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Aspects of certain exobiotic and endobiotic *Audouinella* spp. (class Florideophyceae, division Rhodophyta).

by

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B.Sc. M.A. Biology

A Thesis submitted for the degree of

Doctor of Philosophy

in the Faculty of Science

Department of Botany

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Scotland, U. K

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I

FLORA SCOTICA

BY

THE REV^{D} JOHN LIGHTFOOT.

a

VOL. II.

LONDON.

MDCCLXXVII.

P. Mazell del. et sculp.

Frontispiece : The Title page of Lightfoot's <u>Flora Scotica</u> which contains the first known description of an Audouinelloid Red Alga.

DECLARATION

I hereby declare that this thesis is composed of work carried out by myself unless otherwise cited or acknowledged and that the thesis is of my own composition. The research was carried out within the period March 1983- October 1987. This thesis has not been previously presented for any other degree in whole or in part.

Signed ....

Abdalla Elmansori

Date: 15·10·87

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SUMMARY

Eleven exo- and endobiotic *Audouinella* species were investigated in this study. Isolates were obtained from supporting and `host' material collected from sites in Scotland and Ireland. Taxa from the Culture Collection of Algae and Protozoa have also been studied for comparative purposes.

For 6 of the endobiotic isolates identification at species level not possible -the field material often being of a few cells, and forms in culture, all obtained from single spore sources, showed significant morphological differences from one another.

Evidence from some preliminary studies on cell wall structure and compositions indicate that the morphological differences observed are those of distinct entities, and comparative studies on cell wall histochemistry and fine structure are suggested as a possible taxonomic approach of value in distinguishing between *Audouinella* species. Calorific values of thalli seem to be linked with some aspects of cell wall structures. Sizes and positions of nuclei within cells may also prove features of taxonomic value. The nature of phycoerythrins proved not to be important.

Endobionts from plant `hosts' were able to effect penetration of substrata of animal nature (chitin, calcium carbonate). All endobionts appear able to penetrate agar substrata and filaments appear to `digest' their way through the gel. The ability of endophytic species to penetrate animal substrates raises questions regarding `host' specificity.

Under various stress conditions there appear to be variety of morphological adaptations. e.g. hair formation, ball-like tufts of fragile filaments, reduction of size forms such as tufts with single filaments and spore production.

Daylength appeared to affect different generations in terms of the tuft size and spore production. Night-breaks also were found to be relevant to vegetative growth and spore

production, but has no effect on development of sexual phases.

Spore release studies showed that these taxa possess an effective spore production mechanism with an effective liberation method which enhances dispersal in marine ecosystems. Spore output can be prodigious, with fertile plants producing a spore number many terms greater than their total cell complement.

Photosynthetic rates studies showed that these plants are `opportunists` taking advantage of any light available in the medium. The species can tolerate periods of darkness and still immediately commence photosynthesis when illuminated . In terms of light spectra they showed preference to the blue and green lights irrespective to the intensity.

The *Audouinella* complex is composed of a wide range variety of species of different living habits. With endobiotic species only a few cells may be seen in nature. Culture studies combining morphological, physiological and histochemical methods may sometimes make contributions to solving the speciation problems in this genus .

General Introduction

Audouinella species are members of the family Acrochaetiaceae (Order Nemaliales, Class Florideophyceae, Division Rhodophyta). These plants inhabit both fresh and marine waters in the Northern and Southern hemispheres. The thalli are of simple construction, filamentous and undifferentiated or with prostrate and erect systems. The prostrate system may be unicellular, discoid or of creeping filaments. The erect system may be simple or branched. The form varies with habitat and substrata. Morphological differences can be seen among species growing attached to rock (epilithic), epiphytically on other plants or epizoically on animals, endophytically inside other plants, or endozoically inside animals i.e of exo- or endobiotic habits.

The genus *Audouinella* as recognized at the present day has had a very confusing taxonomical history during the past two centuries. Our knowledge of audouinelloid algae starts with Lightfoot (1777). The Rev. John Lightfoot (1735-88), Chaplain to the Dowager Duchess of Portland, travelled through Scotland in 1772 in the company of Thomas Pennant (1726-98) Zoologist and Antiquary. Lightfoot published his *Flora Scotica* in 1777 based on collections made in his 1772 visit. In this publication (pp. 1000-1001) he described *Audouinella purpurea* (Lightf.) Woelkerling for the first time as Byssus *purpurea* .

> *Purpurea* 4. **BYSSUS** *purpurea* capillacea perennis. (*Michel. Gen. p.211. n. 13. tab. 90.f. 2?*)
>
> Crimson Byssus. *Anglis* .
>
> Upon the base of the abbot *Mackinnon's* tomb, in the ruin'd abby at *J. Columb-kill* It consists of extremely short filaments, crowded together so as to form a mat or crust extremely like a piece of crimson plush or velvet.

> When moisten'd it yields a sweet scent, something like violets, which induced me at first to think it the **BYSSUS** *Jolithus. Lin.* but that is farinaceous, and the kind just describ'd is capillary.
>
> *Linnaeus* however, (in his *Flor. Suec. p. 438.*) says, that he has perceiv'd red filaments in the B. *Jolithus* , growing upon saffron-color'd farinaceous crust, so that ours may possibly be considered yet as a variety of the B. *Jolithus. Lin. sp. pl. 1638.*
>
> *Haller* ranks the B. *Jolithus* amongst the crustaceous Lichens, and says the could discover in it no filaments, but only a red crust slightly farinaceous, turning to a yellowish-green colar in decay. *Helvet. hist. III. p. 103. n. 2090* .
>
> In the midst of these uncertainties, we judged it most advisable to describe ours as a distinct species, in order to avoid confusion.'

Lightfoot's description thus includes a clear reference to the filamentous nature of his *Byssus purpurea* .

In the summer of 1948 Kathleen M. Drew (Mrs. H. Wright Baker) (1901- 1957) went to same place searching for the type locality of this species and confirmed the presence of this alga very close to the site reported by Lightfoot in the Abbey Church of St. Columba on the Island of Iona, close to the tomb of Archbishop Mackinnon (quoted in Dr. K. M. Drew's Obituary, *British Phycological Bulletin* Vol. 1 (6) pp.1-12. 1958). The search for the Lightfoot's type locality of this species was carried out in

order to ensure the identity of *Rhodochorton rothii* (Turton) Nageli which was thought to be *Rhodochorton purpureum* (Lightfoot) Rosenvinge (= *Byssus purpurea*).

Woelkerling's (1971) review listed 20 generic names which have been used to designate taxa now included in *Audouinella.* Table 1.1 shows these and other generic names with the authorities and dates when first used. Eleven of the generic names listed by Woelkerling (1971) were rejected by the International Code of Botanical Nomenclature. Of the remaining nine generic names three have been most frequently applied to these plants in the literature (viz. *Audouinella* Bory (1823), *Acrochaetium* Nageli (1858), and *Rhodochorton* Nageli (1861)). Drew (1928) found *Acrochaetium* and *Rhodochorton* to be linked by so many intermediate species that she suggested it would be best to unite these two genera under the one name *Rhodochorton.* On the basis of the chromatophore (chloroplast) shape, Papenfuss (1945) added a fourth genus *Chromastrum* to include all taxa with stellate chloroplasts and used it as a replacement for *Kylinia.* Woelkerling (1971) stated that differences in chloroplast shape appear to be generally unreliable for taxonomic purposes at the generic level, and relegated *Chromastrum* and *Rhodochorton* to the synonymy of *Audouinella.* In addition he rejected *Acrochaetium* as a distinct genus. He proposed that all existing generic names should be brought within an *Audouinella* complex, so incorporating *Acrochaetium-Rhodochorton* . Dixon and Irvine (1976) and Garbary (1979b) have made a similar proposal. However, the generic names *Acrochaetium* and *Rhodochorton* are still used on occasions.

Woelkerling (1983) stated : " The generic classification of acrochaetoid algae is in a disgraceful state of disarray.... some generic names have been used in so many different ways that it has become difficult to associate the name with a particular concept". This is sufficient commentary on the state of affairs, and in the present work the one genus *Audouinella* is accepted as based on the most recent investigations.

Table 1.1 Generic names, authority and dates of taxa.

Name	First Authority	Date first published
Byssus	Lightfoot	1777
Chantransia	De Candolle	1801
Ceramium	Dillwyn	1809
Coferva	C. Agardh	1817
Trentepohlia	Martius	1817
Callithamnion	Lyngbye	1819
Audouinella	Bory	1823
Acrochaetium	Nageli	1858
Rhodochorton	Nageli	1861
Thamnidium	Thuret in Le Jolis	1863
Balbiania	Sirodot	1876
Colaconema	Batters	1896
Pseudochantransia	Brand	1897
Grania	Rosenvinge	1909
Kylinia	Rosenvinge	1909
Chantransiella	Brebner (as stated by De Toni	1924)
Liagorophila	Yamada	1944
Rhodochortonopsis	Yamada	1944
Chromastrum	Papenfuss	1945
Rhodothamniella	Feldmann	1954
Colacodictyon	Feldmann	1955
Pseudacrochaetium	von Stoch	1965

Speciation of this genus, on the other hand, is even more complicated. The speciation is based entirely on morphological features, e.g. chloroplast number and shape, sporangia dimensions and arrangements on the branches, the morphology of the prostrate system and the erect system including modes of branching, cell size dimensions, and the sexual phase (if known) in the life history. No reliable taxonomical key is as yet available. Woelkerling (1983) listed thirteen different classification systems as having been used since 1927 (namely Hamel 1927; Drew 1928; Papenfuss 1945, 1947; Feldmann 1954, 1962; Woelkerling 1971, Kuhnemann 1972; Levring 1974; Abbott & Hollenberg 1976; Dixon & Irvine 1976; Cordero 1977; and Guiry 1978).

Boney and White (1967) suggested that caution should be used in speciation of such simple life forms. West (1968) stated that few morphological features are available upon which to base a taxonomic system. Later in 1978 he described speciation in these plants as being "traumatic". Woelkerling (1971) concluded that numerous species have been established on minute differences without regard for possible intraspecific variations. Dixon and Irvine (1977) have concluded that species concepts for the taxa in the British Isles are the most confused of all the marine Rhodophyta in the British Flora.

As a result of so many generic names being used the number of species in this complex is high. The list increased from 110 species (prior to 1900) to 390 species in 1980 (Woelkerling 1983). However, the validity of many of these species is uncertain and has been questioned by many authors. Garbary (1979b) studied 170 descriptions as 112 species. In this study he used computerized cluster analysis and so combined the 112 species into 64 species only.

The enigmatic nature of the classification of these algae is apparently due to their small size, the similarity of their simple morphology, the lack of recognizable sexual stages of the endobiotic species, and, above all, their phenotypic plasticity. As these algae inhabit a wide range of habitats with different environmental factors such as

temperature, light intensity and duration, salinity, nutrients and substrata, their morphology tends to change as the habitats or the environmental factors change. Boney (1972), Abbott and Hollenberg (1976), Dixon and Irvine (1977), Rueness (1977), Feldmann (1981), have all reported variations in the form range of single plants from field populations. West (1970) has noted that in cultures, plastid appearance tend to vary with cell age. White and Boney (1968) found that free living forms are morphologically very different from their appearance when endobiotic. Woelkerling (1971) and Stegenga (1979) reported the differences in the chloroplast shape in separate parts of the same erect system. Boney (1975) stated that such simple plants can modify their growth forms in response to a variety of habitat conditions. The possibility has thus arisen that different names have been given to one species, and this may be a common phenomenon among species of this genus.

Life history in the algae as defined by Dixon (1973) includes all different successive phases with their morphological and nuclear (haploid and diploid stages) differences. Information on the life history of most audouinelloid algae remains deplorably incomplete (Woelkerling 1971). However, life history in audouinelloid algae was been used in the early stages of classification of these plants when Nageli (1861) treated *Acrochaetium* as a separate genus on the basis that this genus reproduced by means of zoospores -obviously a mistaken observation for a red alga.

Sexual and asexual reproducing forms of *Audouinella* were found separately on occasions and usually new genera were created, e.g. Bornet (1904) proposed that the genus *Chantransia* should be limited only to those species which had sexual reproduction, and Rosenvinge (1909) described a new genus *Kylinia* on the basis that he observed specialized sexual reproductive structures in this genus - hair-like androphores producing spermatia. Later it was confirmed that those structures he observed as androphores were hyaline cells (Kylin 1944 cited by White 1968). However, Feldmann (1962) stated that Rosenvinge was right as he had ascertained by

the study of living material. In later studies on *Audouinella rosulata* (Rosenv.) Dixon by Boney and White (1967) and Stegenga and Wissen (1979) as *Kylinia rosulata* Rosenv. the presence of Rosenvinge's androphore structures was confirmed. Feldmann (1962) assumed that Kylin did not study the true *Kylina rosulata*. Instead he studied *Acrochaetium kylinioides* nov. sp.. Boney (1970) described carpogonium-like structures in *Audouinella infestans* (Howe et Hoyt) Dixon (=*Acrochaetium infestans* Howe et Hoyt) but stated that it would seem essential that any reference to a sexual phase in the life history should be well authenticated. Therefore, studies of these plants with sexual stages are required to assure the validity of the presence of such stages and structures, i.e. that fertilization does take place, and that post-fertilization phases can be recognized. Woelkerling (1971) on field observations confirmed the presence of sexual phases in *Audouinella* species in Southern Australia (viz. *A. botryocarpa*, *A. daviesii* , and *A. liagorae*). Other culture studies have revealed more audouinelloid algae having life histories with sexual and tetrasporophyte generations Such alternations have been described by West (1968, 1969) for *Audouinella pectinata* (Kylin) Papenfuss (= *Acrochaetium pectinatum* (Kylin) Hamel) and *Audouinella purpurea* (Lightf.) Woelkerling (= *Rhodochorton purpureum* (Lightfoot) Rosenvinge (and = *R. tenue* Kylin); Boillot & Magne (1973) for *Audouinella rosulata* (Rosenv.) Dixon (= *Kylina rosulata* Rosenvinge); Borsje (1973) for *Audouinella virgatula* (Harv.) Dixon (=*Acrochaetium virgatulum* (Harv.) J. Ag.); Stegenga & Vorman (1976) for *Audouinella densum* (=*Acrochaetium densum* (Drew) Papenfuss); Stegenga & Mulder (1979) for *Audouinella moniliforme* (Rosenvinge) Boergesen (=*Chromastrum humile* (Rosenvinge) Papenfuss); Stegenga & van Wissen (1979) for *Audouinella kylinoides* (Feldm.) comb. nov. (=*Acrochaetium reductum*; Stegenga & Mol (1980) for *Audouinella virgatula* (Harv.) Dixon (=*Chromastrum secundatum* (Lyngbye) Papenfuss); Abdel-Rahman (1982a and 1982b) for *Audouinella asparagopsis*

(Chemin) Dixon (=*Acrochaetium asparagopsis* (Chemin) Papenfuss); Dring & West (1983) for *Audouinella purpurea* (Lightf.) Woelkering (=*Rhodochorton purpureum* (Lightfoot) Rosenvinge); and Kuiper (1983) for *Audouinella alariae* (Jonss.) Woelkerling (=*Chromastrum alariae* (Jonsson) Papenfuss); Hansen & Garbary (1984) for *Audouinella arcuata* (Drew) Garbary, Hansen et Scagel.

The life history studies were carried mainly in a search for the identity of these plants as it is very frequently questioned whether such simple forms may not be part of a life history of another alga. Some species have been proved to be part of life history of another species in the same genus or other genera. Sirodot (1873) found that *Audouinella chalybaea* was a juvenile stage of a species of *Batrachospermum*. Kylln (1906) established that the tetrasporic *Rhodochorton chantransioides* Reinke? was but the asexual generation of *Audouinella efflorescens* (J. Ag.) Papenf. Drew (1935) found that the fresh water *Audouinella violacea* (=*Rhodochorton violaceum*) (Kuetz.) reproduces sexually and asexually, and moreover, both male and female plants produce monospores (asexual reproductive structures). Israelson (1942) concluded that *Audouinella hermanni* and *A. violacea* (=*Rhodochorton violaceum*) were conspecific species. Papenfuss (1945) stated "It seems probable that in the greater portion of monosporic species there is no method of reproduction other than by monospores. In such forms sexuality may not as yet have developed, or it may have been lost". von Stosch (1965) found an *Acrochaetium*-like phase in the life history of *Liagora farinosa* Lamour.. West (1969) concluded that *Rhodochorton purpureum* (Lightfoot) Rosenvinge and *R. tenue* Kylin are synonymys after he studied their life histories in culture. Another comparative study of the life histories of *A. purpurea* (Lightf.) Woelkerling (=*R. purpureum*) and *A. floridula* (Dillw.) Woelkerling (=*R. floridulum*) under culture conditions carried by Stegenga (1978) showed the distinction between these two species in terms of their response to temperature and daylength. On the other hand, other *Audouinella*

species, after been studied in culture, were found to be part of life history of another *Audouinella* species. Boillot & Magne (1973) found that *A. pectinata* (Kylin) Papenfuss was a tetrasporophyte generation of *A. rosulata* (Rosenv.) Dixon, Lee and Kurogi (1983) found that two different audouinelloid algae, namely *A. alariae* (Jonss) Woelkerling and *Rhodochorton repens* Jonsson (=*Acrochaetium jonssonii* Papenfuss), are alternate stages of the same species. i.e they were gametophyte and tetrasporophyte respectively in the life history of *A. alariae*, and other cases were listed by Woelkerling (1983).

At least four distinctive sexual cycles involving gametangial phases of audouinelloid morphology are now known, triphasic dimorphic in which three phases, 1 haploid (gametes) and 2 diploid (carposporophyte and tetrásporophyte) are produced on two morphologically different plants; triphasic trimorphic in which three phases (as above) are produced on three morphologically different plants, and diphasic dimorphic in which only one haploid and one diploid phase are produced on two morphologically different plants and taxa with diphasic dimorphic sexual cycle producing gametes but not tetrasporangia (Woelkerling 1983).

The triphasic dimorphic sexual cycle, as in *Audouinella botryocarpa* (Harvey) comb. nov. (=*Acrochaetium botryocarpum* (Harv.) J. Ag. is one in which free living more or less isomorphic gametangial and tetrasporangial phases (both producing multicellular prostrate systems) occur along with a carposporophyte phase which develops on and remains attached to the gametangial plant but is distinctly different morphologically. Gametangial plants are presumably haploid while carposporangial and tetrasporangial stages are presumably diploid (Woelkerling 1970).

A triphasic, trimorphic sexual cycle has been described in *Audouinella rosulata* (Rosenv.) Dixon (=*Kylinia rosulata*) Rosenv. and *Audouinella virgatula* (Harv.) Dixon (=*Chromastrum virgatulum* Collins by Stegenga & van Wissen (1979) and Stegenga Mulder(1979)respectively, in which the free-living gametangial phase

differs morphologically from the free-living tetrasporangial phase primarily in possessing a unicellular rather than a multicellular prostrate system, and the carposporangial phase, which develops on and remains attached to the gametangial plant differs from both.

Diphasic, dimorphic sexual cycles are known in *Audouinella purpurea* (Lightf.) Woelkerling and described by West (1969) as *Rhodochorton purpureum* (Lightfoot) Rosenvinge. This alga under culture conditions developed into comparatively small (< 1 mm tall) haploid gametangial plants which alternate with much larger (up to 25 mm tall) diploid tetrasporangial plants.

The fourth type was reported by Lee & Kurogi (1978) in *Rhodochorton subimmersum* Setchell et Gardner. In this taxon a free-living tetrasporangial plant apparently does not occur and the haploid gametangial phase is dominant in terms of size. After karyogamy, a small group of 2 or 3 celled gonimoplast filaments develop and these eventually produce terminal sporangia, each containing four spores. Woelkerling (1983) thought that this species possesses a carpotetrasporophyte and carpotetraspores and stated that, presumably these spores give rise to new haploid gametangial plants.

The life history and the reproductive structures appear to be affected by environmental factors (Knaggs 1967; Borsje 1973; Garbary 1978c; and Abdel-Rahman 1982a & 1982b). West (1972) reported that day length is the main factor affecting tetrasporangia formation in *Audouinella purpurea* (=*Rhodochorton purpureum*). Borsje found that not all *Audouinella virgatula* (Harv.) Dixon populations produce tetraspores under similar culture conditions. He found that one of the three clones he used (Dutch clone) did not produce any tetraspores under conditions where other Dutch and French clones produced tetraspores. However, he found that all clones produced monospores. Garbary (1978c) found that *Audouinella virgatula* produces more tetrasporangia at high light intensities, and *Audouinella parvula* produces more monospores at lower temperature (8 ^{0} C). Abdel-Rahman 1982a & 1982b found that

night breaks after 14 hours of darkness increased the tetraspore production in *Audouinella asparagopsis* (=*Acrochaetium asparagopsis*).

Audouinella species in the British Isles have not been extensively studied in the past. The few studies carried out were mainly concened with morphological features of these plants in the field and the culture (Knaggs 1965; White & Boney 1969, 1970; Boney 1972; Garbary 1978c). Knaggs (1967) suggested from the results he obtained on *Audouinella floridula* (Dillw.) Woelkerling (=*Rhodochorton floridulum* (Dillwn.) Nag. that in nature, nitrate and phosphate concentrations must attain a certain critical upper level in relation to falling energy flux before sporulation can occur, while in culture under uniform flux, the nutrient concentration must fall to an upper threshold level considerably lower than that in the standard medium before conditions are favourable for reproduction, and under uniform flux, the higher the initial level of nutrient concentration the greater will be the lag-time before the onset of sporangial formation. White and Boney (1969, 1970) and Boney (1972) successfully isolated some *Audouinella* species from their host and grew them free from their hosts, and they were able to re-infect their hosts under culture conditions and the plants retained the same appearance within the 'host'. Boney (1972) suggested that before two endophytes could be synonymized they should be shown to be similar in unialgal culture and the cross infection of the alternate host carried out. Garbary (1979b) did not lay stress on the latter condition. Tam *et al* (1987) synonymized *Audouinella vaga* (Drew) Garbary *et al.* with *A. porphyrea* (Drew) Garbary (endophytes in *Pterosiphonia bipinnata* (Post. et Rupr.) Falk. and *Porphyra* respectively) on morphological similarities under culture conditions while no successful re-infection was attained. In these studies and other studies listed below, these plants showed their phenotypic plasticity, i.e, the effect of the environmental factors on morphology and development. Garbary (1979a) investigated the effect of temperature on the morphology of six *Audouinella* species. He found

that high temperature (23 ^{0}C) caused the decrease in cell length and branching frequency in these species. Garbary (1979b) studied the cell elongation pattern in *Audouinella* species, and concluded that it is unlikely that patterns of intracelluar elongation, as those present in Ceramiaceae (Rhodophyta), are present in *Audouinella* species.

Physiological and biochemical studies involving this group of algae are also few in number and data so far obtained have again been considered from phylogenetic or taxonomic viewpoints. Boney (1972b) has investigated fluorescent extracellular substances in five audouinelloid algae, and Boney and White (1968) investigated the phycoerythrins of four audouinelloid algae, but no conclusions towards taxonomical criteria were drawn from these studies. Glazer *et al* (1982) studied *Audouinella* species searching for chemotaxonomic markers for these algae. They found that both B- and R-phycoerythrins are present in *Audouinella* species but were unable to distiguish any taxonomical significance. Boney (1978) emphasized the need for a continuing search for suitable biochemical criteria but discouraged using differences in the amounts of compounds present for making taxonomic distinctions. Mallery & Richardson (1971, 1972) examined the biliproteins and soluble proteins of one *Acrochaetium* species and concluded that *Acrochaetium* was considered generally as the most primitive genus of the class Florideophyceae and showed some strong biochemical affinities with several taxa of the Class Bangiophyceae. The conclusion regarding the phylogenetic relationships among sexually reproducing acrochaetioid species was also reached in a further study by Richadson & Mallery (1973) when they analysed one species of *Rhodochorton* and three species of *Acrochaetium* for soluble proteins, biliproteins and esterase patterns from polyacrylamide discontinuous pH gel electrophoresis data.

Poor fixation and preparation, and diagrams from herbaria specimens records, add a measurable difficulty in studying and comparing these algae. West (1970)

found that when plants are fixed in alcohol or formalin, plastids may lose their identity.

For all the stated reasons, the classification and number of species remain unstable. The number of species changes as more culture and field studies take place. Dixon (1963) suggested that 20% of species from the British Isles reported by Parke (1958) would ultimately be discarded.

Dixon and Irvine (1977) listed 33 marine species of *Audouinella* from the British Isles. Uncertainty still remained about many taxa and more cultural and field studies were suggested. Of all the areas of taxonomic uncertainty, that involving the endobiotic taxa seems the most difficult. The life histories and sexual cycles which have been described have in the main been for exobiotic taxa. Most endobiotic taxa fit Papenfuss's (1945) description of being monosporic. In addition; within the 'host' organisms they show great morphological simplicity. There seem to be a number of dimensions, especially in the experimental sense, not so far explored with these audouinelloid algae. The objective of the present study was to explore the extent to which a number of experimental techniques would render additional information on the biology of these simple algae, and perhaps indicate further lines of approach to help resolve what is a taxonomic situation of continuing confusion. Further, the understandable emphasis on taxonomic studies with this puzzling genus has obscured consideration of the implications of its primitiveness within the Class Florideophyceae as proposed by some investigators. There would seem to be room for experimental studies designed to examine this aspect of the biology of a genus about which inumerable questions seem to be asked at present.

2. Materials and Methods

2.1. Methods of obtaining cultures of exo- and endobiotic *Audouinella* species

As the aim of this study was to investigate a number of species of *Audouinella* from natural habitats, host plants or animals that were suspected to have exobiotic or endobiotic forms were collected from different sites (Figs. 2.1-2). Whilst a wide range of plants were examined, certain species were found to be particularly good sources of *Audouinella* species.

