

In the presence of nitrogen fertilizer, blue-green algal N_2-fixing
activity is inhibited or at least affected (Roger & Kulasooriya 1980).
Alimagno & Yoshida (1977) estimated about 18-33 Kg N ha^{-1} $crop^{-1}$ are fixed in the
unfertilized lowland rice soil compared to 2.3-5.7 kg N ha^{-1} $crop^{-1}$ in
the fertilized one. In contrast, it has been estimated that the
efficiency of N_2-fixation does not seem to have been affected even when
algae were applied in combination with 75 kg N ha^{-1} chemical fertilizer
and that highest increase in grain yield was obtained in the third
season, suggesting that the benefits accumulate over the years
(Mudholkar et al. 1973). It has been estimated that about 14%
additional energy yield (in terms of nitrogen contribution in the range
of 20-30 kg ha^{-1}) could be obtained by blue-green algal complementation
with nitrogen fertilizer (Venkataraman 1981). Seasonal variation in
N_2-fixing activity has been attributed to the succession of blue-green
algae (Watanabe, Lee & Alimagno 1978), shading by the plant canopy
(Yoshida & Ancajas 1973, Boddey & Ahmad 1981), or a predominant effect
of light intensity in relation to both season and plant canopy (Roger &
Reynaud 1979). In the wet season N_2-fixing activity can decrease to 20%
(3 kg N ha^{-1} $crop^{-1}$) in the flooded rice field compared to the dry
season (15 kg N ha^{-1} $crop^{-1}$) (Yoshida & Ancajas 1973). Two peaks have
been observed by Watanabe, Lee & De Guzman (1978), one at an early stage
of rice cultivation and another after harvesting, in both dry and wet
seasons, which the authors considered as the light effect. However,
peak N_2-fixing activity may occur at any time during the cultivation
cycle (Roger & Reynaud 1979).

A considerable increase of N_2-fixation by blue-green algae in the
presence of the rice crop due to the increased CO_2 supply, has been

documented in a laboratory study (De & Sulaiman 1950). Little or no information is available on the effect of changes of pH (with the change of CO_2) and O_2 in the rice field on N_2-fixing activity by blue-green algae. Rarely, temperature could be a limiting factor for blue-green algal N_2-fixation, particularly in deepwater rice fields which are relatively well temperature buffered, but in dryland, under weak plant cover, a high temperature in the middle of the day may inhibit blue-green algal N_2-fixation (Roger & Reynaud 1979).

1.4 Growth and development in Rivulariaceae

1.4.1 Growth pattern and trichome development

Trichomes of members of Rivulariaceae are broad at one end while towards the other they taper more or less markedly and are commonly produced into a colourless multicellular hair (Geitler 1925, 1932, Fritsch 1945). Tiwari et al. (1979) described the rivularian trichomes as unidirectional and monopolar with a basal heterocyst and gradual tapering end and this unidirectionality results in the corresponding modes of cellular growth, division and ultimate trichome morphology. Schwendener (1894) studied development of Gloeotrichia pisum, Rivularia polyotes and some other members of Rivulariaceae. He described an obvious change at the terminal end of the filament, by the formation of a hair and the meristematic zone behind it, where the most frequent divisions take place. This type of growth initiated by a meristem at the base of the hair has subsequently been described as trichothallic (Geitler, 1925, 1932, Fritsch 1945). Schwendener observed changes throughout the filament and found that long before the hair has completed its development, the division proceeds gradually towards the base and extends finally to the cell next to the heterocyst. Poliansky (1930) observed in Gloeotrichia natans, intensive divisions after akinete formation, in the lower vegetative cells of the trichome, and disappearance of the intercalary meristematic zone (narrowing of meristem cells and at the same time losing its function), which makes the predominant intercalary growth into predominantly basal. Total lack of an intercalary meristem had been observed in G. ghosei (Singh 1939). However, most authors have described the growth of Rivulariaceae members as meristematic, the meristem lying just below the hair as shorter and wider disc-like cells (Schwendener 1894, Geitler 1925,1932, Fritsch 1945, Desikachary 1959, Jeeji-Bai 1977, Fogg et al. 1973, Chang 1979a, 1983, Cmiech et al. 1984). Following the division pattern of a cell in

the meristematic zone, Schwendener (1894) found as many as 8-16 cells descending from a mother cell. A sheath develops around the developing trichome after the development of hair which does not take part in the sheath formation, and as a result lies free in the surrounding water (Schwendener 1894). Cell length and breadth varies tremendously depending on position and the stages of growth, being markedly long and narrow at the base of trichome while below the hair, cells may be considerably shorter and wider or like the cells in the basal part (Schwendener 1894, Poliansky 1930). However, Schwendener (1894) observed all the above phenomena while checking various representatives of the Rivulariaceae.

1.4.2 Hormogonia

Hormogonia are short lengths of trichome with rounded ends and without differentiation of cells; they secrete mucilage during their movement and eventually develop into trichomes (Fritsch 1945). Hormogonia are produced from the intercalary meristematic zone after the hair portion is thrown off (Desikachary 1959, Chang 1979a, 1983). On the other hand, it has been observed in Calothrix parietina that in the medium used, hormogonia release may be continued typically producing successively about five gas vacuolated hormogonia per filament, ultimately leaving only a short basal length of trichome with the heterocyst (Livingstone & Whitton 1983). Fritsch (1945) described the delimitation and liberation of hormognia by the modification and ultimate death of occasional cells in the trichome. Lemont (1969) observed that the necridium ensures trichome breakage. Hormogonia are without any definite sheath and cell division (Schwendener 1894). Singh & Tiwari (1970) observed in Gloeotrichia ghosei fragmentation of the hormogonium into small pieces, having granules and gas vacuoles. Highly gas-vacuolated hormogonia with abundant cyanophycin and polyphosphate granules have been reported in Calothrix (Wood 1984). The maximum net rate of movement has been recorded to be about 0.06 μm s^{-1} over a period of 16 h under uniform light of 150 μmol m^{-2} s^{-1} (Livingstone & Whitton 1983). Hormogonia are usually 45-60 μm long (Darley 1968, Livingstone & Whitton 1983) consisting of usually 8-16 cells (De Bary 1863, Schwendener 1894, Wood 1984). A terminal cell differentiates into a basal heterocyst after the hormogonium settles down (Schwendener 1894, Singh & Tiwari 1970) and then disappearance of gas vacuoles (Livingstone & Whitton 1983) or both gas vacuoles and

granules (Singh & Tiwari 1970) occurs. The life span of a hormogonium from the time of liberation to germination is ca 24h in C. parietina (Livingstone & Whitton 1983) while Rai et al. (1978) found heterocyst formation within 12-14 h in C. brevissima, when ammonium nitrogen-grown undifferentiated filaments were transferred to a medium without nitrogen, maximum heterocyst frequency being obtained after 48 to 72 h of incubation.

1.4.3 Heterocysts

Heterocysts are characterized by having a well defined thickened wall and homogeneous and pale yellow cell contents with polar nodule(s) adjoining the adjacent vegetative cell(s) (Tyagi 1975). In the trichomes of Rivulariaceae the most typical condition is the possession of a single basal heterocyst, but further basal and or intercalary heterocysts may also be produced (Geitler 1932). Tiwari et al. (1979) described the basal heterocysts of Rivulariaceae as unidirectional and monopolar and this unidirectionality results in the corresponding modes of cellular growth, division and ultimate trichome morphology (Section 1.4.1). Terminal heterocysts may also develop in an intercalary position, particularly in association with the production of false branches (Fritsch 1945, Jeeji-Bai 1977, Rai et al. 1978). Heterocysts may be produced alternating with the akinete (Poliansky 1930, Desikachary 1959) or at both ends of the trichome (Claassen 1973). Two basal heterocysts followed by an akinete were also observed in Gloeotrichia natans (Poliansky 1930). Weber (1933) and Chang (1983) observed many successive basal heterocysts in Calothrix fusca and Gloeotrichia echinulata respectively and each developed with the death of the preceding heterocysts. In a mutant form of G. ghosei, Tiwari et al. (1979) also observed a chain of basal heterocysts. However, heterocysts are extremely variable in shape and size, even in the same colony of natural population of G. natans (Poliansky 1930). Germination of heterocysts has been observed in several taxa, liberating motile hormogonia (Desikachary 1946, Singh & Tiwari 1970). The latter authors concluded that the controlling factor for heterocyst germination appears to be the concentration of ammoniacal nitrogen in the medium.

1.4.4 Hairs

The hair of the Rivulariaceae is a region of the trichome where the cells are narrow, elongated, highly vacuolated and usually apparently colourless (Sinclair & Whitton 1977). These authors found

that the phosphorus deficiency in the environment leads to the development of hairs at the end of trichomes of many members of this group. Schwendener (1894) observed the development of a hair in Gloeotrichia pisum from the early stage of trichome formation and described it as follows:

"One can observe the clear stretching of the filament at the terminal end where the hair will develop. At this period the end cell stretches first and then 2-3 cells behind it. In each case, the stretching is accompanied by simultaneous narrowing. Somewhat later, when the participating members have developed double the original length or even longer, the first new cross wall appears usually first in the end cell and then in the neighbouring cell. Afterwards there follows further stretching of the component cells until reaching to the manifold of its cross diameter. With that the construction of the hair end finishes: later the contents become more faded and at the same time more vacuolated and at the end contents disappear completely.."

Recently, hairs have been shown to develop from apical cells which taper to a colourless multicellular hair, and arise through considerable elongation and vacuolization (Livingstone & Whitton 1983, Cmiech et al. 1984). Sinclair (1977: 157, Fig. 5.4 a-c) observed in two Calothrix strains that the transition between the chlorophyll-containing vegetative cells and the adjacent hair cells was abrupt, an examination of the figures of Schwendener (1894: Fig. 21), Desikachary (1959: Pl. 117, Fig. 9), Frémy (1972: Pls 42 and 44) & Chang (1983) also gives this impression. However, contrary to the formation of hair as a response to phosphorus deficiency, Sinclair (1977) observed many hairs in media containing high a concentration of phosphorus in Gloeotrichia echinulata, G. ghosei and Rivularia sp. Following an ultrastructural study of Gloeotrichia echinulata hairs, Cmiech et al. (1984) stated that progressively older cells display increased thylakoidal disorganization, a loss of cytoplasmic matrix, marked vacuolization and rupture of plasmalemma, and considering all these facts, they suggested that the hair cells are more indicative of "degeneration" than "differentiation".

1.4.5 Akinetes

Akinetes are specialized whole cells usually larger with thicker than vegetative cells cell walls and highly granular cytoplasm, representing the usual method of perennation (Nichols & Carr 1978, Nichols & Adams 1982).

Gloeotrichia and several species of Calothrix have the ability to produce an akinete usually next to the heterocyst (Geitler 1932). Three modes of akinete formation have been recognized :

(a) the cell next to_a heterocyst undergoes repeated division, of which the lowermost cell gradually increases in size to many times the length of other cells, forms a thick wall and converts into an akinete (Desikachary 1959);

(b) more than one vegetative cell may take part in akinete formation by the decomposition of cross walls (Geitler 1925, Claassen 1973);

(c) transformation of cells into akinetes alternating with heterocysts (Desikachary 1959, Chang & Blauw 1980).

Simultaneous with the formation of the akinete, a thick sheath begins to form, particularly around the akinete and its adjoining vegetative cells (Desikachary 1959, Claassen 1973).

No clearly defined environmental trigger has been recognized for akinete formation although many nutrients have been implicated (Adams & Carr 1981, Nichols & Adams 1982). Roelfs & Oglesby (1970) noted that in Gloeotrichia echinulata spores seemed to be produced when the colony reached a particular size, even when conditions were apparently favourable for growth in nature and growing logarithmically. In the laboratory, organisms may not develop akinetes (Singh & Tiwari 1970, Maxwell 1974) or the akinete may not express the same shape and size as in nature (Chang 1979a, 1983). Forest & Khan (1972) while discussing morphological plasticity of Rivulariaceae noted that akinetes were never found in the planktonic alga traditionally known as G. echinulata, in a well known natural habitat. In G. ghosei, a deficiency of iron decreased akinete frequency (Nichols & Adams 1982). However, light is expected to have considerable influence on akinete formation by the fact that decreasing the light intensity results in akinete development at a lower cell density and vice versa (Nichols & Adams 1982). No work has been done on the effect of light on akinete formation of Gloeotrichia.

1.4.6 Gas-vacuoles

Gas-vacuoles are bundles of gas vesicles within the protoplasm and appear as irregular and indefinite reddish structures under the light microscope (Smith & Peat 1967, Fogg 1972). These are produced more abundantly at low than at high light intensities and provide a buoyancy regulating mechanism (Walsby 1969, 1978, Fogg 1972, Walsby & Booker 1980) rather than light shielding (Van Liere & Walsby 1982).

In _Gloeotrichia echinulata_, the gas vacuoles could occupy 0.7-0.8% of the cell volume which enables the alga to just float in water (Klebahn 1922) or 22% which would make the alga extremely buoyant (Smith & Peat 1967). Highly gas vacuolated hormogonia have been recorded in _G. ghosei_ (Singh & Tiwari 1970) and _Calothrix parietina_ (Livingstone & Whitton 1983) in the laboratory condition. It has been pointed out that in some forms of blue-green algae, isolated gas vesicles may remain in cells, these have so far remained undetected by light microscopy (Whitton 1972).

1.4.7 Colonies

Species of _Gloeotrichia_ and _Rivularia_ form ordered colonies in nature where the component tapered filaments are radially arranged with the basal heterocysts directed towards the centre and hairs extending beyond the colonial mucilage (Schwendener 1894, Geitler 1932, Desikachary 1959). In most cases this filamentous organization forming an ordered colony does not appear in the laboratory (Darley 1968, Maxwell 1974, Chang 1979a, 1983), other than in a few cases described by De Bary (1863), Schwendener (1894) and Singh & Tiwari (1970). Tiwari _et al_. (1979) observed that in the presence of combined nitrogen a mutant strain of _Gloeotrichia ghosei_ showed complete suppression of heterocysts and developed into a spindle-shaped colony rather than a radiating pattern like the parental clone.

De Bary (1863) described the early stage of _Rivularia_ colony formation. He observed grouping of several hormogonia side by side forming a small bundle as the first sign of colonization. Later, heterocysts develop$_{ed}$ and trichomes elongated giving a sort of radiating appearance with heterocysts at the centre. However, the mechanism which controls the aggregation of hormogonia is unknown (Lazaroff & Vishniac 1964).

In nature, heavy colonization by _Gloeotrichia_ has been reported on the older, submerged plant parts (Kulasooriya _et al_. 1981b).

1.4.8 Morphological variations by the laboratory strains

From the preceding sections it will be apparent that species of Rivulariaceae may exhibit a wide range of morphological forms both in the laboratory and in the field as a result of their rather complex life cycle (De Bary 1863, Schwendener 1894, Poliansky 1930, Singh & Tiwari 1970, Maxwell 1974, Chang 1983). In addition to the inherent variability, some forms may also show morphological variations in the

laboratory which seem not to be apparently directly related to the normal developmental cycle. However, the need for the study of morphological variability has been emphasized, particularly in solving taxonomic problems and understanding the phylogenetic affinities of a taxon (Prowse 1972, Desikachary 1973). One obvious change in morphology is the production of mostly single or sometimes double false branches in old cultures (Jeeji-Bai 1977). Rai et al. (1978) noted that the formation of false branches is dependent on nutritional conditions (particularly presence of low level ammonium nitrogen) and seems to be correlated with the presence or absence of heterocysts, and suggested that false branches emerge due to the hitherto unrecognized pattern of the localization of rapidly dividing cells along the algal filament and the integrity of algal trichomes with the surrounding sheath.

In the older cultures increase in thickness of sheath as well as looplike twisting trichome(s) within the sheath and also helically twisted "ropes" of filaments are usually evident (Pearson & Kingsbury 1966, Lange 1975, Jeeji-Bai 1977, Chang 1983).

1.5 Influence of light on morphology

Gas vacuolation (Walsby & Booker 1980), akinete formation (Wolk 1965, Fernandes & Thomas 1982) and heterocyst differentiation (Kale 1972) have been shown to be influenced by light. It is well established that at low light intensity gas vacuole formation is most active while at high light intensity they collapse (Walsby 1969, 1972, Fogg 1972).

Incandescent light, but not fluorescent, greatly stimulated sporulation, suggesting possible involvement of red light in the akinete differentiation in Anabaena torulosa (Fernandes & Thomas 1982). They observed enhanced sporulation with the increasing incandescent light intensities of 1000, 3000 and 5000 lux. Wolk (1965) reported an inhibitory effect of high light intensity on akinete formation.

No information exists about the effect of light on the morphology of hairs.

1.6 N$_2$-fixation by blue-green algae

Heterocysts are the site of N$_2$-fixation under aerobic conditions (Fay et al. 1968, Stewart et al. 1969, Van Gorkom & Donze 1971) and the nitrogenase activity is not manifest until heterocysts are fully differentiated (Lang 1965, Kulasooriya et al. 1972, Wolk 1981). In batch culture, maximum nitrogenase activity occurs during logarithmic growth and is fairly directly correlated with highest heterocyst frequency (Jewell

& Kulasooriya 1970, Chang 1979b, Antarikanonda & Lorenzen 1982). On the other hand, Stewart & Lex (1970) found in Plectonema boryanum, a non-heterocystous blue-green alga, that the peak specific nitrogenase activity occurred when the phycocyanin content decreased markedly but the exact implications of this phenomenon in relation to N_2-fixation is not known. Phycocyanin may be used as a source of nitrogen during periods of nitrogen starvation (Allen & Smith 1969) or may degrade (Van Gorkom & Donze 1971, Schenk et al. 1983). Reformation of this pigment showed that the organism was no longer deficient in nitrogen (Van Gorkom & Donze 1971). Studies with two colonial types of Gloeotrichia echinulata (a Rivularia-like young and active short trichome with high heterocyst frequency which forms a mesh, and a typical Gloeotrichia-like long trichome with a low heterocyst frequency which forms cluster-like colonies) in axenic culture indicated that N_2-fixing activity is variable in different developmental stages, being higher in the Rivularia-like mesh (Chang & Blauw 1980). They obtained a rate of about 0.3 nmol C_2H_4 mg d. wt^{-1} min^{-1} at 3 klux intensity by the 6 week old algal mesh.

The rate of N_2-fixation by blue-green algae is usually light-dependent and has close relationship with photosynthesis (Fogg 1974, Bothe 1982). The electrons and ATP for N_2-fixation are generated via photosynthesis (Lex & Stewart 1973) and both the rate and duration of nitrogenase activity in the dark are affected by the rate of photosynthesis and assimilation during the light period (Dugdale & Dugdale 1962, Stewart et al.1967, Fay 1976). Lex & Stewart (1973) demonstrated that in cells with high carbohydrate reserves, DCMU (3'-[3,4-dichlorophenyl]-1',1'-dimethylurea) did not affect ARA but upon depletion of these reserves, inhibition progressively appeared. ATP can be supplied to nitrogenase either by photophosphorylation or by terminal respiration, but in carbon starved Anabaena cylindrica, it is the reductant rather than ATP which limits the rate of ARA (Lex & Stewart 1973, Donze et al. 1974). Upon carbon starvation, the formation of reductant becomes progessively more dependent on light and in severely starved cells almost all reductant can be generated via photosystem I (Donze et al.1974).

Axenic strains of Anabaena showed different saturating light intensities for ARA, some at about 5 klux (Chen 1983) while others at about 15 klux (Antarikanonda & Lorenzen 1982). In the field condition also the saturating light intensity for N_2-fixing activity is variable,

e.g. 2000 μmol m^{-2} s^{-1} for a thermal <u>Calothrix</u> (Wickstrom 1980), 900 μmol m^{-2} s^{-1} for <u>Nostoc muscorum</u> (Coxson & Kershaw 1983), and <u>ca</u> 5 klux for <u>Gloeotrichia</u> in a rice field (Roger & Watanabe 1982). N_2-fixing response of light grown algae to darkness is variable, e.g. <u>ca</u> 4% of the light ARA after 60 min dark incubation by <u>G. echinulata</u> (Stewart <u>et al.</u> 1967), 10-25% of light ARA after 60 min dark incubation by <u>G. echinulata</u> in culture (Chang & Blauw 1980) and about 50% loss of ARA after 100 min dark incubation by intertidal lagoon blue-green algae (Potts & Whitton 1977). However, in freshwater bodies the N_2-fixing activity varies with depth, with inhibition at the surface in full sunlight, a maximum some way below the surface and light limitation below this (Dugdale & Dugdale 1962, Goering & Neess 1964, Horne & Fogg 1970, Lewis & Levine 1984).

1.7 Aims

Field observations at Sonargaon from 1981 to 1983 revealed that abundance of <u>Gloeotrichia pisum</u> varies from year to year and from field to field. In 1983 it occurred abundantly, smothering deepwater rice tillers in some fields. The literature about the biology of this organism is scarce. The present study was therefore planned to increase understanding of the biology of <u>G. pisum</u> in the field and in the laboratory. Though the morphology of <u>G. pisum</u> D613 (Section 2.2.2.1) differs in some respects from that of field <u>G. pisum</u>, it was decided to consider this strain for laboratory study.

The literature reviewed in Section 1.4 indicates that members of Rivulariaceae show a wide range of morphologies. It was thus of interest to study the developmental characteristics of the alga from the natural populations with the hope of understanding the pattern of growth of colonies, filaments and akinetes.

Though plenty of research has been done elsewhere on N_2-fixation by blue-green algae in rice fields, no work has been carried out on N_2-fixation by blue-green algae associated with deepwater rice in Bangladesh. All reports in the literature have shown that N_2-fixation by blue-green algae is related to light (Sections 1.3, 1.6). In the deepwater rice field ecosystem, where at any one time light quantity varies markedly, the organism's ability to respond to changes in light flux may have a significant impact on N_2-fixation. It was therefore planned to estimate changes in N_2-fixing activity due to changes in light flux in the field.

Several reports in the literature have shown that some important morphological characters which occur in the natural habitats, such as colonies and akinetes, usually do not appear in the laboratory (Section 1.4). Early studies (De Bary 1863) noted the initial aggregation of hormogonia and formation of colony, but the mechanism which controls aggregation is not known. Detailed morphological studies of G. pisum D613 were therefore planned to elucidate colonization, and filament, akinete and hair formation. Some morphological changes, such as akinete formation have been shown to be affected by the light. It was thus of interest to examine the effect of quantity and quality of light flux on the formation of hairs, gas vacuoles and akinetes.

Nitrogen-fixing activity may vary with developmental stage (Section 1.6). As G. pisum D613 produces colonies at an early stage, it seemed worthwhile to make a subjective estimate of different developmental stages along with other cytological changes and compare this with the nitrogenase activity. It was hoped that this may also reflect the N_2-fixing capacity of G. pisum at different stages of the developmental cycle in nature. A detailed laboratory study was planned to quantify changes in ARA due to changes in light flux.

2 MATERIALS AND METHODS

2.1 Field

2.1.1 Description of study area

All _in situ_ studies were carried out at Sonargaon, which is situated at 23° 29' 25" N and 90° 35' 15" E, in the old Meghna floodplain (Figs 1.1, 2.1). The area has moderate to deep floods (50% over 1 m to 30% over 2 m during peak flood in 1983) and is part of an extensive deepwater rice growing region. As the appearance and disappearance of _Gloeotrichia pisum_ colonies and perhaps N_2-fixing activity in deepwater rice fields appears to be related to flooding, the detailed pattern of flooding was studied (Table 2.1, Fig. 2.2). Like most years (Section 1.2.1), two flood peaks were observed in 1982 and 1983.

Table 2.1   Flooding at Sonargaon

flooding conditions	1982	1983
started	22 Jun	23 Jun
1st peak	early Aug	_ca_ 7 Aug
2nd peak	mid Sep	_ca_ 20 Sep
receded		early Nov

2.1.2 Collection and preservation of algae

For morphological and taxonomic studies _G. pisum_ colonies, epiphytic on deepwater rice or other hydrophytes, were collected and preserved with 4% buffered (pH 7.0) formalin immediately after collection. Live algal materials for culturing or morphological study were kept in an ice box for transfer to the laboratory. Collection of the alga for ARA is described in Section 2.1.4.

2.1.3 Physical variables

In order to specify the effect of light in relation to other physical variables, water temperature, light, pH and O_2 were measured and cloud cover was estimated during the collection of alga for ARA. Light attenuation was measured using a Biospherical light meter (model QSP-170A) and underwater probe (model QSP-200), lowered to the desired depth. When measurements were made from a boat, care was taken to avoid

Fig. 2.1   Sonargaon showing the study site ($\triangle$).

Fig. 2.2   Flooding pattern at the study site, Sonargaon
                during 1983.
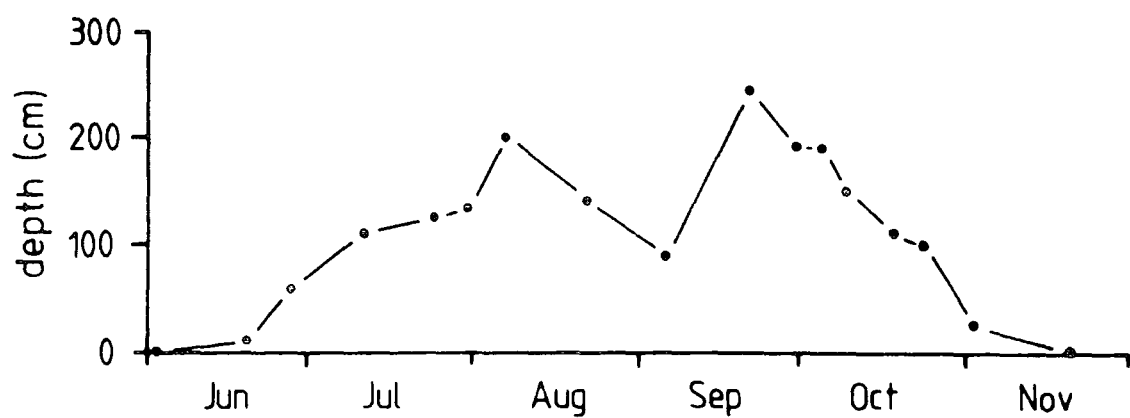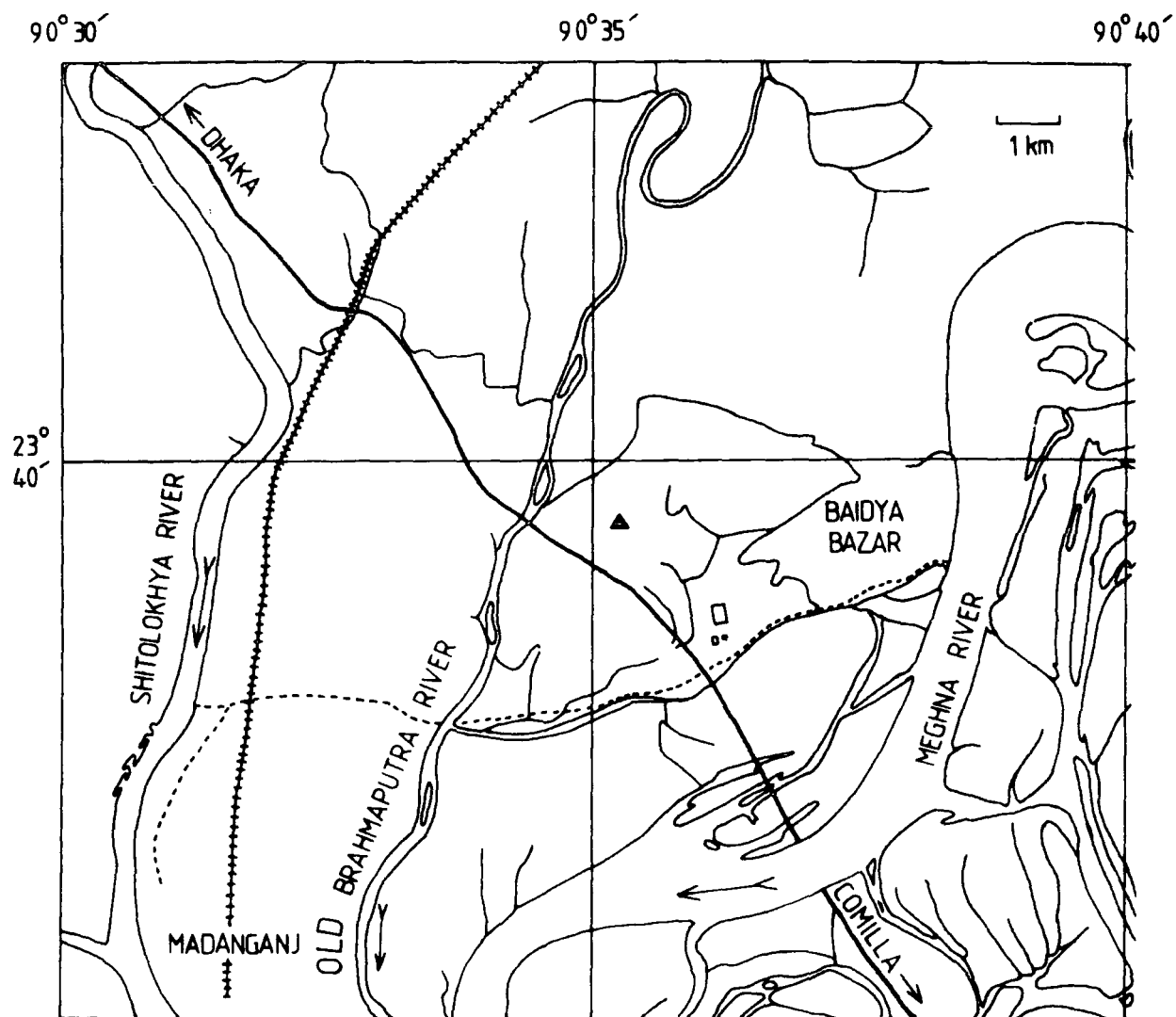
any shade by the boat. Surface light flux was measured simultaneously by putting the reference probe (model QSP-240) on the boat or on the soil (during incubation of alga for ARA). Light flux during ARA was measured at 10 min intervals and the value integrated to calculate the mean light experienced by the alga. Light flux is expressed as μmol photon m^{-2} s^{-1} PAR.

Surface (-1 to -5 cm depth) water temperature, pH and O_2 were measured by lowering a thermometer, pH probe or O_2 probe directly into the water. For measuring these parameters at greater depths, water samples (about 500 ml) were collected from that depth using screw-capped polypropylene bottles. pH was measured using an Orion (Model 407A) pH/specific ion meter fitted with GX electrode 91-05. Dissolved O_2 was measured using the same meter, fitted with an Orion oxygen probe (Model 97-08).

2.1.4 Acetylene reduction assay

N_2-fixation was studied using acetylene reduction assay (Hardy et al. 1973). G. pisum colonies were collected from usually -20 to -40 cm depth, along with parts of the rice plant, and immersed into chak water contained in a 2 l plastic beaker. The collected material was kept away from direct sunlight. To estimate the rate of N_2-fixation by the alga or plant part, G. pisum colonies were scraped (except for one experiment: Section 4.3) from the rice plant parts whilst immersed. For most experiments, about 0.1 ml of algal material was incubated in 8.0 ml serum bottles containing 1 ml chak water. For larger samples, McCartney bottles (26.0 ml capacity) or transparent plastic pots (180 ml capacity) were used.

After inoculation, the incubation vessels were sealed with rubber lined perforated screw caps or suba seals (W.Freeman & Co. Barnsley, U.K.). C_2H_2 gas was injected with plastic syringes (Becton-Dickinson Co., Ireland). After gassing, the incubation vessel was shaken lightly and the gas pressure was equilibrated by another needle. The concentration of C_2H_2 in the gas mixture was kept above 10%. Care was taken to keep the gas phase similar, in different sets of experiments. C_2H_2 gas was carried to the field in football bladders (filled every day), with the adaptor temporarily removed. Collection and preparation of the alga, injection of C_2H_2, equilibration and start of incubation altogether required about 30 min. Material was collected afresh for each incubation. Incubations were made in a channel by the main road, except for one experiment (Section 4.3).

The alga with C_2H_2 gas was incubated under two light regimes - full light (just beneath water surface) and attenuated light (using neutral density filters or incubating vessels to a certain depth) to give approximately the same PAR as colonies at the original field sites; dark incubations were wrapped in several layers of aluminium foil. In all cases incubation was done under water for 60 or 90 min with the tube held on wire racks. Mid-time of incubation was considered as the time for nitrogenase activity.

At the end of the incubation, the gas mixture was collected in 5 ml evacuated blood collection tubes, (Ezee-draw, England). Analysis of the gas was carried out at the University of Durham, England, about $1-1\frac{1}{2}$ months later (see Section 2.2.8).

After evacuation of the gas mixture, the incubation vessels plus algae were kept in the ice box for transfers to the laboratory where they were then stored in the deep freezer until chl a assay (Section 2.2.7). On the day of chl a assay, the samples were thawed and the supernatant was decanted as much as possible to minimize the possible dilution of methanol used in extraction. Absorbance was measured using a Perkin-Elmer spectrophotometer at Bangladesh Rice Research Institute (BRRI), Joydebpur, Dhaka. ARA is expressed as nmol C_2H_4 µg chl $\underline{a}^{-1}$ min^{-1}.

2.2 Laboratory

2.2.1 Physical variables

Temperature was measured using a thermometer. Light flux as PAR was measured by Macam light meter (model Q101) and is expressed as µmol photon m^{-2} s^{-1}. In order to compare results with the existing literature, light flux (as lux) has also been measured in the shaker tank simultaneously with PAR. PAR used in the present study and corresponding lux values are shown in Table 2.2. In both cases light was measured by cutting the bottom part of a flask and placing the respective probe inside. Light attenuation was achieved by wrapping the flask with different grades of neutral density filters. Green and red light was obtained by using the respective filters (Lee Filters Ltd). pH was measured using an EIL pH meter (model 7050) fitted with a Pye Unicam combination electrode (type No.401).

Table 2.2 PAR and corresponding lux value provided by warm white
(light)
fluorescent tubes in the tank (±= variation due to position)

PAR (μmol photon m^{-2} s^{-1})			lux		
200	$\pm$	10	19000	$\pm$	1100
150	$\pm$	8	14500	$\pm$	900
105	$\pm$	7	11000	$\pm$	750
100	$\pm$	5	9400	$\pm$	700
80	$\pm$	5	8000	$\pm$	500
50	$\pm$	2	5800	$\pm$	250
25	$\pm$	1	2300	$\pm$	150
12.5	$\pm$	0.5	1250	$\pm$	100
6.3	$\pm$	0.5	700	$\pm$	50
2.0	$\pm$	0.5	180	$\pm$	50

2.2.2 Experimental materials

2.2.2.1 Origin of cultures

The strain Gloeotrichia pisum D613 was obtained from a dry nodal
root (with colonies which appeared as black beads) of a deepwater rice
plant, collected on 29 November 1981 from Sonargaon (Figs 1.1, 2.1).
Other blue-green algae were obtained from the same area and also from
other regions (Appendix A). The enrichment cultures were brought to
Durham and subsequently many of these were made bacteria-free (Section
2.2.2.2).

2.2.2.2 Isolation and purification

The algae were inoculated on the day of collection (Section
2.2.2.1) into petri dishes containing Chu 10D-N liquid medium and
incubated on a window sill (out of direct sunlight), Department of
Botany, University of Dhaka, Bangladesh, at ca 15-26°C in ca 10: 14 h
light and dark periods. After about 7 days different forms of algal
growth (including greens) were visually obvious. Among blue-greens three
distinct forms appeared in one petri dish: (i) Fischerella sp.,(ii) G.
pisum as aggregates and (iii) Calothrix sp. as free-living filaments.
No further attempts were made to isolate these forms till
1 April 1982, when this culture (and others, Appendix A) was brought to
the University of Durham and subsequently isolated and purified by the
following methods:

Repeated subculturing on agar medium alternating with the liquid
medium (Section 2.2.5.3) and selection of colonies resulting in quick

separation of G. pisum D613 as unialgal isolate. In order to obtain clonal D613, young (2-3 day old), liquid-grown cultures were transferred to the agar medium and observed after 24 h with Nikon (type 102) dissecting microscope. Many isolated hormogonia were found away from the parent inoculum. A single hormogonium was picked up on a small agar block and inoculated into the liquid medium. The resulting clonal culture had only one type of associated bacteria. In order to obtain an axenic strain, care was taken to transfer an apparently bacteria-free hormogonium on a small block of agar to the liquid medium. Repeated use of similar techniques with alternating liquid and agar media led eventually to axenic culture.

2.2.3 Morphology and taxonomy

2.2.3.1 Microscopy and photomicrography

Samples were mounted in a drop of the medium or liquid in which organisms had been growing or preserved. They were then spread or teased apart with needles and covered with No.1 cover slips. As Gloeotrichia pisum D613 is colonial it was difficult to achieve sufficient separation of the filaments without causing them to break, which caused problems for trichome measurement and morphological study. The dissection of G. pisum colonies (collected from field) for morphological study has been described in Section 2.2.2. Morphogenesis of D613 on agar medium was studied by mounting the agar plates directly on the microscope stage after covering the material with cover slips. Light was provided from the inbuilt light source or from an anglepoise lamp.

All drawings were done by camera-lucida (Nachet, France) fitted on to a Vickers microscope. Materials for drawing were mounted on a drop of 4% glycerine, at least 15 min before study to settle down on the slide.

Photomicrography was done using Nikon Fluophot (type 109) microscope fitted with autoexposed Nikon (M-350) camera. Kodak "Ektachrome" 50 (Tungsten) slide films were used for this.

2.2.3.2 Morphology of field Gloetrichia pisum

For detailed morphological studies G. pisum colonies were sectioned in two ways :

(i) The colony was placed on a grooved potato tuber block and gently sectioned by hand using a razor blade, so that colonial organization was not deformed. A median section was mounted and studied as in Section 2.2.3.1.

(ii) The preserved material (Section 2.1.2) was dehydrated by passing through a graded ethanol series of 25, 50, 75 and 100%, with three changes of 15 min at each concentration. The material was then infiltrated for 24 h with a 50:50 mixture of 100% ethanol : Spurr epoxy resin followed by two changes of 24 h each, in 100% resin. It was then embedded in Spurr resin in plastic capsules and hardened for about 15 h at 70°C (Spurr 1969), cut on LKB "Ultrotrome" into 1 μm thick sections, mounted on glass slides and stained with toluidine blue (1% in 1% borax).

2.2.3.3 <u>Morphogenesis of Gloeotrichia pisum D613</u>

Almost all morphogenetic studies were made in standing condition at 32° C, 60 μmol m^{-2} s^{-1} in Chu 10D-N (liquid or agar) unless stated otherwise. Study of growth stages was initially done by picking up algal filaments from the surface of the liquid medium with a wire loop after 1-5 days growth. These were mounted and studied as in Section 2.2.3.1. Subsequently detailed developmental study was carried out on agar plates.

2.2.3.4 <u>Taxonomy</u>

Algae were identified by matching the descriptions, diagrams and ecological conditions with the existing literature (Schwendener 1894, Geitler 1932, Fritsch 1945, Desikachary 1959, Starmach 1966, Islam and Uddin 1973).

2.2.4 <u>Scoring morphological characters</u>

2.2.4.1 <u>Heterocyst frequency and density</u>

Heterocyst frequency and density were studied during the studies of morphogenesis and ARA. Heterocyst frequency is defined as the number of heterocysts per total number of cells and is expressed as a percentage. Heterocyst frequency at a particular stage of filament growth was studied by counting total number of cells per intact filament, while during growth and ARA studies, heterocyst frequency and density were counted in sonicated material using a haemacytometer (improved Neubauer ruling, 0.1 mm depth) as follows:

An aliquot of algal sample was transferred to a snap-cap vial and preserved in 4% buffered (pH 7.0) formalin. The alga was sonicated (Soniprep 150, MSE) at 20 μm amplitude, 5-10 times (depending on culture age) each of 45 s duration, at one min intervals (during which vials were kept on ice). The material was then observed under the microscope to check whether the one-celled condition was achieved. A few 2-3 celled

fragments were always present. When this sample was sonicated again, fragmentation of the individual cells occurred, resulting in a false increase in the number of cells, so cells were counted without further sonication. About 0.05 ml of suspension was pipetted (Finnpipette) to each chamber. Only cells with distinct polar nodules and a thick wall were considered as heterocysts.

2.2.4.2 Cell inclusions

Cyanophycin granules were usually identified without staining by their characteristic refractive appearance. To confirm the presence of granules in the young filaments they were stained with Schneider's acetocarmine (saturated solution of carmine in 45% acetic acid). Two drops of acetocarmine were placed over a drop of dense algal suspension and kept for 5 min for post vital staining. After placing cover slips cyanophycin granules were examined at high magnification (X1000).

Polyphosphate granules were identified by the staining method of Ebel et al. (1958) with minor modifications, using fresh material without pre-fixation. Samples on a slide were soaked for 15 min in 10% (weight/volume) $Pb(NO_3)_2$ in 0.1 N HNO_3 (concentration reduced from 1.0 N in the original method). The treated material was washed thoroughly (about 5 times) with distilled water and treated with 10% $(NH_4)_2SO_4$ for 30 s and again rinsed thoroughly. Polyphosphate granules were stained dark-brown to black.

2.2.5 Culturing

2.2.5.1 Culture vessels and glassware

100 ml Erlenmeyer flasks were used unless stated otherwise. The flasks were plugged with non-absorbant cotton wool stoppers(see Appendix B). Pre-sterilized plastic petri dishes (Sterilin, England) were used for solid media. All glassware was of Pyrex glass, unless stated otherwise.

2.2.5.2 Cleaning glassware

The flasks were cleaned by scrubbing to remove all algal materials and washed with detergent. They were then rinsed with tap water and soaked in 10% HCl for at least one hour. Other glassware was soaked directly in 10% HCl for the same period. Glassware for phosphate analysis was cleaned with tap water (without detergent) and then soaked in 10% H_2SO_4 solution for at least one hour. In all cases glassware was rinsed with distilled water 8 times and then oven dried at 100° C.

2.2.5.3 Media

The water chemistry of the original location (Section 2.1.1) was taken into consideration when developing growth media. Of the available media, Chu 10D (Sinclair & Whitton 1977) was most suited to the water chemistry in the field. As the laboratory study was planned mainly to investigate the nitrogen fixation rate, a nitrogen-free version of Chu 10D, with some other modifications (Table 2.3) was used for all experiments (basal medium), unless stated otherwise. This was done by substituting $CaCl_2.2H_2O$ for $Ca(NO_3)_2.4H_2O$ and omitting $Na_2SiO_3.5H_2O$ (Na concentration was compensated by adding more $NaHCO_3$). $Na_2SiO_3.5H_2O$ was omitted for two reasons : (i) HEPES was used for buffering medium, (ii) Na_2SiO_3 might form complex compounds in the medium. Chu 10D was a modification of the formulation of Gerloff et al. (1950). The composition of the present medium and also concentration of each element, in relation to Chu 10D and the medium of Gerloff et al. (1950) are shown in Tables 2.3 and 2.4. Iron and EDTA was added as a single solution. Stock solutions were stored in a refrigerator. Medium was made up freshly as required using glass-distilled water.

To prepare 1 litre of medium, ca 600 ml distilled water was taken in a 1 litre volumetric flask and stock solutions were then added. 250 ml distilled water was then buffered with 0.6 g HEPES in a beaker. pH dropped to ca 4.4 and this was adjusted to 7.2 by adding ca 1.48 ml 1M NaOH. This buffered aliquot was then mixed with 600 ml mineral medium in the volumetric flask and then adjusted to 1 litre final volume by adding distilled water. The medium in the culture flasks was sterilized immediately (Section 2.2.5.4), stored in the dark and used between 12 and 24 h after autoclaving. pH remained the same after autoclaving. 1% agar (weight/volume) medium was also autoclaved, cooled to ca 50° C, poured into petri dishes aseptically, solidified and stored in the refrigerator.

Thus, the liquid medium used in this study is not an absolute inorganic medium but with two organic compounds, the EDTA as a chelating agent and HEPES as a buffer. In addition to the basal medium, a modified Chu 10D-N, with 0.5 mg l^{-1} Fe and 0.9 mg l^{-1} P (Chu $10D_{X2}Fe_{0.5}P_{0.9}-N$) was also used (Section 5.5).

2.2.5.4 Sterilization

Medium, pipette tips (wrapped with aluminium foil and plugged with cotton wool) and other glassware were sterilized by autoclaving at 121° C (10.35 K Pa) for 15 min.

Table 2.3 Composition of medium (mg l^{-1} of salts) used in the present
study and comparison with Chu 10D (Sinclair & Whitton 1977)
and medium of Gerloff et al. (1950).

salts	medium used in the present study	Chu 10D	Gerloff et al.(1950)
$Ca(NO_3)_2$	-	-	40.0
$Ca(NO_3)_2.4H_2O$	-	57.6	-
K_2HPO_4	-	-	10.0
KH_2PO_4	7.8	7.8	-
$MgSO_4.7H_2O$	25.0	25.0	25.0
$Na_2SiO_3.5H_2O$	-	10.88	25.0
$NaHCO_3$	23.73	15.85	-
Na_2CO_3	-	-	20.0
$CaCl_2.2H_2O$	35.84	-	-
$FeCl_3.6H_2O$	2.42	2.42	-
Na EDTA.$2H_2O$	3.18	3.18	-
Ferric citrate	-	-	3,0
Citric acid	-	-	3.0
$MnCl_2.4H_2O$	0.045	0.045	-
Na $MoO_4.2H_2O$	0.007	0.007	-
$ZnSO_4.7H_2O$	0.056	0.056	-
$CuSO_4.5H_2O$	0.02	0.02	-
$CoSO_4.7H_2O$	0.01	0.01	-
H_3BO_4	0.72	0.72	-
NaOH *	ca 59.0	-	-

* added as 1M NaOH solution (ca 1.48 ml l^{-1} medium) during buffering
with HEPES to pH 7.2.

Table 2.4 Composition of medium (mg 1^{-1} of elements) used in the present study and comparison with Chu 10D (Sinclair & Whitton 1977) and medium of Gerloff et al. (1950).

elements	medium used in the present study	Chu 10D	Gerloff et al.(1950)
N	-	6.83	6.83
P	1.78	1.78	1.78
K	2.24	2.24	4.49
Na	ca 40.0 *	6.69	14.1
Ca	9.78	9.78	9.78
Mg	2.47	2.47	2.47
S	3.25	3.25	3.25
Fe	0.5	0.5	0.5
Si	-	1.44	3.31
Cl	17.26	0.016	-
Mn	0.012	0.012	-
Mo	0.0028	0.0028	-
Zn	0.013	0.013	-
Cu	0.005	0.005	-
Co	0.002	0.002	-
B	0.125	0.125	-

* Na concentration increased from 6.69 to 40.0 mg 1^{-1} due to the addition of 1 M NaOH solution (ca 1.48 ml 1^{-1}) during buffering with HEPES to pH 7.2.

2.2.5.5 Maintenance and subculturing

Stock cultures were maintained in 50 ml liquid medium incubated standing in the 25° C growth room under continuous light ($\underline{ca}$ 40 μmol m^{-2} s^{-1}). Subcultures to fresh medium were made after about three months. Stocks for experimental purposes were maintained at 32° C in the shaker under continuous light of 105 μmol photon m^{-2} s^{-1} PAR, unless stated otherwise.

Subculturing for stock cultures was done by picking up a small aliquot of the alga with a wire loop and inoculating to fresh medium aseptically using a laminar flow cabinet (Microflow Pathfinder). Subculturing for experiments was done by inoculating a standard amount of homogenized algal material. The alga was scrubbed from the glass surface using a glass rod fitted with rubber tube presoaked in absolute alcohol for one hour. The algal mass with the parent medium was then homogenized by taking it in sterile plastic syringes and passing through 50, 25 and 16 mm sterile needles (Gillette Surgical Ltd, U.K.) till an almost homogeneous algal suspension was attained. The homogenate was then taken in a sterile beaker containing$_{\wedge}^{a}$ magnet and placed over a magnetic stirrer. Depending on the algal concentration in the homogenate, 0.5 to 1.0 ml was pipetted at a concentration of 6-8 mg d. wt l^{-1} and inoculated in each flask by automatic pipette fitted with sterile pipette tips. The flasks were randomized during inoculation of alga to have approximately equal inocula for all flasks.

2.2.5.6 Incubation

Experiments were carried out in batch culture under continuous light, unless stated otherwise. In the growth room illumination was provided by white fluorescent tubes above or below the alga . All N$_2$-fixation experiments were carried out in the thermostatically controlled tanks illuminated from below by warm white fluorescent tubes. Darkness was achieved by wrapping flasks with aluminium foil and black polythene. 12:12 h light and dark conditions were provided by Gallenkamp cooled incubators (model lH-270); illumination from above supplied by white fluorescent tubes. The flasks were usually randomized at 12 h intervals. The temperature was 32° C ($\pm$ 0.5). Flasks in the tank were shaken at 60 oscillations min^{-1} and at an amplitude of $\underline{ca}$ 30 mm. The gas phase of the incubated flasks varied from experiment to experiment. All N$_2$-fixation experiments were conducted in the Erlenmyer flasks with 123.8 $\pm$ 1.1, n = 30) ml gas volume with the cotton wool or

suba seal fitted. The gas phase of flasks with 25 ml medium was therefore about 99.0 ml.

2.2.5.7 Tests for purity

Purity of the organisms was tested following the test media described by Hoshaw and Rosowski (1973) and also by examining materials by phase-contrast microscopy (Nikon, Fluophot, type 109).

2.2.6 Chemicals and gases

All chemicals used in medium were of Analar grade and obtained from British Drug House Ltd (BDH), Poole, England, except HEPES (Sigma), Agar (Difco Laboratories) and potassium persulphate analoid compressed tablets (Ridsdale & Co.Ltd, Middlesbrough, England).

Acetylene used in Bangladesh was supplied by Bangladesh Oxygen Co. Ltd, Dhaka, while in Durham, acetylene was supplied by British Oxygen Co., Ltd, and ethylene (99.8%) by BDH Laboratory gas service.

2.2.7 Estimation of growth

Both dry weight and chl $\underline{a}$ have been used to estimate growth. During early stages of growth the whole contents of each flask were used for either dry weight or chl $\underline{a}$. In the growth curve experiment, equal numbers of replicates were therefore taken to estimate biomass after 0, 12 and 24 h. Subsequently a single flask could be used for both estimates, the contents being homogenized (Section 2.2.5.5). The homogenate was adjusted to 25 ml final volume and placed on a magnetic stirrer. Equal aliquots pipetted for both dry weight and chl $\underline{a}$ and collected on glass microfibre filters (GF/C, Whatman) by vacuum filtration.

A sample for dry weight was collected on a pre-weighed GF/C filter, kept in a glass petri dish and oven dried at 105° C for about 24 h. The dried sample was then placed in a desiccator, cooled to room temperature and re-weighed using a Mettler H51 balance.

Samples for chl $\underline{a}$ were deep frozen for about 24 h. Chl $\underline{a}$ was estimated by following the procedure based on the recommendations of Marker et al. (1980). Extraction was done with 5-10 ml 90% hot (70°C) methanol for 10 min. Extracts were then cooled down, kept out of direct light, filtered through GF/C filters and absorbance was measured at 665 and 750 nm using a Shimadzu digital double-beam spectrophotometer (UV-150-02). Absorbance was again measured at 665 and 750 nm after acidification with 0.1 M HCl (final concentration of 10^{-3} HCl) for 60

min and chl $\underline{a}$ was calculated from the following equations:

$$\mu g\ chl\ a = (A_b - A_a) x \ \frac{R}{R-1} \ x\ K\ x\ \frac{V}{L}$$

where A_b = extract absorbance at 665 nm before acidification deducting absorbance at 750 nm

A_a = extract absorbance at 665 nm after acidification deducting absorbance at 750 nm

R = maximum acid ratio (A_b/A_a)

K = 1000 x the reciprocal of the specific absorption coefficient (SAC) of chl $\underline{a}$ at 665 nm in 90% methanol

V = volume of solvent used to extract chl $\underline{a}$ in ml

L = path length of the cuvette used in cm

Marker et al. (1980) recommended a specific absorption coefficient of chl $\underline{a}$ in 90% methanol of 77, and a maximum acid ratio of 1.59 for 90% methanol in 10^{-3} M HCl.

$\mu g\ chl\ \underline{a} = 34.94\ (A_b - A_a)\ x\ V/L$.

Growth rate has been expressed in terms of the relative growth constant or specific growth constant $(\underline{k'})$ (Fogg 1975):

$$\frac{log_{10}\ N_t - log_{10}\ N_o}{t}$$

where t = time in days, from the time of incubation

N_t = biomass after t days

N_o = biomass at zero-time, i.e. at the time of incubation.

Maximum growth rate is defined as the maximum growth rate under light saturation at a specified temperature. The mean generation time or doubling time (G) has been calculated from specific growth constant $\underline{K'}$:

$$G = \frac{0.301}{\underline{K'}}\ day^{-1}$$

2.2.8 Acetylene reduction assay

N_2-fixation rates were estimated using the acetylene reduction assay technique (Hardy et al. 1973). The alga was always kept and maintained in the parent medium and flask, so that it did not experience a major shift in environmental variables. A shift of light during C_2H_2 injection and equilibration, could not be overcome because of the ARA technique itself.

The cotton wool stopper was replaced by a suba seal immediately prior to gassing. 15 ml of acetylene was injected into each flask and was shaken slightly before equilibration of pressures by another needle. After equilibration, flasks were incubated in the experimental conditions. This step took about 45 s. A control, with autoclaved medium only, was used to estimate C_2H_4 contamination.

After incubation with C_2H_2 for 60 min, the gas mixture was collected in a 7 ml Vacutainer (evacuated blood collection tube, Becton-Dickinson Co., New Jersey, U.S.A). Analysis of the gas mixture was made by injecting 1 ml into the Varian Aerograph 1400 gas chromatography equipped with a hydrogen flame ionization detector. N_2 was used as the carrier gas at a rate of 45 ml min^{-1} through a 3.0 mm x 2.0 m column packed with "Porapak" R (Waters Associates Inc, U.S.A). Other operating conditions were: detector temperature, 150° C; column temperature 105° C; air and hydrogen flow rates were 300 and 30 ml min^{-1} respectively. C_2H_4 and C_2H_2 (as a check of accuracy of method) peaks were recorded from chart recorder, identified by the retention time. The gas chromatograph was calibrated on each day of use, with high purity ethylene standards. As in situ ARA was expressed as nmol C_2H_4 µg chl a^{-1} min^{-1} and on the other hand, laboratory experiments were centred on the effect of light flux (which might affect chl a) on ARA, the results of laboratory ARA were expressed on the basis of chl a as well as dry weight.

2.2.9 Chemical analytical procedure

2.2.9.1 Phosphorus fractions in medium

Analysis of the phosphorus fractions in the medium, termed "filtrable reactive phosphorus" and "filtrable total phosphorus" (organic and orthophosphate) were made according to the modifications of

Eisenreich et al. (1975) by D. Livingstone (pers. comm.). In a suitably acidified solution, sodium molybdate and potassium antimonyl tartrate react with orthophosphate to form molybdo-phosphoric acid which is then reduced to the intensely coloured molybdenum blue complex by ascorbic acid and determined spectrophotometrically at 880 nm wavelength. In all cases absorbance was measured strictly after 12 min (as absorbance increased with time); a 5-cm cell was used.

Reagents:

1. Acid-antimony-molybdate reagent

0.57 g potassium antimony tartrate, $K(SbO)C_4H_4O_6\frac{1}{2}H_2O$ was dissolved in 500 ml distilled water and 45 ml of conc. H_2SO_4 (sp. gr. 1.84) was added, with continuous mixing. 8.52 g sodium molybdate ($Na_2MoO_4.2H_2O$) was dissolved in 400 ml distilled water in another volumetric flask. The two solutions were then mixed, cooled and adjusted to 1000 ml with distilled water. The solution was stored in a dark bottle, refrigerated and used within 4 weeks.

2. Sulphuric acid, H_2SO_4, 1N
3. Potassium persulphate, $K_2S_2O_8$, 0.7 g analoid compressed tablets.
4. Phosphate standard solution, K_2HPO_4, 4.39 g dissolved and made to 1 litre with distilled water;   contains 1000 mg 1^{-1} P.
5. Mixed reagent: 0.62 g L-ascorbic acid was dissolved in 100 ml of acid-antimony-molybdate reagent (reagent 1) on the day of analysis.

Procedure:

I. FRP

A suitable aliquot of sample was diluted to 25 ml with distilled water in a 125 ml conical flask. Then 5 ml of mixed reagent was added and mixed thoroughly.

II. FTP

A suitable aliquot of sample was diluted to 100 ml with distilled water in a 250 ml conical flask. 5 ml 1N H_2SO_4 was added to the diluted sample and one persulphate tablet was added . The flask top was covered with aluminium foil and autoclaved at 121°C (10.35 K Pa) for 30 min. The sample was cooled to room temperature and 5 ml of the mixed reagent was added.

Calibration curves were prepared by treating two series of phosphate standards, as for FRP and FTP.

2.2.9.2 Total algal phosphorus

Total algal phosphorus was determined according to Batterton & Van Baalen (1968), with the following modifications :

Algal material was separated from the growth medium by centrifugation of an aliquot of algal homogenate for 15 min at 5000 x g, and washed three times to remove salts by resuspending in distilled water and centrifuging as before. The washed algal pellet was transferred to an acid-washed, pre-dried and pre-weighed snap-cap glass vial and dried for 24 h at 105°C. After estimating dry weight (to express algal-P as μg P mg d. wt^{-1}), 10 ml distilled water was added to the vial, followed by the addition of one persulphate tablet (o.7 g). The sample was then autoclaved as with FTP (Section 2.2.9.1). After cooling to room temperature the digested sample was transferred to a 125 ml conical flask and the volume was made to 25 ml with distilled water. 5 ml of mixed reagent (Section 2.2.9.1) was added to the above solution.