Epiphytes were isolated from samples of *Porphyra umbilicalis* J. G. Ag. (Rhodophyta) from St. Andrews Scotland, *Cladophora rupestris* Kutz. (Chlorophyta) and *P. umbilicalis* from the shores of the Isle of Cumbrae (Firth of Clyde) and Portencross on the Ayrshire coast, and *Ceramium rubrum* (Huds.) C. Ag. (Rhodophyta) from Ardmore Point in the Clyde Estuary. Samples of the host plants were brought to the laboratory, immediately examined under the light microscope for the presence of exo- or endobiotic filamentous red algae, or stored in cold room for examination next day. Fragments of the host plant bearing epiphytes or endophytes were placed on washed coverslips with a drop or two of Enriched Seawater Medium (Boney and Burrows's Medium, **Appendix 1**), and these placed on wet filter papers in petri dishes. The petri dishes were placed in growth chambers with conditions described in 2.1.2. Drops of fresh medium were added, and filter papers were watered daily. The fragments were examined to see if spore release from epiphytic *Audouinella* species had taken place. This was usually accomplished after 72 hours. The host plant fragments were removed. Coverslips with attached spores were put into petri dishes containing ESW and placed in the growth chambers.

Endophytes were isolated from *Delesseria sanguinea* (Huds.) Lamour. (Rhodophyta) from the shores of the Isle of Cumbrae (Firth of Clyde), *Polysiphonia*

lanosa (L.) Tandy (Rhodophyta) from Ardmore Point in the Clyde Estuary, and *P. elongata* (Huds.) Spreng. from Galway Bay, Ireland. Hand and freeze microtome sections from the holdfast of *Delesseria* and thick basal regions of *P. elongata* were found to contain endophytic filaments of red algae. These sections and fragments of *P. lanosa* were put into petri dishes containing **ESW** and incubated in the growth chambers.

Eventually filaments of the endophyte grew from the 'host' material. Those external filaments which were continous with those within the 'host' were removed with sterile glass needles and placed in drops of culture medium on cover glass as described above, and isolates obtained either from released spores or fragments of filaments.

Endozoic forms were isolated from *Obelia geniculata* L. . These hydroid colonies were growing on stipes and fronds of *Laminaria saccharina* (L.) Lamour. (Phaeophyta) brought from the Isle of Cumbrae. Pieces of these hydroids containing endozoic red filaments were put into petri dishes containing **ESW** and incubated in the growth chambers. Filaments growing out from the 'host' were then isolated as described above. After crude cultures of all the above isolates were established, reduction of diatom contaminants were carried out where necessary by adding Germanium dioxide GeO_4 (0.5 mg. l^{-1}), and by using the micropipette technique, using sterilized glassware, and by repeated washing and picking out of clean filaments followed by spore settlement, unialgal cultures of the isolates were obtained.

2.1.1 Culture media

All cultures were carried out in enriched seawater medium (Boney and Burrows, 1966; **Appendix 1**). The salinity of the **ESW** was 27.4‰ while that of seawater was 31.3 ‰.

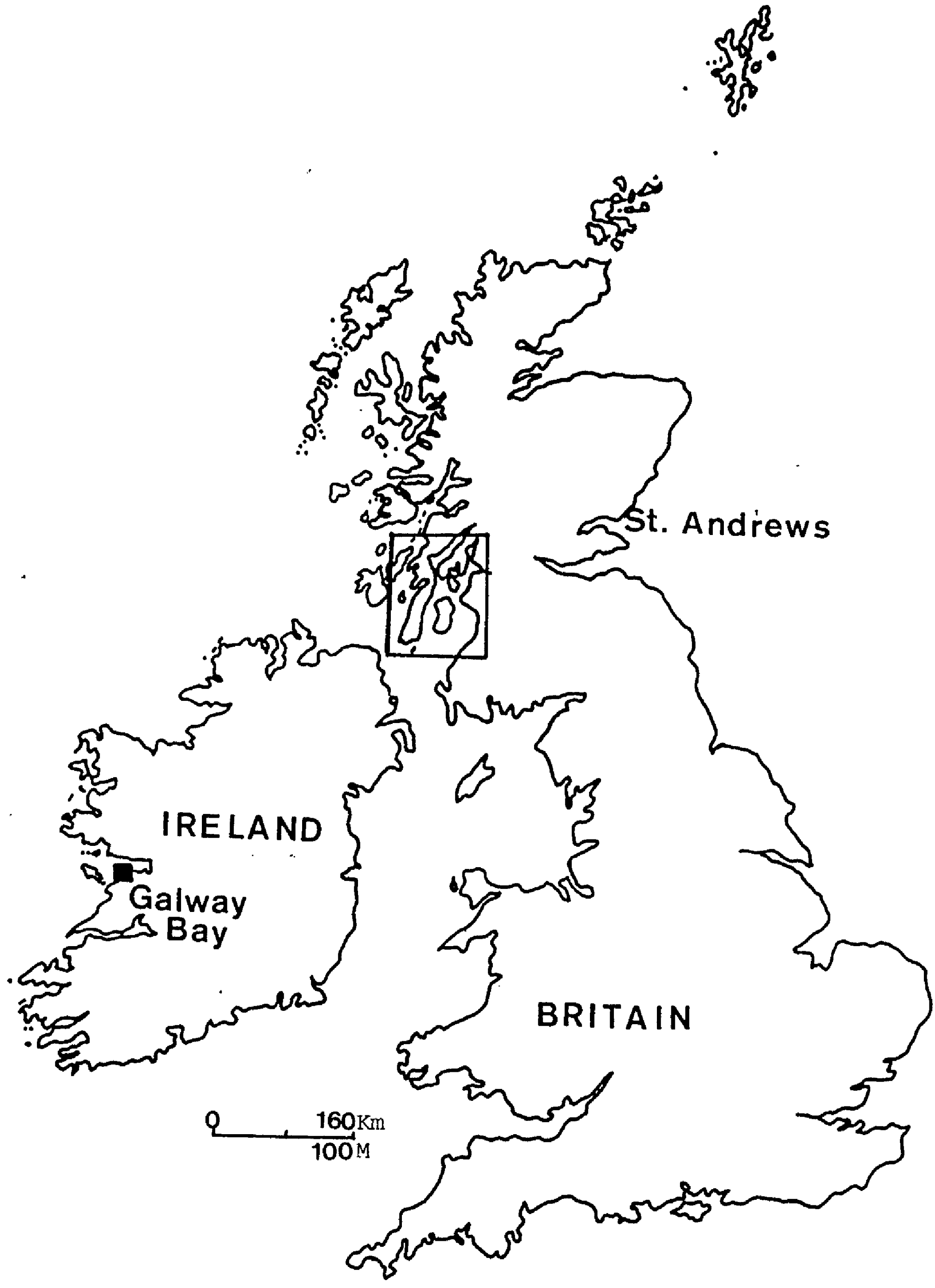


Fig. 2.1. Sampling cites in the British Isles.

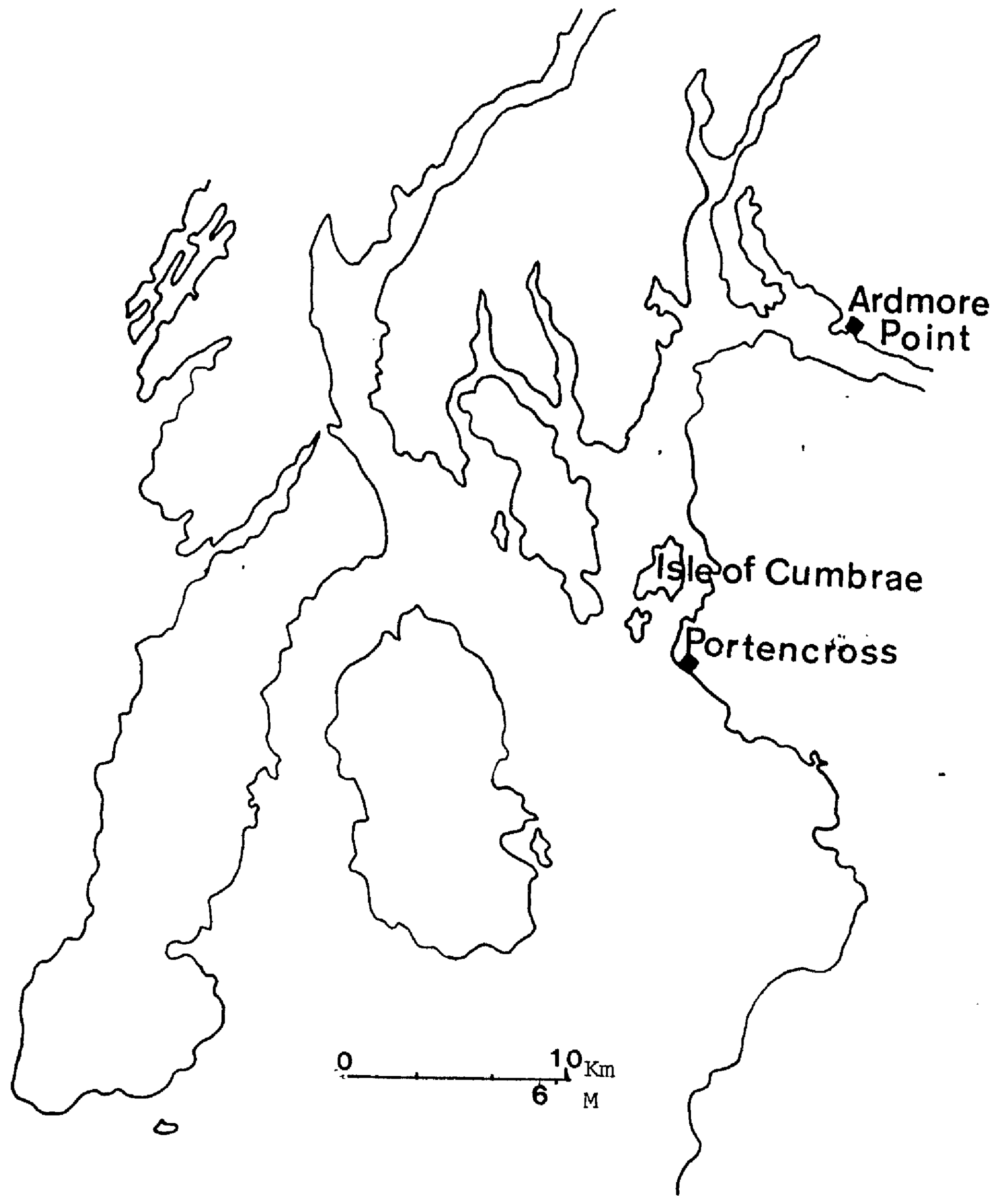


Fig. 2.2. Enlarged area of sampling cites in the Firth of Clyde and Isle of Cumbrae.

2.1.2 Growth conditions

All cultures were grown in growth chambers thermostatically controlled at temperatures of 10-15^0 C, and with a 16:8 Light and Dark cycle. Light intensity (Photosynthetic Active Radiation **PAR)** was varied from one shelf to another ranging between 9.1-22.4 $\mu E\ m^{-2}\ s^{-1}$ measured by SKP 200 radiometer (Skye Instruments Ltd.) calibrated with sensor SKP. This variance was due to their distance from light source (white cool fluorescent).

These variations in light intensities were beneficial and necessary to these plants as some of them are very sensitive to light and were found to die or to lose their colour at high light intensities. Especially sensitive isolates were kept under layers of muslin or covered by a paper towel when required.

Stock cultures were also kept in cool short day growth chambers at temperatures of 2-5^0 C and 8:16 L. D cycle, or on the bench in the laboratory at room temperature and relatively low light intensity.

2.2 Histochemical Studies

2.2. a. Nuclei

Tufts of *Audouinella* species were stained as described by Prakasa Rao (1953). Diameters of nuclei, and lengths and diameters of cells were recorded from freshly prepared slides. Up to 100 cells and nuclei were usually measured. The volumes of the nuclei and cells were calculated from equations $V = 4/3\ \pi R^3$ and $V = \pi R^2 H$ (where R = radius of nuclei and cells and H is the length of cells respectively).

Staining procedure

1. The material was fixed in 3 : 1 absolute alcohol-acetic acid for 10 minutes.
2. The fixed material was brought down to water through 90, 70, 50, and 30 per

cent alcohols and then washed with 2-3 changes of distilled water.

3. The material was put into freshly prepared 4% ferric alum solution as mordant for 5-20 minutes and then washed with 2-3 changes of distilled water.

4. The material was placed on a slide and acetocarmine added. with the coverslip in position, the stain and plant material was heated to boiling twice or thrice.

5. Fresh acetocarmine was added at margin of coverslip, the slide heated again and kept warm on hot plate for 10 minutes.

6. Slides were examined directly and measurements were made.

b. Cell wall

Different chemical stains and reagents, as described below, were applied to fixed or fresh material of *Audouinella* species under investigation.

1. Alcian blue and Alcian yellow

These two compounds stain sulphated and carboxylated poly- saccharides blue and yellow respectively. If the wall contains both types of polysaccharide a green colour is obtained (Parker and Diboll, 1966). The material was fixed as described by Waugh (1960), under reduced pressure for 15 minutes in formalin-acetic acid (formalin 5 parts, glacial acetic acid 5 parts, and 50% alcohol 90 parts). Then the materials were washed in distilled water and stained as follows.

1. Alcian blue (0.5% pH 0.5 adjusted with N HCl) for 60 minutes.
2. Wash in distilled water adjusted to pH 0.5 for 10 minutes.
3. Wash well in distilled water.
4. Alcian yellow (0.5% pH 2.5 adjusted with N HCl) for 60 minutes.
5. Wash in water.

Then the materials were dehydrated in a series of alcohols.

50% for 15 minutes

70% for 15 minutes

95% for 5 minutes

absolute for 5 minutes

The materials then were cleared in xylol for 30 minutes and mounted in Canada Balsam. However the time of staining in most cases was increased up to two hours as tufts of some species were compacted. Thus the dyes required more time to reach every cell in the tuft.

2. Aniline blue

This dye stains cellulose in cell walls blue. It was prepared by dissolving one gram of aniline blue in 100 ml of distilled water. The dye was applied to fresh material for a few minutes, then the stained material washed in distilled water or in 1% aqueus HCl solution and washed again, and mounted in glycerine jelly.

3. Chloral hydrate-Iodine stain

This mixture stains cellulose in cell walls blue after treatment with strong sulphuric acid.

The reagent was prepared by dissolving of 80 grams of Chloral hydrate in 50 ml of distilled water, and adding the iodine crystals to the solution. The fresh material was stained and then transferred to concentrated or diluted sulphuric acid (H_2SO_4). Use of this combination proved unsuccessful, however.

4. Chlorazol Black E stain

A saturated solution was prepared in 70% alcohol. The dye was applied to fresh material for few minutes, and then was used to test for chitin. It stains chitin in cell walls green.

5. Ruthenium red

The solution was made by dissolving 0.1 gram of ruthenium red in 500 ml of distilled water, and was then stored in the dark. Before application of the stain, the material was fixed in acid alcohol fixative (Hydrochloric acid 1 : alcohol 3), and the material was then placed in dilute ammonium hydroxide solution. This dye was used to stain pectic material in the cell wall, which gives a deep red colour.

6. Thionine

The dye solution was made by dissolving 1 gram of thionine and 2.5 gram of phenol in 100 ml of distilled water. The stain was applied to fresh material. Cellulosic materials in the cell wall become purple-red in the presence of the stain.

7. Toluidine blue stain

This dye was not freshly prepared and was applied to fresh material for a few minutes. It was used to stain sulphated and carboxylated polysaccharides, and phenolics in the cell wall pink and blue respectively.

2.3. Processing of materials for examination by electron microscopy

a. Scanning Electron Microscope

a.1. Fixation

Tufts of different endo- and exobiotic forms of *Audouinella* species growing on chitin, calcium carbonate (mollusc shells) or glass substrata (smooth and rough) were fixed in Glutaraldehyde-sodium cacodylate-seawater (prepared as in **Appendix 2**) for one hour.

a.2. Washing

The fixed materials then were given 3 changes of sodium cacodylate in seawater buffer for 15 minutes each.

a.3. Post fixation

The materials were post fixed in 4% Osmium tetroxide buffer for one hour then topped with distilled water twice for 10 minutes each.

a.4. Dehydration

The materials were dehydrated in a graded series of acetone concentrations at room temperature.

30%	10 minutes
50%	10 minutes
70%	10 minutes
90%	10 minutes
Analar acetone	2 for 10 minutes
Dried analar acetone	10 minutes

The materials were critical point dried, gold coated using **SEM** coating unit E5000 (Polaron Equipments Limited), and examined under the scanning electron microscope (Philips SEM 500).

b. Transmission electron microscope

b.1. Processing the plant material

1. Fixation.

Tufts of *Audouinella* species were cut into small pieces and fixed in 5% Glutaraldehyde in 0.1 cacodylate buffer containing 0.25M sucrose at pH 7 on ice for 24 hours (Evans and Christie, 1970).

2. Washing.

The materials were then given three 30-minute washes of 0.1M cacodylate buffer

with decreasing sucrose concentrations (0.25, 0.125 and zero) on ice.

3. Post fixation.

The materials were post-fixed in 1% OsO_4 (buffered as above) for 5 hours on ice.

4. Dehydration.

The materials were dehydrated in a graded series of ethyl alcohol on ice:

30% ethyl alcohol for 30 minutes

50% " " " " "

70% " " " " "

85% " " " " "

95% " " " " "

100% " " " " "

100% " " " " " at room temperature

100% propylene oxide for 30 minutes (2 changes) at room temperature.

5. Infiltration.

Materials for infiltration in resin were placed in a 1:1 mixture of resin : propylene oxide at room temperature. The amount of propylene oxide in the mixture was gradually decreased through evaporation by leaving the vials open overnight in the fume cupboard.

6. Embedding.

When infiltration of the materials was completed, the samples were dried by paper tissues and embedded in a pure resin in small aluminium dishes. The formula of the resin was as follows:

Epon (Epicote) 812 40 cm^3 | |
Agar 100 Resin
DDSA 40 cm^3 | mixed well | mixed well
Dodecenyl Succinic anhydride
MNA 20 cm^3 | |
Dodecenyl Succinic anhydride 'de
MNA 20 cm^3 | |
Methyl nadic anhydride

The resin was prepared during the infiltration and stored in a deep freeze to be used the following day.

Supplied by Agar Aids 66a Cambridge Rd. Stansted, Essex.

7. Polymerization.

Polymerization was carried out in ovens at three different temperatures:

35^0 C for 12 hours

45^0 C for 12 hours

60^0 C for 12 hours

b.2. Sectioning and staining

Ribbons of ultra-thin sections were cut with the LKB 8800 ultratome III using 6 mm thick glass knives made with the LKB 7800 knife maker. The ribbons were floated on to the surface of water in a bath made from silver self-adhesive tapes attached to the knife. The ribbons were then flattened with chloroform and collected on copper grids coated with a thin collodion support film (2% cellulose nitrate in amylacetate).

A double staining procedure with uranyl acetate and lead citrate was used as follows.

1. The grids were floated (sections side downwards) on the meniscus of solution of saturated uranyl acetate in water (7.5%) at room temperature for 30 minutes. The stain was contained in an overfilled polythene cup (6 mm in diameter). The grids were then washed with distilled water and dried on velin tissue.

2. The grids were floated on drops (sections side downwards) of lead citrate (Reynolds, 1963) in a petri dish. The lid of this dish was lined with dental wax to make

an air-tight seal and pellets of KOH surrounded the drops of the stain on the wax seal, so as to absorb CO_2 and hence prevent contamination of the specimens with lead carbonate crystals which are insoluble and extremely hydrophobic (Hayat, 1970).

The sections then were observed by a Philips 301 electron microscope.

2.4. Phycoerythrin pigments in *Audouinella* species

Samples of *Audouinella* species and a *Callithamnion* sp. (used for comparative reasons) were extracted as described by Glazer *et al.* (1982):-

The samples were suspended in 0.1 ml 0.05M Na-phosphate buffer (at pH 7) containing 1 mM NaN_3 and 1.2% Triton X-100 (v/v). The samples were ground at room temperature in glass homogenizer. The extract was centrifuged for 2 minutes. The supernatant was applied to a sucrose density gradient. The sucrose density gradient consisted of 1.5 ml quantities of 0.5M and 0.3M -sucrose, and 1.0 ml 0.1M sucrose, all in 0.05M Na-phosphate plus 1mM NaN_3, (pH7). Centrifugation was performed in MSE high speed 25 centrifuge (Measuring and Scientific Equipment Ltd. London) at 30.000 rpm for 15-16 hours at 10^0 C. The phycoerythrins were isolated by pipetting the other pigments for the top of the gradient. Visible absorption spectra were recorded with Unicam SP 8000 UV Recording Spectrophotometer.

2.5. Calorific values of some *Audouinella* species

Tufts of Audouinella species were freeze dried using liquid nitrogen and vacuum refrigeration. Then the material was immediately made into small pellets. Because of softness of the material it did not require grinding as in hard material, and the whole tufts were homogeneous. The pellets were kept in a desiccator until use.

The prepared material were combusted in Phillipson's microbomb (Model A.H.9 Micro-Bomb Calorimeter Newham Electronics Ltd) Fig. 2.3 assembled as in Fig. 2.4, and following the firing technique described in **Appendix 3.**

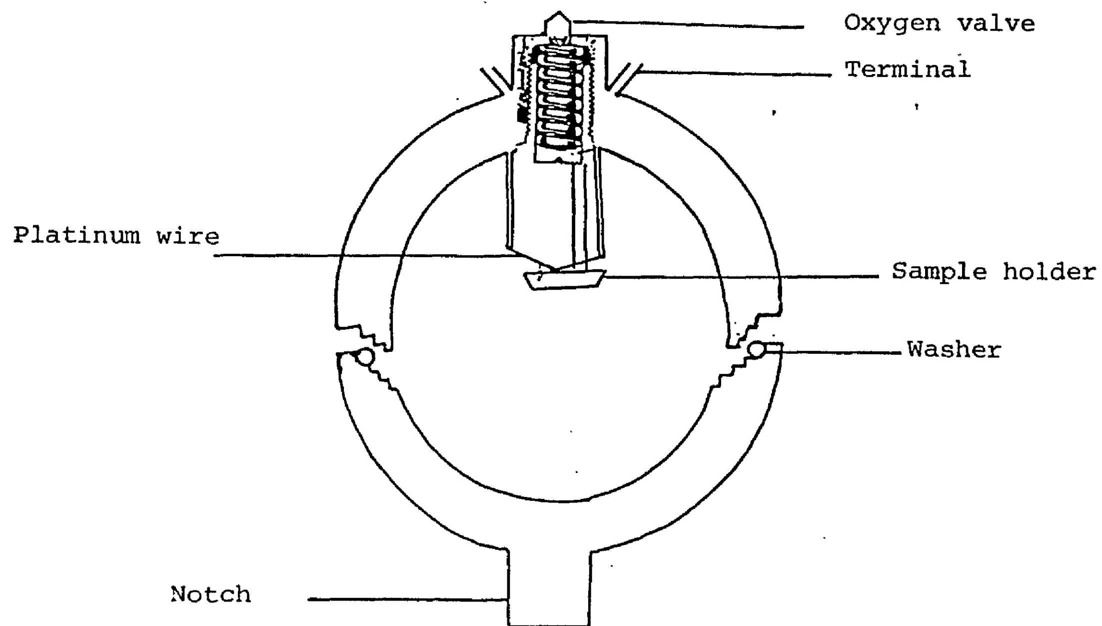


Fig. 2.3 shows a diagram of Phillipson`s Micro-bomb.

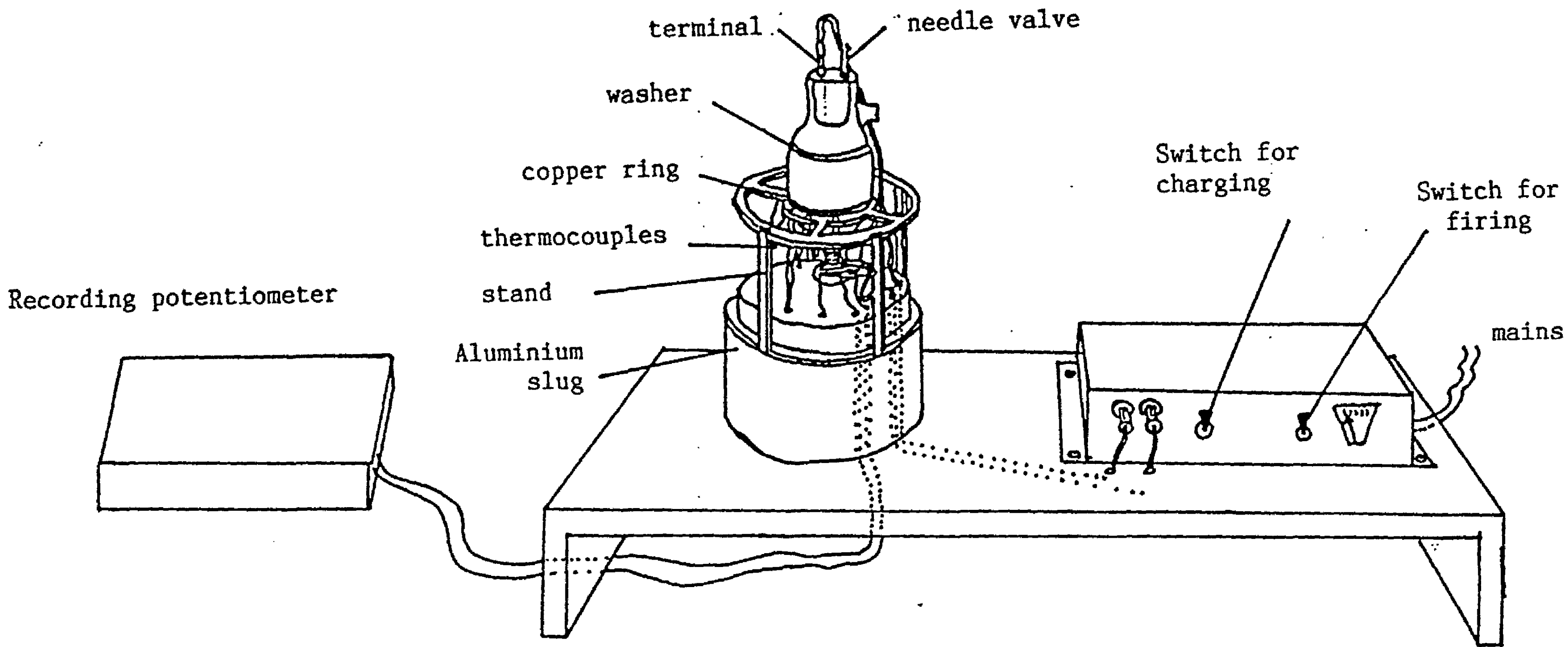


Fig. 4.4. shows the firing assembly.

The bomb calorimeter is made of stainless steel and consists of two parts which can be screwed together, the washer between these parts being of a 2 mm thick "teflon" sheet. When the bomb is assembled, the internal cavity is spherical and has a capacity of approximately 8 ml, with an outer diameter of 34 mm and height of 86 mm. The upper part of the bomb carries the pressurizing valve, the positive and negative terminals of the firing circuit on the outer side, and the sample holder and internal firing terminals to which platinum wire of 0.1 mm thick is connected. One of the terminals is insulated by means of glass bead fused onto the wire and the other terminal is connected to the bomb body. The base of the lower part of the bomb has a square notch to stabilize the bomb in the vice when tightening its two parts together.

In use the bomb rests on the stand which consists of the aluminium slug, copper ring and thermocouples join the aluminium slug and the ring.

The bomb and the stand were connected to a recording potentiometer (RE 54/20 Potentiometer Recorder, Venture).

The bomb was calibrated using benzoic acid as standard material, following the same procedure used in firing technique and using a series of pure and dry benzoic acid bellets varying in weight from 5-20 mg. Then a calibration graph of pen recorder chart divisions against energy content was drawn using the relationship 1 mg benzoic acid = 6.324 calories.

In each combustion, whether of *Audouinella* material or benzoic acid, a typical curve, (Fig. 2.5), representing the maximum temperature detected by thermocouples, was produced. From these curves ΔH (the amount of heat produced by the combustion of the material) was calculated. A correction was applied in each case, on the assumption that the bomb continues to cool or warm up (pre-firing condition) for a short while, and that heat starts to be conducted away before it reaches the peak of the increase in temperature. By convention it is assumed that the initial cooling (or warming) continues until 60% of the measured peak is achieved. Therefore, the true peak of heat output is higher than that indicated by the pen recorder.

Thus to obtain a corrected ΔH for each sample the following calculation was followed in each case.

1. The line **AB** was drawn, then from the peak of the curve (point **C**) the line **CD** parallel to **AB** was drawn.

2. 60% of the value of the apparent ΔH was calculated (peak point - baseline) . 60/100 and added to the baseline.

3. The corrected point was marked on the curve and then the vertical line **EF** was drawn through this point.

4. The line **GH** was drawn to extend the original baseline, to account for the gradual change in the baseline (due either to heat loss or gain by the calorimeter) which occurred before firing.

5. The points at which the lines **CD** and **GH** intersect the line **EF** (**K** and **L** respectively) were recorded.

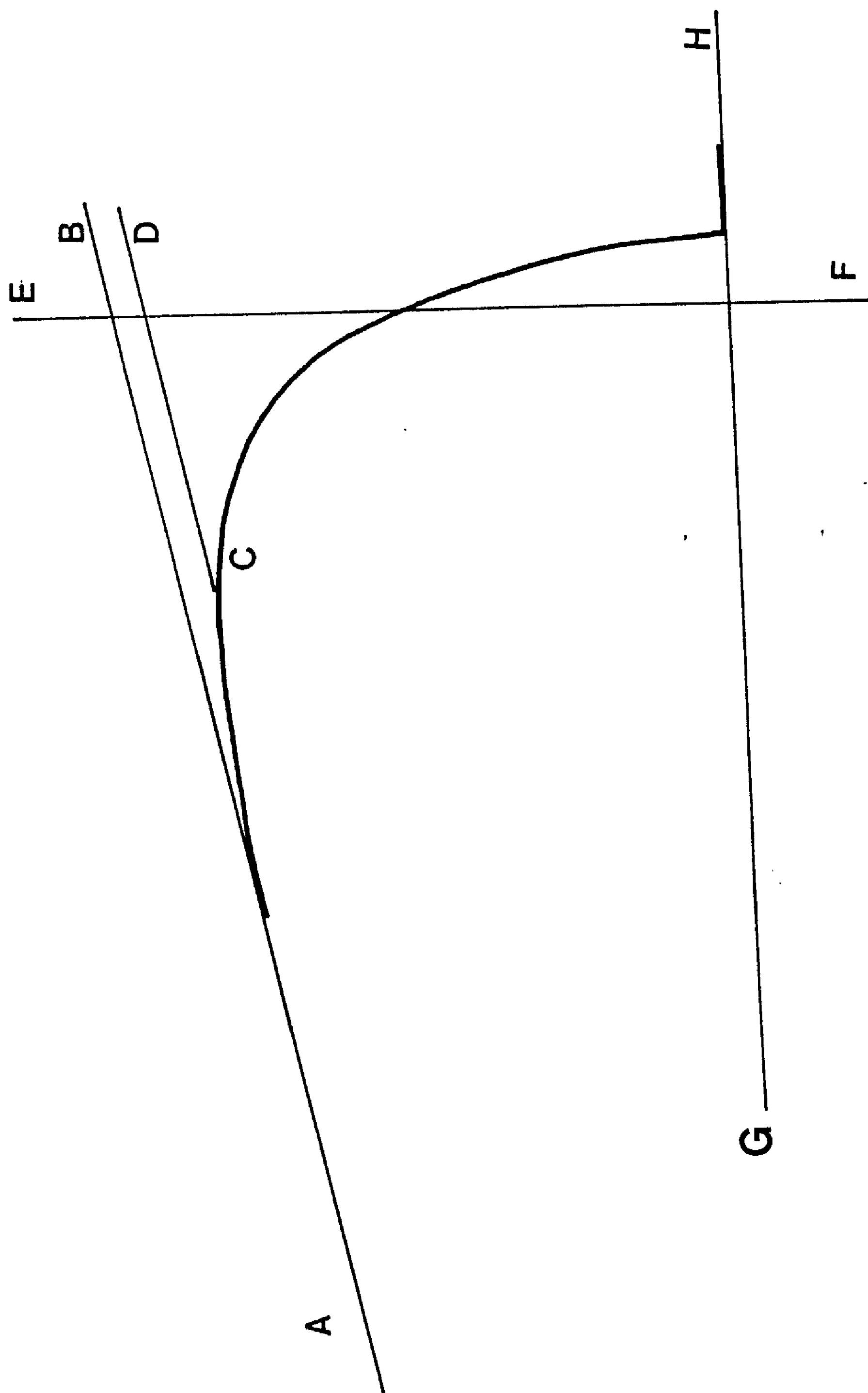


Fig. 2.5 shows the typical curve obtained with combustion of benzoic acid.

6. The actual ΔH (corrected for heat loss) was calculated (i.e **K** - **L**) units.

7. Using the calibration line of the benzoic acid the weights of benzoic acid which would produce the same values ΔH were determined. These weights (in mg) were converted into calories by multiplying these weights by 6.324 since 1 mg of benzoic acid yields 6.324 calories on combustion.

8. Finally, the calorific content of each sample expressed as calories per mg ash-free material was calculated.

2.6. Video recording of monospore release in *Audouinella* species

The apparatus consisted of a microscope (C. Baker London), a Camera (Philips), a television monitor (Rediffusion Stirling) and a videocassette recorder (Sony U.matic) recorder connected together. Mature tufts of epiphytic *Audouinella* species bearing monosporangia were placed on slides with a drop or two of fresh **ESW** medium. The slides were put under the microscope, and spore releases were viewed on the monitor and simultaneously recorded on tape by the videocassette recorder. The emission of the monospores was then viewed on a JVC CP5500 E video player connected to a 21" Sony television monitor and the process timed and slowed down so that the fine details of the process could be closely observed and photographed frame by frame. Photographs of single frames were taken from the monitor by a Nikon F3 Camera with a 55 mm micro lense using an Ilford black and white film. Hand drawings were made according to different sequences of the release of different spores viewed on the monitor.

2.7. Adaptation of exo- and endobiotic *Audouinella* species to substrata

Five species of endophytic and endozoic *Audouinella* were isolated from hosts described earlier. Unialgal cultures of these species were introduced to cultures of algae species described below or to fresh, dried or autoclaved *Obelia geniculata*

isolated from *Laminaria saccharina* (Phaeophyta).

Audouinella species under study were introduced to the 'host' culture as spores where possible, or as fragments of the alga.

Cultures of *Fucus spiralis* L. (Phaeophyta) and *F. serratus* L. were started as germlings of each species, *Gigartina stellata* (Rhodophyta) was started as spores, *Cladophora rupestris* (Chlorophyta), *Ceramium ruprum* (Rhodophyta) and *Polysiphonia lanosa* (Rhodophyta) were obtained by excision and transfer of apical fragments clean of epiphytic growths to a fresh sterilized media. All cultures were carried out in the enriched seawater medium **ESW** (described earlier) at irradiance of 17.5 μm^{-2} s^{-1} at 16:8 Light and Dark cycle and at 15^0 C.

The experiments were carried out in tissue culture multi-well plates of 3.5 ml per well, or in small petri dishes.

Small filaments, fragments or spores where possible of the epiphytic, endophytic, or endozoic *Audouinella* species were placed on pieces of chitin obtained from crab shells (Sigma Chemical Company), or dried and autoclaved pieces of *Obelia geniculata* isolated from colonies on stipes of *Laminaria* species. *Audouinella* species fragments and chitin hydroid pieces were placed in small petri dishes containing **ESW** medium, and left in growth chamber at the same conditions described above for several days. The petri dishes were daily checked after the first five days, the germinating time of spores. After attachment to or the invasion of the chitin was achieved, and tufts were well grown, the tufts were lifted or scraped from the surface of the chitin pieces in order to reveal the growth inside the chitin.

Filamentous fragments, or monospores where possible, were placed on the above sterilized material with a drop or two of **ESW** medium and placed into small petri dishes with moistened filter paper. The petri dishes were kept into growth chambers for few days and fresh medium was added to the material and few drops of distilled water were added to the filter papers.

The calcium carbonate and aragonite with attached filaments or germinated spores were transferred to petri dishes containing fresh **ESW** medium and incubated in the growth chambers with conditions described above. After 2 to 4 weeks the materials with its surface growths and internal filaments were processed for Scanning Electron Microscopy as described earlier (2.3.a.). Photographs by **SEM** of the material were taken.

Observations were recorded by Orthomat Camera (Leitz Wetzlar) mounted on an Ortholux Microscope (Leitz Wetzlar Germany). Drawings were made using Sankei Tube (Sankei Co., Ltd. Tokyo Japan). Observations were also made by Scanning Electron Microscopy. The materials for **SEM** were processed as described earlier (2.3.a.).

Filaments of *Audouinella* species were placed in agar (1% agar + **ESW**) in petri dishes. The petri dishes were kept in growth chambers with conditions described above. The growing cultures were observed under low power magnification of light microscopy. Small pieces of agar containing growing filaments were cut by razor blade, examined, after the agar had been squashed in order to use higher magnification, and photographed.

2.8.a. Hair formation

Mature tufts of *Audouinella secundata* and *A. virgatula* were placed on glass coverslips for spore release. Drops of fresh **ESW** medium were added daily to the tufts for five days. As soon as spores were released, the tufts were removed from the coverslips. After 5-7 days the spores were found to attach to the coverslips and had started to germinate. Coverslips with the germinated spores were transferred to small petri dishes containing fresh enriched sea water (**ESW**) medium (described earlier) with different enrichments. These media were either lacking nitrate **N**, lacking phosphate **P** or lacking both nitrate and phosphate. Those coverslips with germinated

spores which were used as controls were placed in petri dishes of the same size containing **ESW** with full **N** and **P** enrichment.

The petri dishes with germinated spores were subjected to different light irradiance 26.6 and 2.66 $\mu m^{-2} s^{-1}$ at 16:8 Light and Dark cycle and different temperatures 15 and 10^0 C.

The experiment was carried in triplicate and for different time periods up to three months.

2.8.b. Regeneration in *Audouinella* species

Mature tufts of exo- and endobiotic *Audouinella* species were broken into small fragments, occasionally 2-3 cells, and placed into plastic petri dishes containing fresh **ESW** medium (described earlier). Petri dishes with these fragments were placed into growth chambers at 16:8 Light and Dark cycle with irradiance of 26.6 μm^{-2} s^{-1}. and temperature of 15^0 C. Observations were made and recorded on a daily basis.

Other cultures of epiphytic *Audouinella* from other experiments were brought back to normal conditions and kept under observation.

2.9. The effect of daylength and night breaks on the life history and morphology of *Audouinella* species

This study was carried into two parts. The first part was concerned with day length and the second was concerned with night breaks in which light periods were used to interrupt dark periods.

Monospores of *A. secundata* and *A. virgatula* were released on clean glass coverslips and left for 3-5 days for attachment and germination. The coverslips with the germinated spores were put into 50 ml shallow plastic petri dishes containing **ESW** medium. The petri dishes then were put into light boxes (Fig. 2.6) which were constructed in the department workshop as described below by Walland *et al.* (1983)

either under 16:8 or 8:16 hours of Light and Dark cycle with Quantum irradiance of 16 or 10 $\mu E\ m^{-2}\ s^{-1}$, and at 15^{0} C.

Light-tight boxes of 30.0 X 10.5 X 11.5 cm (Length, width and height respectively) dimentions with hinged, counterbalanced lids were constructed from plywood and painted with several coats of white epoxy paint, and foam rubber was used to provide a light tight closure. Hinges used on the lid were free swinging and relatively loose in their action to prevent binding. Two heavy duty push-pull solenoids were fit to each box and connected with relays which were connected with programmable microprocessor suplied by (RS Components Limited, London). When energized by the relay, the plunger of a solenoid makes a sudden, forceful excursion. This kicks the freely hinged, easily swung, counterbalanced lid open or closed depending upon which solenoid was energized.

The apparatus was used in a walk-in growth chamber with continous light and thermostatically temperature controlled.

The experiment was carried for more than one generation, where possible, using spores obtained from cultures grown under each regime. Observations were made on spore production; the time required to reach spore formation; measurement of cell size of five consecutive cells per erect filament; and the number of erect filaments per attaching system were recorded wherever possible.

In the second part two endo- and one exobiotic *Audouinella* species were investigated.

Monospores of *A. virgatula* or sporelings of *A. infestans* and *A. asparagopsis* were grown in the light boxes described above, 8:40 hours Light and Dark cycle with Quanta irradiances of 14, 24, or 60 $\mu E\ m^{-2}\ s^{-1}$. Two hours of light breaks were given after 14 hours of darkness in each experiment.

Cultures were checked, after spores were seen to be released, for any specific reproductive structures formed under these conditions.

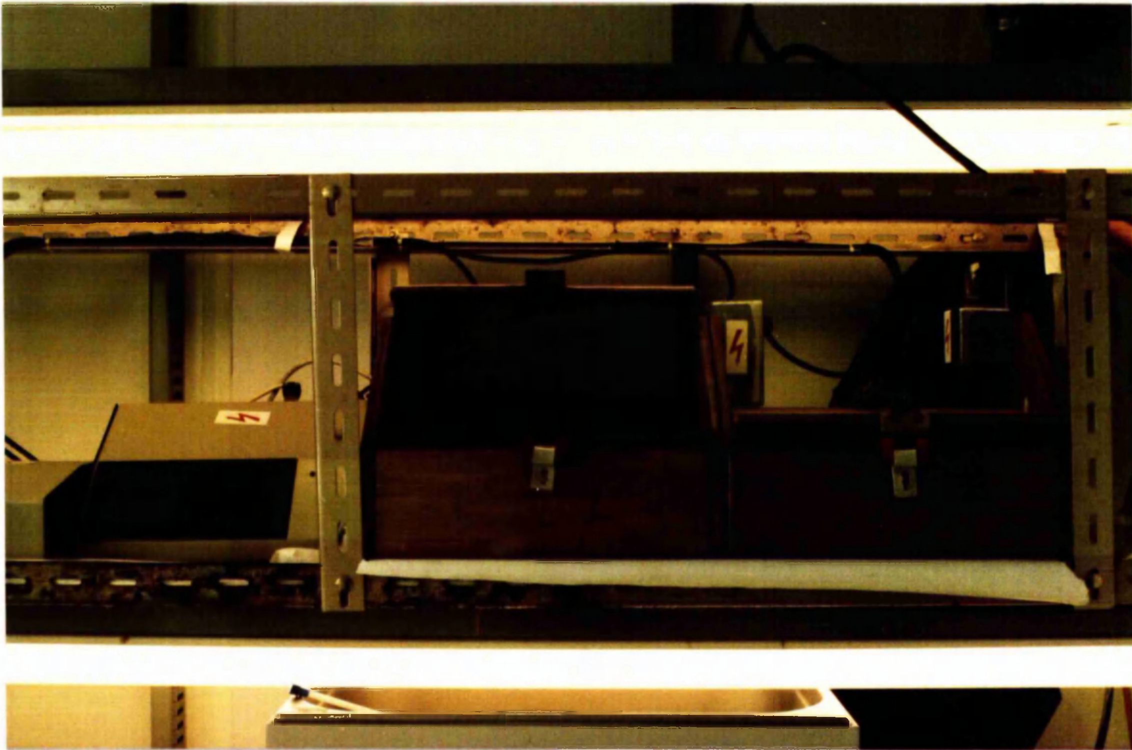


Fig. 2.6 shows the light boxes assembled in the growth chambers.

2.10 Photosynthesis and chromatic adaptation in *Audouinella* species

Measurement of photosynthesis by the use of the oxygen electrode:

The oxygen evolution was measured in a modified Clark oxygen electrode (Hansatech) of the type described by Dilieu and Walker (1972). The apparatus consisted of a platinum wire sealed in plastic as the cathode, and a circular anode of silver wire bathed in a saturated potassium chloride (KCl) solution. The electrodes were separated from the reaction mixture by an oxygen permeable teflon membrane. The reaction mixture in the central cell was stirred constantly with a small magnetic stirring rod. When a voltage was applied across the two electrodes using the polarising meter, the platinum electrode became negative with respect to the reference electrode and the oxygen in the solution is thought to undergo electrolytic reduction at the cathode:-

$$4H^{+} + O_2 + 4e^{-} = 2H_2O$$

The flow of current in the circuit when the polarising voltage was set between 0.5 and 0.8V varied as a linear relationship to the partial pressure of the oxygen in solution. The instrument was usually operated at a polarising voltage of about 0.65V. The current flowing was measured by connecting the electrode to a sensitive potentiometric chart recorder.

The reaction chamber was kept at a constant temperature by circulating water from a temperature-controlled water bath and illuminated with a light source (desk lamp) when needed and was focussed on the reaction chamber by a round flask filled with water. A diagram of the layout of the apparatus is shown in Figure 2.7 and a diagram of the electrode is shown in Figure 2.8.

Calibration of the oxygen electrode was carried out by determining the zero oxygen level (by adding sodium dithionite) and the concentration of oxygen in air-saturated ESW medium. This was related to the oxygen content of the ESW

determined by titration (Winkler method described by Mackereth *et al.* 1978). The number of divisions between saturation and zero level on the chart recorder were counted and correlated with the amount of oxygen present in the medium before starting the experiment, thus the oxygen released by the plant material is calculated accordingly.

The principle of the Winkler method is that when concentrated solutions of divalent manganese and alkaline potassium iodide are added to the water sample, white manganous hydroxide is first formed and then oxidised to manganic hydroxide by the molecularly dissolved oxygen. The brown manganic hydroxide settles to the bottom of the bottle. Sulphuric acid is then added and this dissolves the manganic hydroxide and iodine is librated. The liberated iodine takes up the excess iodine and forms I^{-3}. This solution is then titrated against thiosulphate and the end-point of the titration is indicated by starch solution.

Measurements of biomass:

Biomass can be determined as units of fresh weight, dry weight or chlorophyll. As the biomass of these *Audouinella* species were very small and the accuracy of dry weight measurements uncertain, and since the chlorophyll concentration is known to correlate linearly with the cell numbers, the chlorophyll method was used as being the only reliable one in these experiments to avoid any loss of the material during centrifugation and drying, in addition to the errors caused by presence of salt in the medium which could not be washed by distilled water because of plasmolysis problems and over all to avoid improper drying.

Chlorophyll content was measured immediately after each experiment by extracting the pigments in identical volumes of hot methanol (Holden 1965). This treatment removed all the chlorophyll from the samples. Tufts of the *Audouinella* sp. with medium taken out from the reaction chamber by pipette were centrifuged for 5

minutes; the supernatant was discarded and the pellet was resuspended in redistilled methanol. The tube containing the methanol and plant material was covered with aluminum foil and placed in a hot water bath (45^0 C) for 10 minutes. The solution was then passed through No. 1 Whatman filter paper and the filtrate collected in an aluminium foil-covered test-tube. The optical density of the resulting chlorophyll solution was measured in a 600 SP spectrophotometer against a redistilled methanol blank at 650 and 665 nm. The equation used to determine the chlorophyll content was:-

$$\textbf{Chlorophyll (mg. ml}^{-1}\textbf{) = 25.5 X O.D.}_{650} \textbf{ + 4 X O.D.}_{665}$$

Photosynthetic activity (in terms of O_2 released) was then equated to the quantity of chlorophyll (mg ml^{-1}).

Experimental procedure

The apparatus was assembled as in Fig. 2.7, which consisted of water path, electrode cell, light source and chart recorder.

Small tufts of about the same age, of exo- and endobiotic *Audouinella* species were put into the cell and subjected to the incident light which passed through the glass flask containing water used to concentrate the light on the cell and on the plant material. The water was either uncoloured and used as unfiltered light source or coloured with different dyes (Table 2.1) to give the different light spectra. (i.e, red, orange, yellow, blue and green) (Table 2.2).

The material used was either directly after incubation at normal conditions (growth chamber conditions described earlier), subjected to long dark periods 2-7 days, or grown into multi-well culture dishes covered with coloured plastic sheets, (green, blue or red) (Table 2.3). The saturation and zero point of oxygen in the medium **ESW** was determined before each experiment was carried out as described earlier.

The amount of oxygen released was expressed in terms of mg oxygen per litre per

mg of chlorophyll. Thus the chlorophyll in each sample was determined as described above.

The experiments were carried out under different light intensities using muslin layers for light intensity reduction (Table 2.4), and at different temperatures as stated below, 5, 10 or 22^{0} C.

When lower temperatures were required they were brought down by using ice bags. Adding ice to the water bath caused fluctuations in the chart readings.

To obtain a sufficient biomass for each experiment called for sizable quantities of *Audouinella* material in identical physiological conditions. It was not always possible to obtain sufficient quantities to adequately replicate measurements of photosynthetic activity, and it was also found that not all the isolates were suitable for use in the electrode.

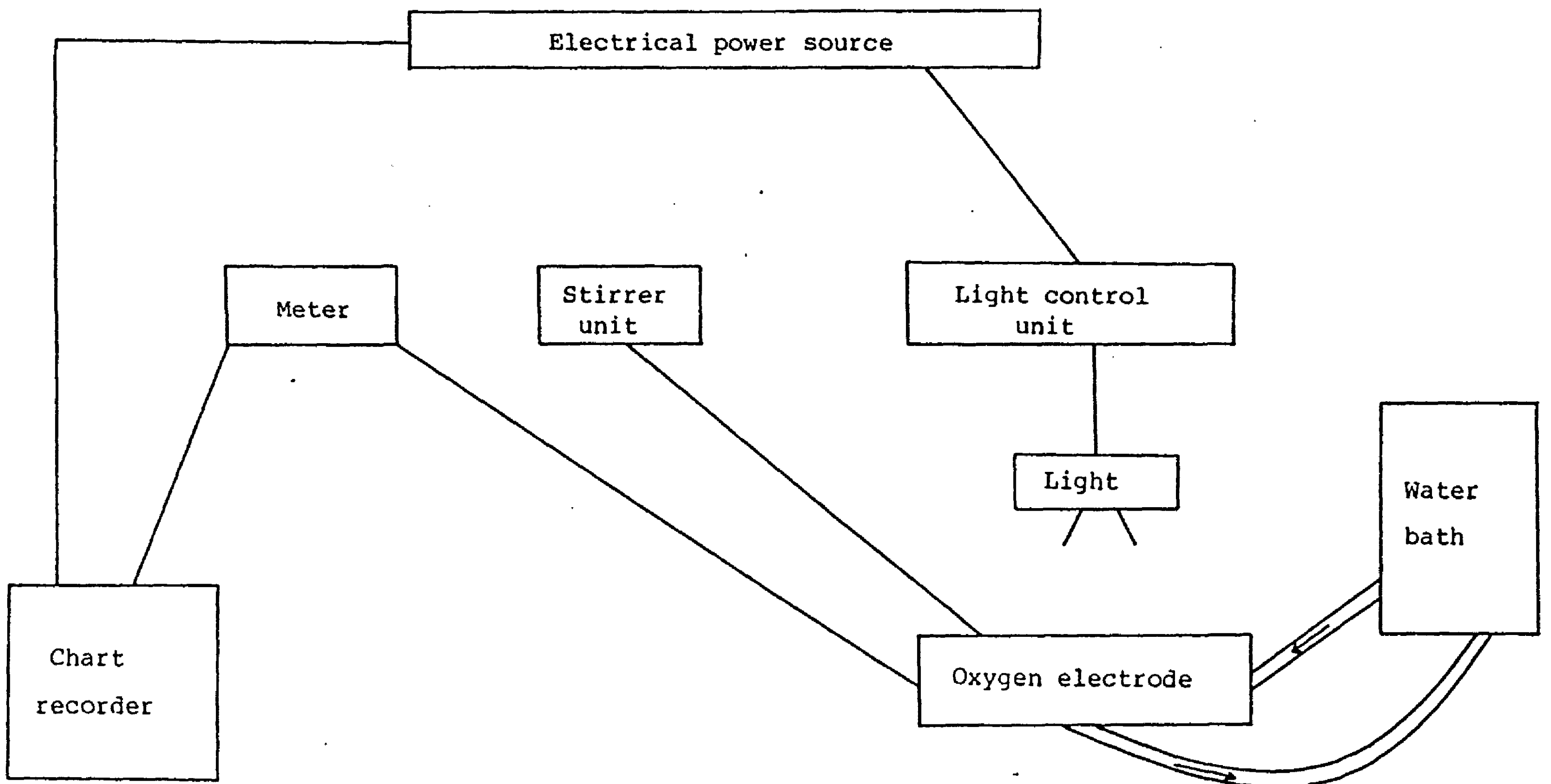


Figure 2.7 The layout of the apparatus used in the oxygen electrode experiment.