A calibration curve was prepared by treating a series of phosphate standards, as for the algal material.

3 MORPHOLOGY OF FIELD Gloeotrichia pisum

3.1 Introduction

The literature reviewed in Section 1.4 indicates that, in nature, species of Gloeotrichia can show distinct morphological changes during development, which can in turn lead to taxonomic confusion. Apart from detailed cytological studies by Palla (1893), few studies have been made on the developmental sequence of colonies, filaments and akinetes of G. pisum from natural habitats. The developmental stages of this alga from natural populations were therefore studied in the hope of understanding the pattern of growth of colonies, filaments and akinetes.

3.2 Structure and development

3.2.1 Colonies

G. pisum colonies epiphytic on deepwater rice and Myriophyllum sp. were studied. All materials were collected from Sonargaon during the period 24.7.83 to 25.8.83 (Section 2.1.2).

The colony is typically firm, blackish-green to dark-brown, hemispherical or spherical and is attached to submerged aquatics (Fig. 3.1A-C). On a few occasions colonies were found epiphytic on larger colonies. In a small area (e.g. 1 cm^{-2}) colonies of a range of sizes and ages were commonly found.

Colonies have a slimy opal-like appearance when young, which becomes rough when filaments transform into akinetes. Colonies are usually 0.5-1.5 mm in diameter, but may sometimes reach 4.5 mm. The trichomes in the smaller colonies are compact, gradually tapered with their heterocysts near the centre of colonies (Fig. 3.1F), but in larger colonies (> 2mm) there is a distinct zonation (Fig. 3.1C), with a central pole zone of loosely packed, narrow, very long trichomes and a peripheral zone of shorter densely packed, tapered trichomes; usually with akinetes (Fig.3.1D-E).

The mucilage has a leathery texture. The hairs extend beyond the thick mucilage (Fig.3.1F-G). A section through the periphery of a colony revealed a thick mucilage sheath around the trichome and intersheath spaces between the mucilage sheath (Fig.3.3H).

The appearance and disappearance of G. pisum colonies at Sonargaon were studied during the flood period in 1983 (Table 2.1, Fig. 2.2).

Fig. 3.1 A-K. Morphology of field Gloeotrichia pisum.

A. Colonies on submerged parts of deepwater rice
    tillers; sections from -50 to -60 cm depth after
    about 50 days of flooding (14.8.83).

B. Colonies on young parts of rice tillers which
    have been exposed due to receding of flood water
    after about a week of submergence ; photographed
    on 14.8.83.

C-E. Anatomy of large spherical colony epiphytic on
     Myriophyllum.
     C. Showing zonation of filaments.
     D. Showing central very long and narrow and
        peripheral short and tapered filaments.
     E. Peripheral filaments, enlarged. Note very long
        akinetes (a) and grouping of cells along trichomes.

F-G. Anatomy of small spherical colonies mounted in Indian
     ink.
     F. Showing compactness of tapered filaments.
     G. Showing hairs (hr), gas vacuoles (g.v.) and
        protrusion of trichomes out of the mucilage (m).

H-J. Heterocysts : variation in shape, size, number and
     position in the same colony.
     H. Spherical and rod-like heterocysts (h).
     I. Two basal heterocysts of different shapes.
     J. Terminal type of heterocyst (h) in between
        akinetes (a).

K. Developing akinete (d.a.); note participating
    cells. Embedded in Spurr low viscosity resin.
    (Scales : A-B = 10 mm ; C,D,F = 300 µm ;
                E,G-K = 20 µm)
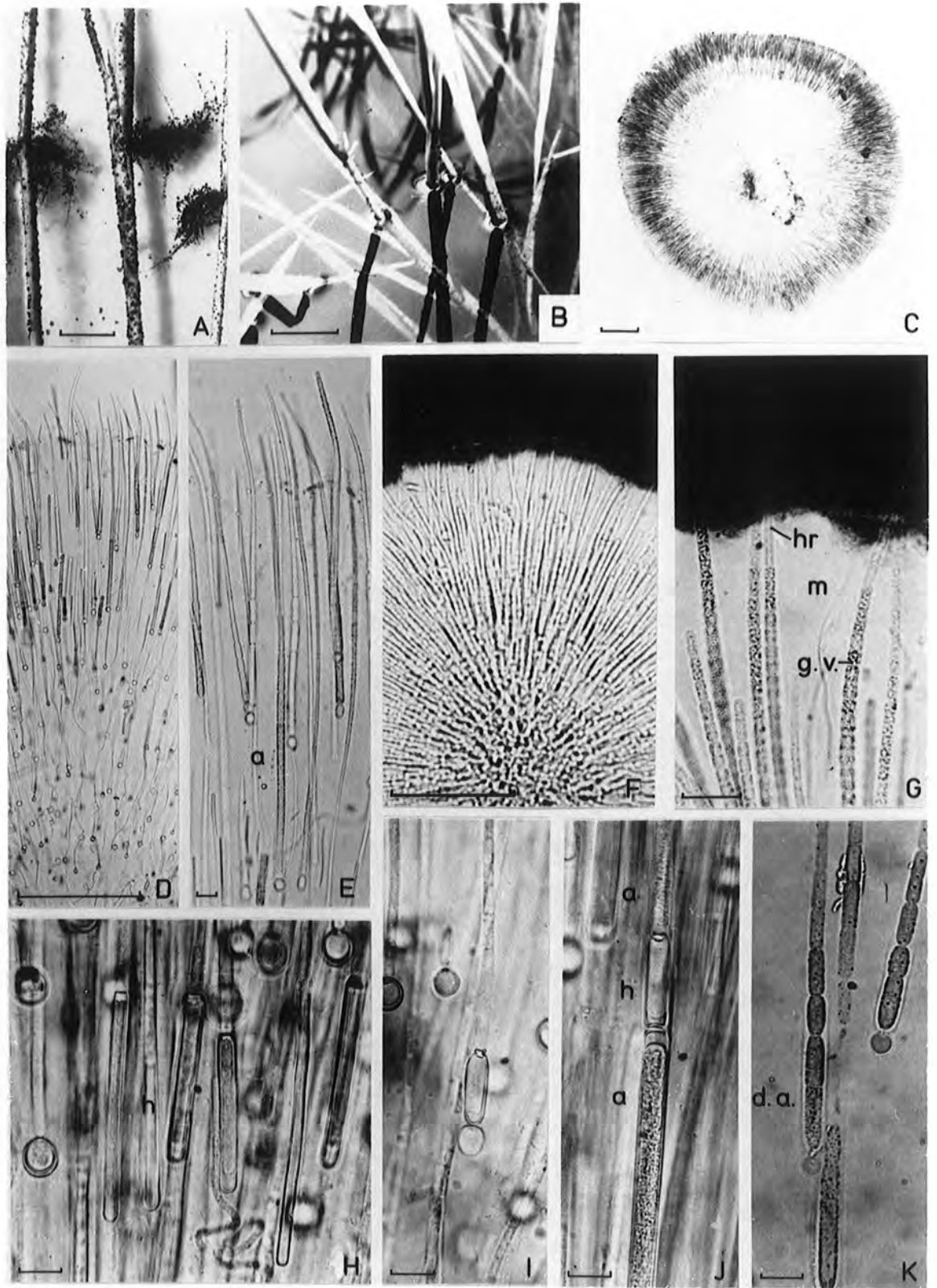
Fig. 3.1

G. pisum colonies became abundant ("first flush" of growth) within two weeks of flooding, i.e. on 6 July when the water depth was about -100 cm. After about a month of flooding (on 24.7.83) when the water depth in the field was about 130 cm, the distribution of colonies down the tiller was studied. Colonies were found from just below the water surface to about -100 cm. Dense colonization (about 25 colonies cm^{-2}) was found at -10 to -60 cm depth. Colonies were absent at -100 to -130 cm.

There was continuous heavy rain on 5 and 6 August and by 7th August the water depth had increased by about 70 cm. The density of tillers in the rice field was about 130 m^{-2} and the canopy was about 30 cm. On 7-10 August there was no rain, but there was cloud cover of about 40-70% throughout the day. The surface light flux was in the range of 2225-2280 $\mu mol \ m^{-2} \ s^{-1}$ at about mid-day. Numerous small pale blue-green and soft colonies were observed ("second flush" of growth) on the freshly submerged parts of the rice tiller on 9th August, i.e. after three to four days of submergence. By 14 August these colonies had attained about 1 mm. By that time, the water column dropped by about 10 cm and as a result the G. pisum colonies that had colonized 8-9 days earlier on the freshly submerged tillers were exposed as distinct black beads (Fig. 3.1B).

Colonies were abundant up to the 1st week of September (i.e. for about two months) after which there was a rapid decline with few colonies from then till the end of the flood period (Section 2.1.1).

3.2.2 Filaments

Filaments are extremely variable, depending on their age, their position in a colony and the size of the colony. A developing trichome consists of a basal heterocyst and a row of vegetative cells which may terminate in a long hair (Fig. 3.2A-C). In this filament distinct groups of cells are found starting from the base of a hair towards the heterocyst. Each of these groups is usually composed of 5-9 cells and appears as a hormogonial segment (Fig. 3.2C). An old filament consists of a basal heterocyst followed by a long cylindrical akinete and vegetative cells. These filaments had no hairs (Figs 3.1E, 3.2G-H). Filaments with mature akinetes may have few hormogonia-like segments (Fig. 3.3A-B) or an empty sheath (Fig. 3.3C) next to the akinete. On a few occasions filaments were found with two terminal heterocysts at the base or with a single terminal heterocyst next to a mature akinete (Figs. 3.1I-J, 3.3D-E). Heterocysts are mostly spherical and sometimes ellipsoidal to very long and rod-like (Figs 3.1H, 3.21-K).

Fig. 3.2A-K.Developmental stages and morphology of filaments of
field <u>Gloeotrichia</u> <u>pisum</u>.

A.   Young filament with hair.

B.   Cell next to the heterocyst dividing.

C.   Two more divisions in cells next to the heterocyst
forming a group of cells with abundant granules ;
filament consisting of several groups, each with 4-9
cells. Note the absence of granules in the rapidly
dividing group of cells ; sharp differences in
granulations between basal cells of the hair and the
group of cells immediately below, and the appearance
of sheath around basal cells.

D-H. Differentiation of akinete.

    D.   Group of five enlarged cells next to the
heterocysts where the basal cell has enlarged
more than the rest. Note abundant gas vacuoles
(g.v.) in cells below the hair (hr).

    E-F. Much enlarged developing akinetes.

    G.   Long akinete. Note faint cross walls (arrows)
of cells next to the akinete.

    H.   Very long akinete with visible cross wall (c.w.)
in the upper half. Note the cells next to the
akinete with abundant granules (as in the
akinete) and also faint cross walls (arrows).

I-K. Heterocysts : variation in shape and size from the
same colony.
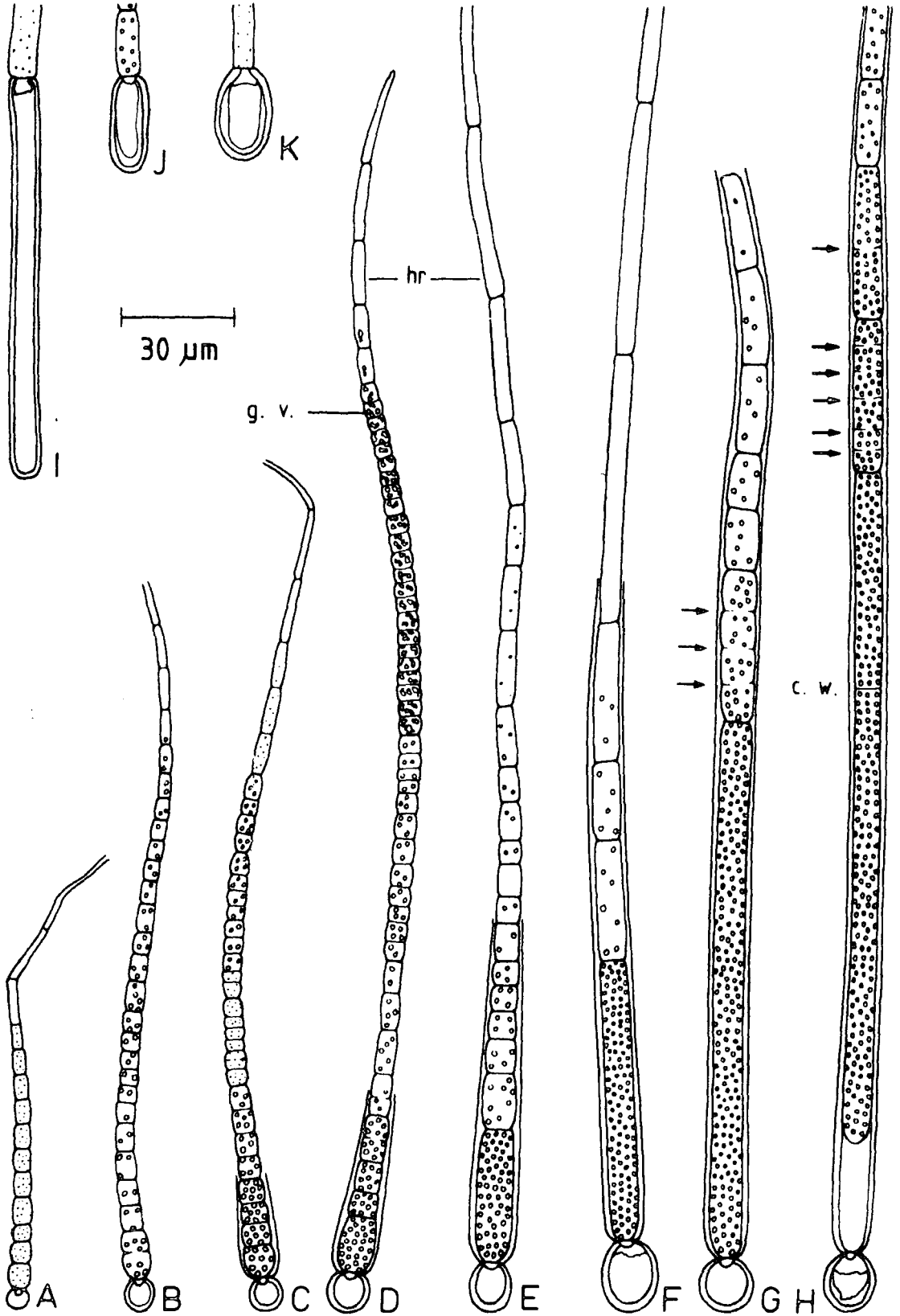
Fig. 3.2

Fig. 3.3A-H. Developmental stages and morphology of filaments
and the colonial morphology of field _Gloeotrichia_
pisum.

A-C. Fully matured akinetes, delimited by thickened cross
walls. Note liberating hormogonia in 'B' leaving
empty sheath in 'C'.

D. Akinetes (A) alternating with terminal
heterocysts (h).

E. Dead basal heterocyst followed by empty sheath.
Note terminal heterocyst and developing akinete
within the parent sheath.

F. Filament from old colony with gigantic akinete
surrounded by stratified sheath. Note the deeply
constricted cross walls next to the akinete.

G. Very long and narrow filament having long and
less pigmented cells at the basal end compared
to the terminal ones.

H. Peripheral section of a spherical colony showing
thick sheath(s) around trichome(t), and inter-
sheath spaces(i.s.). Embedded in Spurr low
viscosity resin.
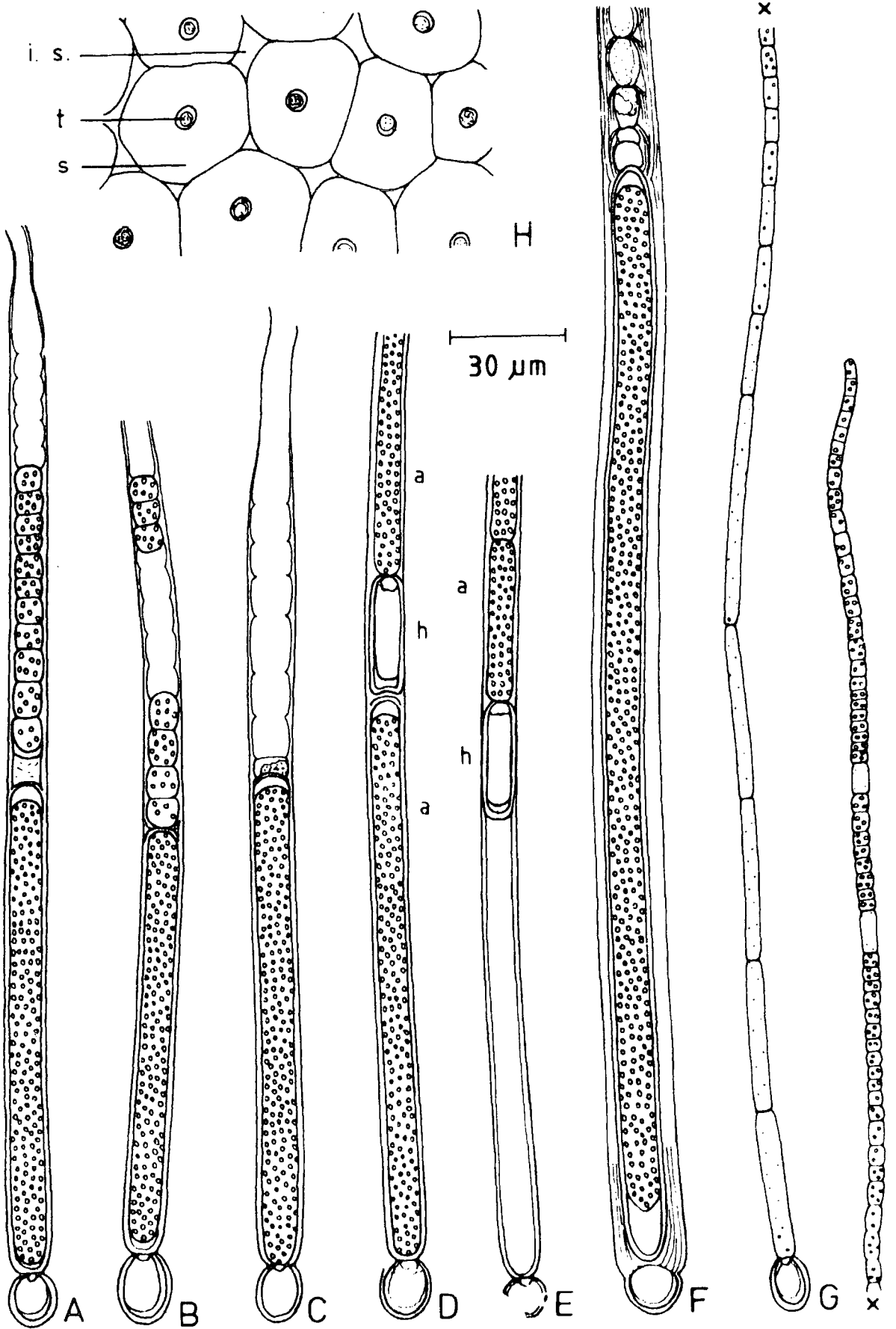
Fig. 3.3

The sheath is hyaline, firm and thin (2.6-5.0 μm) at the basal end
(becomes stratified in old filaments: Fig. 3.3F) and very thick
(8.5-20.0 μm) at the terminal end (Fig. 3.3H). Cells of the narrow, very
long filament type have less pigmentation and cyanophycin granules at
the basal end compared to the terminal end (Fig. 3.3G). Cells of the
tapered filament (except very young: Fig.3.2A) have abundant cyanophycin
granules except in the hair cells and in the rapidly dividing portion of
a filament (Fig. 3.2C). Gas vacuoles occur in cells below the hair
(Figs 3.1G, 3.2D).

Tapered trichomes are usually up to 350 μm long and may be more
than 520 μm in large colonies. Trichomes are 5-7.7 μm in diameter at the
base during the vegetative phase. Dimensions of vegetative cells are
extremely variable along the trichome. Akinetes are usually 8-8.6 μm in
diameter (without sheath) and 60-125 μm long. The akinete in an old
colony may attain a length of more than 240 μm. Spherical heterocysts
are 5.5-12.8 μm in diameter. Rod-like heterocysts vary from 20 to 105
μm in length.

3.2.3 Akinete formation

A vegetative trichome consists of a basal heterocyst and a chain
of vegetative cells which may terminate in a long hair (Fig. 3.2A). At
the onset of akinete formation the cell next to the heterocyst enlarges
and divides (Fig. 3.2B). The process continues, producing a group of
5-7 cells, larger and denser than the rest of the trichome (Fig.
3.2C-D). Subsequent changes seem to take place in one of the following
ways :

(i)   The cell next to the heterocyst appears to elongate,
        accumulate granules and form a thick wall delimiting it
        from the rest of trichome ; this is the mature akinete
        (Fig. 3.3A-C,F). It is not clear whether this long
        akinete is the product of elongation of a single basal
        cell or of several cells in a group. Vegetative cells
        next to the akinete may be released as hormogonia
        (Fig. 3.3A-C).

(ii)   The akinete is not delimited from the rest of the
        trichome by a thick wall. The cell next to this akinete
        is usually as wide as the adjacent akinete with faint
        intervening wall and abundant granules (Fig. 3.2G-H). In
        a few cases a cross wall was observed in the upper half

of a long akinete (Fig. 3.2H). It appears that this akinete is the product of two independent cells where ^the mother cross wall still remained.

(iii)   A variant of the first method of akinete formation occurs by the formation of a heterocyst next to an akinete. In this case cell(s) thereafter transform into an akinete resulting in a chain of akinetes alternating with hetero- cysts (Figs 3.1J, 3.3D).

(iv)   During the early stage of filament development, the trichome next to the heterocyst occasionally becomes dissociated and the basal cell subsequently transformed into a heterocyst. The cell next to this new heterocyst may transform into an akinete (Fig. 3.3E).

3.3   Summary

A large colony from the field shows distinct zonation of filaments. On the periphery of the colony, intersheath spaces exist in between ^the thick hyaline mucilage sheaths. Rapid colonization of G. pisum may occur on freshly submerged and young rice culms. The alga was much more abundant during the first half of the flood period.

Cell divisions are not restricted to the base of a hair. Trichomes are seen to be composed of several groups, each with usually 5-9 cells. There are indications of participation of more than one cell in akinete formation. Akinetes may be formed alternating with heterocysts. Heterocyst shape, number and position are variable.

4 ARA (NITROGENASE ACTIVITY) BY <u>Gloeotrichia pisum</u> AT SONARGAON

4.1 Introduction

The literature reviewed in Sections 1.3 and 1.6 indicates that the rate of nitrogenase activity by blue-green algae can be influenced by environmental factors such as light flux. In Bangladesh light flux may change very rapidly during the monsoon period (Section 1.2.1). Inside the deepwater rice fields light flux varies markedly according to density of rice plants, depth of water, presence of hydrophytes (floating or submerged), centre or edge of a field etc. and hence <u>G. pisum</u> colonizing the rice plant also experiences marked differences in light flux with time and space. Experiments were therefore planned to investigate the influence on ARA of light flux and distribution of ARA down the water column. Studies of the diel changes in ARA and environmental variables were also included.

4.2 Influence of light flux on ARA

The alga was incubated for 90 min in serum bottles within 30 min of collection (Section 2.1.4). Light attenuation was achieved by wrapping the incubation vessels with neutral density filters. The influence of light flux on ARA is shown in Table 4.1. Transfer from high to lower light flux brought about a marked reduction in ARA during the first 1.5 h incubation. A reduction of light flux by 39 and 74% brought about a reduction in ARA by 30 and 62% respectively. (A dark incubation for 1 h resulted in a decrease of ARA by about 83% (result not shown : see Whitton 1984).)

4.3 ARA down the water column

Colonies of <u>G. pisum</u> were quite conspicuous on deepwater rice in a number of fields for about two months (July and August) during 1983. In the first half of August no other visible algal growths were noticed on tillers. Experiments were therefore planned to establish the distribution of ARA on submerged parts of tillers due to <u>G. pisum</u> alone. The experiment was carried out on 10.8.83 during peak flood (-2 m, Fig. 2.2). Surface PAR during the incubation was about 2255 μmol m^{-2} s^{-1} with <u>ca</u> 10% cloud cover. A tiller (deepwater rice cultivar <u>duli aman</u>) was cut into 20-30 cm long sections, each consisting of one node and one internode. Each section was further divided into an internode (with or without leaf) and nodal root mass. Each individual section was incubated at approximately the same depth from which it originated.

Table 4.1 Relationship between changes in light flux and ARA for <u>G. pisum</u> <u>in</u> <u>situ</u> (n = 6)

<u>date</u>	mid-time	light flux (μmol m^{-2} s^{-1})			ARA (nmol C$_2$H$_4$ μg chla^{-1} min^{-1})				%of full
		full	<u>reduced</u>	<u>(% reduction)</u>	full	light	reduced	light	<u>light</u>
	<u>(h)</u>	(at -4cm)			$\overline{x}$	$\pm$	$\overline{x}$	$\pm$	
29.7.83	1115	1847	1121	39	0.234	0.083	0.163	0.027	70
"	1645	1289	526	59	0.256	0.038	0.164	0.020	64
7.8.83	1045	1825	475	74	0.352	0.090	0.133	0.025	38
"	1245	1890	443	77	0.276	0.044	0.138	0.022	50
22.8.83	1345	985	394	60	0.161	0.030	0.086	0.010	53

Table 4.2 Distribution of ARA on individual sections of a tiller with

G. pisum, of deepwater rice and changes down the water

column on 10.8.83 at 1030 h (mid-time). (i) internode ;

-1,-2 etc. are nodal root masses, -1 being just below the

water surface ; each section was incubated at approximately

the same depth from which it originated ; n = 1

section of tiller	ARA (nmol C_2H_4 min^{-1})
i -1	2.46
i	3.38
-2	0.24
i	10.12
-3	6.02
i	9.10
-4	11.67
i	18.72
-5	6.75
i	6.80
-6	1.69
i	0.20
-7 i -8	0.31

whole tiller	=	77.44 nmol C_2H_4 min^{-1}*

* partitioning of ARA between internodes and nodal roots:

all internodes = 49.59 nmol C_2H_4 min^{-1}

all nodal roots = 27.85 nmol C_2H_4 min^{-1}

Table 4.3 Changes of ARA down a tiller with attached G. pisum, and changes in the physical environment down the water column on 10.8.83 at 1030 h. Physical variables were recorded in the water column, where alga was incubated. The rates are expressed for section of a tiller which include node with the internode immediately above

depth (cm)							
incubation at	sample from	sections of a tiller	light flux $(\mu\text{mol m}^{-2}\text{ s}^{-1})$	temperature $(^\circ\text{C})$	O_2 (mg l^{-1})	pH	ARA(nmol C_2H_4 min^{-1})
-10	0 to -50	node -1 + internode and leaf	1885	32.7	9.1	6.40	2.46
-75	-51 to -75	node -2 + internode and leaf	210	31.5	9.5	6.35	3.62
-85	-76 to -85	node -3 + internode and leaf	178	31.5	9.5	6.30	16.14
-90	-86 to -90	node -4 + internode	178	31.0	9.0	6.30	20.77
-130	-91 to -130	node -5 + internode and leaf	106	31.0	8.6	6.25	25.47
-155	-131 to -155	node -6 + internode	53	31.0	7.9	6.20	8.48
-180	-156 to -200	nodes-7 & -8 + 2 internodes	27	30.7	8.0	6.10	0.51

Fig. 4.1   Distribution of ARA on a tiller with $\underline{G.\ pisum}$
            and changes of environmental variables down the
            water column on 10.8.83 at 1030 h (the rates
            are expressed for section of a tiller which
            includes node with the internode immediately above).
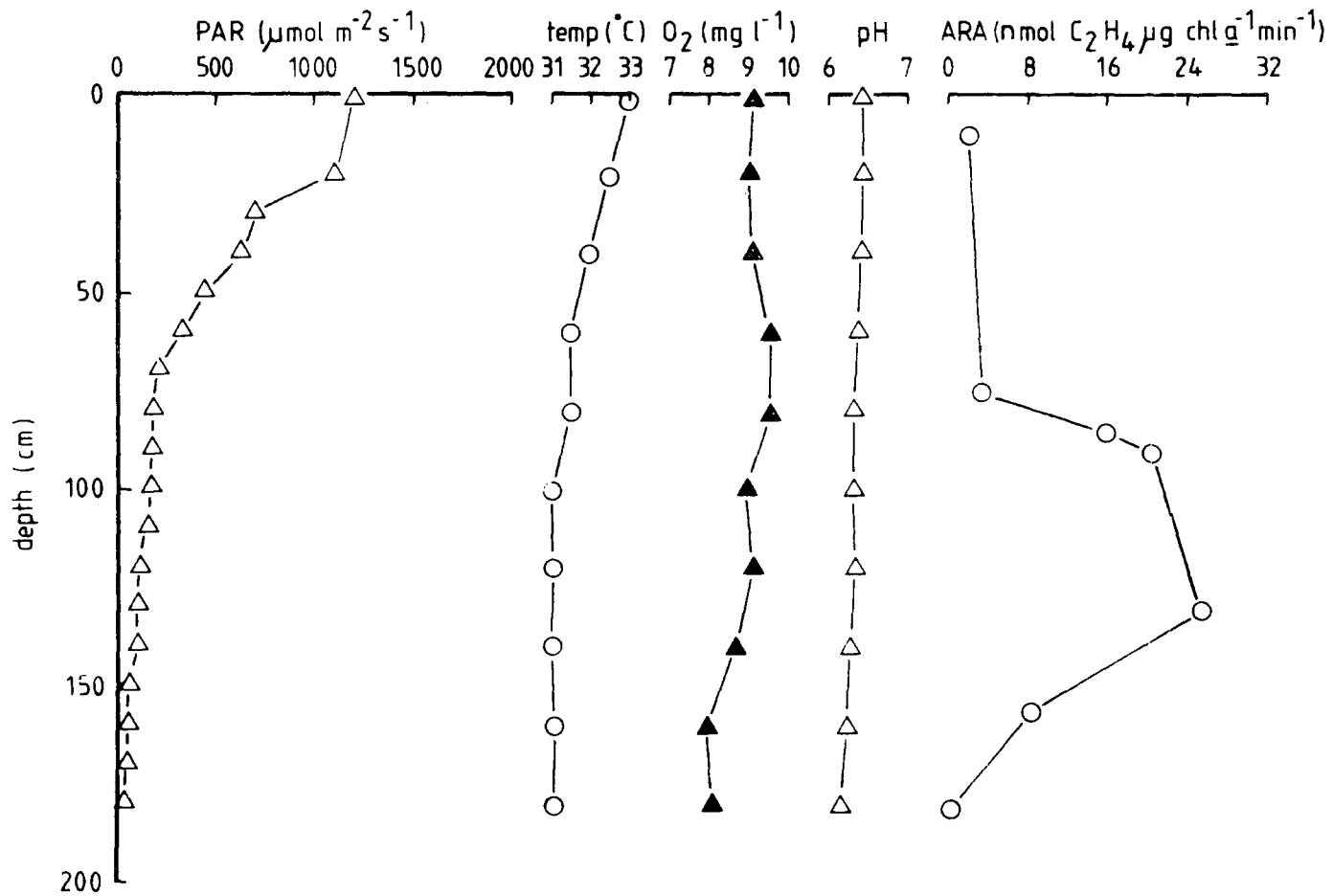
Incubations were done in plastic pots for 60 min (Section 2.1.4) inside the deepwater rice field.

Distribution of ARA on individual sections of a tiller with G. pisum and changes down the water column are shown in Tables 4.2, 4.3 and Fig. 4.1. During the period of study, ARA was quantitatively important down to node -6 and depth -155 cm. The total ARA for internodes (with or without leaves) was higher than that of nodal roots (Table 4.2).

An experiment was planned to measure the percentage contribution of ARA by G. pisum on rice internodes. Six tillers were collected from the same area with approximately equal G. pisum cover. Internode between nodes -3 and -4 was used, cut into 2 cm sections and incubated (treated) as described in Table 4.4. Care was taken during scraping of the alga not to damage the internode or to leave visible G. pisum colonies on the internode. Incubations were done in McCartney bottles in a channel by the main road.

The relative contribution of ARA by G. pisum on a section of rice internode is shown in Table 4.4. The alga constituted 91% of the total ARA by the rice internode with attached G. pisum (cf. treatments 1 and 3).

Table 4.4 Relative contribution of ARA by G. pisum on a section of rice internode on 7.8.83 at 1300 h (mid-time)(34° C; surface light flux 2230 μmol m^{-2} s^{-1}, with ca 70% thin cloud cover;incubation light at 443 μmol m^{-2} s^{-1} ; n = 6)

materials incubated (treatment)	biomass[a]		ARA
	d. wt (mg)	chl a (μg)	(nmol C_2H_4 min^{-1})
1. internode with attached G. pisum	521 (internode)	35.28 (G. pisum)	12.12
2. scraped G. pisum		35.28	5.31
3. internode without G. pisum	521		0.99

a
  little differences were found in d. wt and chl a determinations of different treatments. Values quoted here are corrected to compare different treatments.

4.4 Diel variation in ARA

Diel changes in ARA and environmental variables were studied in situ over 24 h on 20/21.8.83. ARA was assayed at 30 min intervals incubating with C_2H_2 for 60 min in serum bottles (Section 2.1.4). Incubation vessels were covered with neutral density filters to give approximately the same light flux as colonies at -30 cm in the field. Incubations were done in a channel beside the main road at -4 cm. The diel variation in ARA and environmental variables are shown in Fig. 4.2 and Table C1. The diel variation in ARA was marked and usually the higher the light flux, the higher activity was observed (Figs 4.2, 4.3) although there was still considerable ARA at night (3.7% of the total ARA).

4.5 Summary

Transfer of G. pisum to reduced light brought about a marked reduction in ARA during the first one and half hours incubation. The nitrogenase activity ranged from 0.234 to 0.352 nmol C_2H_4 µg chl $\underline{a}^{-1}$ min^{-1} at around mid-day, by the alga being scraped from the rice tillers (Table 4.1). Within the deepwater rice field, ARA was quantitatively important down to about -155 cm and node -6 of a rice tiller. About 91% of the total ARA was due to the G. pisum. Usually the higher the light flux, the higher ARA was observed. At night, 3.7% of the total ARA occurred.

Fig. 4.2   Diel variation of environmental variables and ARA
for G. pisum in situ on 20/21.8.83. Temp., O_2 and p^H
at -30 cm, the depth from where alga originated;
PAR at incubations (each point is the mid-time of
incubations; n=6).
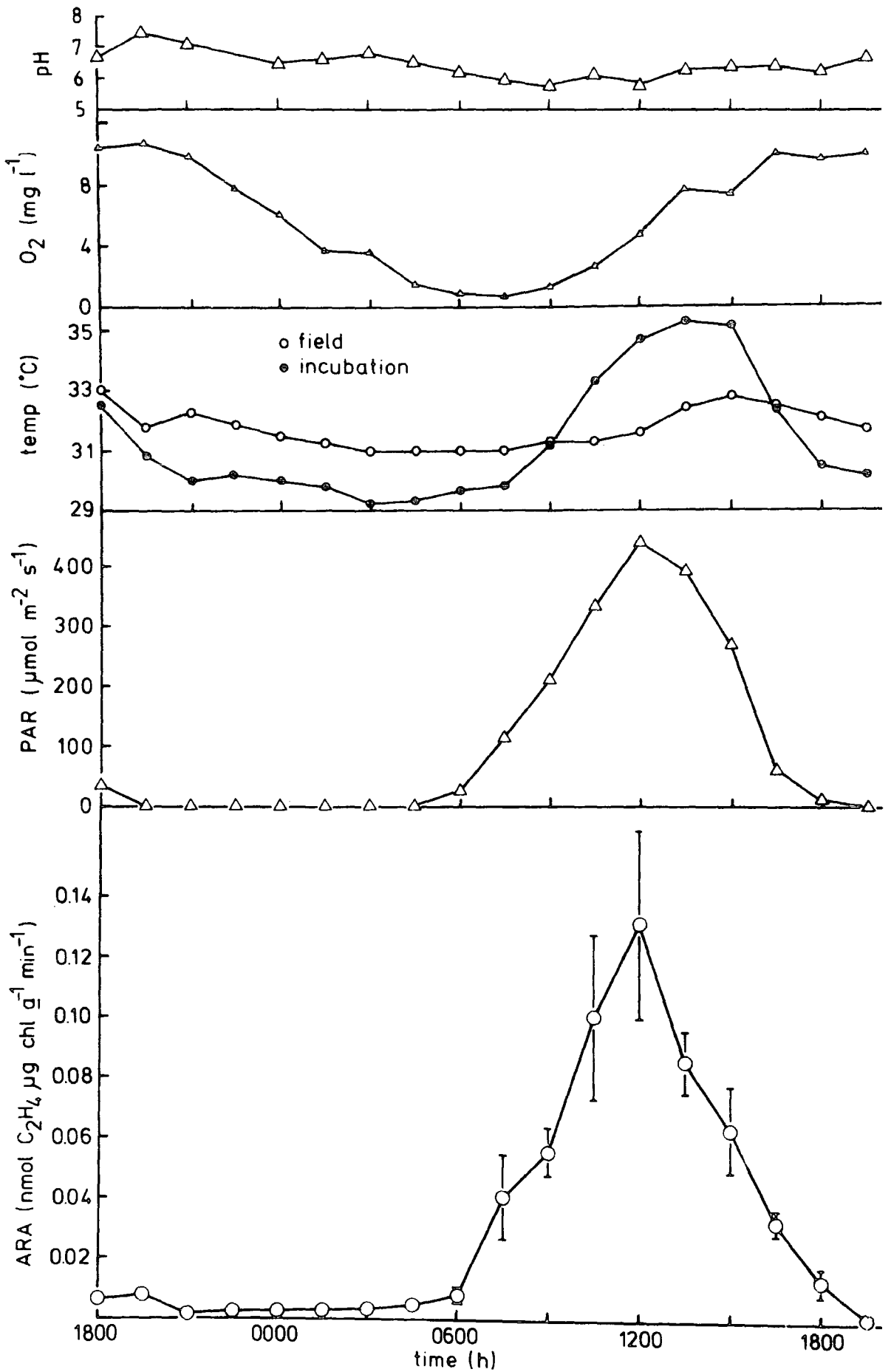
Fig. 4.3   Relationship between ARA and light flux during

the course of the day (on 21.8.83) ; light flux

is for the point where the alga was incubated (derived

from Fig.4.2).

ARA (nmol C_2H_4 µg chl a^{-1} min^{-1})

PAR (µmol $m^{-2}s^{-1}$)

number	incubation mid-point
0	0430 h
1	0600
2	0730
3	0900
4	1030
5	1200
6	1330
7	1500
8	1630
9	1800
10	1930

0.14
0.12
0.10
0.08
0.06
0.04
0.02

0   100   200   300   400
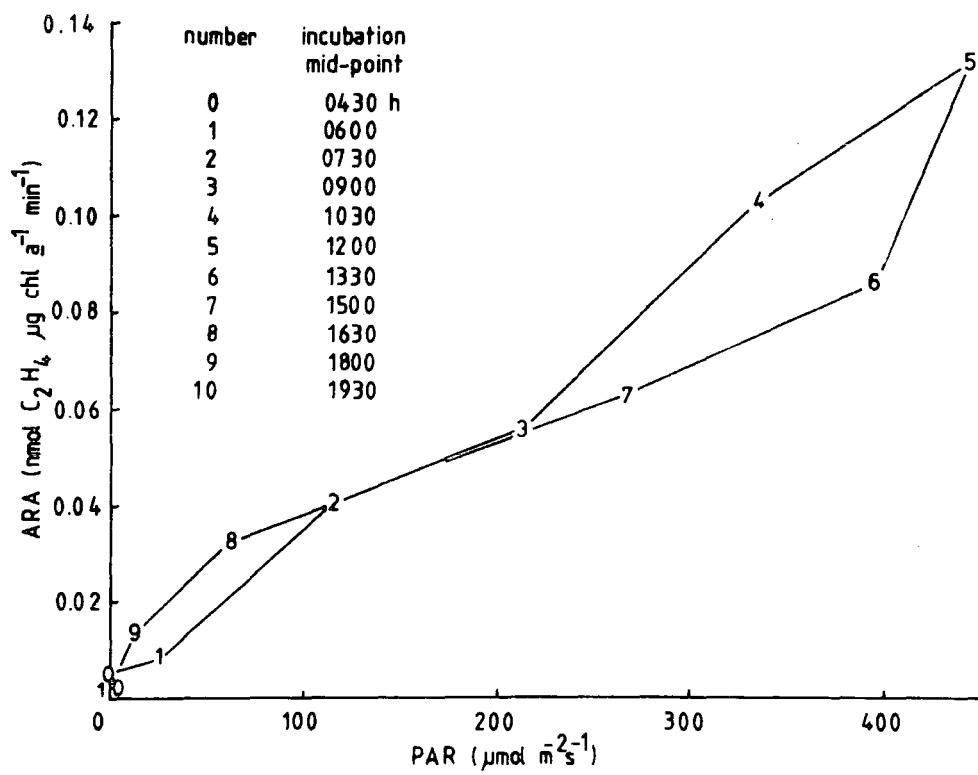
5 MORPHOLOGY OF <u>Gloeotrichia pisum</u> D613

5.1   Introduction

The field studies (Section 3.2) showed that <u>G. pisum</u> could form a colony, hair, gas vacuole and akinete, and that cell division was not restricted to the base of a hair.   Developing filaments of field <u>G. pisum</u> were composed of several 5-9 celled groups, which looked like hormogonia (Fig. 3.2C).   Whether these groups subsequently act as hormogonia is not known. Akinetes may be formed in different ways (Section 3.2.3). The significance of participation of several cells in the formation of a long akinete is also not known.   However, from the literature review (Section 1.4.5) and from studies on field material, the following questions arise:

(i)   does a meristematic zone exist ?

(ii)   which portion of a trichome develops into a hormogonium ?

(iii)   which cell of a trichome is "sacrificed" for the release of a hormogonium ?

(iv)   which cell of a hormogonium differentiates into a heterocyst ?

(v)   how are akinetes and hairs formed ?

(vi)   how does colonization take place ?

5.2   Structure

5.2.1 Hormogonia

The alga was grown in the liquid medium, in the standing condition and under continuous light flux of 60 μmol m^{-2} s^{-1} (Section 2.2.5.6). Three to four day old culture was studied (unless stated otherwise).

Hormogonia are short segments of filament consisting of a variable number of cells, usually 5-8 ($\bar{x}$ = 7.0, ± 1.6, n = 25), rarely as low as 2 or as high as 20 (Figs 5.1A, 5.2A).   The length of hormogonia varies from 30-65 μm ($\bar{x}$ = 44, ± 6.7, n = 25) and breadth from 2.5-5.0 μm ($\bar{x}$ = 3.3, ± 0.6, n = 25).   The basal cell of a newly liberated hormogonium is usually shorter than the tip cell.   Hormogonia do not have gas vacuoles and polyphosphate granules but do have abundant cyanophycin granules. There is no mucilage sheath around the hormogonium, but mucilage is secreted during its movement (Fig. 5.7C).

Hormogonia show gliding movements by clockwise (forward) and anticlockwise (backward) rotations.   On a glass slide at 60 μmol m^{-2} s^{-1}

light flux and 32° C, newly released hormogonia move at the rate of 1.61 $\mu m \ s^{-1}$ (± 0.27, n = 25) whilst on 1% agar the rate is 1.38 $\mu m \ s^{-1}$ (± 0.17, n = 25). To observe whether or not the basal cell of a hormogonium differentiates into a heterocyst, a three day old colony was transferred onto the agar with a wire loop. From the time of release, the hormogonium was kept under continuous observation until it became non-motile, to keep track of the basal cell. The hormogonium gradually became sluggish and then non-motile, 2-4 h after release. No visual changes were observed during this period. Within the next 10 h, cross walls became deeply constricted, cyanophycin granules disappear[ed] and the basal cell (which had been towards the heterocyst [of the parent trichome]) differentiated into a heterocyst. The time period for heterocyst differentiation varied from 14 to 24 h (n = 4). Cell divisions do not occur before heterocyst differentiation.

5.2.2 Filaments

A chain of cells with a fully differentiated heterocyst and the same number of cells as in the parent hormogonium can be regarded as a juvenile filament (Figs 5.1B, 5.2B). Division and redivision of cells (filament at this stage, but before hormogonium formation, is regarded as developing filament : Figs 5.1C-E, 5.2C-E) produce a mature filament with a hormogonium at the apex (Figs 5.1F, 5.2F-G). Mature filaments range from 138 to 213 μm in length ($\bar{x}$ = 174, ± 18, n = 25) and usually appear segmented. In old cultures the filament can be much longer. The number of cells in a mature filament is variable ; it depends on the state of hormogonia development and release, and ranges from 33 to 48 ($\bar{x}$ = 38, ± 4.5, n = 25). Heterocyst frequency at this stage ranges from 2-3% ($\bar{x}$ = 2.73, ± 0.48, n = 25) while at the juvenile stage it was 15.2% (± 3.7, n = 25). Cell length and breadth are extremely variable depending upon the developmental stage and culture age, being longer and narrower in the older culture. Heterocysts are 7-10 μm ($\bar{x}$ = 8.0, ± 0.9, n = 25) in diameter and usually spherical to slightly conical in shape.

The sheath is hyaline and may extend slightly beyond the apex of the trichome (Fig. 5.7M). The sheath gradually becomes thicker as the alga becomes older. In cultures of more than 30 days old there are obvious changes in filament morphology which include formation of false branches and bulging of trichomes (Fig. 5.6A-D). These changes were

Fig. 5.1A-F. Developmental stages of filaments of <u>Gloeotrichia</u> <u>pisum</u>
D613; stages selected from 3-4 day old batch culture (liquid).

A. Hormogonium; note abundant granulations.

B. Juvenile filament, with heterocyst. Note enlarged
cells with constricted cross walls but no cell
division or granulations.

C. First division in the tip cell.

D. Developing filament with two more divisions in
terminal cells (0_7-0_8 & tip cells) producing two
groups of four cells each. Note that the 0_6-0_7 cell
did not divide but the 0_5-0_6 cell has divided;
granules appeared.

E. Repeated divisions of cells of each group,
particularly terminal ones, producing a long and
distinctly segmented filament. Division pattern in
each group of cells has been adapted from agar plate
study (Fig. 5.3 F-G); sheath extended up to the tip
but only partly shown.

F. Mature filament with terminal group of cells being
released as a hormogonium, leaving behind the necridium.
Note the differentiating necridium(arrow) from the
basal cell of 0_7-0_8 group; cell next to the heterocyst
(0_1-0_2) also undergoing division.
(0_1, 0_2...0_8 indicate mother cross walls; successive
divisions in each group are shown by numerals).
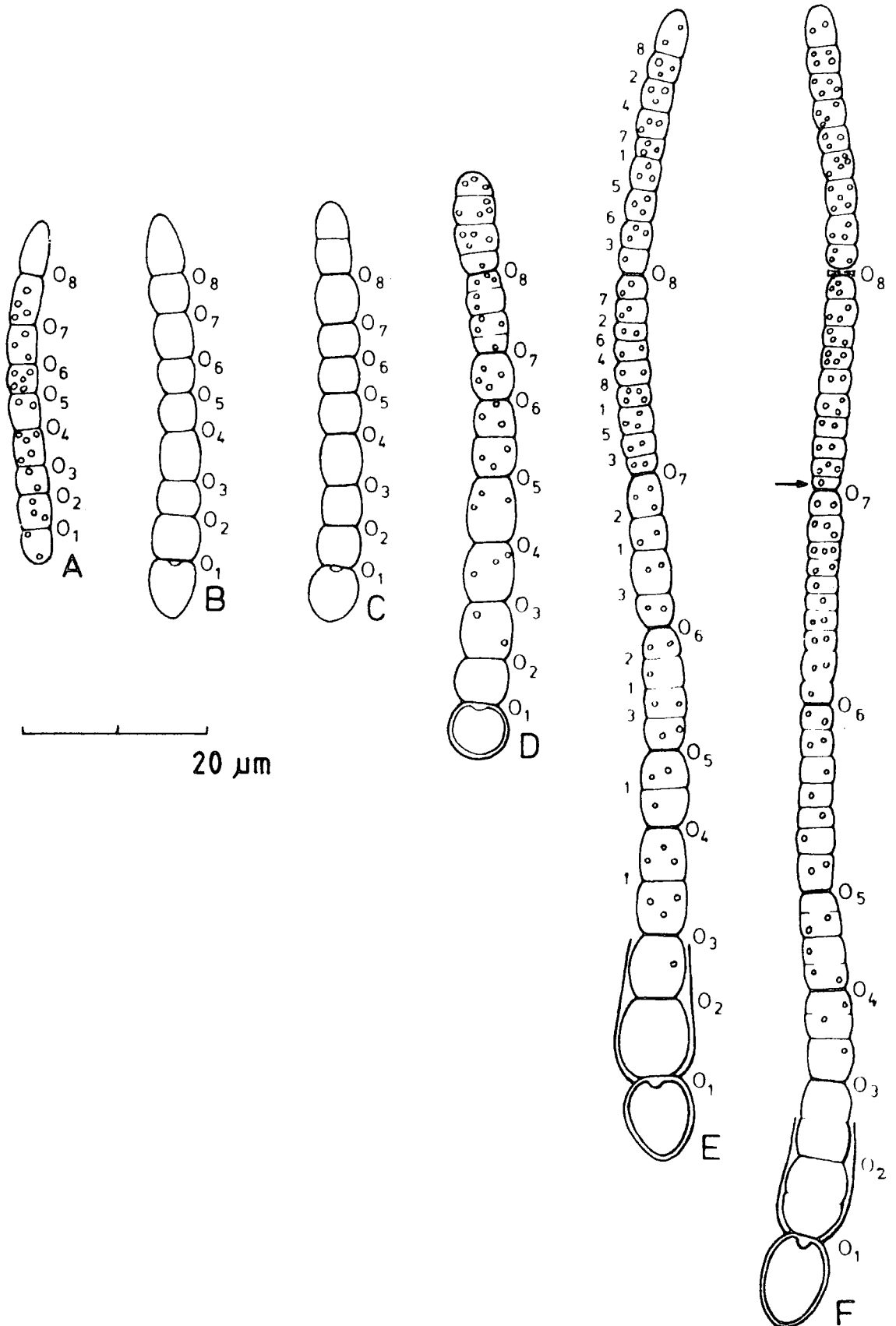
Fig. 5.1

Fig. 5.2A-J.Developmental stages of filaments of <u>Gloeotrichia</u>
<u>pisum</u> D613 ; stages selected and photomicrographed
from 3-4 day old batch culture (liquid).

A. Hormogonium ; note abundant granulations

B. Juvenile filament with heterocyst ; note much
    less granulation.

C. In the upper filament, the tip cell (arrow) has
    divided once, whilst in the lower filament, the
    tip cell has divided three times producing a
    terminal group of four cells. Note the cell below
    has divided once (arrow); granules appearing.

D. Cell division has progressed down to 6th cell
    (arrows).

E. Three more divisions in the terminal group of cells
    producing a seven-celled group. Note a four-celled
    group below the terminal group of cells.

F. Long filament with groups of cells in a row,
    virtually similar in length.

G. Mature filament. Note division of basal cell
    of the terminal group of cells producing necridium
    (arrow) and that cell division has progressed down
    to cell next to heterocyst ; the whole trichome
    appearing segmented because of the deeply
    constricted mother cross walls.

H. Two terminal groups of cells, showing the
    differentiating necridium (arrow), formed by
    division of basal cell of the terminal groups
    of cells. Note the enlargement of cells and the
    unequal cell divisions (d).

I. Terminal group of cells being liberated as
    hormogonium (ho) leaving behind the necridium (n).

J. Filament after two subculturings to fresh medium at
    24 h interval. Note that the heterocyst and only a
    basal four-celled group remained after liberating
    all the groups as hormogonia.

    (Scale: 10 μm)
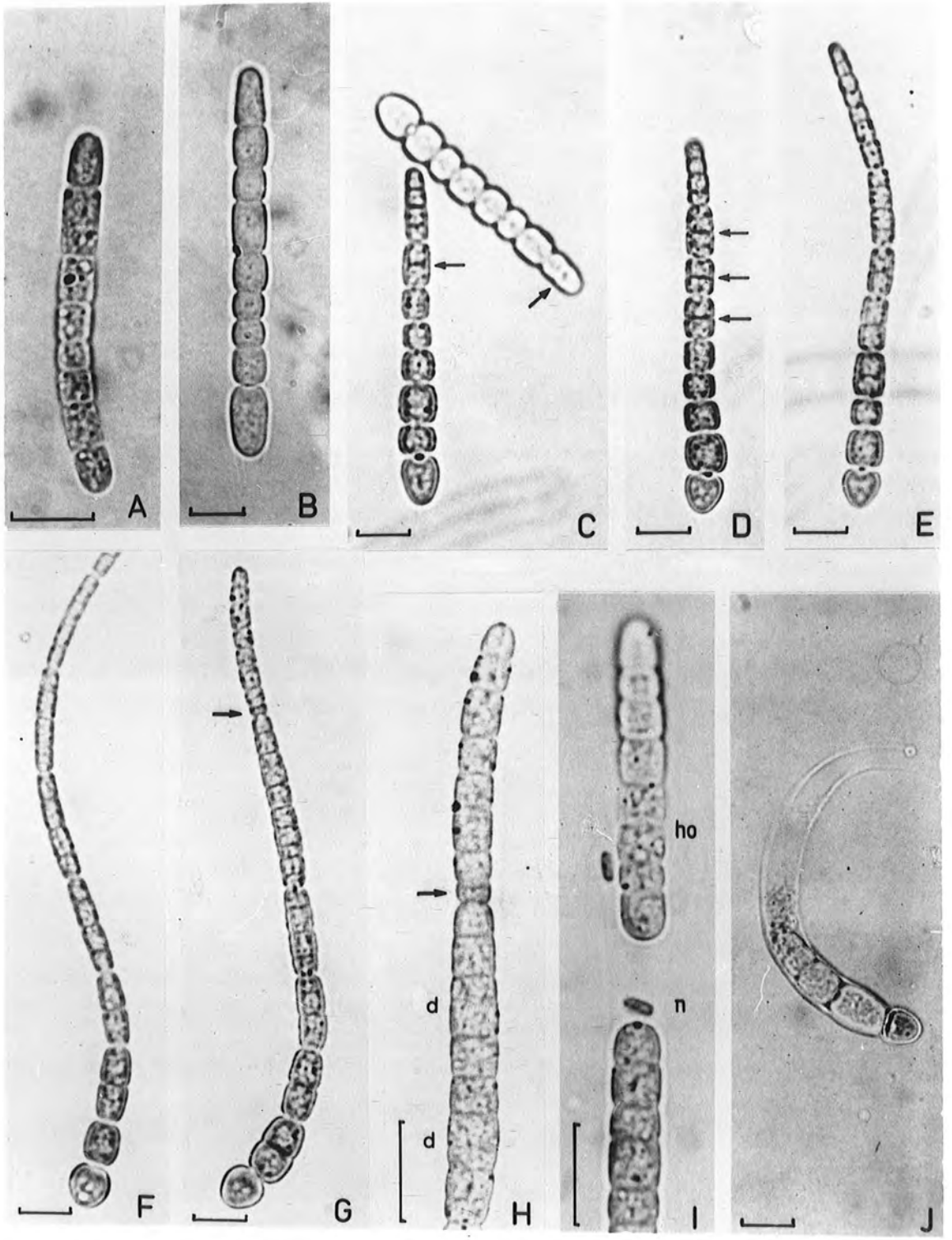
Fig. 5.2

presumably due to the _in situ_ germination of hormogonia trapped within the thick sheath. Divisions in terminal cells of juvenile filaments (see Section 5.3.1) create pressure on the surrounding sheath. Ultimately the growing apex comes out by bursting the sheath as a single false branch (Fig. 5.6A, C-D). Several hormogonia can germinate in this way producing several false branches (Fig. 5.6C). Cells in the median region of the developing trichome may divide more than the terminal cells resulting in the formation of loops (Fig. 5.6A-C). This happened due to the inability of the growing trichome to slide through the thick sheath, presumably due to constrictions of the sheath around cross walls (Fig.5.6B). However, the basal cell in each case develops into a terminal heterocyst and ultimately appears as a tolypothricoid filament (Fig. 5.6C).

In addition to the basal heterocyst, a terminal heterocyst may also be developed at the apex (Fig. 5.6G). In old cultures the heterocyst is also observed to germinate (Fig. 5.6E-F). Akinetes and hairs were not found in the laboratory.

5.2.3 Colonies

Under standing conditions (in the growth room : Section 2.2.5.6) the alga forms patches of floating colonies which appear as small dark-brown beads after about two days' growth (Fig. 5.7A). After first sub-culturing to the shaking tank, circular colonies appear on the side of flasks (Fig. 5.7B) as well as some floating colonies. After several more sub-cultures, colonies appear as long "ropes", attached to the bottom of flasks. Each small bead is usually larger than 0.5 mm and appears as a distinct radiating structure with hanging "rope" (Fig. 5.7H-J). Ropeless conical (in side view) colonies were also observed occasionally (Fig. 5.7M). The colonial structure at different stages is described and illustrated in detail in Section 5.3.2.