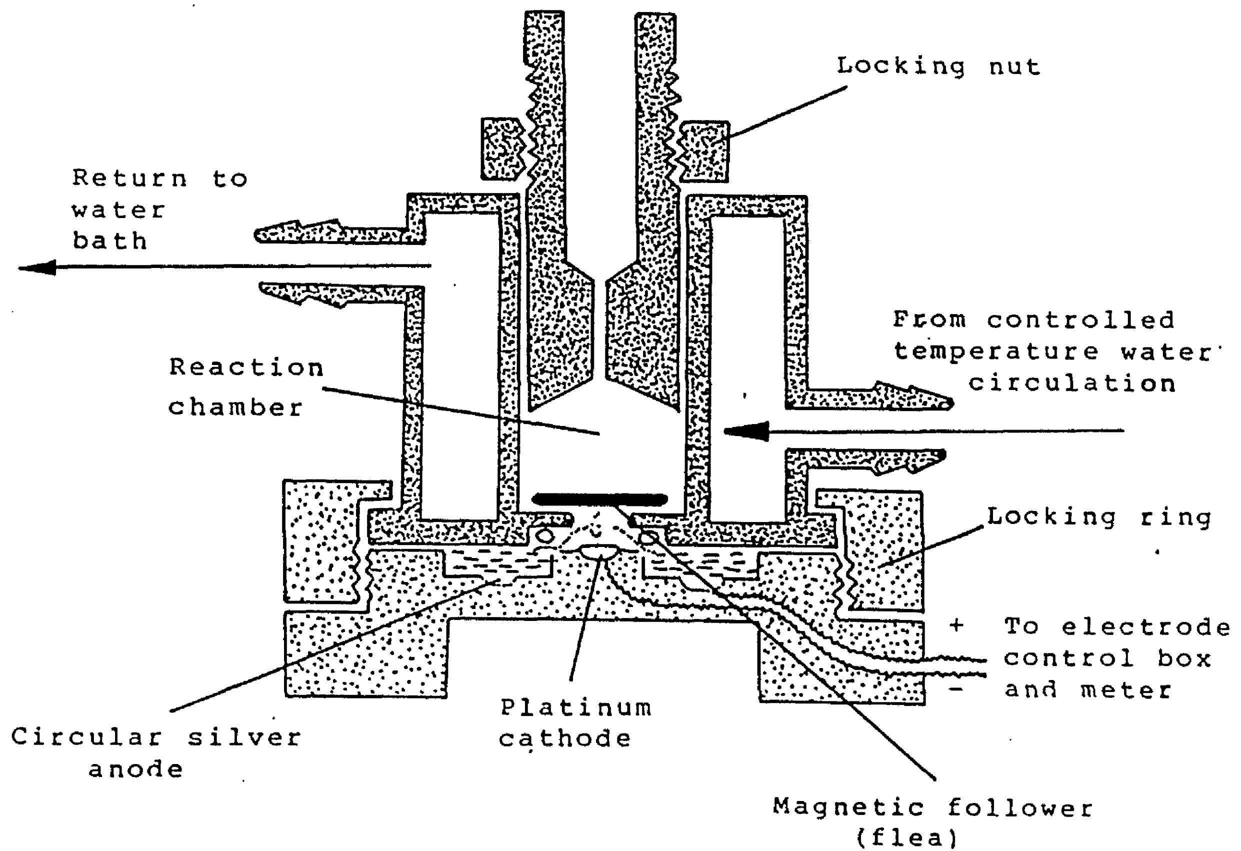


Figure 2.8 A diagram of the oxygen electrode.

Table 2.1 Chemicals and dyes used for colouring the water filters as 0.1 gm per 100 ml distilled water

Water colour	dye or chemical used		λ max (nm)
Red_1	Rose Bengal (dye)		533
Red_2	Cobalt nitrate	$(Co(NO_3)_2$	540
Orange	Potassium dichromate	$(K_2 Cr_2 O_7)$	340
Yellow	Potassium chromate	$(K_2 CrO_4)$	367
Green	Nickel sulphate	$(NiSO_4)$	390
Blue	Copper sulphate	$(CuSO_4)$	<270

Table 2.2 coloured water filters and related Photosynthetic Active Irradiances (PAR).

Water colour	Photosynthetically Active Irradiance (**PAR**) $\mu Em^{-2}s^{-1}$
Red_2	66.0
Red_1	78.3
Orange	109.4
Yellow	138.0
Green	45.2
Blue	23.0

Table 2.3 coloured plastic filters and corresponding irradiances

Plastic colour	Irradiances $\mu E\ m^{-2}\ s^{-1}$ (PAR)
Green	12.0
Blue	14.0
Red	20.0

Table 2.4 layers of muslin and irradiances

Layers of muslin	Photosynthetic Active Irradiance (**PAR**) $\mu Em^{-2}s^{-1}$
0	141.0
1	81.0
2	68.8
3	59.1
4	45.0

3. Morphology and identification of isolates.

The morphology of *Audouinella* species, as has been described earlier **(Ch.1)**, is very simple yet at the same time creates great confusion in their taxonomy. Dixon & Irvine (1977) separated the species in the flora of Britain firstly on whether they were exo- or endobiotic in habitat, and secondly on their morphology.

Acrochaetiaceae, in general, are considered as a group with great similarity and there is no logical basis for genera delineation (Dixon & Irvine 1976,1977; Russell 1978; Garbary 1979a; Stegenga 1979). Speciation, on the other hand, has proved to be confusing, because as already stated so many taxonomical characters have to be considered, i.e., morphology of prostrate and erect systems of the thallus; reproductive structures, their morphology and arrangements; germination and post germination modes; cell size and chloroplast shape; and host specificity.

The above taxonomical characters are considered to be the end-product of multi-environmental factors, such as substrata, temperature, light intensity and day length, and salinity. Thus, studies on material in nature and in culture are required for collective data on these species which may enhance our understanding of them.

Morphology of exo- and endobiotic *Audouinella* spp. in nature and in culture.

The study of these plants in nature is very difficult especially with the endobiotic species because of their microscopic size, their presence often in small quantities, their similarities, and the difficulty of keeping their hosts alive in the laboratory for long periods. Therefore, detailed study of these plants in nature is rarely obtained, and only short notes such as cell sizes, shape and branching habit, have been recorded.

Culture studies, on the other hand, provide more time and the opportunities for

close and detailed observation. Moreover, subculturing of crude cultures often shows that they contain more growth forms (and possibly different species) than what had been thought at the time of isolation. Therefore the study of these algae in culture offers more scope since they can be isolated and grown free from their 'host' organism, except for those which are proven to be host dependent. In this study one of the endophytic *Audouinella* sp. found in *Polysiphonia elongata* proved to be host dependent as all attempts to grow it free from the 'host' plant failed.

In addition to the isolation of species, cultures also produce a greater biomass of each species in contrast to nature where only a small biomass is often found. Moreover, algal species under culture conditions can be subjected to the same environmental conditions for similar periods so removing problems of different ages and physiological conditions, as in case of epiphytic *Audouinella* spp. (viz. *A. secundata* and *A. virgatula*) where their relationship has been questioned many times because of their morphological similarities in nature (Dixon & Irvine 1977). However, although more time was spent on observing these *Audouinella* spp. in culture, speciation of some of these isolates was not immediately possible using the most recent diagnostic key of Dixon & Irvine (1977).

3.1. Exobiotic species.

a. Audouinella secundata (Lyngbye) Dixon.

In nature the plants are red or brownish-red in colour, differentiated into erect and prostrate axes. Erect axes are usually unbranched near the base but with relatively abundant branching in the upper portions. The branching is usually unilateral or secund arising from every cell of the erect axes. Prostrate axes up to 2mm, abundantly branched and aggregated into a compact disc, often more than one layer in thickness. The cells are cylindrical 12-24 µm in length, 6-8 µm in diameter and increasing in size with age to reach up to 45 µm in length and up to 15 µm in

diameter, each with single, axial, and stellate or lobed chloroplast with one pyrenoid (Dixon & Irvine 1977). This alga in this study was found growing epiphytically on *Porphyra umbilicalis*, *Cladophora rupestris* and *Ceramium rubrum*. It was found to have a remarkably varied morphology on the different 'host' plants depending on its position on the 'host', and the time of collection during the year. These differences were observed in terms of tuft and cell size. The erect system cells were cylindrical, 10-18 µm in length and 10-12 µm in diameter, erect branched filaments consisted of 3-5 filaments, with unbranched filaments a few cells long, and number of cells in mature erect filaments were 12-18 cells. The upper parts of 2-3 erect filaments were secundly or unilaterally branched but not abundantly and compoundly.

In culture, on the other hand, the early stages grown from spores of this species were very similar to *A. virgatula* and *A. seiriolana*. Mature tufts (Fig. 3.1) were smaller than tufts of both of the above species. Tufts of this alga with a smaller prostrate system and with irregular cell shapes can become compacted together to form a ball like mass with outgrowths which formed the erect system. The erect system consisted of short filaments 6-13 cells long, and 3-7 erect filaments per basal mass when growing attached to a substratum and up to 11 filaments when growing free. Branching secund forming lateral branches of 1-3 cells were usually terminated by sporangia. Sporangia sizes 12-18 µm in length and 9-14 µm in width. The cylindrical cells of the erect system 12 µm in length and 10 µm in diameter, with occasionally their length equal to their diameter (10 µm) when cultured for more than one generation under the same conditions. Reproduction in this alga is by monospores which are produced at any time of the year. Their size is 12-13.5 µm in diameter. Dixon and Irvine (1977) described tetrasporangia as another form of reproduction, but these were not observed in the cultures. The plants described in this study are different in cell size from those described by Dixon & Irvine (1977) and by Garbary (1978c). This is probably because of the supporting plant or the locality.

The above observations suggest that differences are due to growth conditions and

generations of these plants, as these plants are expected to produce more than one generation at a short time in every growth season.

These short periods may have the same environmental conditions for long or short periods depending on geographical site. Therefore, culture studies of these algae from different geographical sites would be necessary before any conclusions can be drawn on morphological differences.

b. *Audouinella virgatula* (Harvey) Dixon.

Plants consist of uniseriate filaments, differentiated into erect and prostrate axes (Fig. 3.2), red or brownish-red in colour. The prostrate axes are frequently branched and aggregated into a compact disc (Fig. 3.3). Erect axes much branched with sparse and highly irregular branching, main and lateral axes often terminating in a hair. Cells cylindrical 15-20 µm in length and 6-12 µm in diameter increasing by age reaching up to 70 µm and 18 µm in length and diameter respectively. Chloroplasts are single, axial, deeply lobed, and containing one pyrenoid. Reproduction by tetra- and monospores (Dixon & Irvine 1977).

In this study *A. vigatula* was found growing epiphytically on *Porphyra umbilicalis.* It was at first overlooked because of its similarity to *A. secundata* growing on the same supporting plant. But the individual plants of *A. virgatula* were observed with hairs on lateral branches, and these individuals were subcultured and produced the experimental population used in this study. This alga under culture conditions developed mature tufts with a large prostrate system spreading on the substratum and producing a single layered encrusting growth with 3-5 erect filaments each with a few branches formed irregularly. The detached free floating tufts produced a ball-like prostrate system (Fig. 3.4) with large numbers of erect filaments (up to 13). The erect system cells cylindrical, 12-18 µm in length and 10-12 µm in diameter. Erect filaments 18-28(37) cells in length. Reproduction by monospores

produced in sporangia on single celled branches. This alga, as it aged, lost its pattern of branching and produced compound lateral branches, with a reduction in the number of erect filaments and extended growth of the prostrate system.

c. ***Audouinella seiriolana*** (Gibson) Dixon.

Rose red plants consisting of uniseriate filaments, differentiated into prostrate and erect axes. The prostrate axes are much branched and tightly compacted into a monostromatic encrusting mass of cells. Erect axes virtually unbranched except occasionally during the formation of tetrasporangia. Tetrasporangia 21-25 µm in length and 18-25 µm in diameter. Cells are cylindrical 15-20 µm in length, 8-11 µm in diameter with a looped chloroplast (Dixon and Irvine 1977).' In this study this alga was found growing epiphytically on *Ceramium rubrum* and *Cladophora rupestris*. In culture, the mature tufts were larger than those of *A. secundata* which it resembles in its secund branching. The prostrate system was also larger than the prostrate system of *A. virgatula*. Large tufts of *A. seiriolana* produce 5-7 erect filaments, 13- 30 cells long. The cells cylindrical, 15-22 (30) µm in length and 8-12 µm in diameter. Reproduction was by means of monospores produced on secund branches. *In situ* germination was also common in this aiga and could again cause confusion with tetraspore formation. On the other hand, cells of the prostrate system, under unfavourable conditiond, tend to die and lose their pigments. The prostrate system may thus appear in filamentous form because of the loss of these cells.

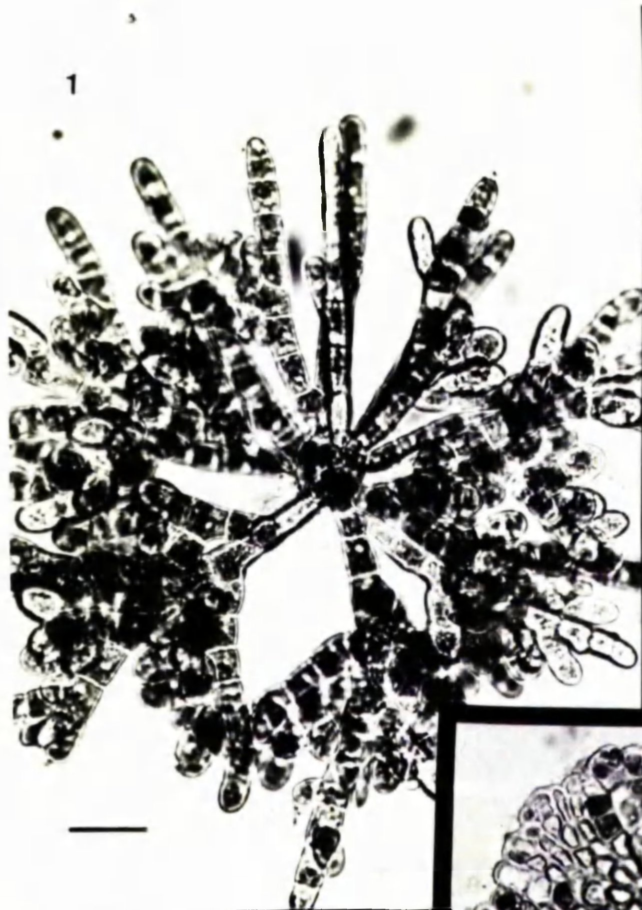
Fig. 3.1 Free living tuft of *Audouinella secundata* (bar = 25 μm).

Fig. 3.2 Free living tuft of *Audouinella virgatula* (bar = 50μm).

Fig. 3.3 The prostrate system of *Audouinella virgatula* Note the form it takes and the aggregation of the prostrate branches when it grows attached to a substratum (bar = 50 μm).

Fig. 3.4 The ball like prostrate system produced in free floating tufts of *Audouinella virgatula* (bar = 50 μm).

Figs. 3.5-6 Jovenile tuft of *Audouinella efflorescens* with persistent tetrasporangium (bar = 100 and 50 μm respectively).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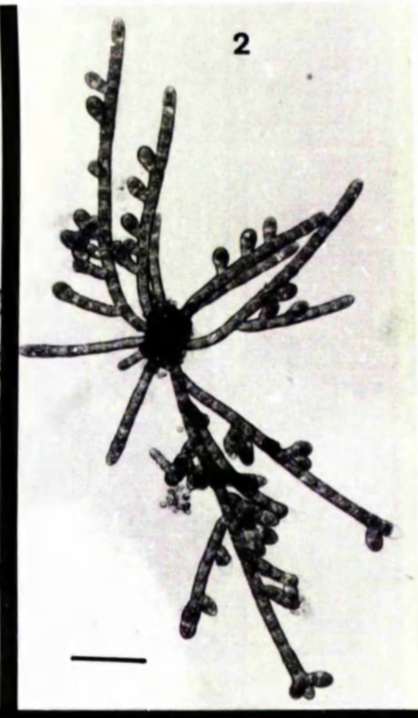
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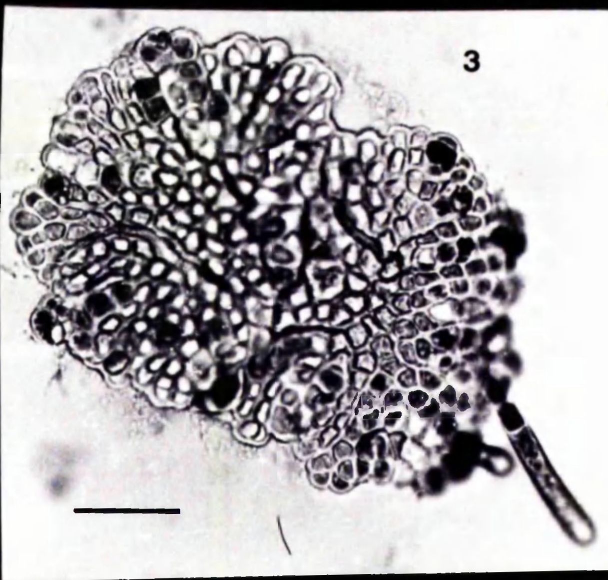
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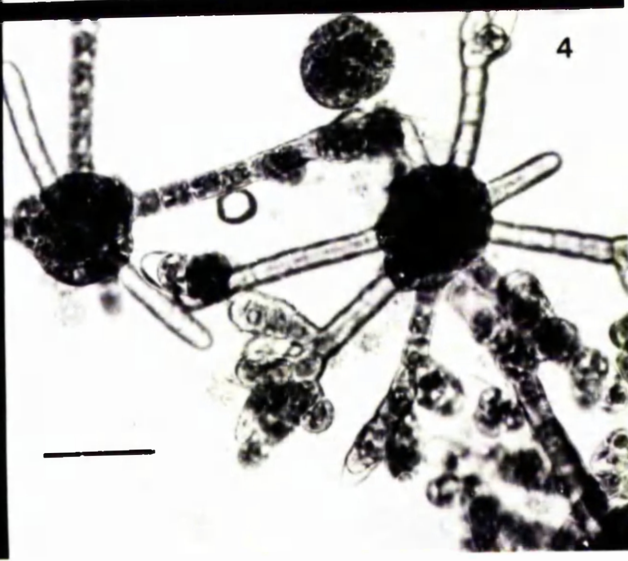
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3.2. Endobiotic species

a. Endozoic

1. Audouinella efflorescens (J. Ag.) Papenf.

Plants consisting of uniseriate filaments, differentiated into erect and prostrate axes, pale red in colour but often completely colourless towards the apices. Prostrate axes branched irregularly and compacted to a varying degree. Erect axes take different forms according to the type of the plant (i.e. gametangial or tetrasporangial). Tetrasporangial plants sparingly branched in the lower parts, increasingly so towards the apices. Cells cylindrical elongate, 45-90 µm in length, 4-7 µm in diameter, each with one or more chloroplasts which are parietal, spiral-shaped, possibly fragmenting or fusing with age. Tetraspoangia ovoid with cruciate tetraspores, formed singly or in pairs on short, usually one-celled stalks (Dixon & Irvine 1977).

This alga was found growing endozoically in *Obelia geniculata.* In culture the filaments are pink-red in colour, uniseriately branched forming felt-like tufts, easily detached from solid substrata, which suggests the absence of prostrate (attaching) system (Figs. 3.5-6). Cells were cylindrical and elongate becoming large and spherical at the ends when aged, chloroplasts parietal and spiral shaped in terminal cells. Cell sizes 30-90 µm in length and 4-9 µm in diameter. Only tetraspores were produced by this alga, found in clusters on a very short branches usually on old protected branches. Tetraspores are spherical or ovoid of 20-25 µm in diameter. This alga was found to be very sensitive to high light intensity so that shading was necessary for its growth.

2. *Audouinella infestans* (Howe et Hoyt) Dixon.

Plants in nature are pale rose-pink in colour, consisting of uniseriate filaments. Endozoic filaments spreading profusely and irregularly branched. Cells of variable outline, 12-60 µm in length, 2-6 µm in diameter. Emergent filaments often secundly branched. Cells 6-12 µm in length, 4-7 µm in diameter, usually with very elongate hairs. Chloroplasts small, discoid, usually with a pyrenoid. Reproduction by monospores produced in ovoid sporangia, born singly or in clusters of 1-3 sporangia (Dixon & Irvine 1977). In this study, on the other hand, the endozoic filaments, growing in *Obelia geniculata,* were oppositely branched (Fig. 3.7) and no side branches of the same type were seen. The cells were of irregular shape and size (17-18 µm in length and 4-8 µm in diameter). The chloroplasts were parietal with a large pyrenoid in the middle. In the areas where the alga infected the hydroid heavily, the filaments appeared interwoven with filaments of diverse cell size which later proved to be another endozoic *Audouinella* species.

In culture, The plants at first thought to be *A. infestans* were pale-rose-red to dark-red, depending on light intensity. Tufts were differentiated into prostrate (attaching) and erect systems, with the prostrate system of short filaments with rounded and barrel shaped cells. The erect system consists of barrel shaped cells with those at the branch ends cylindrical (Fig. 3.8). Cell sizes 15-23 µm in length, the shorter cells being in the prostrate system, (average 18.2 µm), and 6-8 µm in diameter (average of 6.6 µm). Reproduction by monospores which are produced in sporangia which are ovoid of 12-16 µm in length and 7-9 µm in width, either on short branches of one to two cells or sessile, and no cluster-like sporangia were seen in this study. However, other reproductive cells were found in the attaching system, which are rounded. These cells when they are separated from the original tuft by any mechanical action, germinate and produce new filaments. When these form new filaments the mother cell retains its form and its contents. This is different from the monospores where the contents migrate into the first formed cell. Monospores are formed at all

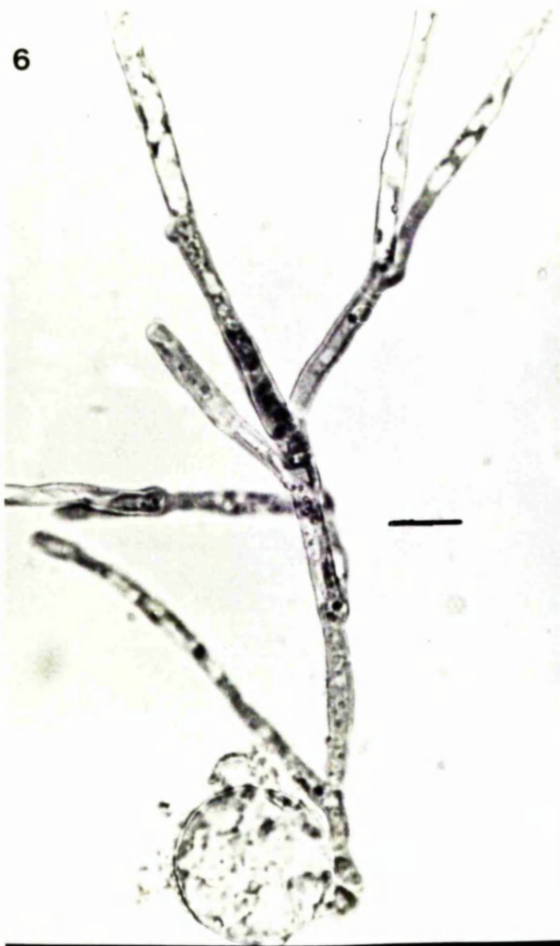
times of the year and can be induced by breaking the tufts and transferring them to new growth conditions such as fresh medium, different temperatures, or different light intensities.

From the above observations, the alga in this study appears to be different from the species described by Dixon & Irvine (1977) in terms of absence of the hair-like cells, the cell size, and the mode of sporangia formation. White (1968) reported this alga with the same features (cell size, absence of hair-like cells), and Aziz (1965) also observed specimens without hairs.

Smaller tufts were found in culture growing very close to tufts of *A. infestans*. These tufts were very similar to larger tufts of *A. infestans* in the same culture in branching and cell shape, but their sizes are much smaller and angular (Fig. 3.9) 12-13 μm in length with average of 12.7 μm, and 4-5 μm in diameter with average of 4.3 μm . These size differences, apparently, are very common in this species as White (1968) reported shorter cells for samples from certain localities.

Figs. 3.7-8 Light microscopy photographs of tufts of *Audouinella infestans* showing the barrel and cylindrical shaped cells. The arrows indicate the sessile sporangia (bars = 50 μm).

Fig. 3.9 shows the morphology of *Audouinella infestans*-like plant with smaller and angular cells. (bar = 25 μm).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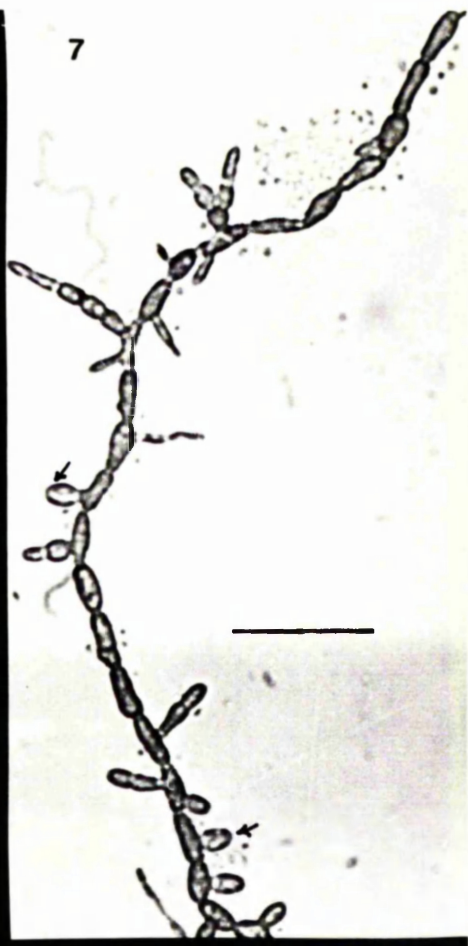
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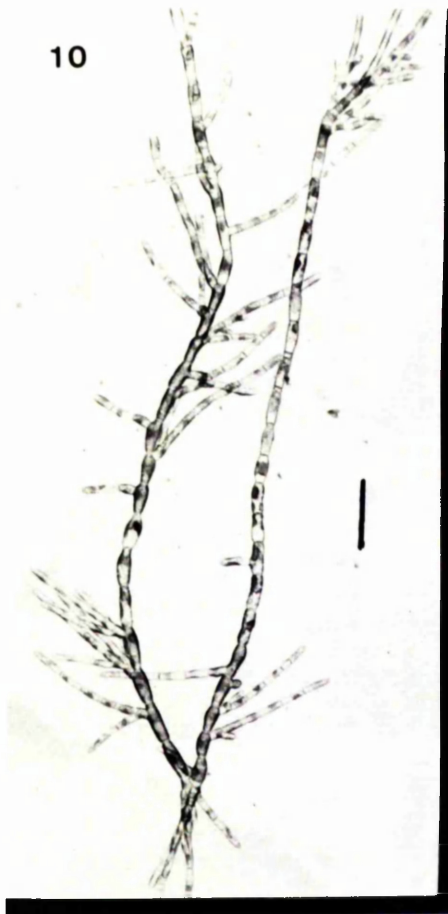
Figs. 3.10-11 show the morphology of tufts of endozoic *Audouinella* species 1. (bar = 100 μm).

Fig. 3.12 show the morphology of endozoic *Audouinella* species 2. (bar = 50 μm).

Fig. 3.13 shows the morphology of endophytic *Audouinella* species 1. (bar = 50 μm).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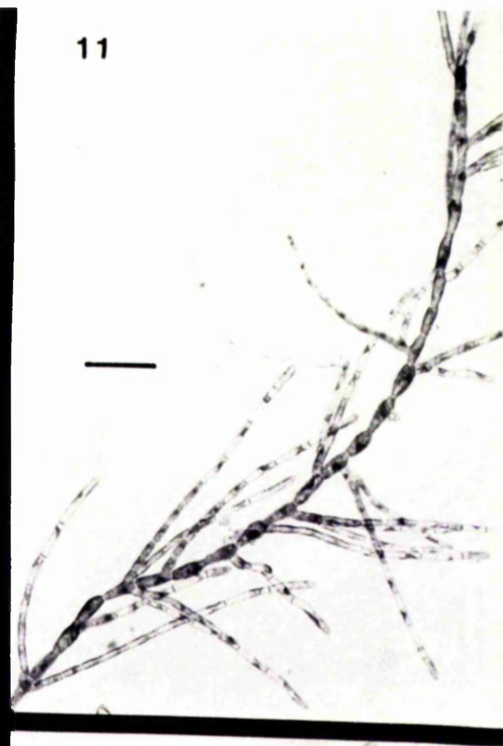
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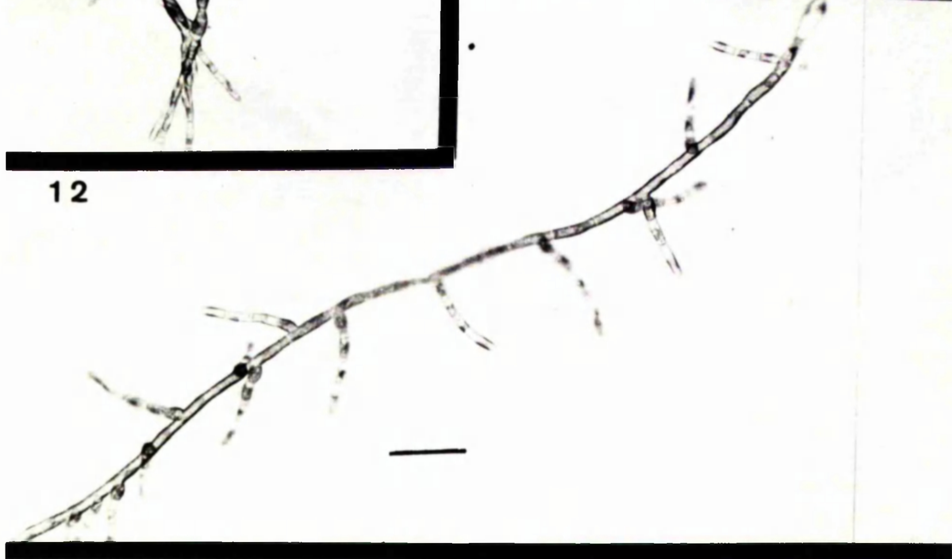
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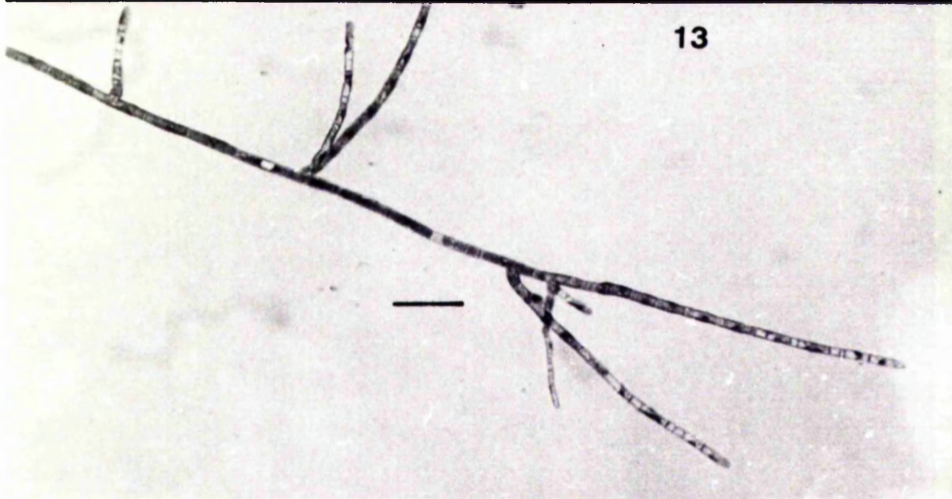
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Fig. 3.14 shows barrel shaped cells in the main axes and cylindrical cells in lateral branches (bar = 25 µm). (Endophytic Audouinella sp. 1)

Fig. 3.15 shows the hair-like cell and prostrate system formed in endophytic *Audouinella* species 1. (bar = 50 µm).

Figs. 3.16-17 show the morphology of endophytic *Audouinella* species 2. note the rounded cells in the prostrate system and the irregular shaped cells in the erect system. Monosporangia are indicated by arrows. (bars = 50 µm).

Fig. 3.18 The second type of reproductive structure in endophytic *Audouinella* species 2. note empty sporangia-like cells. (bar = 25 µm).

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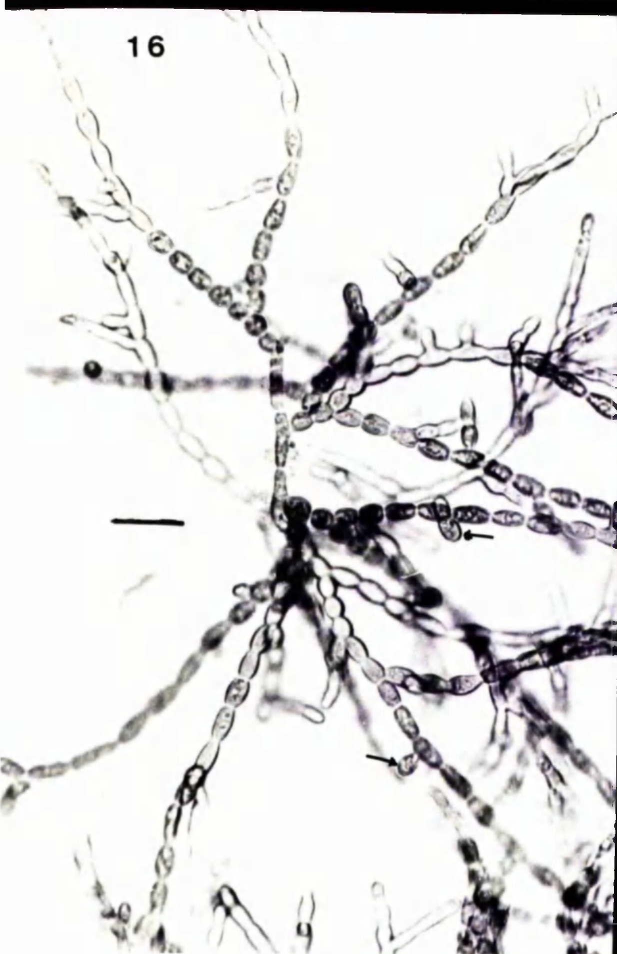
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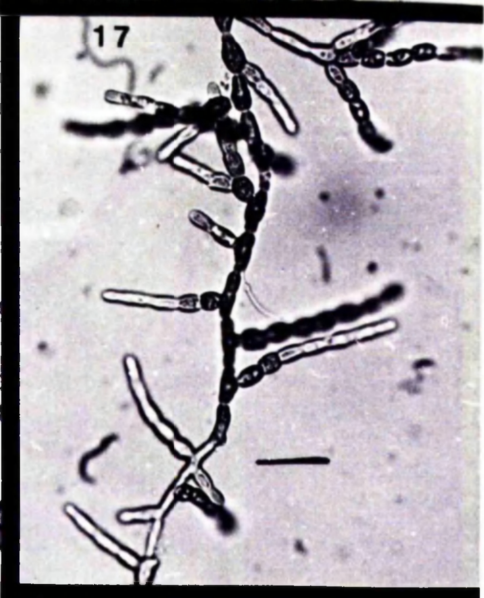
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Figs. 3.19-20 Tufts of endophytic *Audouinella* species 3. (bar = 50 μm).

Fig. 3.21 Lateral and sessile monosporangium formed by endophytic *Audouinella* species 3. (bar = 100 μm).

Figs. 3.23-25. Show the morphology of the endophytic *Audouinella* species 4. note the contracted chloroplast occupying part of the cell. (bars = 25 μm in 23-24 and 10 μm in 25).

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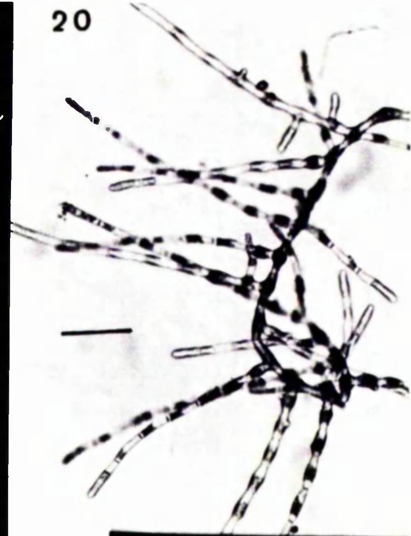
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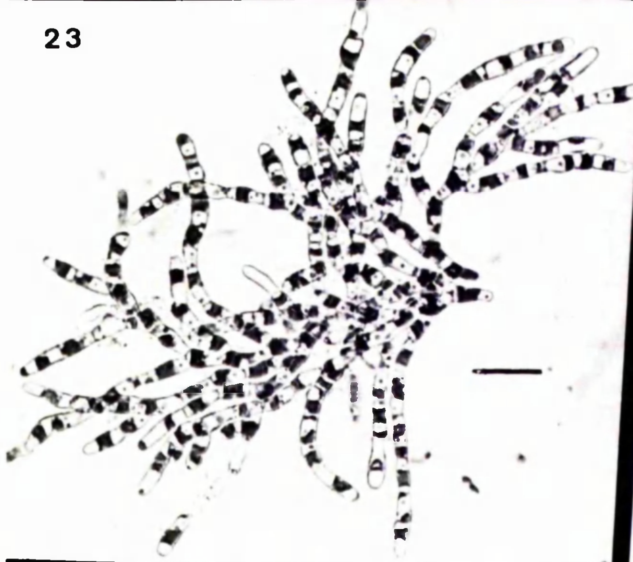
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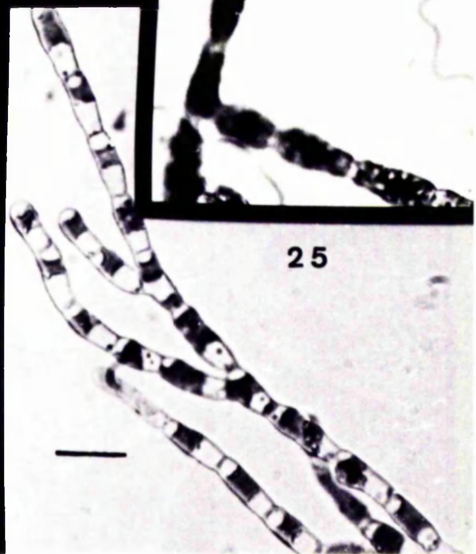
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3. Endozoic *Audouinella* sp. 1.

This alga was also isolated from endozoic filaments in *Obelia geniculata.* The plants, in culture, were pale rose-red to dark red in colour, uniseriately, occasionally alternately branched (Figs. 3.10-11). Tufts were not differentiated into erect and prostrate system. Cells were cylindrical and slightly swollen in the middle. Cells sizes 37.5-50 µm with average of 40.9 µm in length, and 7.5-9 µm with average of 8.3 µm in diameter. Chloroplasts parietal with pyrenoids. Reproduction by monospores produced in ovoid sporangia. Sporangia produced either singly and sessile or in clusters on single-celled branches of 15-20 µm in length and 7-12 µm in width. The latter type of sporangia were produced on long filaments growing from the middle of the tuft. Later in this study it was found that the sessile sporangià produced spores which grew into small tufts within the large tufts. These small tufts later produced these long filaments under favourable conditions and then produced large numbers of spores which on germination grew to produce the large tufts. It was also found that when these small tufts were broken into small fragments, each fragment grew and produced these long filaments which in turn produced spores in clusters. These spores form the dominant large tufts after germination.

The first type of spore (from sessile sporangia) and their germination were apparently induced by a long period of stagnation of the cultures. In this study they were observed in cultures of large tufts left on laboratory bench at room temperature and relative low light intensity (laboratory illumination) for several months. The **ESW** medium was not changed but frequently added as the level of the medium in the petri dish decreased.

Mode of spore germination in the second type of spore is not constant with persistent and non-persistent spores.

4. Endozoic *Audouinella* sp. 2

This alga was also isolated from *Obelia geniculata*. In culture the plants are

pale-rose-red to dark red in colour. Tufts were differentiated into erect and prostrate systems, with the prostrate system composed of short and single filaments attached to the substratum. Erect systems consisted of long filaments branched alternately in the terminal region. Cells cylindrical and swollen in the middle, with cell sizes 22.5-40 µm (average of 25.9 µm) in length, and 5.8-7.5 µm (average of 6.7 µm) in diameter (Fig. 3.12). Reproduction by monospores produced in ovoid sporangia 10-15 µm in length and 6-10 µm in width. Sporangia were produced only in early summer, and in clusters on short branches of long filaments similar to those described for endozoic *Audouinella* sp.1. The differences between these two species are in terms of cell sizes and presence of erect and prostrate systems in the representative just described.

b. Endophytic

1. Endophytic *Audouinella* sp.1

This alga was found growing in the holdfast region of *Delesseria sanguinea*. In nature, the cells were barrel shaped, very swollen in the middle up to three times the diameter of the cell ends. Cell size were 24.2-25 µm in length and 2.8-4.7 µm in diameter at joining points. The chloroplast occupies most of the cell with several pyrenoids. No branches were observed in the 'host' material.

In culture, the plants were rose-red in colour, and not differentiated into erect and prostrate system. Filaments were long and irregularly branched. Cells cylindrical (not barrel shaped) with a parietal chloroplast occupying only part of the cell. Cells sizes range from 11- 26 µm in length (average 19.9 µm), and 3.7-5.6 µm (average 4.4 µm)in diameter (Figs. 3.13-14). Reproduction was apparently by monospores only. These structures were seen in the first year of isolation only once. Sporangia ovoid usually formed in clusters of 2-3 on short branches (usually single celled). Sporangia sizes are 14.8-22 µm in length (average 17.85 µm) and 7.4-11.1 µm

(average 10.3 µm) in diameter. Spores are not persistant after germination. The alga showed polarity when grown from filament fragments. Occasionally this alga produced long hair like cells (Fig. 3.15). This type of cell was seen only in old tufts and were possibly formed because of exhaustion or nutrient depletion.

2. Endophytic *Audouinella* sp. 2

This alga was also isolated from the basal region of *Delesseria sanguinea*. Filaments were not seen in quantity in 'host' material. Plants in culture were dark-red in colour, irregularly branched. Tufts were differentiated into erect and prostrate systems. The prostrate system is characterized by barrel to round-shaped cells, usually smaller than the long erect filament cells. The erect system contained barrel shaped, cylindrical, and occasionally angular cells with parietal chloroplasts. Cells sizes 11-20 µm in length (average 15.5 µm) and 5-7 µm in diameter (average 5.7 µm) (Figs. 3.16-17). Reproduction by monospores which are produced at any time of the year and can be induced as in *Audouinella infestans*. Sporangia were produced singly and are sessile, ovoid in shape, their size 13- 14 µm in length (average of 13.6 µm) and 8-9 µm in diameter (average of 8.6 µm).

Other forms of reproductive cells were seen during subculturing of this alga. These cells were rounded in shape and originated from the prostrate system (Fig. 3.18). They were similar to those stated above for *A. infestans* in their modes of germination.

3. Endophytic *Audouinella* sp. 3

Endophytic in *Polysiphonia elongata*. In nature, the plants were dark-red in colour. In culture, they are brown red to light brown in colour when subjected to high light intensity, darker red in lower light intensity,with irregularly branched, barrel-shaped to irregular cells in the prostrate system and cylindrical cells in the erect system. Chloroplasts parietal, stellate or of irregular shape. Cells sizes were 22-39 µm in length (average of 27.4 µm), and 9-12 µm (average 10.4 µm) in diameter (Figs.

3.19-20). Sporangia ovoid of 15-22 µm in length and 8-10 µm in width, formed laterally and sessile (Fig. 2.21).

4. Endophytic *Audouinella* sp. 4

Endophytic in *P. lanosa.* In culture, brown-red in colour, alternately or irregularly branched forming small ball like tufts without any attaching system. Cells were cylindrical with an irregular chloroplast occupying only part of the cell. Cell sizes 8-13 µm in length (average 11.5 µm), and 3-4 µm (average 3.7 µm) in diameter (Figs. 3.23-25). Reproduction by monospores produced in ovoid sporangia of 6-10 µm in length and 2-3 µm in diameter, which are formed singly and sessile. This alga was not seen in quantity in nature and was found to be sensitive to light intensity, high temperature (15^0 C) and salinity changes. This sensitivity limited the availability of the isolate in investigation, because most experiments required a larger biomass which was not possible under these culture conditions. However, these observations on sensitivity and its slow growth under low light intensity and lower temperature ($2\text{-}5^0$ C) suggest that this alga may be restricted to Northern and colder areas, therefore it is unlikely to be a conspecific with *Audouinella emergens* (Rosenvinge) Dixon which is reported to be limited to spring and summer (Dixon and Irvine 1977). Other morphological differences are the smaller chloroplast and the cylindrical cells formed by this endophyte.

C. *Audouinella* spp. in culture from the Culture Collection of the Algae and Protozoa.

For comparative purposes, some species of Audouinella, originally endobiotic or exobiotic, and now growing in culture obtained from the Culture Collection of Algae and Protozoa.

1. ***A.* *asparagopsis*** (Chemin) Dixon **(CCAP 1360/1)**. Original authority and description. Chemin, E., 1926 *C. R. Acad. Sci.* Paris, Vol. 183, P. 902, Figs. 1-4.

This alga was most recently described by Garbary (1979c). Plants in culture were rose-pink in colour, consisting of uniseriate filaments. These filaments are profusely and alternately branched. Cells are of irregular shape. Cell sizes 13-17 µm in length and 5-6 µm in diameter. Reproduction by monospores produced in sporangia which are found either singly and sessile, or in clusters of two on single celled branches. Sporangia are ovoid or subspherical 6-8 µm in diameter.

2. ***A.* *endophytica*** (Batt.) Dixon **(CCAP 1360/5)** Original authority and description Batters, E. A. L., 1896 *J. Bot., Lond.*, Vol. 34, p. 386.

The plants in culture were red in colour. The base of the thallus is composed of interwoven branches which give off at the edge free branching filaments. Filaments are laterally and alternately branched. Cells elongate, cylindrical, and with parietal chloroplasts. Pyrenoids not visible. Cells sizes were 4-12(14) µm in length and 2-6 µm in diameter. Reproduction by monospores produced in spherical or slightly ovoid monosporangia, occasionally these sporangia are either terminal or lateral, often on short branches. Monospores are 6 µm in diameter (White 1968).

3. ***A.* *newtonii*** Garbary **(CCAP 1360/8)**

This alga in culture showed at early stages two distinct parts, a small multicellular disc-like prostrate system and 3-5 erect filaments. Filaments were secundly to irregularly branched. Branches arise from every cell in the main erect filament. In culture as lateral branches developed the tufts usually became detached. The plants were brownish-red in colour. Cells were cylindrical occasionally producing lateral long hairs. Cells sizes 30-40(59) µm in length and 12-13 µm in diameter. Reproduction by monospores produced in sporangia, on one celled branches or sessile on main axes, of 20 µm in length and 15 µm in diameter. Monospores have single stellate chloroplasts

each with a large pyrenoid.

Discussion

Exo- and endobiotic *Audouinella* have been found to be hosted by a wide range of host plants and animals. Their small size appear to be the main factor of their being often overlooked by phycologists. Isolation of Audouinella species as unialgal cultures in previous studies and in this study was carried successfully but not without difficulties. These difficulties were related to the similarities among different species and the dissimilarities within the same species when they were growing on or in different 'host' organisms and observed at different times of the year. The latter problem was easily solved when different isolates of *Audouinella secundata* from different hosts and sites (described earlier) were grown under the same culture conditions. But this problem contributes most to the taxonomical confusion of this genus as many authors classified these species in relation their host organism in addition to morphological features.

Similarities of *Audouinella* species especially when they were first observed in or on the 'host' organism proved to be the most difficult and their classification based on information on their features within the 'host' was found almost impossible, as these species, such as endophytic *Audouinella* species 1 and 2, or endozoic *Audouinella* species 1 and 2, were found to inhabit the same 'host' at the same time. Their filaments were found to be intermixed and multicellular filaments rarely found in the same plane of the section. Thus crude cultures of these species were found to be very essential for their isolation and consequently for their identification.

However, it was found that one species was completely dependent on its host, e.g an endophytic *Audouinella* species found in *Polysiphonia elongata*. The manner in which it depends on its 'host' organism was not established in this study. All attempts to isolate and grow it free from the 'host' plant failed.

Identification of these isolates, where possible, was made using morphological

features reported in the literature (viz. Dixon and Irvine 1977). However, for endophytic *Audouinella* species 1, 2, 3, and 4 and endozoic *Audouinella* species 1 and 2 morphological features were found not to fit in any of those reported from the British Isles by Dixon and Irvine (1977) nor those reported from elsewhere by Drew (1928) and Papenfuss (1945; 1947). This was due to the differences in the morphology of these plants when growing in natural habitats and when growing under culture conditions.

Those species which were identified were found to differ slightly from those reported in the literature. These differences either in reproductive structures formed by the species or in reproductive structures and morphological features, e.g. *Audouinella efflorescens* was found to form tetrasporangia only rather than monosporangia and tetrasporangia, and *A. virgatula* also was found to produce monosporangia only under culture conditions rather than both mono- and tetrasporangia. The latter may be mistaken for *in situ* germination. In case morphological features and reproductive structures formed by the organism, it was found that *Audouinella infestanse* differ from these reported by Dixon and Irvine (1977) that it produced no hair-like cells, cells are smaller and monosporangia never been found in clusters.