5.3   Growth and development

5.3.1 Filaments

In a four-day old liquid culture (standing, continuous light flux of 60 μmol m^{-2} s^{-1}) different stages of filament development were studied (Figs 5.1A-F, 5.2A-J). In young filaments repeated divisions (transverse) at the distal end produced several daughter cells and these remain together as small groups (Figs 5.1C-D, 5.2C-F). Divisions were

restricted not only to the tip, but also to cells towards the heterocyst (Figs 5.1F, 5.2G). In all cases, daughter cells of each parent cell appeared to remain together forming several groups in a row (Figs 5.1E-F, 5.2F-G). In a mature filament, the basal cell of a terminal group was found to divide producing a basal necridium (Fig. 5.2G-H), the death of which resulted in the release of the terminal group as a hormogonium (Figs 5.1F, 5.2I). It was also observed that several such groups can be released successively, leaving only a very short section of the trichome next to the heterocyst (Fig. 5.2J). However, it appears from the above observations that growth in this alga was diffuse i.e. there was no meristematic zone ; the hormogonium was a group of cells formed by the division and redivision of a single cell of a parent hormogonium and the basal cell of each group divided to produce a necridium for the release of the rest cells as a hormogonium. The developmental sequence of juvenile filaments of G. pisum D613 was therefore studied on agar plates (Section 2.2.5.3) to confirm these observations and also to observe the pattern of cell division that formed each group (Figs 5.3A-G, 5.4A-D, 5.5A-C). Incubation was done under continuous light flux of 60 μmol m^{-2} s^{-1}. The filament on agar was covered with a cover slip to study under high magnification (Section 2.2.3.1).

The first obvious change after heterocyst differentiation (Fig. 5.3A) is the division of distal cells (Fig. 5.3B). The division proceeds towards the base, but one intercalary cell remained undivided for some time (Figs 5.3B-D, 5.4A-B). Simultaneous division of the basal and tip cells is also observed resulting in a row of cells in groups along the length of the trichome (Fig. 5.5A-C). Each of these groups is separated from the others by cross walls of the mother cell and in most cases both ends are conspicuously constricted, giving a segmented appearance to the whole trichome (Figs 5.3D-G, 5.4A). The number of cells in each group is usually 6-9. One obvious feature is that the cell which will divide next is usually distinctly larger than adjacent cells and that the division is unequal (see also Fig. 5.2H).

The cell division in the formation of a group of cells appears to follow a definite pattern, at least in the early stage of development. The second and third divisions occur in the upper and lower daughter cells respectively (Fig. 5.3C). In later stages of growth and

Fig. 5.3A-G.Sequential changes during growth and development of a
trichome of <u>Gloeotrichia</u> <u>pisum</u> D613 grown on agar.

A.   Filament with proheterocyst ; note no cell division.

B.   Young developing filament. Note almost simultaneous
      divisions in some intercalary cells (O_3-O_4, O_4-O_5)
      and tip cell.

C.   Two more divisions producing three groups of four
      cells each. Note the second and third divisions
      in the upper and lower daughter cells respectively.

D.   Filament with more divisions, producing distinct
      groups of cells with deeply constricted mother
      cross walls (O_1, O_2 etc.). Note that the division
      pattern in the terminal group of cells is not similar
      to the lower groups of cells ; cell division has
      extended up to the base, while one intercalary cell
      (O_5-O_6) has not yet divided (see Fig. 5.4A).

E.   Elongation and more division of cells. Note second
      division in the lower daughter cell of group O_2-O_3
      (see Fig. 5.4B-C): some blue-green rings appeared
      terminally, perhaps due to > 32° C temperature;
      petri dish was transferred to 25° C growth room;
      note also that the terminal group of cells has
      broken down at 5th division cross wall and that the
      terminal part is moving away.

F.   Assymmetric divisions in cells of group O_3-O_4 and
      also second division in the lower daughter cell of
      group O_1-O_2. Note the portion of filament above
      O_5 has dropped.

G.   Almost mature filament with more divisions in the
      upper half of each group of cells. Note three-celled
      group next to the heterocyst (see Fig. 5.4D).
      (O_1, O_2....O_6 indicate mother cross walls ; successive
      divisions in each group are shown by numerals ; cell
      inclusions omitted for simplicity).
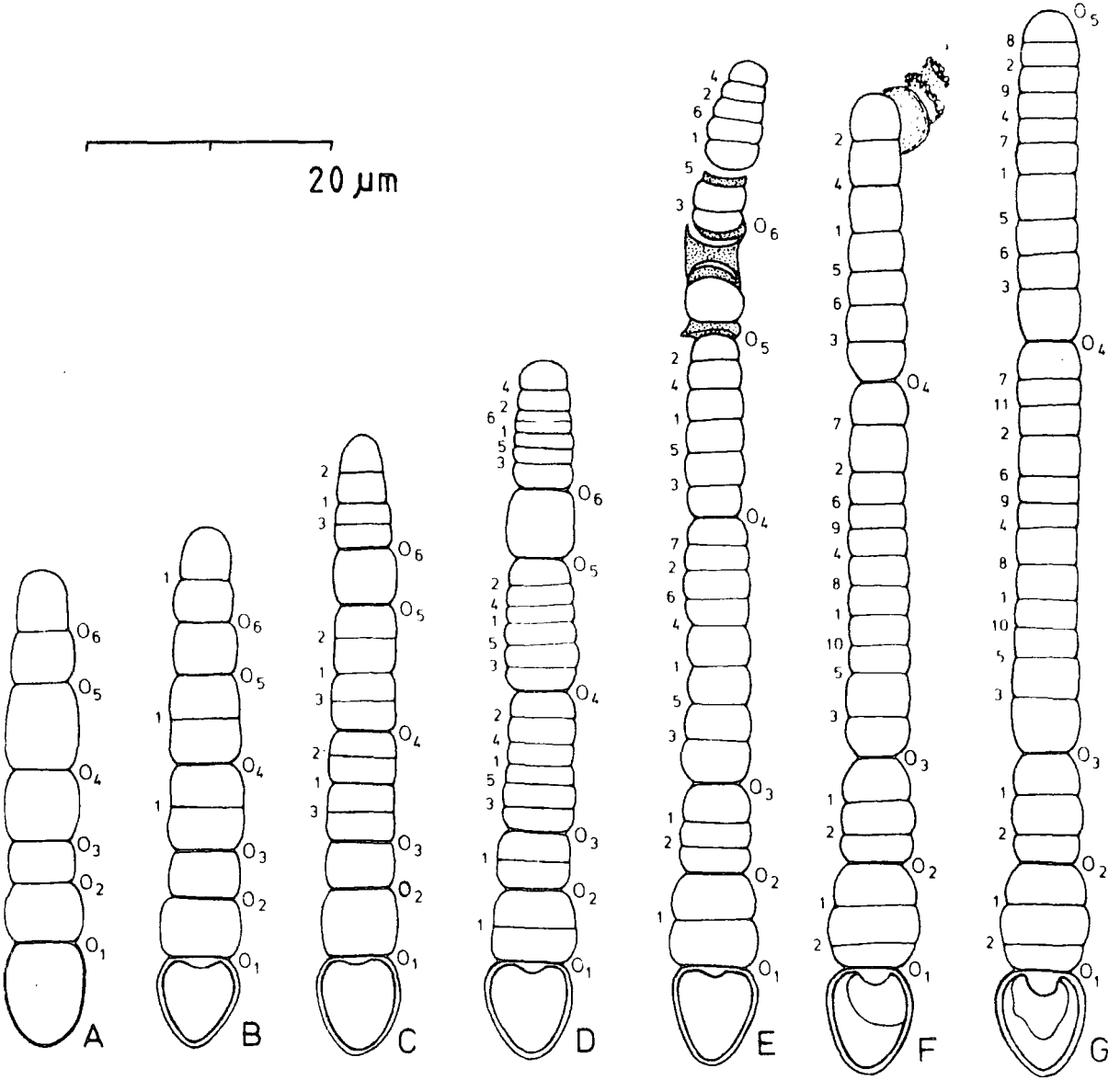
Fig. 5.3

Fig. 5.4A-D.Photomicrographs of sequential changes during growth
and development of a trichome of <u>Gloeotrichia</u> <u>pisum</u>
D613 grown on agar.

A.   Developing trichome consisting of several groups
     of cells. Note deeply constricted mother cross
     walls resulting in segmented appearance of the
     whole trichome (see Fig. 5.3D for division pattern).

B.   More divisions and elongation of daughter cells of
     each group, resulting in elongation of trichome.
     Note division of cells next to the heterocyst.

C.   Second division in the lower daughter cell of the
     2nd group   ; terminal group of cells has broken
     down due to ring formation (see Fig. 5.3E for
     division pattern).

D.   Almost mature filament, consisting of four groups
     of cells. Note three-celled group next to the
     heterocyst (see Fig. 5.3G for division pattern).
     (Scale : 10 μm)
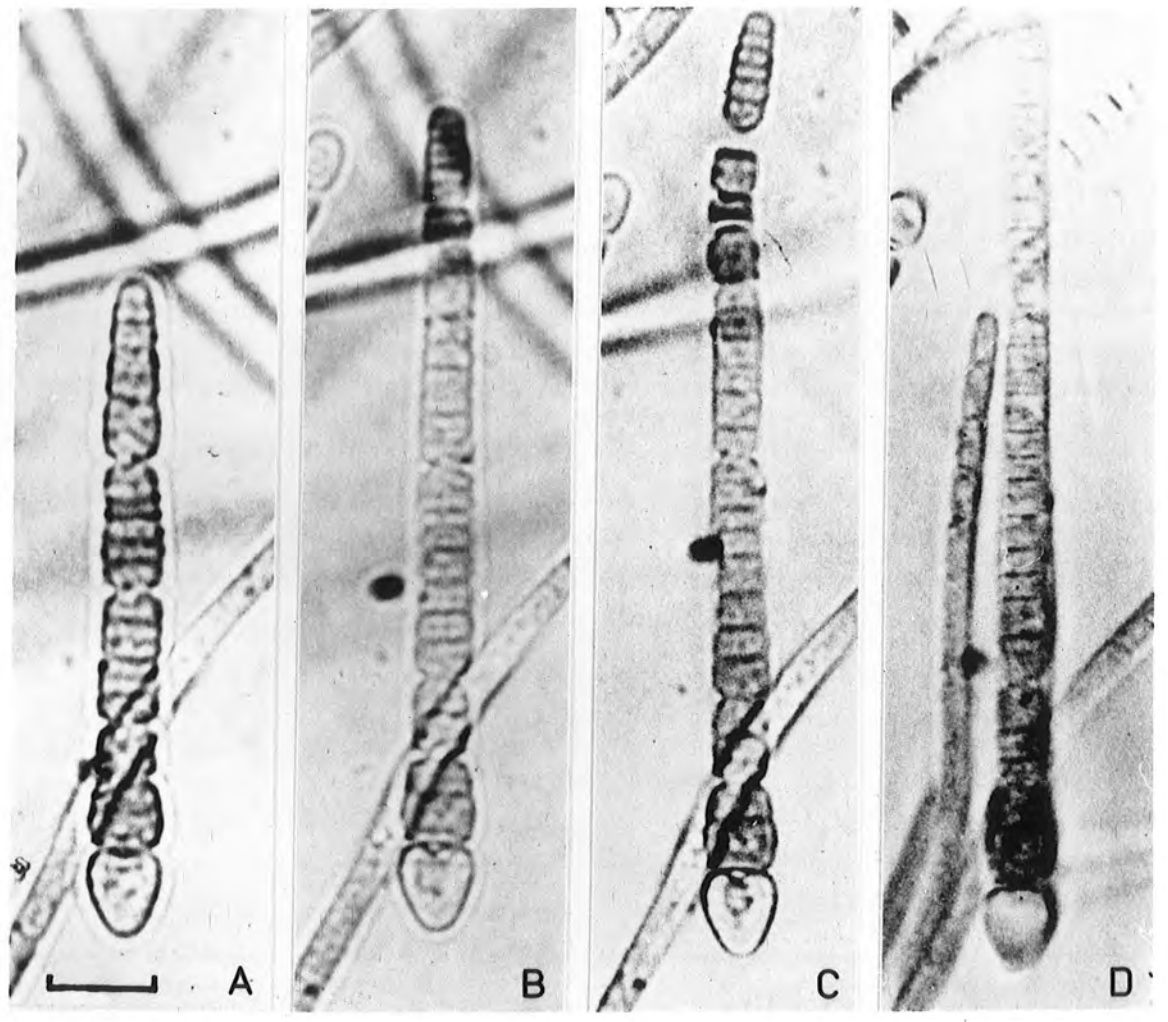
Fig. 5.4

Fig. 5.5A-C.Growth and development of a trichome of <u>Gloeotrichia</u>
<u>pisum</u> D613 showing that some intercalary groups of
cells can be formed faster than the terminal group of
cells.

D-I. Fate of a trichome of a mature filament grown on
agar.

D. Mature filament after transfer to agar from three
day old liquid culture ; filament at this stage
is considered as at zero-time. Note the basal cell
of the terminal group of cells dividing producing
basal necridium (arrow).

E. Filament after 14 h; 4th hormogonium (h) being
released leaving behind necridium (n).

F. Filament after 24 h ; seven hormogonia released.
Note very long empty sheath ; cells at this stage
had abundant granulations and as a result divisions
were difficult to observe.

G. Filament after 36 h ; another hormogonia released.
Note elongation of all three groups of cells.

H. Filament after 50 h ; trichome almost filling the
sheath.

I. Filament after 62 h ; trichome filling the sheath
and is longer than at zero-time (cf. D). Note that
all the groups of cells have elongated several times
the length at zero-time.

30 μm
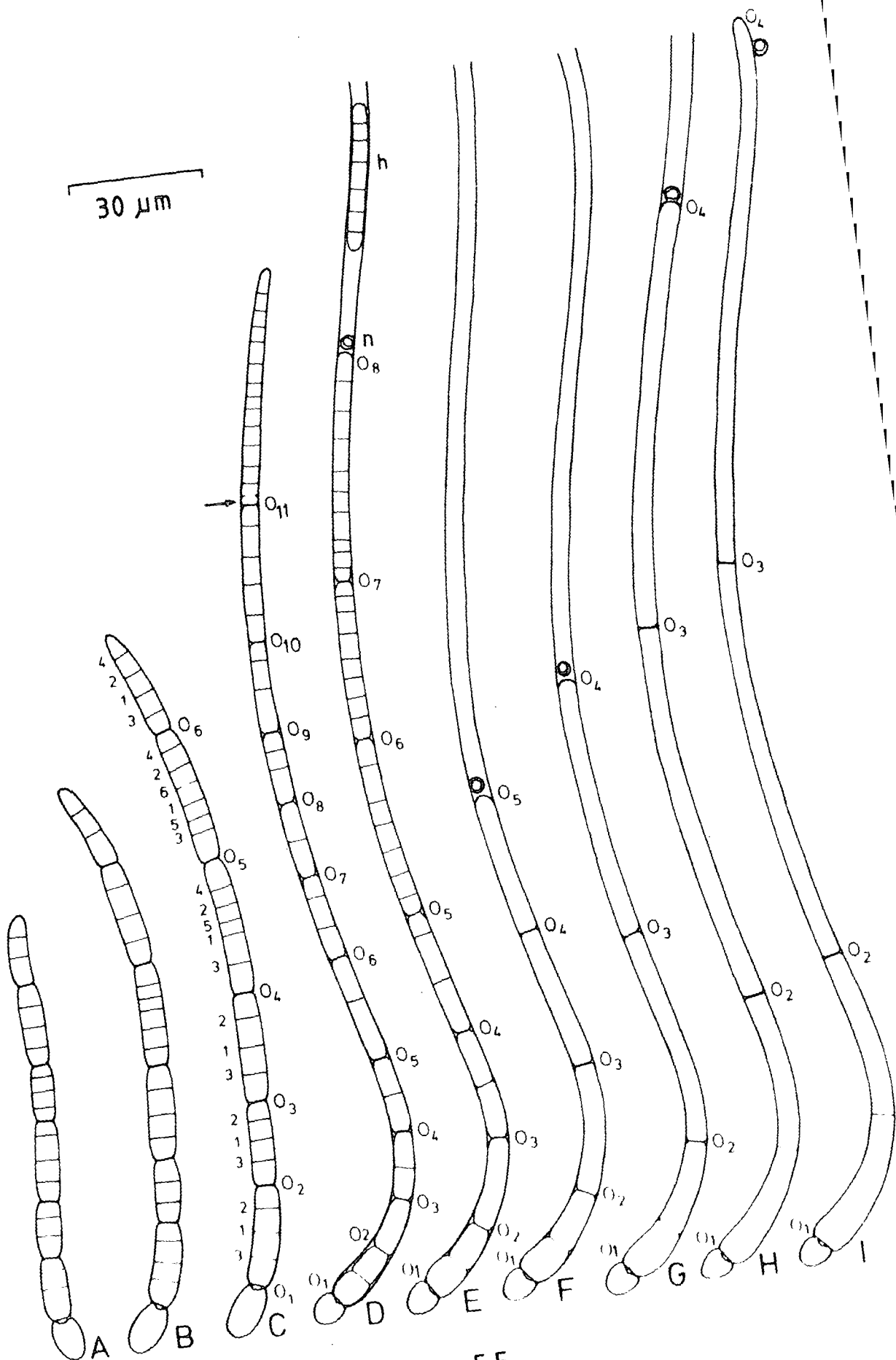
Fig. 5.5

Fig. 5.6A-G. Morphology of <u>Gloeotrichia</u> <u>pisum</u> D613
in old cultures.

A.   Single false branch (f. b.);   note bulging (arrow)
of trichome and terminal heterocysts (h) at the point
of branching.

B.   Bulging trichome;   note thick sheath (arrow) around
trichome.

C.   Repeated false branching (f.b.).

D.   Geminate and single false branches.

E-F. Germination of heterocysts, in 50 day old culture.

G.   Terminal heterocysts (h) at both ends;   note
paired heterocysts at the base.

(Scale : 20 μm)
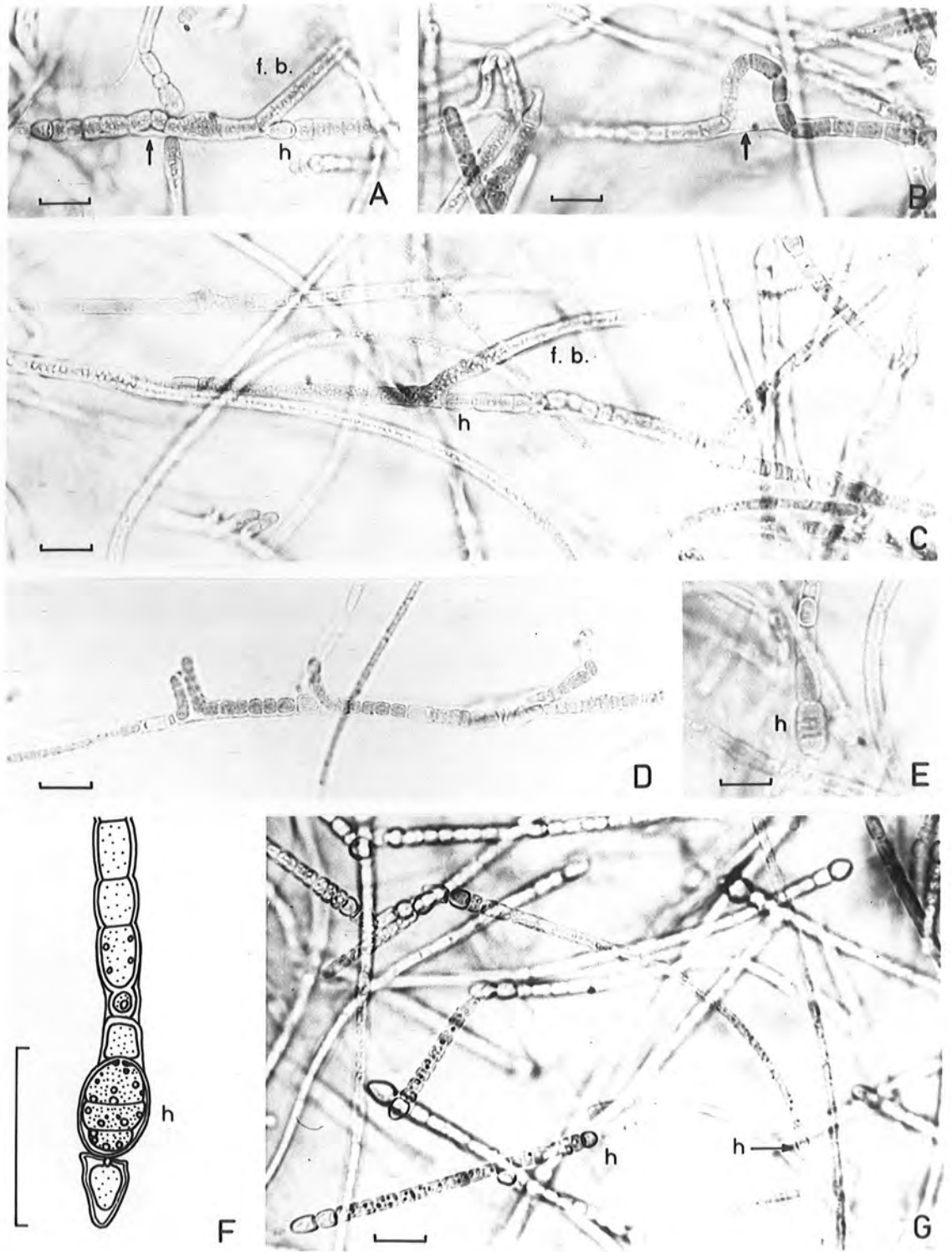
Fig. 5.6

particularly in cells towards the base, a second division is observed in the lower daughter cell (Fig. 5.3E-F, groups O_2-O_3 and O_1-O_2). Fourth and fifth divisions usually occur in daughter cells adjacent to the first division cross wall (Fig. 5.3D), resulting in three daughter cells on either side. However, in the formation of a group of eight or more cells, cell division takes place preferentially in the upper half, producing an asymmetric group (Fig. 5.3E-G). Further growth and release of each group could not be followed, because of the development of a large air bubble around the filament. However, in the development of a mature filament all cells except the heterocyst undergo division and redivision and daughter cells remain together forming groups of cells. These groups maintain their identity throughout the growth of a trichome.

A mature filament was then studied on agar (Section 2.2.5.3) to follow the fate of a trichome and in particular changes in the terminal and basal (next to the heterocyst) groups of cells with the hope of understanding hair and akinete formation, respectively (Fig. 5.5D-I). A typical mature filament is shown in Fig. 5.5D. The basal cell of the terminal group has divided to form a necridium. The cell next to the heterocyst (group O_1-O_2) has divided once and attained a length of 15 μm. However, in the following description this filament has been considered to be a filament at zero-time. In the first 24 h rapid release of hormogonia occurred leaving four groups of cells next to the heterocyst (Fig. 5.5F). The eighth hormogonium was released within the next 12 h (Fig. 5.5G). Under the present conditions, hormogonia were released at a rate of one every 4.6 h. During this 36 h period the basal group of cells (O_1-O_2) doubled in length. However, after that no hormogonia were released but the remaining groups elongated, filling the whole parent sheath (Fig. 5.5H-I). The whole trichome was very granular with no vacuolations. At this stage the basal group increases its length to about five times that at the start. After about 60 h this old filament (Fig. 5.5I) is longer (298 μm) than that at zero-time (272 μm). The filament then died and hence further change could not be studied.

5.3.2 Colonies

The alga was studied on agar or liquid medium, grown in standing condition and under continuous light flux of 60 μmol m^{-2} s^{-1} (unless stated otherwise). A hormogonium secretes mucilage during its movement

and becomes surrounded by mucilage of its own after becoming non-motile (Fig. 5.7C). Both on agar and at the liquid surface few hormogonia aggregate and subsequently many more come together and become entangled within the secreted mucilage. Aggregation of hormogonia at the initial stage is irregular, parallel to or at right angles to each other, with the basal end not always directed towards the centre of a growing colony (Fig. 5.7D-F). However, it is observed that in a developing colony the central portion is surrounded by colonial mucilage (Fig. 5.7L) out of which distal ends of filaments extend (Fig. 5.7J). The abundance of mucilage at the basal end has also been observed in solitary filaments (Fig. 5.7K). It is possible that the differentiation of the hormogonium into a juvenile filament and subsequent diffuse growth starting from the distal end (Section 5.3.1) would result in the protrusion of filament tips out of the colonial mucilage, giving a radiating appearance (Fig. 5.7H,J,M). Attachment of hormogonia always occurred at the centre and often underneath the developing colony resulting in a "V"-shaped or rope-like pendant structure (Fig. 5.7G-J). However, it appears that hormogonia preferentially attach to tips of these "ropes" (Fig. 5.7I-J). Transfer of developing colonies (without "ropes") to fresh media (2-5 colonies transferred to the liquid surface by a wire loop and the flasks placed over aluminium foil) in the standing condition also resulted in "rope" formation indicating that the liberated hormogonia (parental or from other colonies) glide over the colonial mucilage and participate in "rope" formation. The liberated hormogonia can also aggregate forming daughter colonies surrounding the parental colony. When the light source from below was removed (wrapping flask base with black polythene) a much shorter "rope" was formed. After several subcultures in the shaking tank (light from below), long "rope"-like colonies and matted growths were found attached to the bottom of flasks.

Fig. 5.7A-M. Photomicrographs of structure and development
of colonies of <u>Gloeotrichia pisum</u> D613. Stages
selected from standing culture (except B) on
different days.

A.   Patches of floating colonies. Note beaded colonies.

B.   Individual colonies on the side of flasks after
first subculturing to shaker from standing
condition.

C-M. Stages of colony formation.

   C. Random movement of hormogonia after 12 h of
growth on 1% agar, lacking P. Note mucilage
tracks along the path of hormogonia and
secretion of mucilage around non-motile
hormogonia. Mounted in Indian ink.

D.   Aggregated hormogonia and juvenile filaments.
Note irregular arrangement of trichomes, and
heterocysts not always directed towards the centre
of the aggregate.

E-F. Aggregation of more hormogonia ; earlier attached
ones developed into long filaments.

G.   Bigger colony, with many mature filaments giving
radiating appearance. Note aggregation of
hormogonia underneath the colony and production
of hormogonia (arrow).

H.   Fully formed radiating colony. Note the long
rope-like structure underneath the radiating part
of the colony.

I.   Side view of a rope, mounted in Indian ink. Note
colonial mucilage.

J.   Rope, where filament tips are emerging through
the colonial mucilage.

K.   Single filament showing thick mucilage around basal
end. Mounted in Indian ink.

L.   Developing colony showing mucilage around the
central aggregate. Mounted in Indian ink.

M.   Side view of a mature conical colony. Note
terminal end of filaments protruding out of the
colonial mucilage ; rope like structure absent.
(Scale : A-B = 10 mm ; C-M = 40 μm).
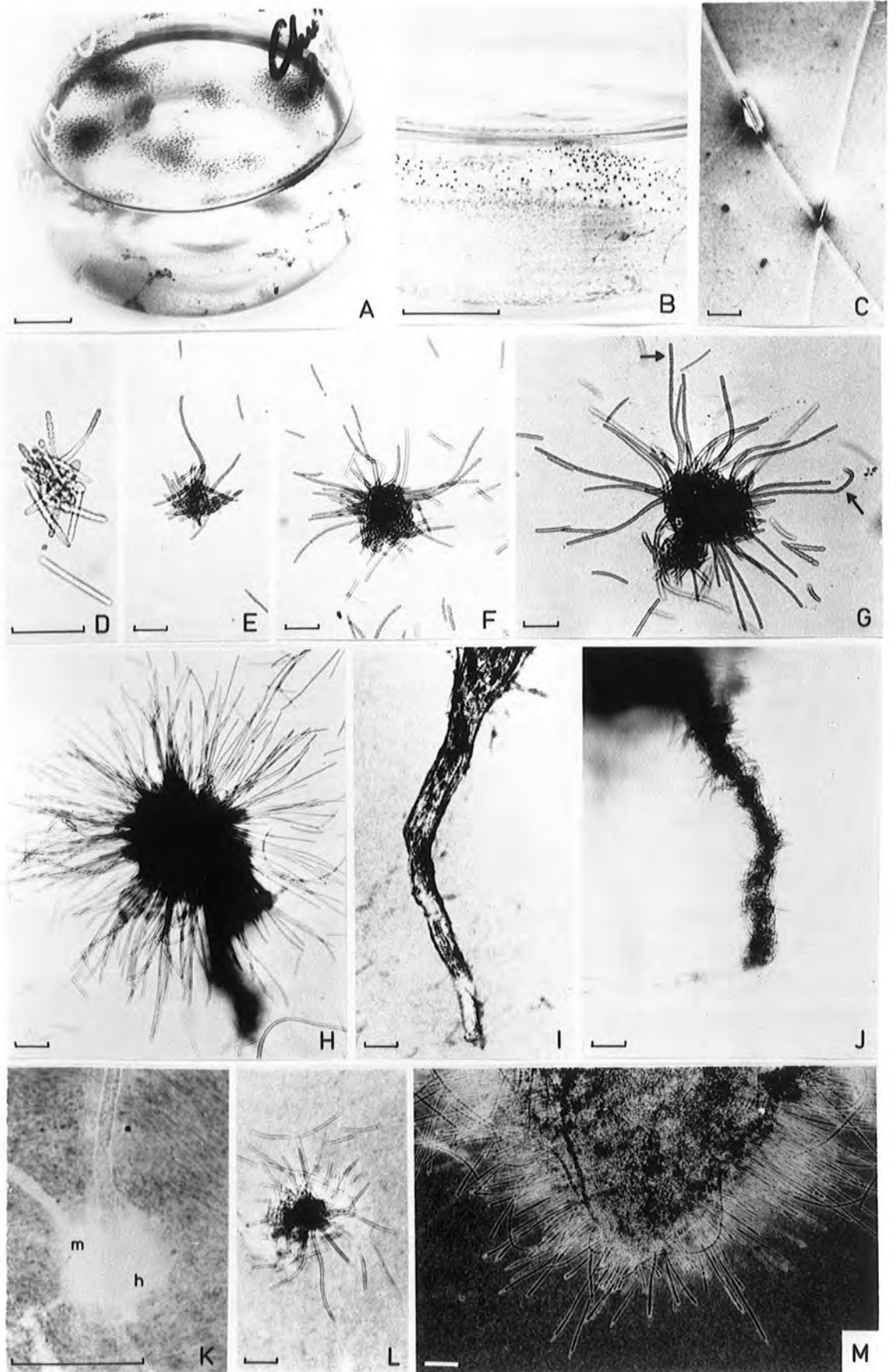
Fig. 5.7

5.4   Influence of light on morphology

G. pisum D613 did not produce hairs, gas vacuoles or akinetes in the previous laboratory study (Sections 5.2.2, 5.3.1). Studies were therefore planned to investigate the influence of quantity and quality of light flux on the formation of these structures. The light variables tested are shown in Table 5.1. White light was provided from above. Red and green lights were provided from underneath (Section 2.2.5.6), flasks were covered by a wooden box with the bottom removed (treatments 2-4). The experiment was carried out in the standing condition. No hair, gas vacuole or akinete was found under any light conditions used.