Other difficulties such as spore production and sensitivity to light were found to slow down the isolation process of these *Audouinella* species in unialgal cultures and their loss occasionally (viz. *A. parvula* and endophytic *Audouinella* species 4). The latter species also was found to grow with contracted chromatophores.

As endophytic *Audouinella* species 2 and *A. infestans* and endozoic *Audouinella* species 1 and 2 were found to have great similarities other confirmatory experiments were carried and discussed in later sections to assure their separate entities.

4. Some histological and biochemical observations

4.1. a. Cell wall constituents

Introduction

Algal cell wall organization has been often studied, and the taxonomic implications remain an important consideration. In general, cell walls are composite materials and at least two components can be identified, a fibrillar and a non-fibrillar one. In red algae, these components basically are formed of polysaccharides such as agaroses, mannans, xylans and cellulose.

Agaroses (agar, carrageenan and porphyran) are all sulphated polysaccharides, essentially linear galactans, consisting mostly of alternating 1,3- and 1,4- linked D- and L- galactose units. Mannans (found only in the cell wall fractions of certain members of the class Bangiophyceae, namely *Porphyra umbilicalis* and *Bangia fuscopurpurea*) occur as B- 1,4- linked mannans (Lewin 1974). Xylans in red algae can be of two types. In one, either as a homoxylan or combined as a heteropolysaccharide, both 1,3- and 1,4- xylosidic linkages occur in a structure which may be linear or branched. In the second type the molecule is essentially linear, with only 1,3 links or only 1,4 links (Turvey and Williams 1970). The presence of cellulose in red algal cell walls is open to question. Studies in many red algae have shown dissimilarities in this material. McCandless (1978) concluded that these materials found in the cell wall of such species should be called xyloglucans, or galactoglucans, or glucomannans. However, Mukai *et al* (1981) have shown that cellulose microfibrils are present in the cell walls of the *Conchocelis*-phase of *Porphyra tenera,* whereas the thalloid form has xylan microfibrils. Gretz *et al* (1986) found that cellulose comprised 5.5% of *Porphyra leucosticta* Thur. cell wall weight.

However, many of the current idea on relationships between polysaccharide molecule

structure, properties and functions have been derived from studies of polysaccharides of algal origin (Mackie and Preston 1974). Different methods were employed in isolation, differentiation and identification of these polysaccharides such as X-ray crystallography, chromatographic separation of sugars, infra-red and nuclear magnetic resonance spectroscopy, immunochemistry, and staining with different dyes. As these methods improve so the picture and our understanding of these constituents changes. McCandless (1978) stated that a proper combination of analytical and histological methods was essential to determine the presence of a particular polysaccharide.

On the other hand, the importance of these polysaccharides in taxonomy is not clear yet, as different generations (sporophytes and gametophytes) of certain families showed different cell wall composition (McCandless & Richer 1971 ; McCandless *et al.* 1973 ; Gordon-Mills & McCandless 1975 ; McCandless 1978). Therefore, Percival (1978) concluded that before a complete picture of possible generic and specific differences can be drawn, a vast amount of chemical work remains to be done on seaweed polysaccharides.

In this study attempts were made to investigate the cell wall components of certain endo- and exobiotic *Audouinella* spp. These attempts were encouraged by observations from the **SEM** studies on erect and prostrate systems of *Audouinella* species, which showed that with some species these appeared to be differences in the texture of their cell surfaces. Garbary (1978b) used the **SEM** to investigate the surface structure of some epiphytic *Audouinella* species. In his investigation he found that three different cell wall surfaces were to be found among audoulnelloid algae. Vegetative cells of *Audouinella secundata* were heavily striated with no defined start or end of these lines, *A. daviesii* (Dillwynn) Woelkerling and other species showed cell wall surfaces with longitudinal ridges but less prominent than those in *A. secundata*, whilst *A. floridula* showed a smooth surface. Thus he suggested that these features might prove to be of taxonomic value. However, he stated that in some samples the striations were present on

most of the cells, whereas in other samples of *A. daviesii* most cells were smooth with only a few cells near the apex displaying striations.

Observations

In this study however, the **SEM** investigations showed that striations of the cell wall, suggested by Garbary (1978b), have no real taxonomic significance. Longitudinal striations and wrinklings of cell walls were found in one sample of *Audouinella virgatula* (Fig. 4.1.1a), and in the other samples showed more wrinklings but lesser longitudinal striations (Fig. 4.1.1b). Endozoic *Audouinella* species 1 and 2 showed some cells with extensive wrinkling of the wall, with enlarged regions at the cell junctions and cells with no surface changes (Figs. 4.1.2a-c, 4.1.3 respectively). *Audouinella infestans* (Figs. 4.1.4, 4.1.5a,b) showed some cells with walls entirely smooth, and others with noticeable surface wrinkling. Endophytic *Audouinella* species 3 (Fig. 4.1.6) and endozic *Audouinella* species 1 (Fig. 4.1.7a,b) showed smooth surfaces on old cells and wrinkled surfaces on some newly produced cells (nearest to the apex). In the present work, however, it was found that the main disadvantage of studying some of these algae by **SEM** was their fragility in the processing (fixation, washing, and dehydration). The material can be easily lost during different steps of fixation, washing, post fixation, dehydration and drying, especially these species which have no real prostrate system growing on smooth surfaces. On many occasions filaments of endophytic forms 1 & 3 were seen to break down when they were subjected to high electron densities in the **SEM** beam.

Table 4.1. and figures 4.1.8A-Q show the cell wall colour reactions with different stains. Certain *Audouinella* spp. had a definite cell wall colour reaction with certain stains, others have occasional colour reactions (such as cell junctions of endozoic *Audouinella* sp. 2). As the study was aimed to investigate the cell wall, definite colour reactions were not always recorded. Endophytic *Audouinella* spp. 1 and 2 and endozoic *Audouinella* sp. 1 showed that their outer cell walls may consist of pectic material.

However, cell walls of some species showed no detectable colour reaction to stains even when these were applied for longer times. On the other hand, where reaction occured the colour reactions showed that there were few common histochemical reactions. *A. infestans, A. efflorescens* and *A. newtonii* which reacted only to Chlorazol Black E stain where their outer cell wall was stained red. *A. asparagopsis* and endophytic *Audouinella* sp. 3 showed the presence of carboxylated polysaccharide in the outer and inner wall respectively (Figs. 4.1.8A and 4.1.8D). Cellulose appeared to be present, mixed with pectic and phenolic compounds, in the cell junctions of the endozoic *Audouinella* sp. 2 (Figs. 4.1.8E, 4.1.8F, 4.1.8N and 4.1.8Q). This may explain the thick rings shown under SEM observations as this area gave positive reactions for cellulosic material and pectin. Endophytic *Audouinella* species 1 and 2 showed the presence of sulphated polysaccharides at cell junctions of the former (Fig. 4.1.8B) and in the outer wall of the latter (Fig. 4.1.8C).

Pectic material was also detected in the outer wall of endozoic *Audouinella* sp. 1 (Fig. 4.1.8M). In this species and endozoic *Audouinella* sp. 2 the colour was darker than that seen in endophytic *Audouinella* species 2 and 3. This phenomenon was common and was clear where no reaction was detected in the cell wall region but occasionally the whole cell contents appeared to be stained which may be due the reflection of the colour on the cell surface due to the stain picked up by the mucilage. This mucilaginous layer can be clearly seen in electron micrographs to be presented, and appeared as an irregular layer occasionally broken into small pieces or debris.

4.1. b. Ultrastructure of the cell wall of some *Audouinella* species.

The ultrastructure of cell walls of *Audouinella* species have not been investigated in detail before. Mandura (1981) briefly described the cell wall of *A. sagraeanum* as approximately 1 µm wide and the microfibrils are probably in a random distribution, and the outermost side of the cell wall is thin (0.06 µm) and electron dense and

probably represents the cuticle. In this study the cell wall staining showed a wide variety of reactions in some species and showed no reactions in others (Table 4.1). Therefore, further investigation using Transmission Electron Microscopy (**TEM**) was thought worthwhile.

Observations

Electron micrographs of different *Audouinella* species (Figs. 4.1.9- 4.1.17) showed that the cell walls of these isolates were composed of multi-layers of different natures and thickness. However, it is evident that the non fibrillar layers tend to be predominant in all species.

Audouinella infestans as shown in Fig. 4.1.9a-c showed a multilayered construction of the cell wall of layers with low electron density much thicker than the layers with high electron density. Fibrillar material appears to be loosely embedded in the abundant inner non fibrillar layer. This species showed no reaction to chemical stains apart from Chlorazol Black E which appeared to be picked up by the surface layer which forms the darker (electron dense) components in the micrograph.

The cell wall of Endophytic *Audouinella* species 1 (Fig. 4.1.10a-c) showed two different layers, an outer and relatively thin layer of higher electron density and a thicker less electron dense region inside. The inner wall showed evidence of layering with some loose fibrillar material while the outer layer was more evenly dense with no layers visible (Fig. 4.1.10a). The cell junction showed the presence of a dark single layer continuous with that separating the two main wall regions (Fig. 4.1.10b) and a much enlarged region corresponding to the inner clearer layer (Figs. 4.1.10b-c). These figures also show the pit connection area with the plug cap which is also has a high electron density and multi-layered structure. This species gave a positive reaction for carboxylated polysaccharide at the cell junctions, and it is possible that the extensive clear non fibrillar material seen in the section consists of this polysaccharide.

Table 4.1 Effect of various reagents on the cell walls of *Audouinella* species

	Species	Alcian blue and Alcian yellow	Figure reference	Aniline blue	Figure reference
1	*A. asparagopsis*	Yellow along the outer wall.	A	No reaction	
2	*A. efflorescens*	No reaction		No reaction	
3	*A. endophytica*	No reaction		No reaction	
4	*A. infestans*	No reaction		No reaction	
5	*A. newtonii*	No reaction		No reaction	
6	Endophyte 1	Blue at cell junctions	B	No reaction	
7	Endophyte 2	Blue along the outer wall	C	No reaction	
8	Endophyte 3	Blue outer wall yellow inner wall	D	No reaction	
9	Endozoic 1	No reaction		No reaction	
10	Endozoic 2	Green at cell junctions	E	Blue at cell junctions	F

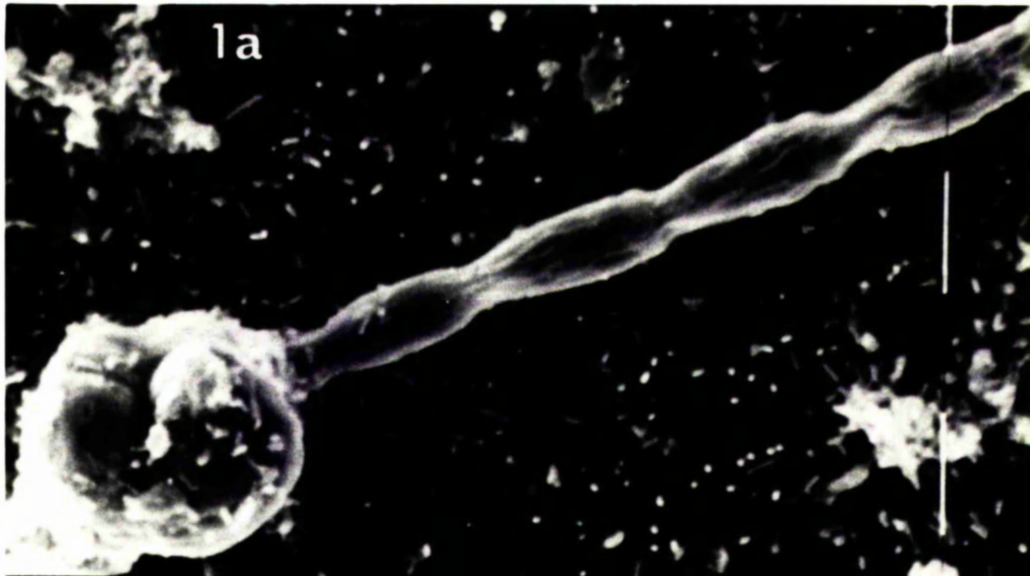
Table 4.1 Continued.....

	Chlorazol Black E	Figure	Ruthenium red	Figure
1	No reaction		No reaction	
2	Outer wall red	G	No reaction	
3	No reaction		No reaction	
4	Outer wall red	H	No reaction	
5	Outer wall red	I	No reaction	
6	Outer wall red	J	No reaction	
7	No reaction		Red along outer wall	K
8	No reaction		Red along outer wall	L
9	No reaction		Dark red outer wall	M
10	No reaction		Red at cell junctions	N

Table 4.1 Continued.

	Thionine	Figure reference	Toludine blue	Figure reference
1	No reaction		No reaction	
2	No reaction		No reaction	
3	No reaction		No reaction	
4	No reaction		No reaction	
5	No reaction		No reaction	
6	No reaction		No reaction	
7	No reaction		No reaction	
8	No reaction		Blue along outer wall	O
9	No reaction		Blue along outer wall	P
10	No reaction		Blue at cell junctions	Q

Figure 4.1.1 *Audouinella virgatula* a. shows longitudinal striations and wrinklings. b. shows more wrinklings and less longitudinal striations.



1b

Figure 4.1.2. Endozoic *Audouinella* species 1. a. shows cells of lateral branches with wrinklings while cells of the main axes showed smooth surfaces. b,c. shows extensive wrinklings in the cell wall and the enlarged regions at cell junctions.

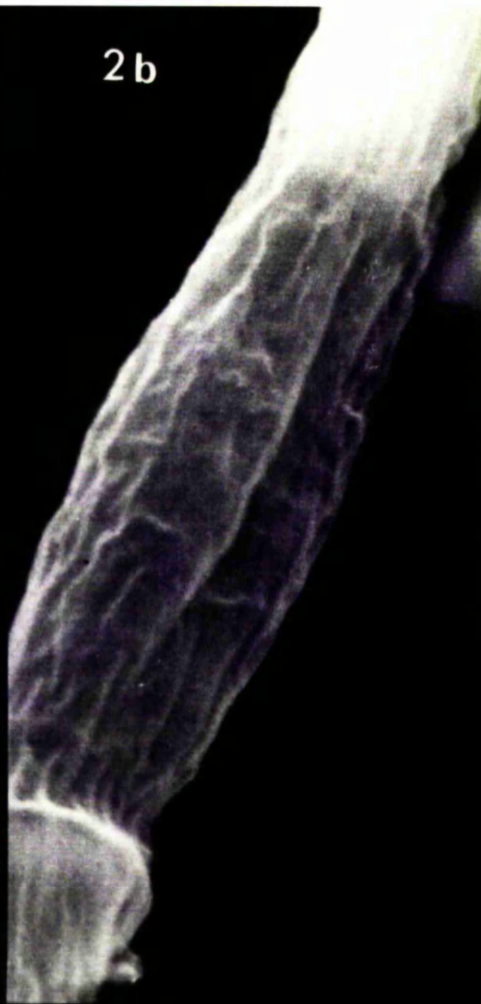
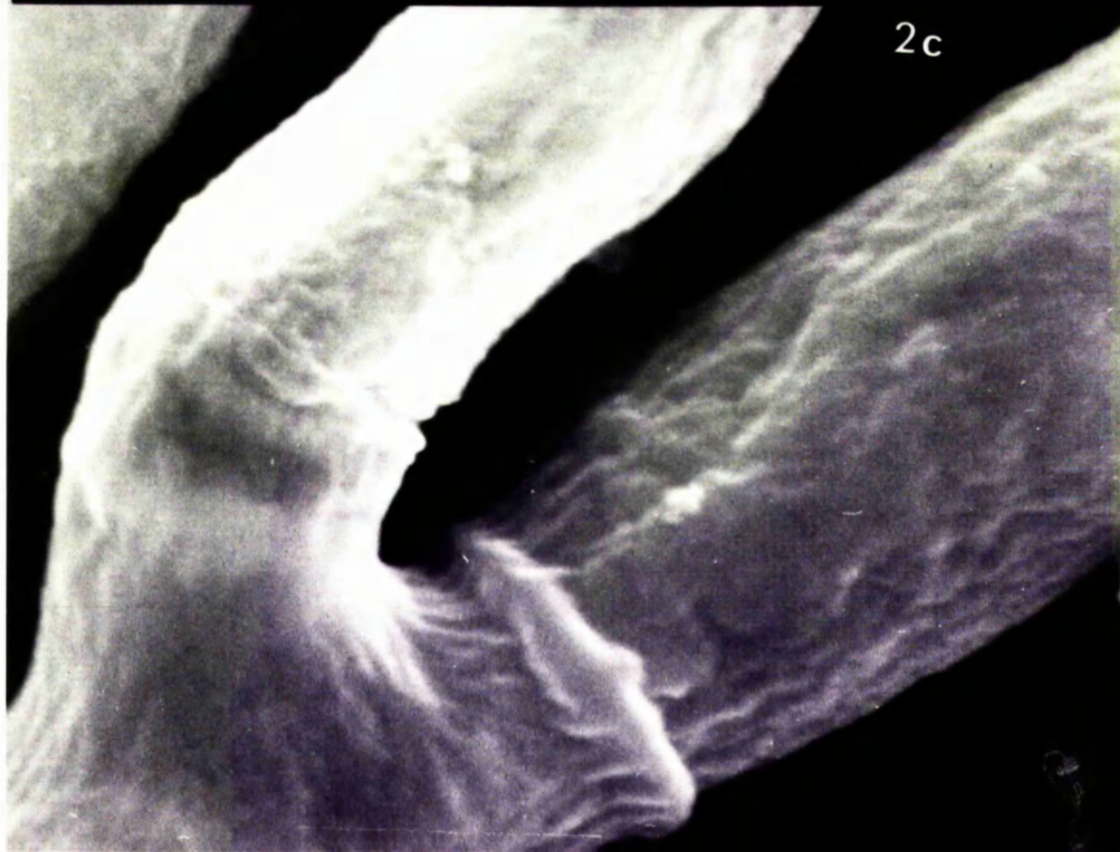
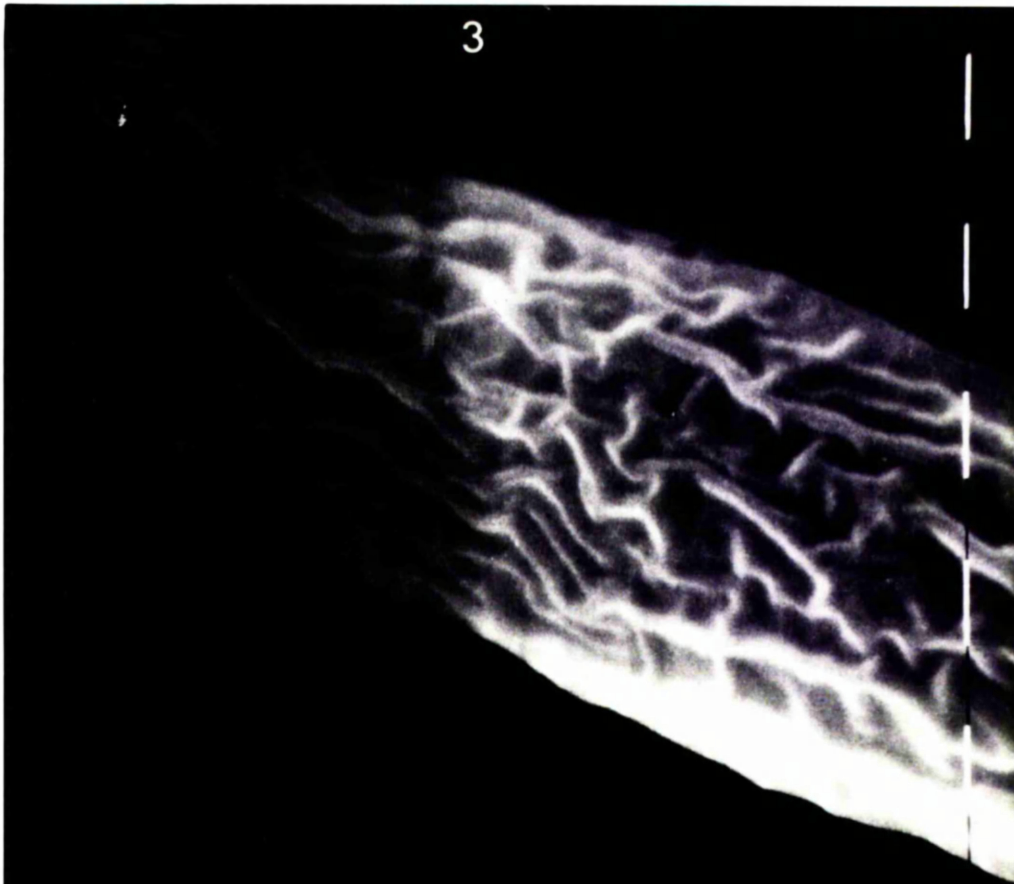
2a

2b

2c

Figure 4.1.3. Shows wrinkles and enlarged region of the cells of Endozoic *Audouinella* species 2.

Figure 4.1.4 Cells of *Audouinella infestans* with walls entirely smooth.


3

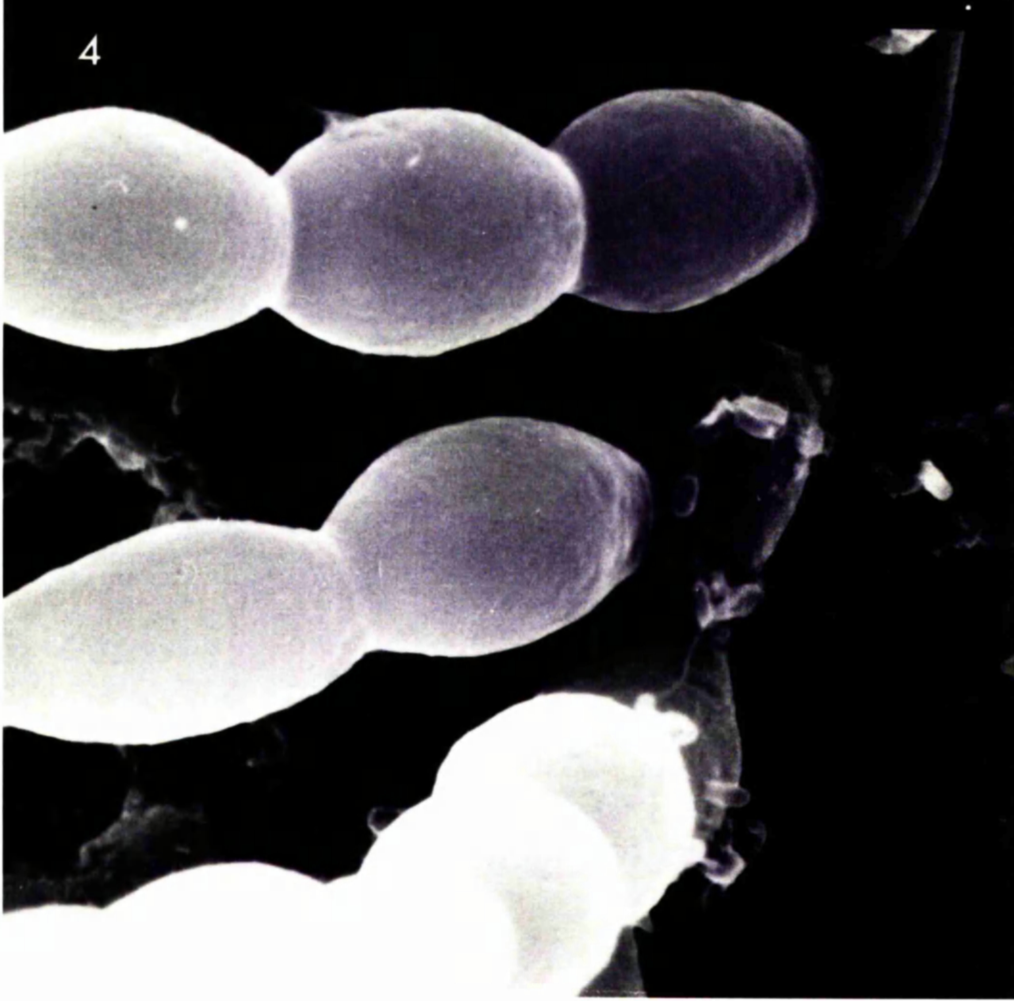
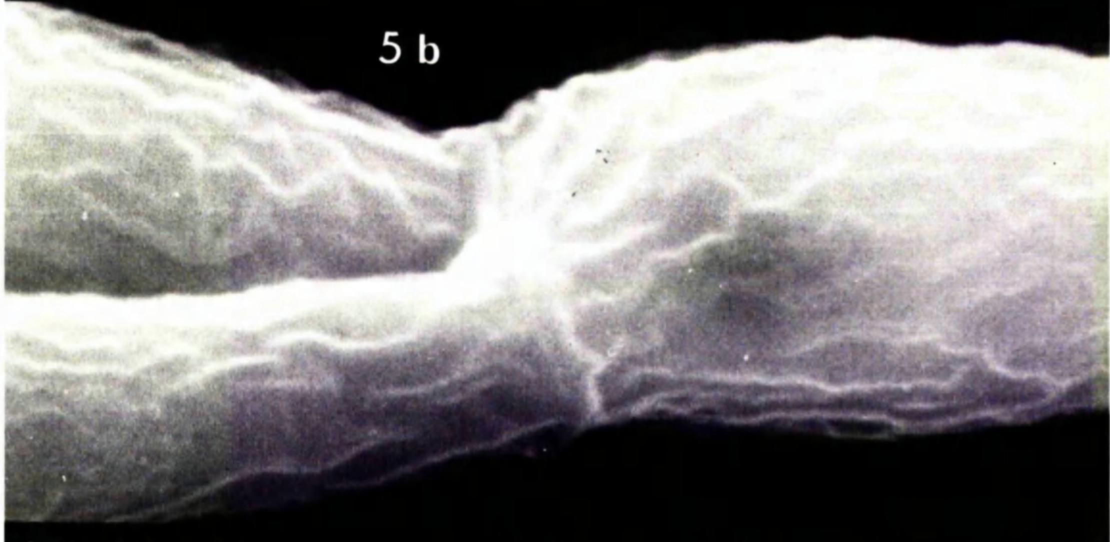
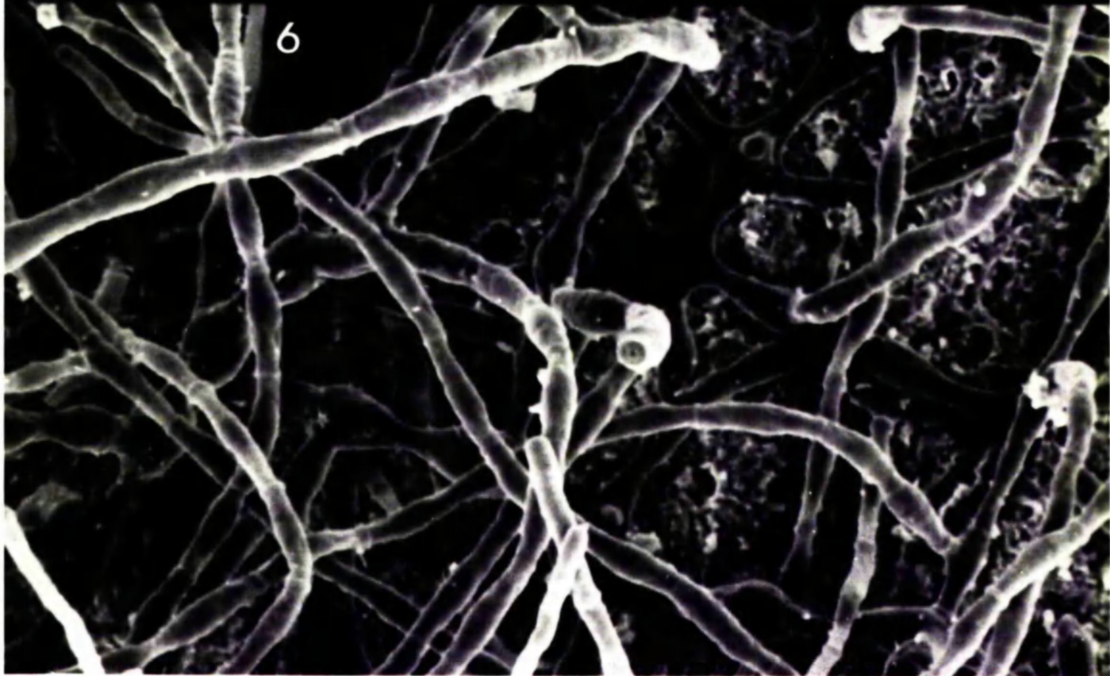

4

Figure 4.1.5a,b Cells of *Audouinella infestans* with walls with wrinklings.