Table 5.1   Quantity and quality of light flux used to induce hair, gas vacuole or akinete formation in G. pisum D613 (control = white light at 60 μmol m^{-2} s^{-1} ; continuous light)

treatment	light quantity (μmol m^{-2} s^{-1} PAR)	light quality	transmission band (nm)
1	gradually transferring the culture to lower lights on different days: from zero-time to 4 d(at 60) → 5 to 6 d(at 40)→7 to 9 d(at 30) 10 to 12 d (at 20)→ end of experiment (at 15)	white	> 330
2	15-20 (before passing through filter was 130 ± 9)	red	> 590
3	15-20 (before passing through filter was 105 ± 7)	green	463-570 and > 700
4	7-9, achieved by placing a neutral density filter over red filter	red	> 590

In another experiment the influence of light and dark cycle (12:12 h: Section 2.2.5.6) on morphology was observed. White (60 μmol m^{-2} s^{-1}) and red (7-9 μmol m^{-2} s^{-1}; light quantity before passing through filter was 60 μmol m^{-2} s^{-1}) light was used. No hair, gas vacuole or akinete was found in either treatment.

The literature (Section 1.6) indicated that gas vacuole formation can be enhanced by low light. In another experiment the alga was therefore acclimatized at a range of low values of light flux, under

continuous and light and dark conditions, and subsequently subcultured to the similar or different light conditions to induce gas vacuole formation (Table 5.2). Only newly liberated hormogonia were studied. Gas vacuoles were not found under any treatment.

Table 5.2 Acclimatization of G. pisum D613 in different low light conditions and subculturing to similar or different light conditions to induce gas vacuole formation

acclimatization (for 15 d)		subsequent growth conditions	
light flux $(\mu mol\ m^{-2}\ s^{-1})$	continuous light/ light and dark (12:12 h)	light flux $(\mu mol\ m^{-2}\ s^{-1})$	continuous light/ light and dark (12:12 h)
2.5	continuous light	2.5	continuous light
5.0	" "	5.0	" "
5.0	" "	2.5	" "
10.0	" "	10.0	light and dark
2.5	" "	2.5	" "
5.0	light and dark	5.0	" "
10.0	" "	2.5	" "
20.0	" "	2.5	" "

5.5 Influence of nutrient deficiency on morphology

G. pisum D613 did not produce hairs or akinetes under different combinations of light quantity and quality (Section 5.4). The influence of nutrient deficiency on the formation of these structures was therefore investigated.

In one experiment the alga was grown in media lacking P or Fe, under continuous light flux of 60 $\mu mol\ m^{-2}\ s^{-1}$. No hair or akinete was formed.

In another experiment a slightly modified medium, Chu $10D_{X2}$ $Fe_{0.5}$ $P_{0.9}$-N (Section 2.2.5.3) was used. It was hypothesized that an excess of some elements and a deficiency of P or Fe or a combined effect with one of the light variables (Table 5.1) may influence hair or akinete differentiation. In addition to the continuous light flux, a light and dark cycle (12:12 h) was also used. Only one filament was found with an enlarged cell, resembling the early stage in akinete differentiation, after 50 days growth under continuous red light at 15-20 $\mu mol\ m^{-2}\ s^{-1}$

PAR (Fig. 5.8). A similar structure was also observed in a 70-day old culture, grown under continuous light at 60 μmol m^{-2} s^{-1}. No hair was observed under any treatment.

5.6 Summary

Growth of G. pisum D613 filaments in the laboratory is diffuse, i.e. there is no meristematic zone. A trichome is composed of several groups of cells attached to the heterocyst. Each group is derived by the division and redivision of a single cell of the parent hormogonium. Each group appears to have been liberated from the tip of a filament as a hormogonium. At the time of release, the basal cell of each group divides. The lower daughter cell, the necridium, dies, the hormogonium is released and the basal cell (youngest) develops into a heterocyst. Under usual experimental conditions (Section 2.2.5.6) akinetes are not produced but the cell next to a heterocyst divides producing a group of cells. Hairs and gas vacuoles were not found. Attempts to induce the formation of akinetes, hairs and gas vacuoles, using different quantities and qualities of light, and nutrient deficient media, were unsuccessful. During the colonization, the initial aggregation of hormogonia seems to occur by chance and is entangled within the mucilage but some other factors may be involved in the subsequent aggregation which is always at the centre of a developing colony. The radiating nature appears to develop due to mucilage at the basal end and diffuse growth usually at the terminal end.

Fig. 5.8   Enlarged cell resembling the early stage in
            akinete formation of G. pisum D613 (grown in
            Chu 10D$_{X2}$ Fe$_{0.5}$ P$_{0.9}$-N under red light
            (continuous) at 15-20 μmol m^{-2} s^{-1} PAR).

6 GROWTH CHARACTERISTICS OF <u>Gloeotrichia</u> <u>pisum</u> D613

6.1   Introduction

The literature (Section 1.6) indicates that ARA of an alga can be related to its growth characteristics, such as developmental stage or heterocyst frequency. These are features which change during growth in batch culture (Section 5.3). Experiments were therefore planned to characterize the growth rate of this alga, such as influence of light flux on growth, morphology of colonies and filaments in batch culture, including heterocyst frequency and cell inclusions.

6.2   Influence of light flux on growth rate

The influence of light flux on growth rate was studied in standard conditions (apart from light flux) in the shaker (Section 2.2.5.6) . The alga was grown in the respective light flux for two subcultures. It was difficult to standardize the inoculum. The range of inocula used was from 7.8 to 14.5 mg l^{-1} dry weight and 0.1 to 0.2 mg l^{-1} chl <u>a</u>. The influence of light flux on growth rate is shown in Fig. 6.1, Tables 6.1 and C2. Light saturation occurred between 50 and 100 μmol m^{-2} s^{-1}. Because of a limited number of points on the graph, it was difficult to identify the exponential phase of growth. Apart from growth at 25 μmol m^{-2} s^{-1}, the alga showed approximately exponential growth from about zero-time to two days at all light fluxes (Fig. 6.1). However, in the calculation of $\underline{k'}$, zero-time to two days and one to two days have been considered and summarized in Table 6.1. At 100 μmol m^{-2} s^{-1} and considering yield from zero-time to two days, the alga had a mean generation time of about 15 h.

Table 6.1   Influence of light flux on specific growth constant ($\underline{k'}$) and
mean generation time (G) of <u>G. pisum</u> D613 (inoculum eight
days old ; 32° C; continuous light; shaking)

light flux	d. wt				chl <u>a</u>			
	$\underline{k'}$		G(h)		$\underline{k'}$		G(h)	
(μmol $m^{-2}s^{-1}$)	0-2 d	1-2 d	0-2 d	1-2 d	0-2 d	1-2 d	0-2 d	1-2 d
25	0.219	0.289	33.0	25.0	0.241	0.228	30.0	31.7
50	0.412	0.438	17.5	16.5	0.42	0.509	17.2	14.2
100	0.462	0.403	15.6	18.0	0.486	0.505	14.9	14.3
150	0.428	0.42	16.9	17.2	0.499	0.526	14.5	13.7
200	0.504	0.573	14.3	12.6	0.507	0.625	14.2	11.6

Fig. 6.1   Influence of light flux on growth rate of
            G. pisum D613 (inoculum eight days old ;
            32° C ; continuous light ; shaking ; n = 4).

Caption area contains legend:

—✳— 200 μmol m^{-2}s^{-1}
—△— 150 " "
—○— 100 " "
—□— 50 " "
—✕— 25 " "
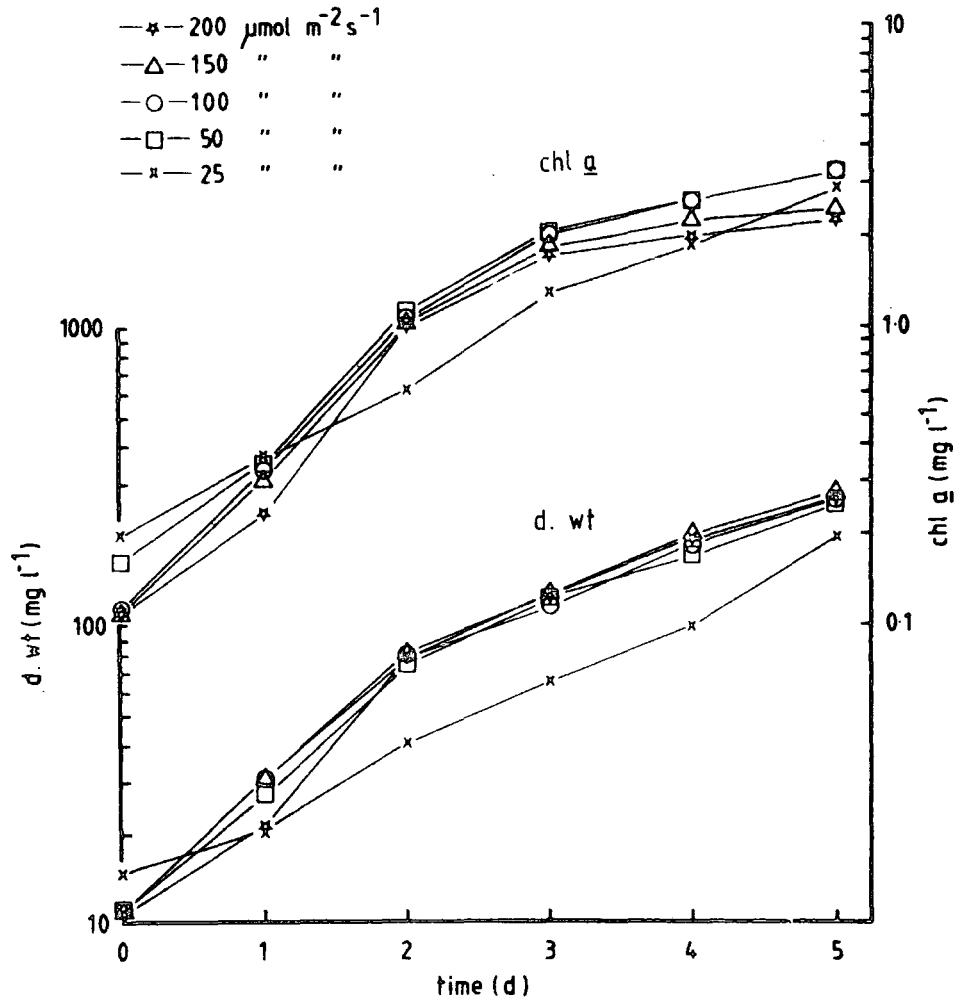
In another experiment, the alga was grown at about 2 μmol m^{-2} s^{-1} for three subcultures, under continuous light and shaking. The alga showed visually obvious growth by 30 days.

It was then decided to find out the compensation point of the alga by growing at low-light flux ($\leqslant$ 2 μmol m^{-2} s^{-1}) directly transferring from 60 μmol m^{-2} s^{-1}. The flasks surfaces were covered with aluminium foil to avoid any incident light other than that coming from underneath. The alga showed a compensation point at about 1.5 μmol m^{-2} s^{-1} (Fig.6.2).

6.3 Change in morphology and chlorophyll a content during growth in batch culture

A detailed study of the changes in morphology, chl a content and other growth characteristics has been made in batch culture. The experiment was carried out under continuous light at 105 μmol m^{-2} s^{-1}. (The slight shift from previous light 100 μmol m^{-2} s^{-1}, was due to differences in arrangement of illumination in the tank.) Six day old alga was used as the inoculum. The growth curve and other growth characteristics are shown in Fig 7.1 and Tables 6.2 and 6.3. (ARA was also measured : Section 7.2.)

The ratio, chl a : d. wt varied according to the growth phases (Fig. 7.1). During "exponential" phase (zero-time to two days) it ranged from 0.007 - 0.012 and the lowest ratio was obtained after about a day.

In order to study the developmental characteristics of the alga, colonies were examined on different days (Table 6.2) and other morphological characteristics were recorded (Table 6.3, see Section 2.2.4). As the alga is colonial, it was difficult to count individual filaments. A subjective visual estimate of different growth stages (see Section 5.2) of filaments was therefore made at low magnification (Section 2.2.3.1). After about a day, colonies appeared, consisting mostly of juvenile filaments and attached hormogonia (Table 6.2). After about five days, colonies were composed of mostly mature filaments. The culture turned light yellow-green after about a day and dark-brown within the next 24 h.

The highest heterocyst frequency (ca 6%: Fig. 7.1) was obtained during exponential growth and this was due to the abundance of juvenile and also developing filaments in the population (see Section 5.2.2). The heterocyst frequency declined thereafter due to the increased number of vegetative cells per filament still combined with only one heterocyst (Section 5.3.1).

Fig. 6.2   Compensation point of <u>G. pisum</u> D613 in
                standing condition and continuous light
                flux (growth measured after 30 days ; light
                from underneath ; n = 3).
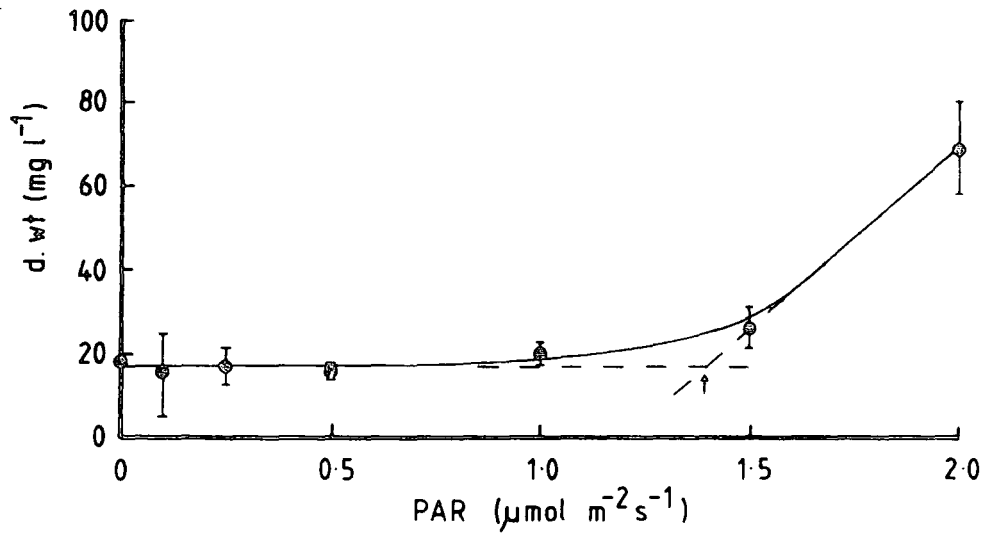
Table 6.2   Developmental characteristics and changes in culture colour
            during growth of G. pisum D613

culture age (d)	developmental characteristics	culture colour
0	colonies composed of long mature filaments; juvenile filaments rare	yellow-green
1	colonies long, cylindrical and rope-like, consisting ^{mostly} of juvenile filaments and completely surrounded by colonial mucilage,i.e without protruding filaments tips ; hormogonia also attached to the rope	light yellow-green
2	part of a colony with mature and developing filaments with tips protruding out of the mucilage giving radiating appearance, while in the rest, juvenile filaments embedded within colonial mucilage and have attached hormogonia as in day one	dark-brown
3	colony with much longer rope, about one half of which composed of radiating mature filaments, while the other half composed of mostly developing filaments, with juvenile filaments found only in the extreme tip of the rope	dark-brown
4	most of colony composed of mature and developing filaments with other stages of filaments at the extreme tip of the rope	dark-brown
5	colonies composed of mostly mature filaments while developing filaments and other stages of filaments rare	greenish
6	colonies as in five day old culture but with much longer filaments	yellow-green

The occurrence of cyanophycin and polyphosphate granules at different stages of filament development is shown in Table 6.3. Hormogonia always had abundant cyanophycin granules but mostly lacked polyphosphate granules (checked after staining: Section 2.2.4.2). On the other hand juvenile filaments were without visible cyanophycin granules, but with abundant polyphosphate granules. Mature filaments in 4 to 6 day old cultures always had both granules.

Table 6.3   The occurrence of cyanophycin and polyphosphate granules at different stages of filament growth of G. pisum D613 (studied during growth curve for 6 days)

developmental stages	cyanophycin	polyphosphate
1. hormogonia	always present	if present, very small
2. juvenile filaments	absent	abundant, gradually decreasing distally
3. developing filaments	rarely present at stationary phase	abundant, gradually decreasing distally
4. mature filaments	always present, few at exponential phase but abundant at stationary phase	abundant, gradually decreasing distally with few or no granules in the hormogonial segments

6.4   Summary

Light saturation for growth occurred at about 100 μmol m^{-2} s^{-1}, at 32° C with a mean generation time of about 15 h. The exponential phase was about two days. The lowest chl a : d. wt ratio occurred after about one day and highest after about three days. Highest heterocyst frequency was about 6% and occurred during exponential phase of growth. Juvenile filaments were abundant after about a day of growth. These were always without visible cyanophycin granules but with abundant polyphosphate granules.

7 ARA (NITROGENASE ACTIVITY) BY Gloeotrichia pisum D613 IN THE LABORATORY

7.1 Introduction

The literature (Section 1.6) indicates that ARA can be related to environmental factors such as light flux. Assays with G. pisum at Sonargaon indicated (Sections 4.2, 4.4) that usually the higher light flux, the higher rate of activity, although there was still considerable ARA in the dark. Laboratory studies on ARA were therefore planned with the following objectives :

(i)   to quantify changes in ARA and identify possible reasons for such changes during batch culture;

(ii)   to quantify percentage changes and speed of response due to change in light flux;

(iii)   to see how pretreatment in the dark influences ARA after transfer to light;

(iv)   to quantify ARA in the dark

7.2 Growth and ARA

ARA was measured parallel to the study of growth characteristics at 105 μmol m^{-2} s^{-1} (Section 6.3) in batch culture. The changes in ARA and their relation to growth characteristics are shown in Fig. 7.1 and Table C3. Maximum ARA occurred after about one day of exponential growth. This was followed by a decrease in ARA (per unit chl a), but with activity still detectable after 20 days. Specific activity and total nmol C_2H_4 l^{-1} min^{-1} declined long before the exhaustion of P from the medium (Table C3).

7.3 Influence of light flux on ARA

The alga was grown at 105 μmol m^{-2} s^{-1} for about 36 h and then incubated at lower values of light flux (Section 2.2.5.6). The wrapping with neutral density filters, C_2H_2 injection etc. required about 45 s. The influence of downshift in light flux on ARA, is shown in Tables 7.1 and 7.2. A transfer to lower values of light flux and dark brought about a marked reduction in ARA during the first hour of incubation. A reduction by about 50% of the initial light brought about a reduction in ARA by about 30%.

Fig. 7.1   Growth characteristics and changes in ARA
            of <u>G. pisum</u> D613 in batch culture (inoculum
            six days old; 32° C; 105 µmol m^{-2} s^{-1}
            continuous light flux; continuous shaking;
            n = 4).
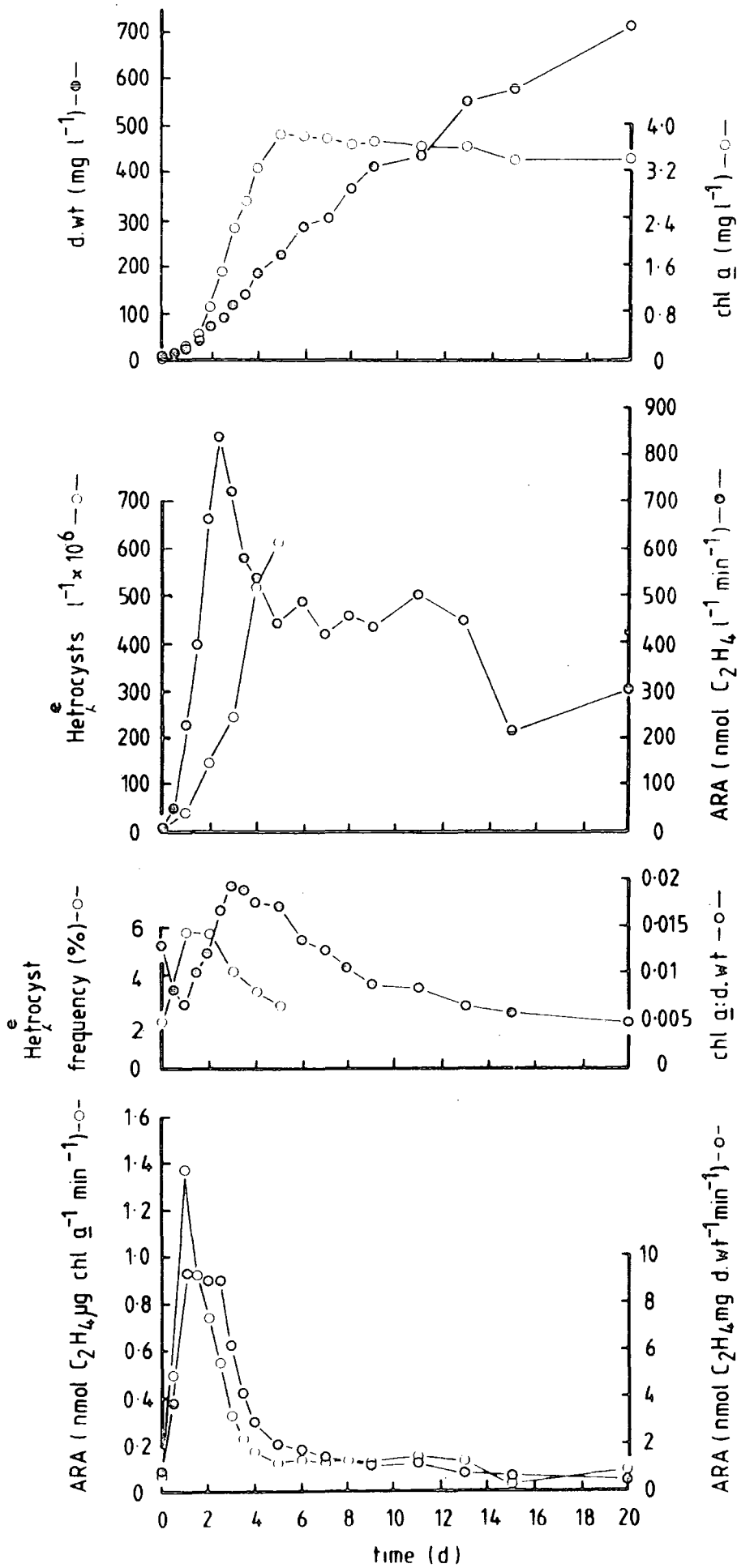
Table 7.1 Influences of downshift in light flux for one hour on ARA
of G. pisum D613 (based on an independent experiment with one
replicate; inoculum three days old; control of 105 μmol m^{-2}
s^{-1})

light flux (μmol m^{-2} s^{-1})	% reduction of light	n	% of control ARA range	$\bar{x}$
80	20	3	86–93	90
50	50	3	66–73	69
25	75	3	37–39	38
12.5	87.5	3	22–28	25
6.3	93.7	3	14–17	16
dark	100	3	11–16	13

In order to study the speed of response to the downshift in light, alga
grown at 105 μmol m^{-2} s^{-1} for 36 h, was incubated at different values of
light flux for about six hours and ARA was measured. The response in
ARA to all reduced lights was fast. The values apparently level off
between one and one and half hours after transfer (Table 7.2).

In order to study the speed of response to an upshift in light
flux, the alga was grown initially at 12.5 μmol m^{-2} s^{-1} for five days
and then acclimatized at 6.3 μmol m^{-2} s^{-1} for one day (considered as
the control). Growth (as dry weight) at this stage was similar to that
obtained after 36 h of growth at 105 μmol m^{-2} s^{-1} in the previous
experiment. When the alga was transferred to higher light, response in
ARA was also fast and appeared to level off between 1.5 and 2 h (Table
7.3).

7.4   Influence of pretreatment in dark on ARA after transfer to light

The alga was grown at 105 μmol m^{-2} s^{-1} for 36 h and then
incubated in the dark for 12, 24 and 48 h. During dark incubation
flasks were wrapped first with neutral density filters according to the
light flux to be used in the subsequent light phase. This ensured the
alga would not be exposed to higher light during C_2H_2 gassing,
equilibration etc. Flasks were finally wrapped with aluminium foil and
black polythene. After dark incubation, alga was exposed to different
light flux and ARA was measured. The influence of pretreatment in the
dark on ARA in the subsequent light phase is shown in Fig. 7.2. It is
clear that the shorter the dark period, the quicker the response of ARA.
This occurred at all values of light flux. Specific activity dropped
markedly within 10 h of light treatment, after reaching maximum between
four and six hours. Dark incubation of 12 h resulted in a decrease in

Table 7.2 Influence of downshift in light flux for periods one to six hours on ARA of G. pisum D613 (initial growth: 105 μmol m^{-2} s^{-1} for 36 h ; n = 1)

light flux (μmol m^{-2} s^{-1})	ARA (nmol C_2H_4 mg d. wt^{-1} min^{-1}) after a period of incubation in different light flux (% of control)					
	1.0 h	1.5 h	2.0 h	2.5 h	3.0 h	6.0 h
105	13.6 (100)	13.7 (100)	12.3 (100)	13.3 (100)	14.2 (100)	11.9 (100)
80	12.6 (93)	12.3 (90)	11.3 (92)	12.6 (95)	14.5 (102)	12.7 (107)
50	9.9 (73)	9.6 (70)	10.0 (81)	9.9 (70)	11.1 (78)	12.9 (108)
25	5.0 (37)	5.2 (38)	4.8 (39)	6.1 (46)	5.0 (35)	8.3 (70)
12.5	3.2 (24)	2.9 (21)	2.9 (24)	3.1 (23)	2.9 (20)	5.0 (42)
6.3	2.3 (17)	2.2 (16)	2.1 (17)	2.3 (17)	2.7 (19)	3.0 (25)
dark	1.8 (13)	1.7 (12)	1.7 (14)	1.6 (12)	2.0 (14)	1.5 (13)

Table 7.3 Influence of upshift in light flux on ARA of G. pisum D613
(control at 6.3 μmol m^{-2} s^{-1}; n = 1)

light flux (μmol m^{-2} s^{-1})	ARA (nmol C_2H_4 mg d. wt^{-1} min^{-1}) after a period of treatment in different light flux					
	1.0 h	1.5 h	2.0 h	2.5 h	3.0 h	6.0 h
105	9.2	11.5	12.7	11.0	10.6	11.5
80	8.5	10.5	12.7	11.5	10.3	12.6
50	7.4	9.8	10.8	10.2	10.2	10.5
25	7.2	8.2	8.3	9.0	9.7	7.7
12.5	5.1	5.5	7.5	7.0	6.8	4.4
6.3	3.1	3.7	2.6	2.7	2.9	2.2
dark	<detection*	<detection*	<detection*	<detection*	<detection*	<detection*

* Value was not more than twice the mean value of blank.

Fig. 7.2   Influence of pretreatment in dark for 12,
            24 and 48 h on ARA, upon reillumination
            by G. pisum D613 (each point is the mid-time
            of incubation;   n = 1).

A
12h dark pretreatment

B
24h dark pretreatment

C
48h dark pretreatment

-○- 105 μmol m^{-2}s^{-1}
-△- 80 "
-□- 50 "
-×- 25 "
-◇- 12·5 "
-⊕- 6·3 "
-●- dark

ARA (μmol C$_2$H$_4$ mg d.wt^{-1} min^{-1})

time (h)
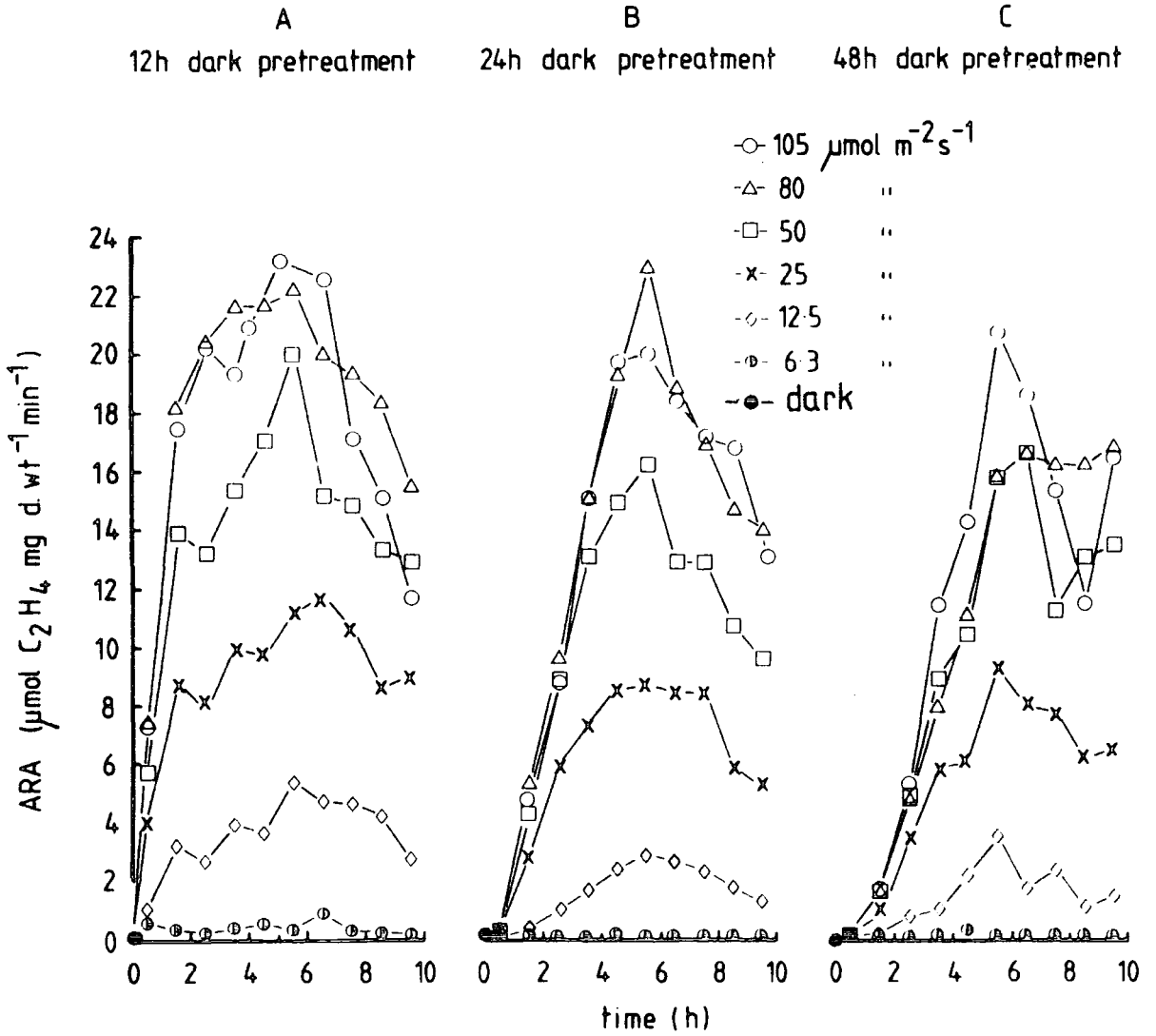
dry weight by about 15% and it remained unchanged after 24 and 48 h (Table 7.4). However, a pretreatment in the dark, resulted in higher ARA than was obtained during the growth under continuous light (cf. Fig. 7.1). An experiment was therefore planned to see how dark treatment had influenced higher ARA in the subsequent light phase (Fig. 7.3, Table C4). As in the earlier experiment, alga was grown at 105 $\mu mol\ m^{-2}\ s^{-1}$ for 36 h, after which flasks were divided into two series, continuous light and dark. After 12 h of dark treatment the alga was exposed to the initial light condition and ARA was measured at regular intervals to record maximum ARA. The ARA was also measured during growth under continuous light to record maximum ARA and compare with the ARA of the dark treated alga. Transfer of dark treated alga to the initial light led to much higher ARA than was found under continuous light. Dark treatment caused ARA per unit d. wt to increase by about 100% in the subsequent light phase compared to continuous light (Fig. 7.3)

Table 7.4 Changes in dry weight due to dark incubation for varying periods in G. pisum D613 (n = 5)

	time (h)			
	zero-time	12 h	24 h	48 h
d. wt (mg l^{-1})	41.4 ± 1.8	35.9 ± 1.7	36.2 ± 0.4	34.6 ± 1.4

7.5 ARA in the dark

In the previous sections (7.3, 7.4) it has been shown that within one hour of transfer to dark, ARA declined sharply to about 13% of the light control. The decline afterwards was slow (Table 7.2) with detectable activity after about 12 h (Table D4). In the present experiment the alga was treated in dark for 48 h to see whether dark ARA still occurred. The alga was grown at 105 $\mu mol\ m^{-2}\ s^{-1}$ for 36 h and then dark incubated. At the end of the experiment, the culture was found to be axenic. Detectable ARA was found after 24 h of dark treatment (Table 7.5).

Table 7.5 ARA (nmol C_2H_4 mg d. wt^{-1} min^{-1}) by G. pisum D613 in the dark

light ARA	dark incubation (mid-time) ARA							
zero-time	0.5 h	1.5 h	2.5 h	5.5 h	8.5 h	11.5 h	23.5 h	47.5 h
14.27	2.28	2.16	2.20	2.39	2.48	0.29	0.30	*
±0.89	±0.17	±0.28	±0.26	±0.09	±0.06	±0.17	±0.08	<detection
n = 5	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3

* Value is not significantly different from the blank

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Fig. 7.3   Influence of pretreatment in dark for 12 h
            on ARA, upon reillumination by G. pisum D613
            (each point is the mid-time of incubation;
            n = 4; -o- continuous light, -●- dark,
            -◉- reillumination)
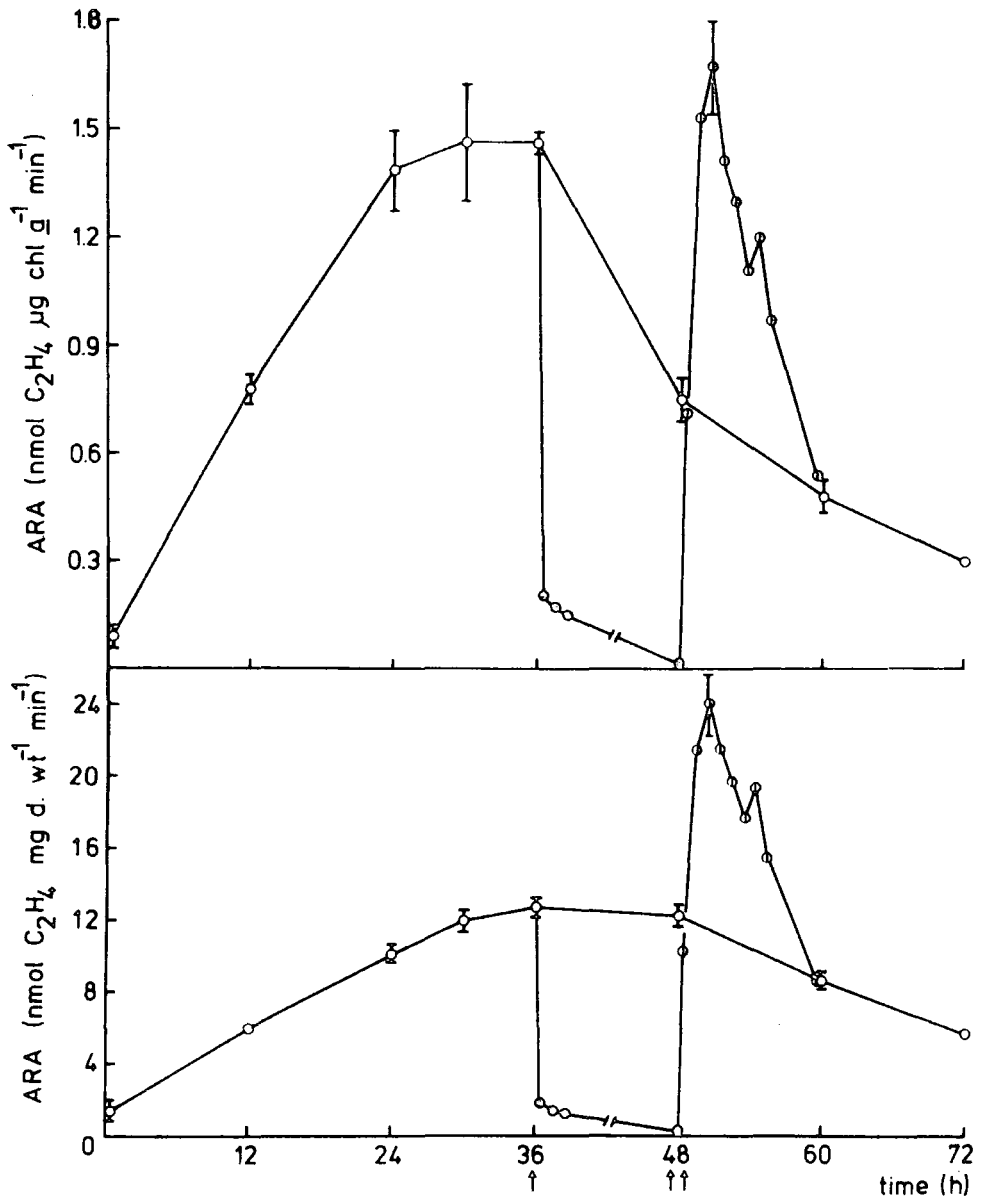
7.6 Summary

Maximum ARA occurred after about one day of exponential growth. A reduction of initial full light ($105 \ \mu mol \ m^{-2} \ s^{-1}$) by about 50% for an hour brought about a decrease in ARA by about 30%. The response of ARA to change in light (downshift or upshift) was fast.

Dark pretreatment led to ARA per unit d. wt being increased by about 100% in the subsequent light phase. The shorter the dark period, the quicker the response of ARA. The ARA was detectable after 24 h of dark treatment.

8 DISCUSSION

8.1 Introduction

In Bangladesh, deepwater rice yield is relatively stable year after year with little or no addition of fertilizers. An important part of this fertility has been attributed to the presence of blue-green algae (Sections 1.2.3, 1.3). Of the various blue-green algae, Gloeotrichia pisum is one of the most widespread, growing as an epiphyte on the deepwater rice. The alga occurred abundantly at Sonargaon in 1983, when intensive field studies were made. G. pisum was therefore considered for the field studies, whilst for laboratory studies, G. pisum D613, an isolate from the same locality (Section 2.2.2.1) was used.

An effort has been made in all parts of this work to coordinate field and laboratory studies. The alga showed obvious similarities and dissimilarities in morphology and nitrogenase activity in the field and the laboratory, and the following discussion deals with some of these features.

8.2 Morphology and growth of Gloeotrichia pisum in the field and the laboratory

Studies of G. pisum from the field showed that akinetes and hairs are widespread and that gas vacuoles were also commonly found in some filaments of some colonies. In batch culture these structures were not observed under the standard experimental conditions (Table 8.1). These structures were not observed even at the beginning of isolation of this alga from enriched culture (Section 2.2.2.2) indicating that an inducible factor or factors (if any) released by the associated organisms, such as bacteria, are probably not responsible for their differentiation.

In the field, hairs were found in filaments during the first half of flooding (Figs 3.1G, 3.2A-F). As mentioned earlier, hairs were not observed (Section 5.2.2) in the laboratory. Hairs in several tapered blue-green algae have been shown to be formed in nutrient deficient media (Sinclair & Whitton 1977). In the present study the alga did not show any sign of hair formation in media lacking P or Fe or slightly modified medium (Section 5.5). When the alga was grown under different combinations of light quantity and quality (Table 5.1), no effect was observed.

In the field, gas vacuoles, when present, were found in cells below the hair (Fig. 3.1G), but were not observed in the laboratory. Whilst various tests were done to induce hair or akinete formation, hormogonia from young cultures were examined for the presence or absence of gas vacuoles (Section 5.4). In addition the alga was grown under low values of light flux since it is well known that this enhances gas vacuole formation in many blue-green algae (Walsby 1978, Walsby & Booker 1980). However, gas vacuoles were not found under any treatment. In standing conditions when light was provided from above, hormogonia came up to the surface and ultimately formed floating mats (the inoculum remained at the bottom of the flask). To come up to the surface, hormogonia either need gas vacuoles for buoyancy (Walsby & Booker 1980) or a semi-solid or solid surface for gliding movements (Section 5.2.1). It may be that hormogonia contain isolated gas vesicles sufficient to float in water (Klebahn 1922) and these were undetectable with the light microscope (Whitton 1972).

Table 8.1 A comparison of field and laboratory morphologies of
    Gloeotrichia pisum

field	laboratory
1. colonies hemispherical or spherical with radiating filaments; attached	colonies not organized as in the field; filaments radiating; colonies attached or floating
2. trichomes tapered, consisting of a basal heterocyst, akinete, row of vegetative cells in groups and may terminate in a hair	trichomes tapered consisting of a basal heterocyst and row of vegetative cells in groups but without akinetes and hairs
3. trichome's growth diffuse	as in the field
4. gas vacuoles present in cells below hair	gas vacuoles not found
5. akinetes very long and appear to be composed of more than one cell	akinetes absent but a group of cells next to the heterocyst showed much elongation in old cultures
6. two heterocysts at the base of a trichome, or heterocysts alternating with akinetes	in old cultures, commonly two to three basal heterocysts were observed; heterocysts at both ends observed occasionally

7. hormogonia liberated successively   as in the field

   leaving empty sheath at the apex

   of filaments

8. colonies appeared about 1 mm diameter   colonies appeared maximum size

   after about 8 days growth   (0.5 mm diameter) after about

                                       4 days growth in batch culture

                                         under continuous light

In the field, the alga produced very long akinetes and appeared to be composed of more than one cell. These were not produced in the laboratory under standard conditions (though - see below). Variables tested in the laboratory were nutrient deficiency and various combinations of light quantity and quality. Only in a slightly modified medium were enlarged cells with slight resemblance to early stages in akinete formation observed in very few filaments after 50 days under continuous light (Section 5.5, Fig. 5.8). Addition of distilled water to this old culture did not induce further development. Akinetes were not observed subsequent to subculture in a medium lacking P or Fe. It seems unlikely therefore that akinete formation in this alga is a response to limitation of either nutrient or light flux. Although no akinetes were formed in the laboratory it is worth noting that the cell next to the heterocyst divided during trichome growth just as it did in field materials (Figs 3.2B-D cf. 5.2G, 5.3E-G, 5.4B-D).

All _Gloeotrichia_ strains that have been isolated from Bangladesh are without hairs, gas vacuoles or akinetes. However, at least one _Gloeotrichia_ isolate in culture (_Gloeotrichia_ D636 isolated from a rice field in Iraq) shows all three features (A. Al-Mousawi: pers.comm.) suggesting the possibility that there is some morphogenetic relationship between these features.

In the field the alga forms hemispherical or spherical colonies, with all filaments distinctly radiating and the heterocysts towards the centre of colonies (Fig. 3.1A-F). Colonies can increase in size and in large colonies zonation of filaments was observed. In the laboratory colonies with radiating filaments were formed but mature colonies were not as organized as in the field, i.e. rope-like structures were formed underneath the radiating part of colonies (Fig. 5.7H-J). It is not at all clear why hormogonia always got attached at the centre and underneath the developing colony. In the beginning of colonization

hormogonia appeared to aggregate by chance and become entangled with the secreted mucilage of their own (Fig. 5.7C-E). After attachment and formation of juvenile filaments, rapid divisions occur usually terminally (Figs 5.1D-F, 5.2C-E, 5.3C-G). The basal end is surrounded by mucilage (Fig. 5.7K). However, mucilage at the basal end and diffuse growth starting from the terminal end results in the protrusion of filaments giving a radiating appearance to the whole aggregate (Fig. 5.7F-H).

In the field, the alga grew ("first flush") abundantly on rice tillers within 15 days of flooding and this coincides with the observation made by Finke & Seeley (1978) for epiphytic Gloeotrichia in lakes near Ithaca, U.S.A. The "second flush" of growth was observed after about 45 days of flooding, i.e. at about the time of the first flood peak. In this case numerous small colonies appeared on the newly submerged rice tillers after three to four days and after about eight days colonies attained about 1 mm diameter (Fig. 3.1B cf. 3.1A). The longer time (15 days) needed for the "first flush" of growth (Section 3.2.1) might be due to the longer time needed to build up inocula from whatever source the Gloeotrichia comes (old Brahmaputra or Meghna river flood, tanks, bils, overwintered akinetes or filaments etc.). In the laboratory on the other hand the alga appeared as small beads after about two days and distinct colonies (about 0.5 mm diameter) after about four days (32° C, 105 μmol m^{-2} s^{-1} continuous light). The slightly longer period required for the "second flush" in the field might be due to the diel cycle of light and dark or lower nutrient level (e.g. P).

8.3 ARA (nitrogenase activity) by Gloetrichia pisum in the field and in the laboratory

The influence of changes in light flux on nitrogenase activity by G. pisum was studied in the field and in the laboratory. The alga responded very similarly in some respects under both conditions (Table 8.2). It showed a rapid response to the changes in light flux and was affected similarly due to a reduction of light flux (cf. Tables 4.1 & 7.1). Transfer to the dark for 1.0 h reduced nitrogenase activity by 83 to 87% (field and laboratory respectively) when compared with parallel incubation in the light. This percentage reduction in activity is consistent with some algae reported in the literature (Section 1.3) though Finke and Seeley (1978) observed 50 to 66% reduction in activity

within 1-2 h dark treatment. Lex & Stewart (1973) demonstrated that photosynthesis promotes N_2-fixation by producing carbon compounds, which act as reductant for nitrogenase. Quick responses and decrease of nitrogenase activity due to a shift down in light flux suggest that N_2-fixation in the light is dependent upon reductant produced during recent photosynthesis (Peterson et al. 1977). Light flux therefore has a tremendous potential influence on the nitrogen input in the deepwater rice field ecosystem, where light flux varies markedly depending on parts of a field, position in the water column (Fig. 4.1), time of day and cloud cover (Whitton 1984). Thus the N_2-fixation by G. pisum in rice fields during the monsoon period is in constant flux even over a short period of time (Stewart et al. 1967).

The other feature of nitrogenase activity common to field and laboratory was the behaviour of the alga in the dark. Considerable nitrogenase activity was observed during the dark period of about 12 h. This is quite similar to the behaviour of Gloeotrichia on Myriophyllum in ponds near Ithaca, U.S.A. (Finke & Seeley 1978), except that a brief night-time maximum did not occur in the present study. Both the rate and duration of enzyme activity in the dark are affected by the rate of photosynthesis and carbon assimilation during the light period (Lex & Stewart 1973, Fay 1976). The night time ARA has also been attributed to the O_2-scavenging bacteria (Finke & Seeley 1978) or to the utilization of suitable organic substances in nature (Fay 1965, 1976). Dark nitrogenase activity by bacteria-free G. pisum (Table 7.5) indicates that these two factors may not be predominant, but rather a pool of carbon compounds.

Table 8.2 A comparison of field and laboratory ARA (nitrogenase activity) of Gloeotrichia pisum

field	laboratory 1.
1. reduction of 1850 μmol m^{-2} s^{-1} light flux by about 40% for about 1.5 h resulted in about 30% reduction in ARA	reduction of 105 μmol m^{-2} s^{-1} light flux by about 50% for about 1.0 h resulted in about 30% reduction in ARA
2. transfer to the dark during day-time (1000 to 1230 h) resulted in 83% reduction in ARA within 1.0 h	transfer to the dark from 105 μmol m^{-2} s^{-1} light flux resulted 87% reduction in ARA within 1.0 h

3. considerable ARA occurred during night-time (total period ca 11.0 h)

detectable ARA occurred even after 24 h in the dark

4. maximum ARA of about 0.352 nmol C_2H_4 µg chl $\underline{a}^{-1}$ min^{-1} at a light flux of about 1825 µmol m^{-2} s^{-1}

maximum ARA of about 1.4 nmol C_2H_4 µg chl $\underline{a}^{-1}$ min^{-1} at a light flux of about 105 µmol m^{-2} s^{-1}

5. not studied

juvenile filaments appeared to be particularly efficient as nitrogen fixers

In the field maximum nitrogenase activity of 0.352 nmol C_2H_4 µg chl $\underline{a}^{-1}$ min^{-1} was observed after about 45 days of flooding by the alga being scraped from the rice tillers at a light flux of about 1825 µmol m^{-2} s^{-1}. (A comparison of nitrogenase activity of young and old colonies was not studied.) In batch culture, on the other hand, the alga showed a maximum activity of about 1.4 nmol C_2H_4 µg chl $\underline{a}^{-1}$ min^{-1} after about a day's growth and a value equivalent to the field, after about three days under continuous light flux of 105 µmol m^{-2} s^{-1}. The low ARA in the field might be due to the alga being scraped from the rice tillers (Table 4.4) and thus perhaps damaging colonies (Finke & Seeley 1978) and microhabitat and due to mixed population of young and old colonies (see below). A preliminary experiment (Section 4.3) indicated that getting colonies by scraping resulted in about 50% reduction in ARA (Table 4.4, cf. treatment no. 1 with combined values of treatments nos. 2 & 3). Accordingly corrected ARA value in the field after about 45 days of flooding is about 0.7 nmol C_2H_4 µg chl $\underline{a}^{-1}$ min^{-1}.

From the laboratory study of young and mature colonies (from the same culture) of G. echinulata, Chang & Blauw (1980) reported much higher activity in young colonies than the mature ones. They attributed the lower activity of mature colonies to the transport of metabolic products being blocked between the vegetative cell and the heterocyst by the akinete. Although this cannot happen in G. pisum D613 because of the absence of an akinete, the activity (per unit chl $\underline{a}$) decreased by about 50% at the end of the exponential phase of growth. In the present study differences in activity were related to the developmental stage of the alga and other growth characteristics (Tables 6.2, 6.3, Fig. 7.1). Maximum ARA occurred after about one day's growth in batch culture. During this period, juvenile filaments were abundant, cyanophycin granules lacking and cultures had the lowest chl $\underline{a}$: d. wt ratio. (It

appeared a light yellow-green.) The last two features indicate nitrogen deficiency (Allen & Smith 1969, Fay 1983). Rapid division of cells occurs in filaments only after heterocyst differentiation (Section 5.2.1, Figs 5.1C, 5.2C, 5.3B) and when the normal level of pigmentation is restored (Allen & Smith 1969). This suggests that nitrogen fixation occurred at a high rate at this stage to meet the nitrogen requirement for cell synthesis (Jewell & Kulasooriya 1970). As the high fixation rate continued, the alga gradually reached a nitrogen-sufficient state. However, total ARA and ARA per unit d. wt started to decline after three days (Fig. 7.1), when a high chl $\underline{a}$: d. wt ratio and abundant developing and mature filaments occurred (Table 6.2). During this period P was not exhausted from the medium (Table C3), indicating that P deficiency was not involved. It appears from the above discussion that the juvenile filament (Figs 5.1B, 5.2B) is the most active nitrogen fixer during the life cycle of the alga. It will be important to study nitrogenase activity in synchronized cultures to confirm this.

Diel studies of nitrogenase activity in the field showed maximum rates at mid-day (Fig. 4.2). For planktonic blue-green algae in lakes, the diel variation of activity with maximum before noon or in the early afternoon has been attributed partly to diel changes in light flux and partly to diel migration of algae (Vanderhoef et al. 1975, Levine & Lewis 1984). However, for epiphytic blue-green algae, such as $\underline{G}$. $\underline{pisum}$ and considering rapid response of ARA due to change in light flux (Section 4.2), the diel variation is probably due mainly to the changes in light flux (Fig. 4.2). The data in Fig. 4.2 show that during the hours before noon, about 51% of the day's total activity occurred in the presence of 49% of the day's total light flux, producing almost a symmetric curve (Alimagno & Yoshida 1977). The short term drop during early afternoon may be due to the high O_2 concentration or photoinhibition (Goering & Neess 1964, Stewart 1971, Vanderhoef et al. 1975, Peterson et al. 1977, Roger & Reynaud 1979).

During the first half of August in 1983, $\underline{G}$. $\underline{pisum}$ was the only visible blue-green alga growing on deepwater rice at Sonargaon. Its contribution to N_2-fixation was estimated from a study on a whole tiller (on 10.8.83). Incubations of sections of the tiller with attached $\underline{G}$. $\underline{pisum}$ at different depths (from where sections originated) showed total ARA of 77.44 nmol C_2H_4 tiller^{-1} min^{-1}. ARA was quantitatively important

from about -76 to -155 cm (Table 4.3, Fig. 4.1). The greater activity at these depths was due to the abundance of the alga (Section 3.2.1). However, considering effects of scraping (Table 4.4) and changing light flux on ARA (Section 4.2) and variation of light flux down the water column (Fig. 4.1, see Whitton 1984), the above value represents a true situation in the field. Estimation of nitrogen contributed over a season based on a single determination becomes difficult because of obvious changes in light flux between days, hours of a day, changes in biomass, spatial distribution of the alga etc. (Dugdale & Dugdale 1962, Vanderhoef et al. 1975, Levine & Lewis 1984). For all the above reasons, estimations during the flood season for the whole chak were made based on visual observations of the abundance of colonies in fields, counting tiller density, etc. plus some approximations throughout the flood period. However, in the calculation, the following approximations and estimates have been included :

1. Number of days in the flood period :

   23 June to 9 November = 140 days

2. Approximate distribution of ARA during flood period :

   Days 1-10 = negligible

   Days 11-75 = 20% value obtained from experiment on 10.8.83 (Section 4.3) because of differences of biomass between fields and also parts of a field.

   Days 76-140 = 20% value obtained for days 11-75, because of rapid decline of biomass, differences of light flux between days, shading by increased number of tillers and kneeing of tillers, etc.

3. Estimate percentage of total ARA at 1030 h by the whole tiller on 24 h basis, based on ARA during diel variation (Section 4.4): this was found to be 12.4%.

4. Estimate ARA ha^{-1} $season^{-1}$ considering 130 tillers m^{-2} and approximate distribution of ARA (see No.2) throughout the flood period.

5. Estimate ARA by G. pisum alone by deducting 9% of the total ARA as a fixation by rice tiller without G. pisum (Section 4.3).

6. Conversion of ARA to N_2 fixed, using 5:1 $C_2H_2:N_2$ ratio (Whitton 1984).

Fixation of 3.87 kg N ha^{-1} $season^{-1}$ by G. pisum seems to be a

reasonable estimate for Sonargaon in 1983. If the density of colonies in the study field (Fig. 3.1A) were similar throughout the <u>chak</u>, including changes in abundance during the season (Section 3.2.1), then the contribution of nitrogen can be estimated as about 19.4 kg ha^{-1} season^{-1}. This value fits well with 10-20 kg N ha^{-1} crop^{-1} reported by Kulasooriya <u>et</u> <u>al</u>. (1981a) for a study of deepwater rice at IRRI, Philippines.

Some comparison can be made between values of ARA found in the present study and values reported in the literature for <u>Gloeotrichia</u> and other blue-green algae. <u>In</u> <u>vivo</u> nitrogenase activity (ARA) in batch cultures, is in a typical range of 1-10 nmol C_2H_4 mg protein^{-1} min^{-1} (Stewart 1973). Assuming that a typical blue-green algal cell during exponential growth phase consists of 50% of d. wt as protein (Collyer & Fogg 1955) and 1% as chl <u>a</u> (Fay 1969), then the above value corresponds to 0.05 -0.5 nmol C_2H_4 µg chl <u>a</u>$^{-1}$ min^{-1}. Accordingly the maximum rate obtained in the present study is higher. Several strains of blue-green algae (other than <u>Gloeotrichia</u>) isolated from rice fields show high rates under optimal growth conditions, ranging from 0.45 to 1.0 nmol C_2H_4 µg chl <u>a</u>$^{-1}$ min^{-1} (= 9-20 nmol C_2H_4 mg protein^{-1} min^{-1} : Antarikanonda & Lorenzen 1982, Chen 1983). However in the present study the <u>G. pisum</u> showed many times higher ARA (about 13 nmol C_2H_4 mg d. wt^{-1} min^{-1} : Fig.7.1, Table 7.2) than those reported for two day old unialgal culture of <u>Gloeotrichia</u> (0.81 nmol C_2H_4 mg d. wt^{-1} min^{-1} at 4000 lux light flux : Finke & Seeley 1978) or the axenic culture of <u>G. echinulata</u> (0.61 nmol C_2H_4 mg d. wt^{-1} min^{-1} : Chang & Blauw 1980).