Figure 4.1.6 Cells of Endophytic *Audouinella* species 3 with walls entirely smooth (attaching system) and cells with wrinklings (erect branches).

5a


5 b


6

Figures 4.1.7a,b Cells of Endozoic *Audouinella* sp. 1.' a. cells of the main axes with smooth walls and cells of lateral branches and apical cell with wrinklings.

7a

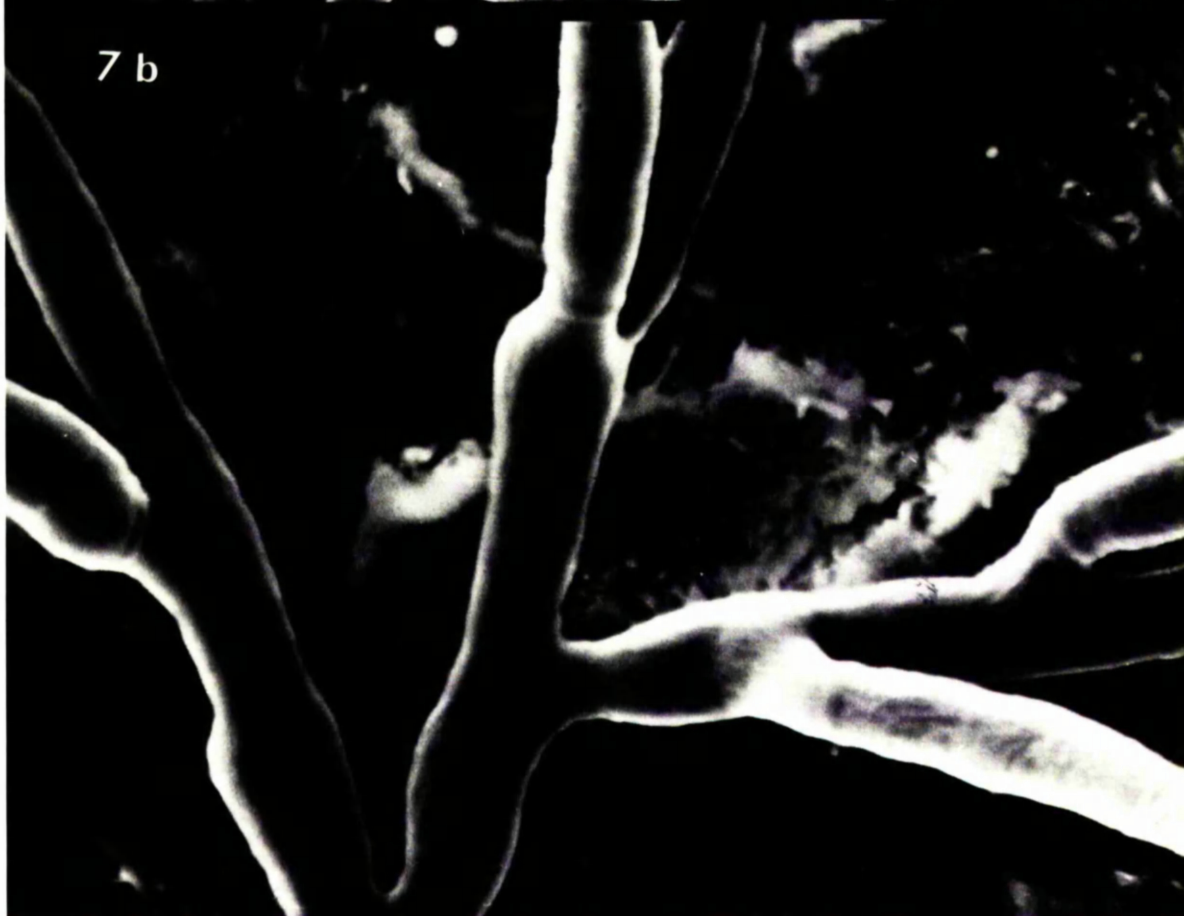

7 b

Figure 4.1.8A-Q Diagrammatic representation of cell walls of different *Audouinella* species and reaction to different stains and dyes (refer to Table 4.1).

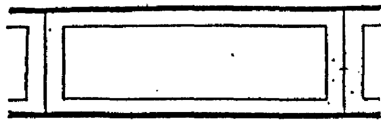


FIG. A

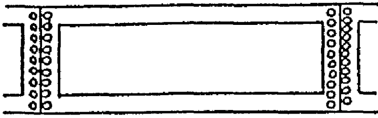


FIG. B

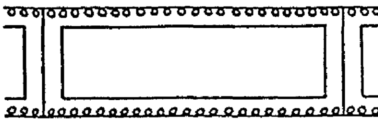


FIG. C

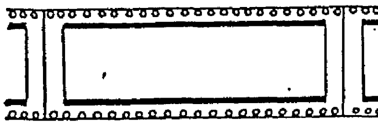


FIG. D

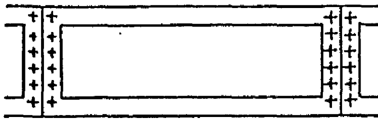


FIG. E

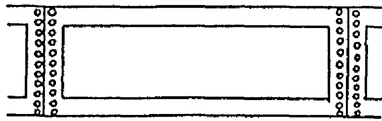


FIG. F

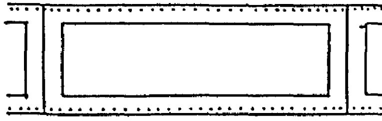


FIG. G

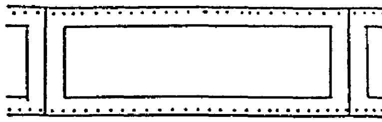


FIG. H

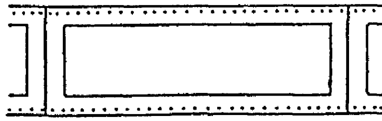


FIG. I

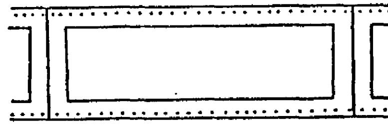


FIG. J

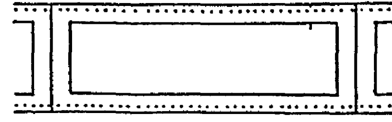


FIG. K

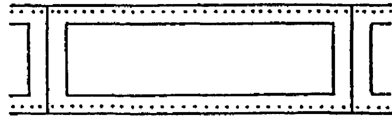


FIG. L

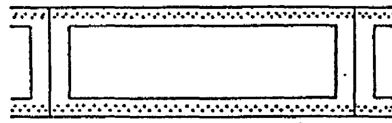


FIG. M

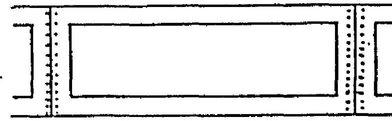


FIG. N

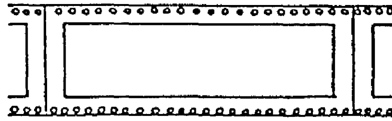


FIG. O

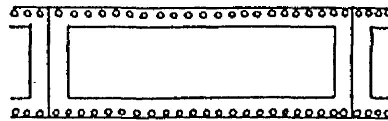


FIG. P

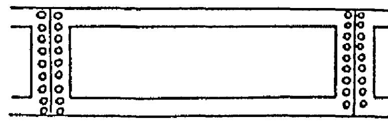
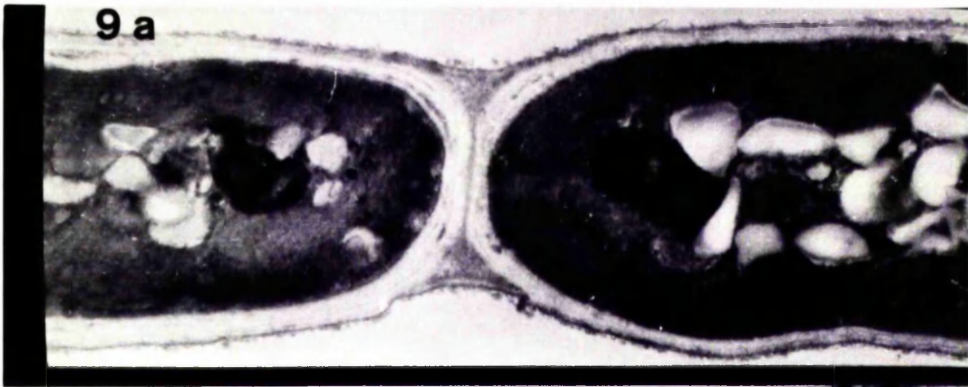
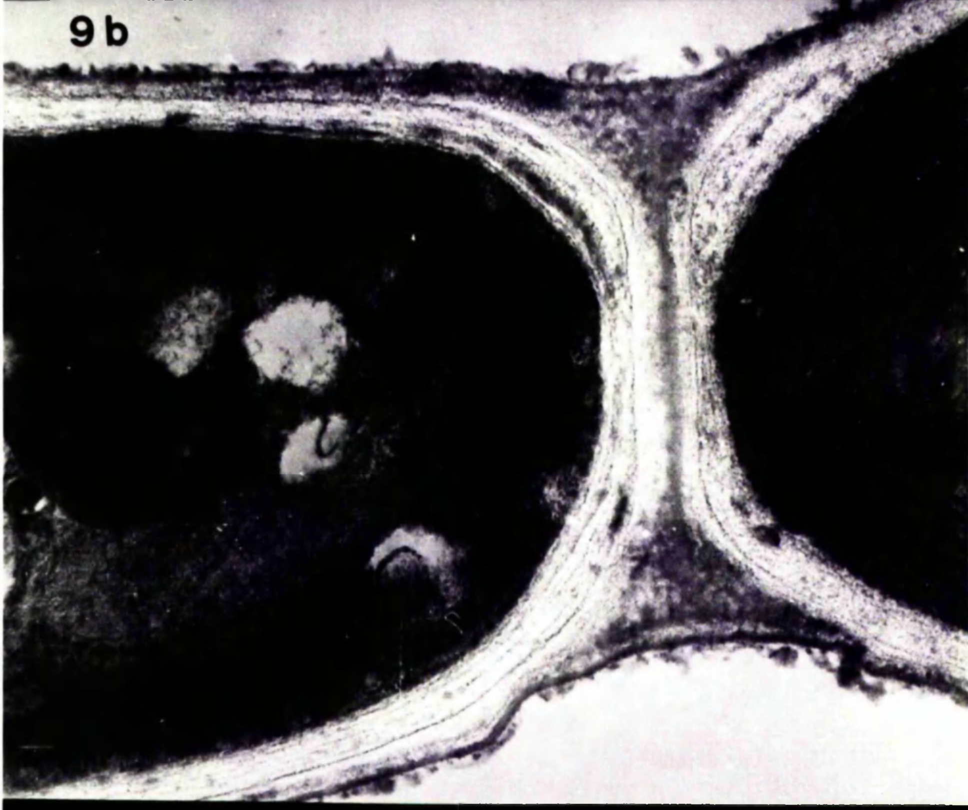


FIG. Q

BLUE ooo
GREEN +++
RED ...
YELLOW —

Figure 4.1.9a-c The multilayred structure of the cell wall of *Audouinella infestans*. (magnification a x19600, b,c x54600)


9 a


9 b

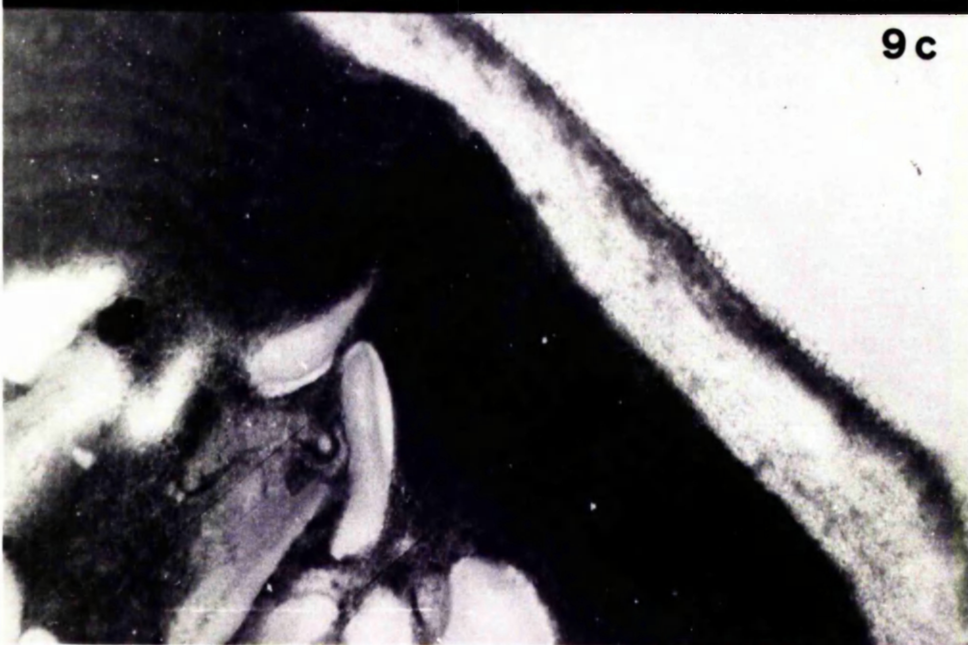
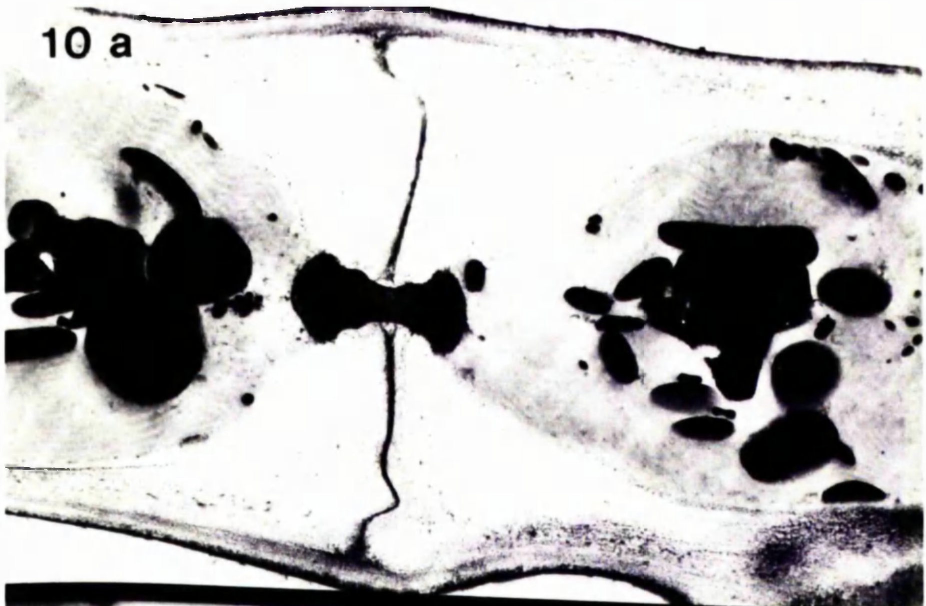
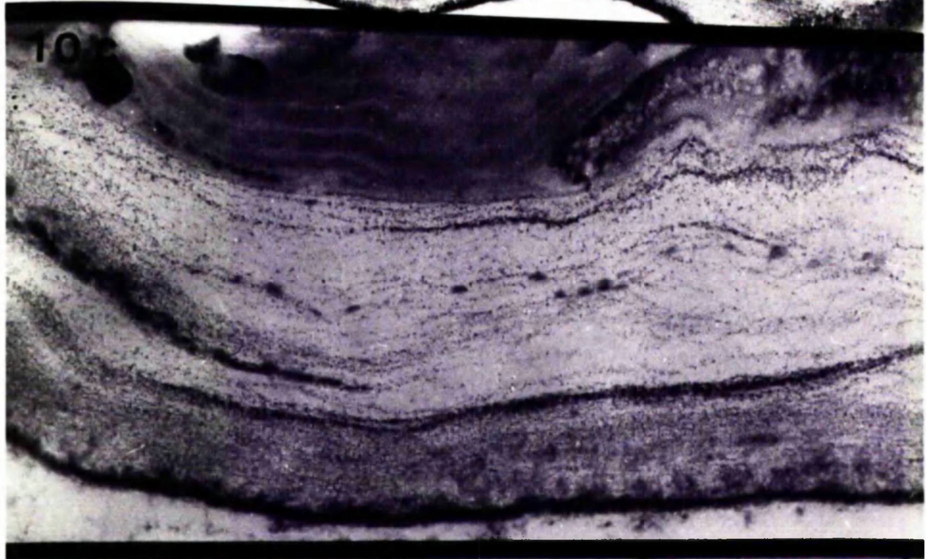

9 c

Figure 4.1.10a-c The cell wall of Endophytic *Audouinella* sp. 1. in section a,b showing the plug cap with multilayered construction. c. shows the two layers of different electron densities. (magnification a x19600, b,c x54600)


10 a



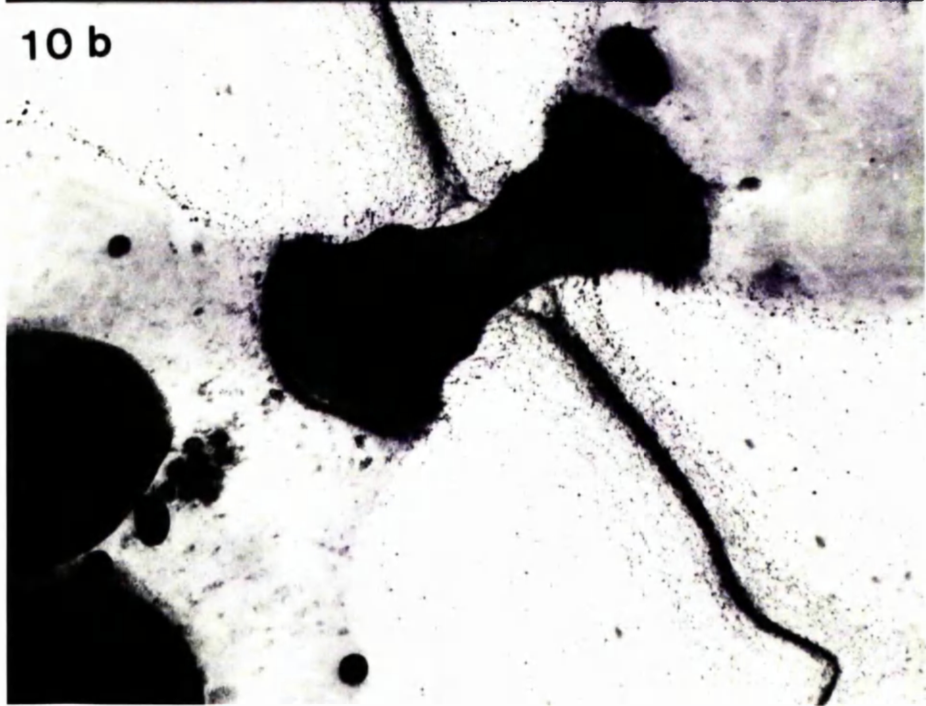
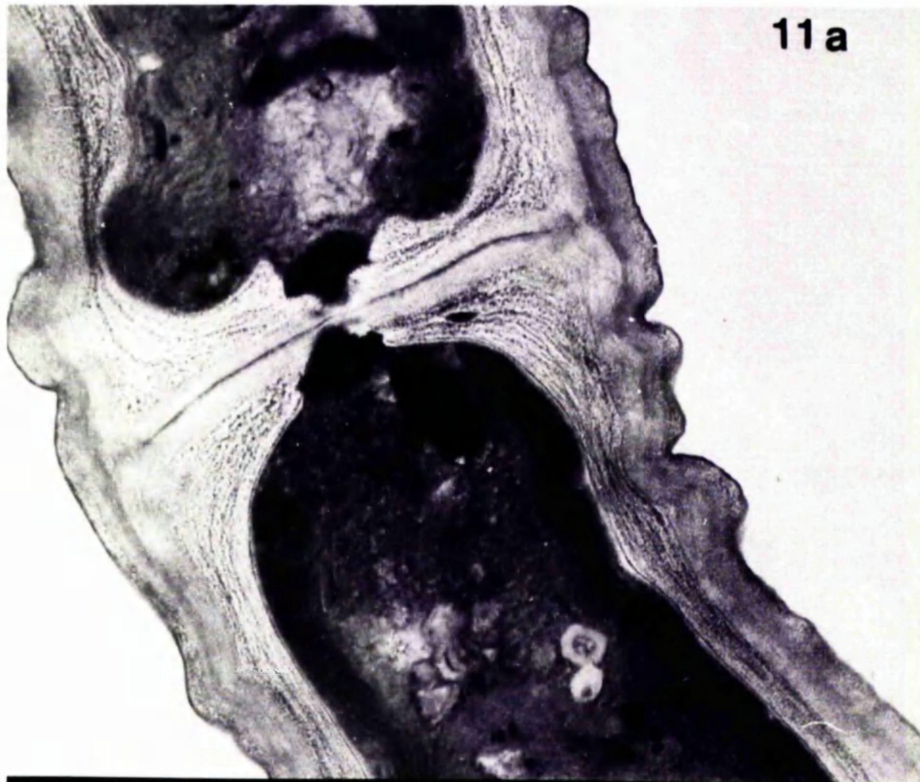

10 b

Figure 4.1.11a,b The two layered cell wall of Endophytic *Audouinella* sp. 2 in section and the pit connection region. (magnification a x19600 , b x54600)