One of the most interesting properties of <u>G. pisum</u> D613 nitrogenase activity (ARA) in the laboratory is the higher activity in the dark-grown alga when reilluminated, than the maximum activity ever found under continuous illumination (Fig. 7.3). Meyer <u>et</u> <u>al</u>. (1978) provided evidence of extremely active biosynthesis of enzyme upon reillumination of <u>Rhodopseudomonas</u> <u>capsulata</u>, a purple nonsulphur bacterium (photosynthetic and facultative anaerobes). In a medium free of combined nitrogen and carbon, they observed that nitrogenase synthesis proceeded at a rate such that within 3-4 h, the activity was equal to that found in light grown cultures. They also observed consistently higher activity under intermittent illumination and the retention of the ability to synthesize nitrogenase over a longer period of time under discontinuous illumination. In <u>G. pisum</u> D613 it has been observed following

reillumination for 10-12 h that maximum nitrogenase activity occurs within 3-6 h, after which it declined to the level obtained under continuous illumination (Fig. 7.3). The longer the dark period, the slower the response to light. It has been mentioned earlier that in this alga considerable ARA occurred during 12 h dark incubation (Table 7.5), contrary to Rhodopseudomonas capsulata, where no activity was observed in the dark (Meyer et al. 1978). From all these facts it is difficult to predict whether the higher activity upon reillumination is due to activation of the existing enzymes or fresh biosynthesis (Meyer et al. 1978), or both, or due to O_2 scavenging by dark respiration (Spiller et al 1981). The lower value for maximum nitrogenase activity under continuous light might be due to the increase in O_2 concentration in the medium by photosynthesis (Spiller et al. 1981). This also raises the possibility that after reillumination the O_2 level gradually goes up and an inhibitory level is reached when nitrogenase activity starts declining and ultimately attains the level under continuous illumination (Fig. 7.3).

Intermittent illumination resembles the natural growth conditions; this perhaps maintains the nitrogenase activity over a longer period as has been observed in the present study. It has also been observed that the synthesis of chl a continues but d. wt decreased by about 15% within 12 h of dark treatment. This chl a synthesis in the dark is advantageous in allowing rapid transition to photosynthesis and N_2-fixation (Meyer et al. 1978).

SUMMARY

1.   In deepwater rice fields of Bangladesh, Gloeotrichia pisum is one
of the most common blue-green algae, occurring as an epiphyte on rice
tillers. Its occurrence varies from year to year, locality to locality
and field to field. In 1983 it was abundant on deepwater rice in some
fields at Sonargaon near Dhaka, where all field studies were carried
out. G. pisum D613, an isolate from the same locality, showed some
morphological similarities with G. pisum in the field.   Laboratory
                              and differences from
studies were therefore made on this strain.

2.   In the field, the alga was found to colonize the newly submerged
rice parts. During the flood period the alga showed obvious changes in
abundance. The pattern in 1983 was: rapid colonization within 15 days
of flooding with "second flush" of growth after about 50 days, abundant
for about two months, rapid decline, with a few colonies remaining till
the end of the flood period.

3.   Studies on the morphology of both field and laboratory
Gloeotrichia revealed some features in common:

     (i)   trichomes composed of basal heterocysts and rows of
             vegetative cells in groups;

     (ii)   growth of trichomes was not obviously localized, i.e.
             no meristematic zone occurred ;

     (iii)   the cell next to the heterocyst divides ;

     (iv)   due to the release of hormogonia, a section of empty
             sheath is left terminally.

4.   Some of the obvious features such as akinetes, hairs and gas
vacuoles which were found in the field were not found in the laboratory.
In the field the long akinete appeared to be formed by the participation
of more than one cell next to the heterocyst.

5.   During colonization in batch culture, the initial aggregation of
hormogonia appeared to occur by chance and then they became entangled
within the mucilage. The radiating nature appeared to develop due to
the production of mucilage at the basal end and diffuse growth usually
at the terminal end.

6.   Four stages of filament development were recognized in the laboratory: hormogonia, juvenile filament, developing filament, mature filament.

Detailed developmental studies of trichomes in the laboratory showed the following features:

(i)   each group of vegetative cells of a trichome was formed by repeated divisions of a single cell of the parent hormogonium;

(ii) these groups were released from the tip of trichomes as hormogonia;

(iii) at the time of release of a hormogonium the basal cell of a group divided once and then the lower daughter cell was sacrificed for the release of the rest of the cells as a hormogonium;

(iv) the basal (youngest) cell of the hormogonium differentiated into a heterocyst.

7.   In batch culture, light saturation for growth (32°C) under continuous light occurred at about 100 μmol m^{-2} s^{-1}; under these conditions the mean generation time was about 15 h. Juvenile filaments were abundant after about one day's growth, when the highest heterocyst frequency was also observed. These filaments had no visible cyanophycin granules, but abundant polyphosphate granules. The lowest chl $\underline{a}$: d. wt ratio was also observed after about a day of growth.

8.   In the field nitrogenase activity measured by ARA showed rapid responses to changes in light flux. A downshift of light flux by 40% (full light 1850 μmol m^{-2} s^{-1}) for 1.5 h reduced activity by about 30%. Transfer to the dark during day time for 1 h led to 83% reduction in activity.

9.   Diel variation in nitrogenase activity in the field was related to light, usually the higher light flux, the higher activity. About 4% of the daily activity occurred at night.

10. After 35 to 45 days of flooding, nitrogenase activity by the alga being scraped from the rice tillers ranged from 0.234 to 0.352 nmol C_2H_4 μg chl $\underline{a}^{-1}$ min^{-1} at around mid-day (light flux from 1825 to 1890 μmol m^{-2} s^{-1}) and at a depth of -4 cm.

11. The alga contributed approximately 4 kg N ha^{-1} season^{-1} (140 days) in the deepwater rice field ecosystem at Sonargaon, Bangladesh in 1983 (extrapolating ARA of 37468 nmol C_2H_4 tiller^{-1} day^{-1} and considering 130 tillers m^{-2} and some approximations, based on a single determination down the water column at 1030 h after about 50 days of flooding).

12. In batch culture, the alga showed maximum nitrogenase activity (ca 1.4 nmol C_2H_4 µg chl a^{-1} min^{-1} or 9.2-12.8 nmol C_2H_4 mg d. wt^{-1} min^{-1}) after about one day of growth under continuous light flux of 105 µmol m^{-2} s^{-1}.

13. In the laboratory, the response of nitrogenase to changes in light flux (down- or up-shift) was fast. Activity was reduced by about 30% when the light flux was reduced by about 50% (full light 105 µmol m^{-2} s^{-1}) for 1 h. At all values for light flux, nitrogenase activity following transfer levelled off after about 1.5 h. Transfer to the dark for 1h from 105 µmol m^{-2} s^{-1} light led to 87% reduction in activity. Subsequent changes in the dark were slow, with detectable activity after 24 h.

14. In batch culture nitrogenase activity was much higher upon illumination after a period of time in the dark than under continuous illumination. The shorter the dark period, the quicker the resumption of nitrogenase activity upon illumination.

15. In the field, maximum nitrogenase activity of 0.352 nmol C_2H_4 µg chl a^{-1} min^{-1} was observed in the alga being scraped from the rice tillers and after about 45 days of flooding at a light flux of 1825 µmol m^{-2} s^{-1}. (A comparison of nitrogenase activity of young and old colonies was not made). In batch culture, on the other hand, the alga showed a maximum rate of 1.4 nmol C_2H_4 µg chl a^{-1} min^{-1} after about one day's growth and a value equivalent to that in the field, after about three days under continuous light flux of 105 µmol m^{-2} s^{-1}. Nitrogenase activity in the laboratory levelled off after about five days, showing a rate of about 0.1 nmol C_2H_4 µg chl a^{-1} min^{-1}.

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APPENDIX A

LIST OF AXENIC AND CLONAL STRAINS ISOLATED FROM DEEPWATER

RICE (DWR) FIELDS OF BANGLADESH
(List in order of Durham strain numbers)

D605 Tolypothrix sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D C1(17)-N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Manikganj   By A. Aziz   On 25/06/81

Isolated by A. Aziz

Made axenic on not   and clonal on 18/05/82

  Collection 4 inoculum moist soil from DWR field.

D606 Nostoc sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D C1(17)-N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Daudkandi   By A. Aziz   On 25/08/81

Isolated by A. Aziz

Made axenic on not   and clonal on 01/06/82

  Collection 14 inoculum Hygroryza root.

D607 Scytonema sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D C1(17) -N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Sonargaon   By A. Aziz   On 25/08/81

Isolated by A. Aziz

Made axenic on not   and clonal on 12/05/82

  Collection 18 inoculum floating mat.

D608 Nostoc sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D C1(17) -N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Agrakhola   By A. Aziz   On 01/09/81

Isolated by A. Aziz

Made axenic on 18/06/82 and clonal on 05/05/82

  Collection 28 inoculum nodal roots of DWR.

D609 Gloeotrichia sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D Cl(17)-N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Sonargaon   By A. Aziz   On 06/11/81

Isolated by A. Aziz

Made axenic on not   and clonal on 03/05/82

 Collection 93 inoculum internode and sheath of DWR.

D 610   Scytonema sp.

Country of sample Bangladesh

Grown at 32°C on Chu 10D Cl(17)-N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Sonargaon   By A. Aziz   On 06/11/81

Isolated by A. Aziz

Made axenic on 17/03/83 and clonal on 09/05/82

 Collection 93 inoculum internode and sheath of DWR.

D611 Nostoc sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D Cl(17) -N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Sonargaon   By A. Aziz   On 29/11/81

Isolated by A. Aziz

Made axenic on 01/06/82 and clonal on   05/05/82

 Collection 94 inoculum dry stem and roots of DWR.

 The alga resembles N. linckia Born et. Thur.

D612 Fischerella sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D Cl(17) -N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Sonargaon   By A. Aziz   On 29/11/81

Isolated by A. Aziz

Made axenic on 18/06/82 and clonal on   05/05/82

 Collection 95 inoculum dry nodal roots of DWR.

D613 <u>Gloeotrichia</u> <u>pisum</u> Thur.

Country of sample Bangladesh

Grown at 32° C on Chu 10D Cl(17)-N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Sonargaon   By A. Aziz   On 29/11/81

Isolated by A. Aziz

Made axenic on 11/09/82 and clonal on 12/05/82

  Collection 95 inoculum dry nodal roots of DWR.

D614 <u>Nostoc</u> sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D Cl(17)-N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Sonargaon   By A. Aziz   On 29/11/81

Isolated by A. Aziz

Made axenic on 18/06/82 and clonal on 05/05/82

  Collection 96 inoculum dried leaf sheath of DWR.

D615 <u>Fischerella</u> sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D Cl(17)-N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Sonargaon   By A. Aziz   On 29/11/81

Isolated by A. Aziz

Made axenic on not   and clonal on 05/05/82

  Collection 96 inoculum dried leaf sheath of DWR.

D616 <u>Nostoc</u> sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D Cl(17)-N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Sonargaon   By A. Aziz   On 29/11/81

Isolated by A. Aziz

Made axenic on not   and clonal on 07/05/82

  Collection 96 inoculum dried leaf sheath of DWR.

The alga resembles <u>N.</u> <u>linckia</u> Born et Thur.

D617 <u>Anabaena</u> sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D Cl(17)-N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Sonargaon   By A. Aziz   On 29/11/81

Isolated by A. Aziz

Made axenic on 01/11/82   and clonal on 12/05/82

 Collection 96 inoculum dry leaf sheath of DWR.

 Pathogen causing lysis ; ? Phage.

D618   <u>Gloeotrichia</u> sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D Cl(17)-N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Agrakhola   By A. Aziz   On 01/09/81

Isolated by A. Aziz

Made axenic on not   and clonal on not

 Collection 28 inoculum nodal roots of DWR.

D625 <u>Anabaena</u> sp.

Country of sample Bangladesh

Grown at 32° C on Chu 10D Cl(17)-N pH 7.0 + HEPES

Stored under liquid nitrogen

Found at Sonargaon   By A. Aziz   On 29/11/81

Isolated by A. Aziz

Made axenic on 07/03/83 and clonal on 06/06/82

 Collection 98 from moist soil

 Trichome lysis frequent; previously identified as <u>Aulosira</u> sp.
(Whitton 1984).

APPENDIX B

SELECTION OF STOPPER FOR PLUGGING CULTURE VESSELS

B1   Introduction

In microbiology laboratories cotton wool stoppers have been used for many years. Recently silicon rubber stoppers have been substituted for these in order to obtain uniform size, easy handling and reusability. In cultures of limited volume, sooner or later exponential growth ceases due to limitation by one or other factor (rate of diffusion of CO_2, nutrient exhaustion, pH alteration, reduction of light by self-shading, autoinhibiton: Fogg 1975). Kratz and Myers (1955) pointed out that use of a blue-green alga as a tool organism for physiological studies becomes difficult because of the inadequate provision of CO_2 inherent in most of the culture methods used. Sinclair (1977) observed no differences in growth rate between flasks with cotton wool stoppers and metal caps, indicating the absence of any growth promoting substance which might be released from cotton wool stoppers during autoclaving. She also observed faster growth rate in aerated flasks than in the non-aerated ones. Experiments were therefore planned to choose the type of stopper which would provide a better yield and also to find out possible factors responsible for differences in the yield.

B2   Materials and Methods

Axenic strains of Nostoc D611 and or Anabaena D617 (Appendix A) were used. Yield was measured (Section 2.2.7) after 7 and 4 days for Nostoc D611 and Anabaena D617 respectively unless stated otherwise. Two light flux values were used, 60 μmol m^{-2} s^{-1} in standing condition under continuous light and light and dark cycle (12:12 h) and 105 μmol m^{-2} s^{-1} in the shaking tank. Silicon rubber stoppers (type S28, Sanko Plastic Co. Ltd) were cleaned with 2% Decon 90 (Decon Laboratories Ltd, England). Differences of light obstruction by the silicon rubber and cotton wool stoppers were negligible. During treatment with $NaHCO_3$, the Na concentration in the cotton wool stoppered flasks was adjusted with NaCl.

B3   Results

B3.1   Yield under different physical environments

Under continuous light and standing conditions the yield was significantly (P <0.001) higher in the cotton wool stoppered flasks than in the silicon rubber stoppered flasks (Table B1).   For Anabaena D617 differences of growth rate became visually obvious after two days. Subsequent studies with Nostoc D611 in   standing, shaking and in the light and dark cycle (Section 2.2.5.6) also showed higher yield in the cotton wool stoppered flasks (Table B2).

Table B1   Yield and pH shift in cultures of Nostoc D611 and
Anabaena D617 grown with two types of stopper.
(Continuous light at 60 μmol m^{-2} s^{-1} in the standing
condition; basal medium ; n = 4 ; *** = p <0.001)

stopper types	Nostoc D611						Anabaena D617					
	chla(mg l^{-1})		d.wt(mg l^{-1})		pH		chla(mg l^{-1})		d.wt(mg l^{-1})		pH	
	$\bar{x}$	±	$\bar{x}$	±			$\bar{x}$	±	$\bar{x}$	±		
cotton wool	*** 2.678	0.307	*** 339	26	7.40		*** 4.586	0.145	*** 106	4	7.38	
silicon rubber	*** 2.076	0.045	*** 248	9	7.32		*** 3.354	0.121	*** 89	7	7.33	

Table B2   Percentage increase of yield of Nostoc D611 in the
cotton wool stoppered flasks compared to the silicon rubber
stoppered flasks under three physical conditions; in light
and dark cycle treatment yield was measured after 10 days; n=4

yield parameters	standing (continuous light)	shaking (continuous light)	standing (light and dark)
chl a (% increase)	29	19	12
d. wt (% increase)	37	17	15

B3.2   Effect of NaHCO₃ on the yield under different physical
        environments

The addition of about 1.9 mM NaHCO$_3$ in the silicon rubber
stoppered flasks under continuous illumination and shaking, resulted
in a yield similar to the cotton wool stoppered flasks having 0.19 mM
NaHCO$_3$ (Table B3). In the light and dark cycle, the addition of 0.47 mM
NaHCO$_3$ in the silicon rubber stoppered flasks compensated the require-
ment (Table B4).

Table B3   Yield of Nostoc D611 under continuous illumination and
            shaking in the presence of different NaHCO$_3$ concentrations;
            n = 4

yield parameter	cotton wool (control)	silicon rubber (0.95 mM NaHCO$_3$)	cotton wool (control)	silicon rubber (1.9 mM NaHCO$_3$)	cotton wool (control)	silicon rubber (3.8 mM NaHCO$_3$)
chl a (mg l^{-1})	2.970 ± 0.094	2.555 ± 0.075	2.916 ± 0.075	3.174 ± 0.126	3.100 ± 0.126	3.470 ± 0.156
d. wt (mg l^{-1})	359 ± 10	298 ± 20	355 ± 35	315 ± 9	372 ± 18	405 ± 18

Table B4   Yield of Nostoc D611 under the light and dark cycle in
            the presence of different NaHCO$_3$ concentrations; yield was
            measured after 10 days; n = 4

yield parameter	cotton wool (control)	silicon rubber (0.47 mM NaHCO$_3$)	cotton wool (control)	silicon rubber (0.95 mM NaHCO$_3$)	cotton wool (control)	silicon rubber (1.9mM NaHCO$_3$)
chl a (mg l^{-1})	3.298 ± 0.031	2.752 ± 0.309	2.861 ± 0,031	3.516 ± 0.092	3.407 ± 0.370	3.757 ± 0.276
d. wt (mg l^{-1})	304 ± 48	300 ± 9	269 ± 13	366 ± 1	396 ± 18	454 ± 45

B4   Summary

A higher yield was obtained with cotton wool stoppers. A lower
yield in the silicon stoppered flasks was due to the limited diffusion
of CO$_2$.

APPENDIX C

SUPPLEMENTARY DATA RELEVANT TO FIGURES IN THE TEXT

Table C1 Diel variation of environmental variables and ARA for $\underline{G}$. $\underline{pisum}$ $\underline{in}$ $\underline{situ}$ on 20/21.8.83 n = 6 (see Fig.4.2)

mid-time (h)	light flux (μmol m^{-2} s^{-1})		temperature (^{o}C)		O$_2$ (mg l^{-1})	pH	ARA (nmol C$_2$H$_4$ μg chl $\underline{a}^{-1}$ min^{-1})	
	surface	at incubation	at -30cm	at incubation	at -30 cm	at -30cm	$\bar{x}$	$\pm$
1800	72.0	35.0	33.3	32.5	10.5	6.74	0.0063	0.0012
1930	<0.2	<0.2	31.8	30.8	10.5	7.50	0.0081	0.0040
2100	<0.2	<0.2	32.3	30.0	9.9	7.10	0.0017	0.0009
2230	<0.2	<0.2	31.9	30.2	7.8	6.80	0.0026	0.0018
0000	<0.2	<0.2	31.5	30.0	6.1	6.50	0.0027	0.0015
0130	<0.2	<0.2	31.3	29.8	3.7	6.60	0.0032	0.0011
0300	<0.2	<0.2	31.0	29.3	3.6	6.80	0.0035	0.0013
0430	<0.2	<0.2	31.0	29.4	1.5	6.50	0.0050	0.0017
0600	129	26.0	31.0	29.7	0.8	6.20	0.0082	0.0027
0730	581	116	31.0	29.8	0.6	5.90	0.0408	0.0140
0900	1062	212	31.3	31.2	1.2	5.70	0.0558	0.0080
1030	1668	334	31.3	33.3	2.5	6.10	0.1030	0.0272
1200	2208	442	31.6	34.7	4.6	5.90	0.1313	0.0313
1330	1969	394	32.4	35.3	7.6	6.10	0.0858	0.0104
1500	1343	267	32.8	35.2	7.3	6.35	0.0631	0.0148
1630	322	62.0	32.5	32.3	10.0	6.35	0.0327	0.0041
1800	61.0	12.0	32.1	30.5	9.6	6.20	0.0135	0.0046
1930	0.2	0.2	31.7	30.2	9.9	6.65	0.0015	0.0005

Table C2 Influence of light flux on growth rate of G. pisum D.613.
Inoculum eight days old ; 32°C ; continuous light flux and shaking ; n = 4 except zero-time (n = 2)
(see Fig.6.1)

growth under light flux (μmol m^{-2} s^{-1})

time (d)		25 d. wt (mg l^{-1})	25 chl a (mg l^{-1})	25 chl a: d. wt	50 d. wt (mg l^{-1})	50 chl a (mg l^{-1})	50 chl a: d. wt	100 d. wt (mg l^{-1})	100 chl a (mg l^{-1})	100 chl a: d. wt	150 d. wt (mg l^{-1})	150 chl a (mg l^{-1})	150 chl a: d. wt	200 d. wt (mg l^{-1})	200 chl a (mg l^{-1})	200 chl a: d. wt
0	x̄	14.5	0.204	0.0140	11.0	0.162	0.0150	9.3	0.114	0.0120	11.3	0.105	0.0090	7.8	0.097	0.0120
1	x̄	20.5	0.366	0.0183	26.8	0.347	0.0128	30.7	0.334	0.0113	30.8	0.311	0.0103	21.2	0.237	0.0113
	±	4.8	0.020	0.0033	1.5	0.026	0.0015	6.8	0.159	0.0030	2.4	0.035	0.0010	3.7	0.0022	0.0021
2	x̄	39.8	0.619	0.0160	73.6	1.121	0.0153	77.6	1.069	0.0135	81.0	1.043	0.0130	79.2	1.000	0.0125
	±	9.8	0.053	0.0030	3.3	0.075	0.0005	2.0	0.058	0.0006	6.3	0.035	0.0008	4.9	0.050	0.0006
3	x̄	64.4	1.031	0.0168	122	2.092	0.0173	115	2.029	0.0173	125	1.878	0.0148	128	1.760	0.0140
	±	11.6	0.118	0.0028	10	0.209	0.0005	7	0.077	0.0005	5	0.197	0.0001	0	0.130	0.0008
4	x̄	97.4	1.856	0.0165	169	2.636	0.0155	186	2.654	0.0143	200	2.271	0.0113	196	2.009	0.0103
	±	14.8	0.176	0.0034	28	0.243	0.0026	6	0.125	0.0005	8	0.213	0.0010	10	0.101	0.0010
5	x̄	194	2.865	0.0145	250	3.276	0.0130	260	3.232	0.0123	276	2.455	0.0090	254	2.266	0.0090
	±	11	0.167	0.0005	8	0.098	0.0000	22	0.035	0.0010	5	0.144	0.0005	14	0.056	0.0010

Table C3 Growth characteristics and changes of ARA of G. pisum D613. Inoculum six days old ; $32°C$; continuous light flux of 105 µmol $m^{-2} s^{-1}$; shaking ; $n = 4$ (see Fig.7.1)

time (d)	d. wt ($mg\ l^{-1}$)	chl a ($mg\ l^{-1}$)	chl a: d. wt	heterocyst frequency	heterocyst number x 10^6	nmol C_2H_4 $l^{-1} min^{-1}$	nmol C_2H_4 mg d.wt$^{-1} min^{-1}$	nmol C_2H_4 µg chl a$^{-1} min^{-1}$	FRP ($mg\ l^{-1}$)	µg algal-P mg d.wt^{-1}
0 x̄	6.1	0.080	0.0131						1.875	7.2
±	0.4	0.004	0.0012						0.008	0.2
0.04 x̄	6.2	0.071	0.0115	2.00	2.86	4.6	0.75	0.07	1.655	41.6
±	0.4	0.003	0.0003	0.44	1.42	2.2	0.37	0.03	0.003	4.0
0.5 x̄	12.6	0.102	0.0083			50.8	3.72	0.49	1.573	
±	2.1	0.004	0.0013			11.6	0.71	0.13	0.011	
1 x̄	25.8	0.171	0.0068	5.78	42.53	233	9.24	1.37	1.440	15.0
±	5.6	0.006	0.0014	0.76	4.26	41	1.12	0.21	0.033	3.2
1.5 x̄	43.7	0.450	0.0104			404	9.19	0.92	1.240	
±	6.8	0.056	0.0010			93	1.22	0.18	0.080	
2 x̄	73.7	0.896	0.0122	5.69	150.88	668	8.98	0.74	1.157	9.7
±	10.4	0.068	0.0009	1.22	52.93	159	1.36	0.13	0.033	1.7
2.5 x̄	91.3	1.529	0.0169			841	9.08	0.55	0.545	
±	10.4	0.048	0.0020			160	1.40	0.14	0.047	
3 x̄	116.3	2.248	0.0194	4.08	247.47	726	6.28	0.32	0.232	14.5
±	8.2	0.070	0.0009	0.42	66.07	63	0.78	0.03	0.046	1.4

Cont'd..

3.5	x̄ 141	2.673	0.0195			587	4.17	0.22	0.022		
	± 10	0.049	0.0016			30	0.47	0.01	0.007		
4	x̄ 186	3.282	0.0177	3.24	522.03	541	2.9	0.165	0.004	11.8	
	± 8	0.139	0.0004	0.61	110.20	47	0.2	0.012	0.001	0.3	
5	x̄ 224	3.844	0.0172	2.66	618.18	441	1.97	0.115	<0.004	8.4	
	± 7	0.095	0.0008	0.46	105.40	92	0.40	0.026		0.2	
6	x̄ 281	3.800	0.0136			491	1.75	0.128	<0.004	6.8	
	± 7	0.156	0.0008			88	0.29	0.023		0.2	
7	x̄ 304	3.764	0.0124			423	1.40	0.116		6.3	
	± 8	0.325	0.0008			46	0.19	0.024		0.2	
8	x̄ 363	3.677	0.0107			460	1.26	0.125		5.3	
	± 8	0.160	0.0011			62	0.16	0.022		0.1	
9	x̄ 413	3.727	0.0090			440	1.07	0.119		4.6	
	± 5	0.244	0.0007			58	0.15	0.021		0.1	
11	x̄ 431	3.620	0.0084			508	1.15	0.139		4.5	
	± 16	0.081	0.0002			20	0.06	0.006		0.3	
13	x̄ 549	3.628	0.0066			446	0.73	0.123		3.5	
	± 8	0.134	0.0003			20	0.18	0.009		0.1	
15	x̄ 575	3.380	0.0059			220	0.39	0.065		3.3	
	± 17	0.162	0.0003			19	0.03	0.003		0.1	
20	x̄ 706	3.400	0.0048			303	0.43	0.090		2.64	
	± 6	0.199	0.0003			16	0.02	0.006		0.02	

Table C4 Influence of pretreatment in dark for 12 h on ARA, after transfer to the light by G. pisum D613 (105 μmol m^{-2} s^{-1}; continuous shaking ; n = 4 ; * = P<0.05;*** = P<0.001

experimental conditions	mid-time (h)	chl a (mg l^{-1})		d. wt (mg l^{-1})		nmol C$_2$H$_4$ μg chl a^{-1} min^{-1}		nmol C$_2$H$_4$ mg d. wt^{-1} min^{-1}	
		x̄	±	x̄	±	x̄	±	x̄	±
continuous light (control)	0.5	0.116	0.003	8.1	1.2	0.09	0.03	1.38	0.60
"	12.0	0.160	0.013	20.6	1.8	0.78	0.04	6.02	0.21
"	24.0	0.263	0.012	35.7	2.4	1.38	0.11	10.16	0.52
"	30.0	0.396	0.013	47.8	3.2	1.46	0.16	12.04	0.63
"	36.0	0.590	0.052	* 66.6	3.5	1.46	0.03	***12.84	0.52
"	48.0	1.473	0.036	90.0	2.5	0.75	0.06	12.33	0.63
"	60.0	2.280	0.073	127.5	17.5	0.48	0.05	8.66	0.52
"	72.0	3.028	0.101	158.5	5.0	0.30	0.03	5.72	0.55
dark incubation of 36 h old culture	0.5	0.616	0.034	63.2	3.8	0.20	0.03	1.96	0.15
"	1.5	0.622	0.049	68.2	1.8	0.17	0.02	1.51	0.03
"	2.5	0.656	0.021	69.2	4.0	0.15	0.01	1.39	0.15
"	12.0	0.750	0.025	48.6	5.9	0.016	0.006	0.26	0.09
transfer of 12 h dark alga to light	0.5	0.792	0.024	53.4	5.2	0.71	0.14	10.36	1.91
"	1.5	0.826	0.078	58.4	5.7	1.53	0.13	21.52	0.75
"	2.5	0.883	0.079	*61.0	3.4	1.67	0.13	***24.02	1.71
"	3.5	0.975	0.036	63.8	4.6	1.41	0.10	21.54	2.24
"	4.5	1.032	0.099	67.4	5.3	1.30	0.15	19.73	1.84
"	5.5	1.161	0.070	72.8	6.2	1.11	0.04	17.68	1.95
"	6.5	1.229	0.050	74.6	6.1	1.20	0.04	19.42	1.49
"	7.5	1.337	0.054	83.2	3.8	0.97	0.04	15.56	0.71
"	11.5	1.775	0.074	109.0	4.7	0.54	0.03	8.75	0.07