11a

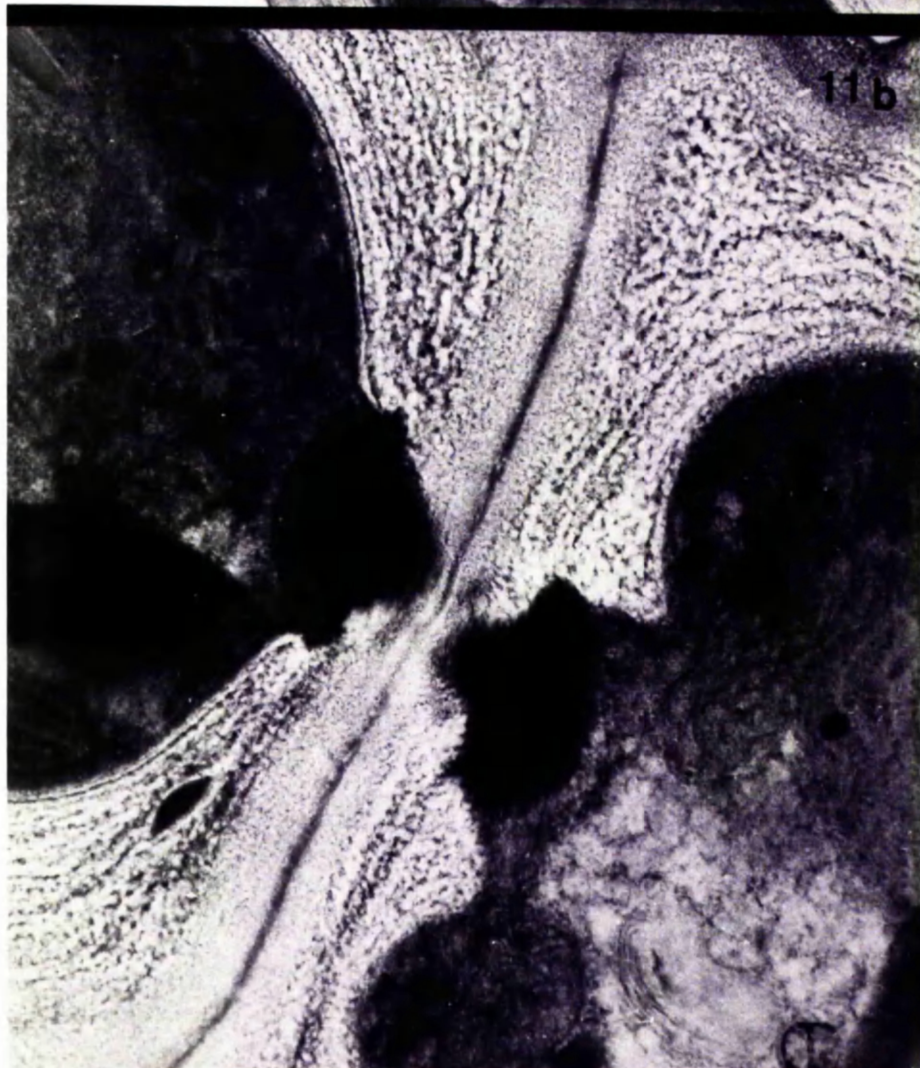
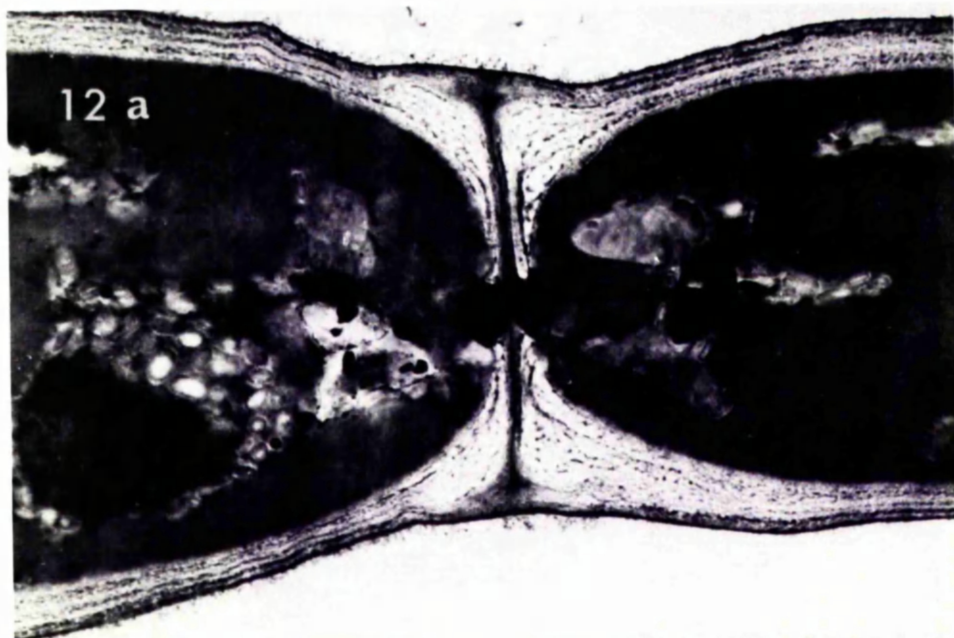
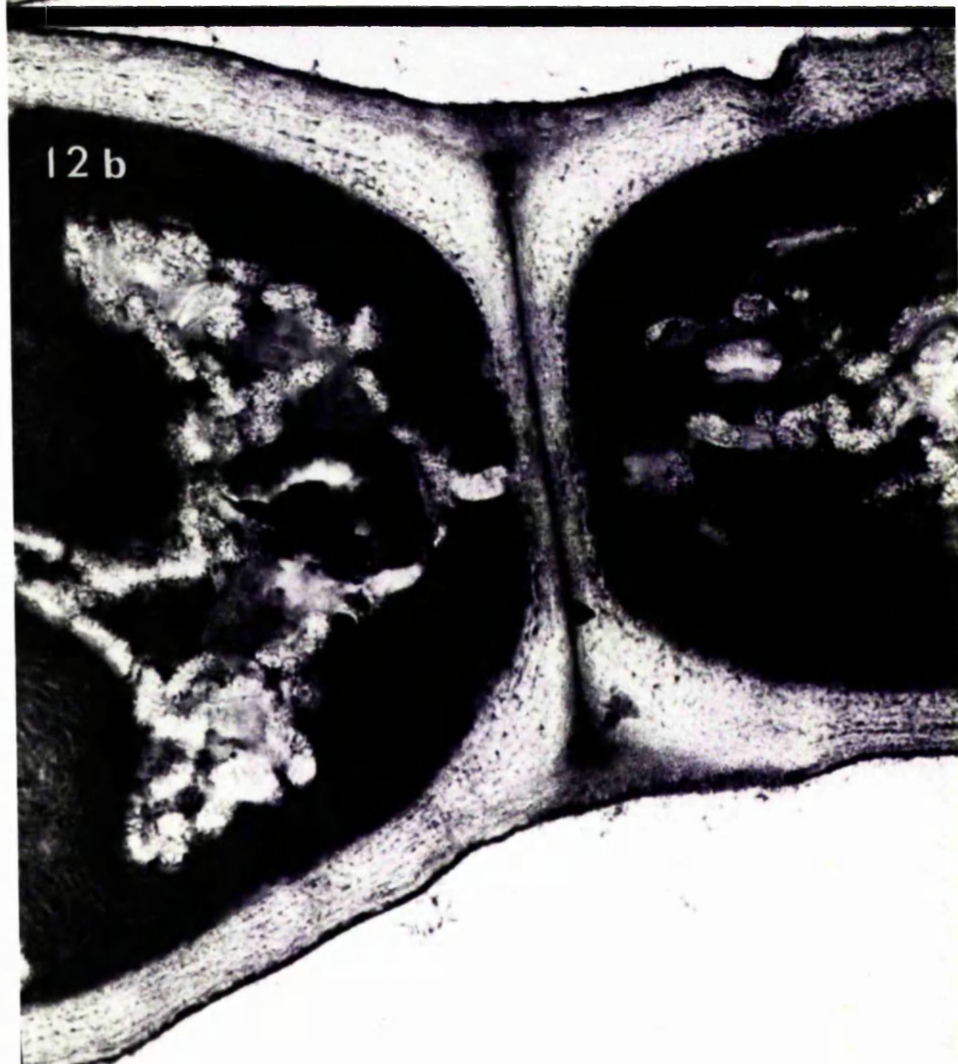
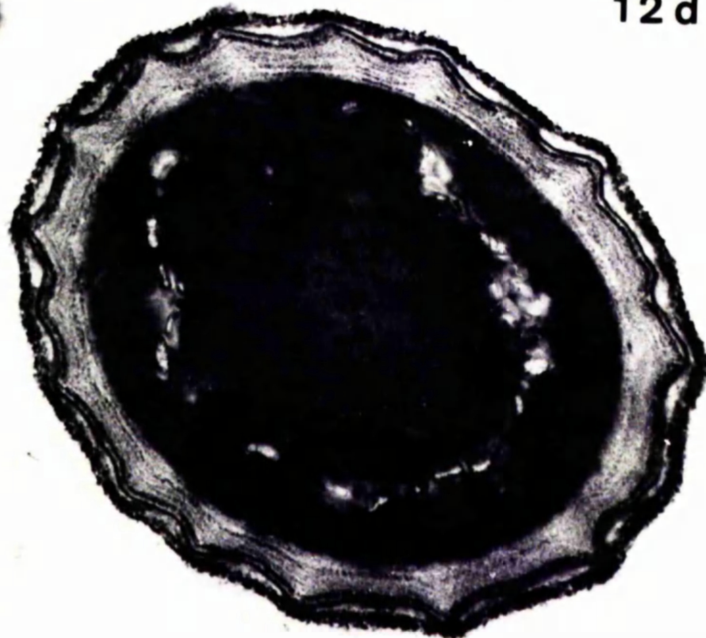

11b

Figure 4.1.12a-d The cell wall of Endophytic *Audouinella* sp. 3. in section a-c show the multilayer construction of the cell wall. d shows the corrugation in the cell wall. (magnification a,d x11900, b x19600, c x44100)


12 a


12 b

12c


12d

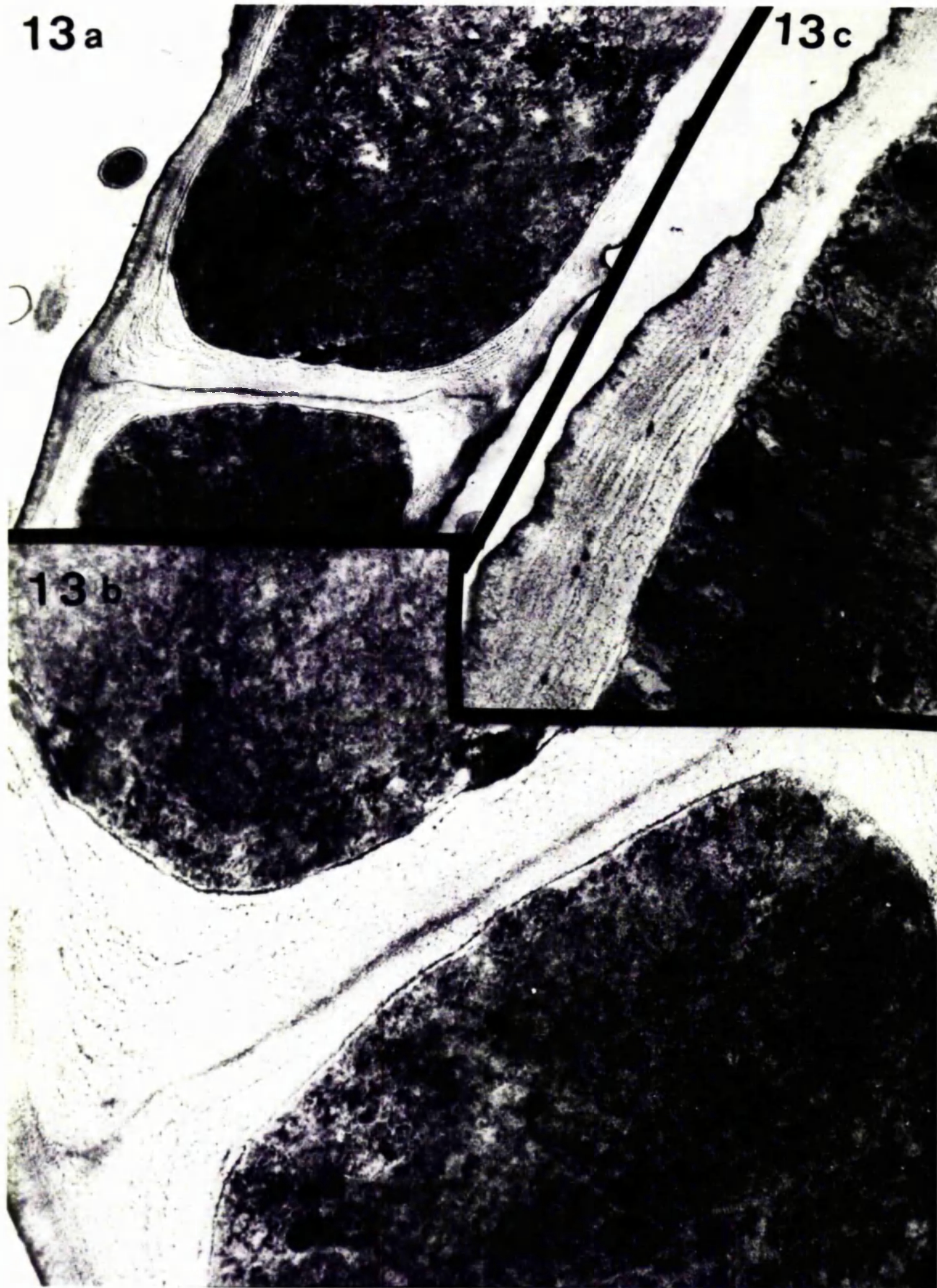

13a
13b
13c

Figure 4.1.13a-c The two layered cell wall of Endozoic *Audouinella* sp. 1; a and c show the outline of high electron density which is thought to be the region reacting with Ruthenium red. (magnification a x19600, b x33600, c x54600)

Figure 4.1.14a-b The two layers in the cell wall of Endozoic *Audouinella* sp. 2. Note the cell junction region with material of high electron density. (magnification a x19600, b x54600)

14a

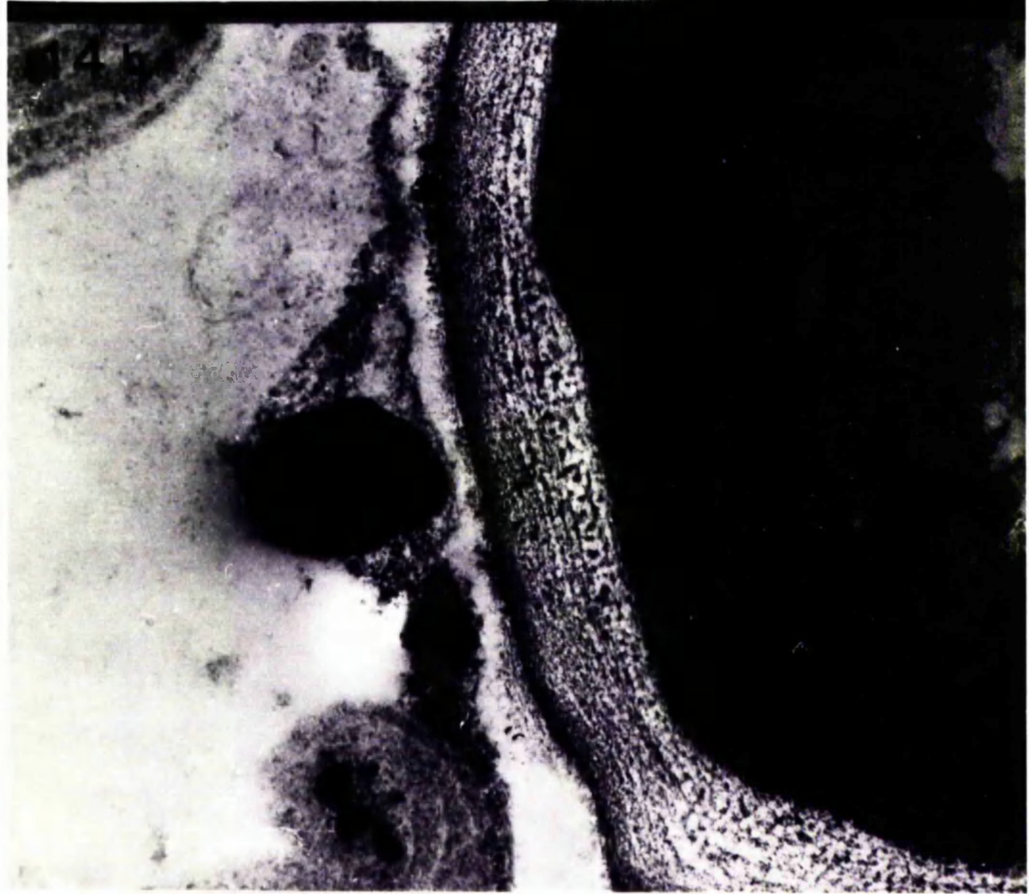
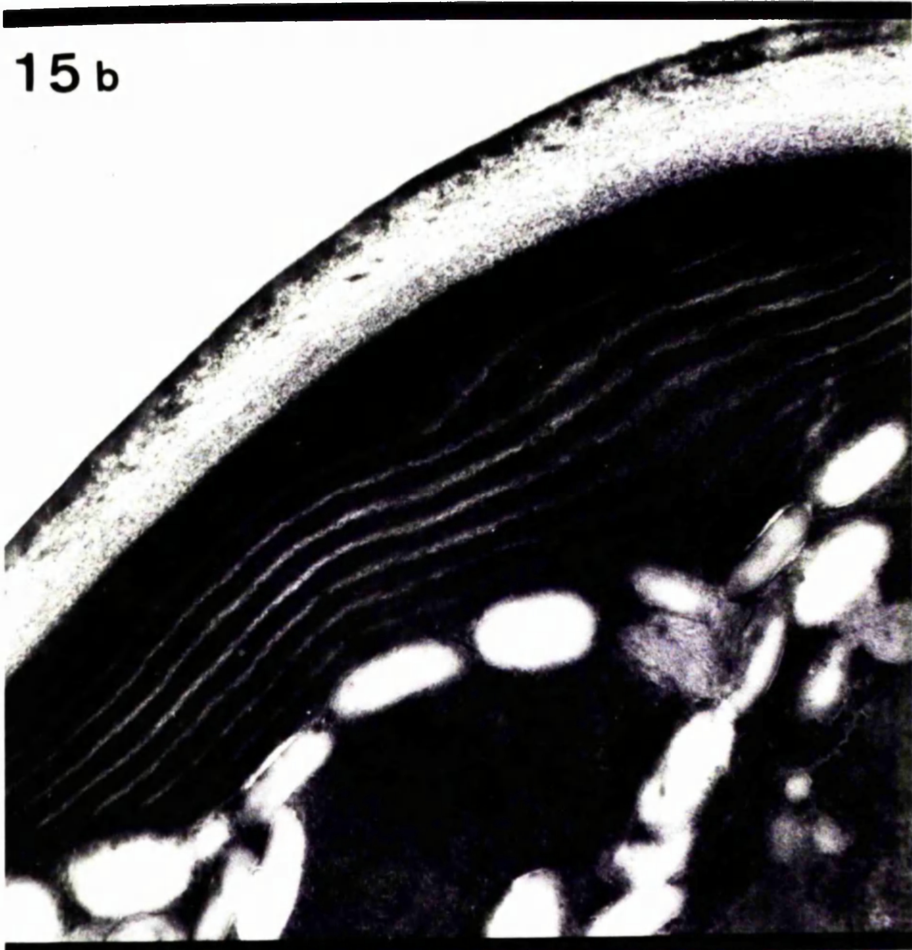


Figure 4.1.15a-c The cell wall structure of *A. asparagopsis*. c shows the cell in cross section. (magnification a x15400, 'b x44100', c x19600)

15a


15b

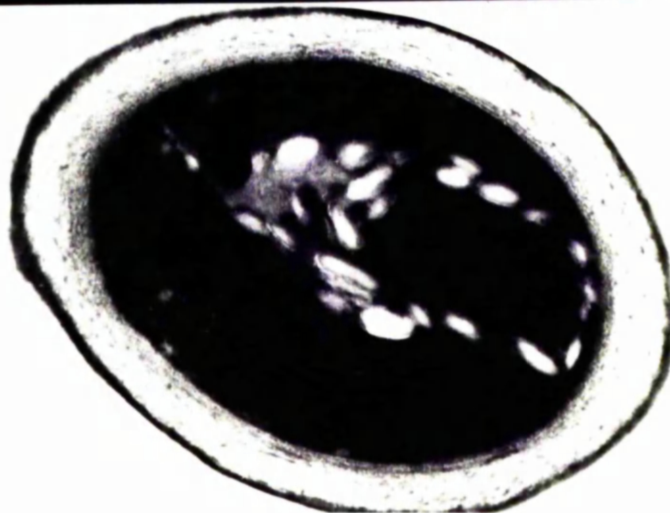

15c

Figure 4.1.16a-c The unevenly electron dense layers in the cell wall of *A. newtonii.* c shows the corrugated region with layers of different electron density. (magnification a x19600, b x54600, c x45600)

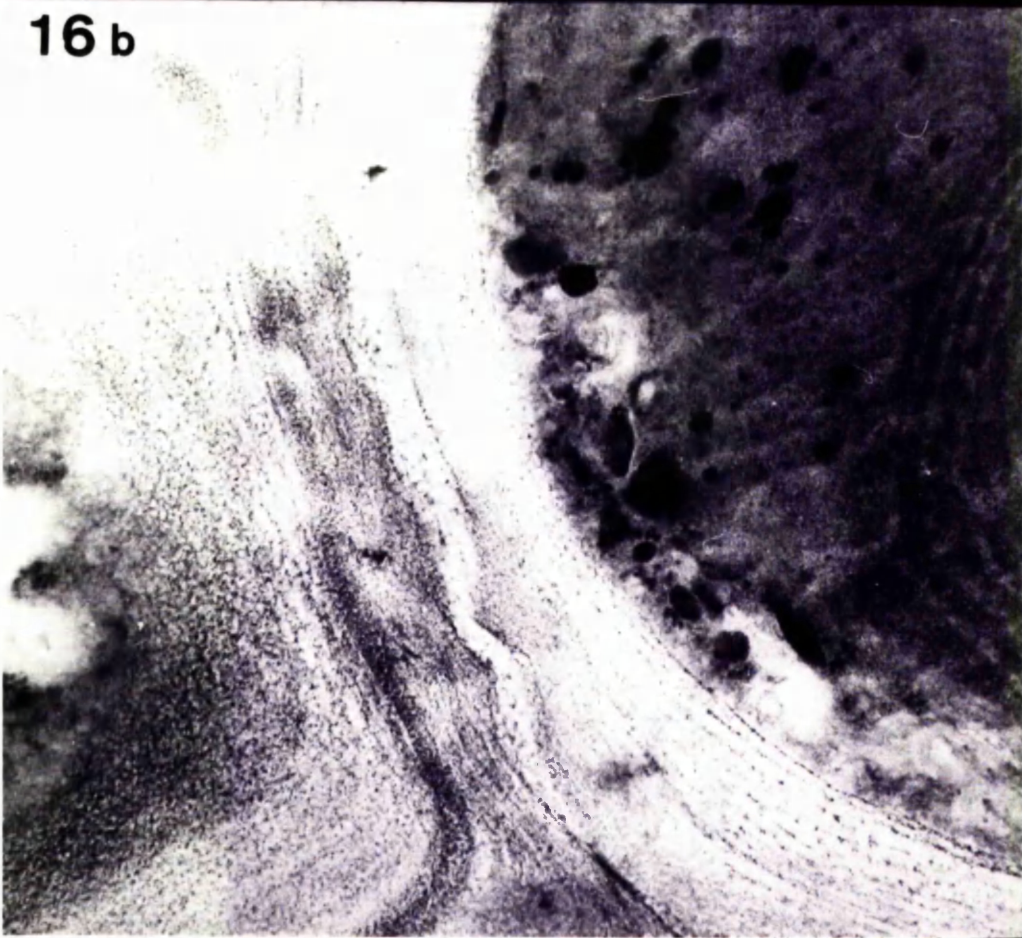
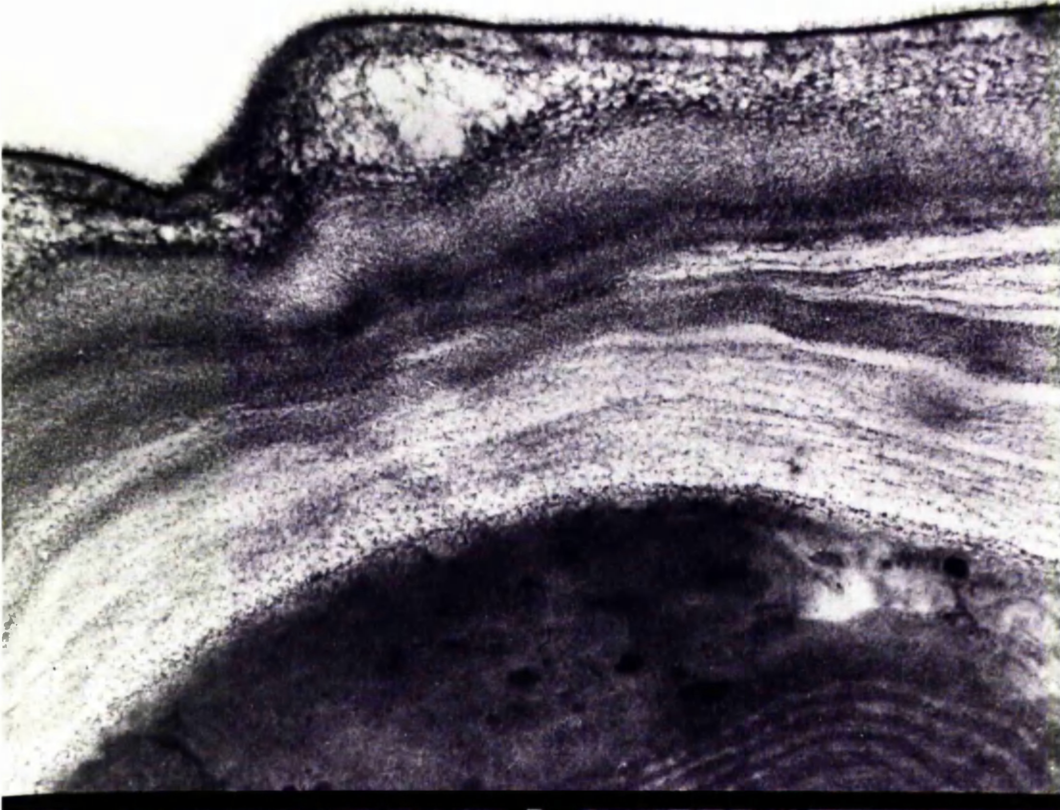
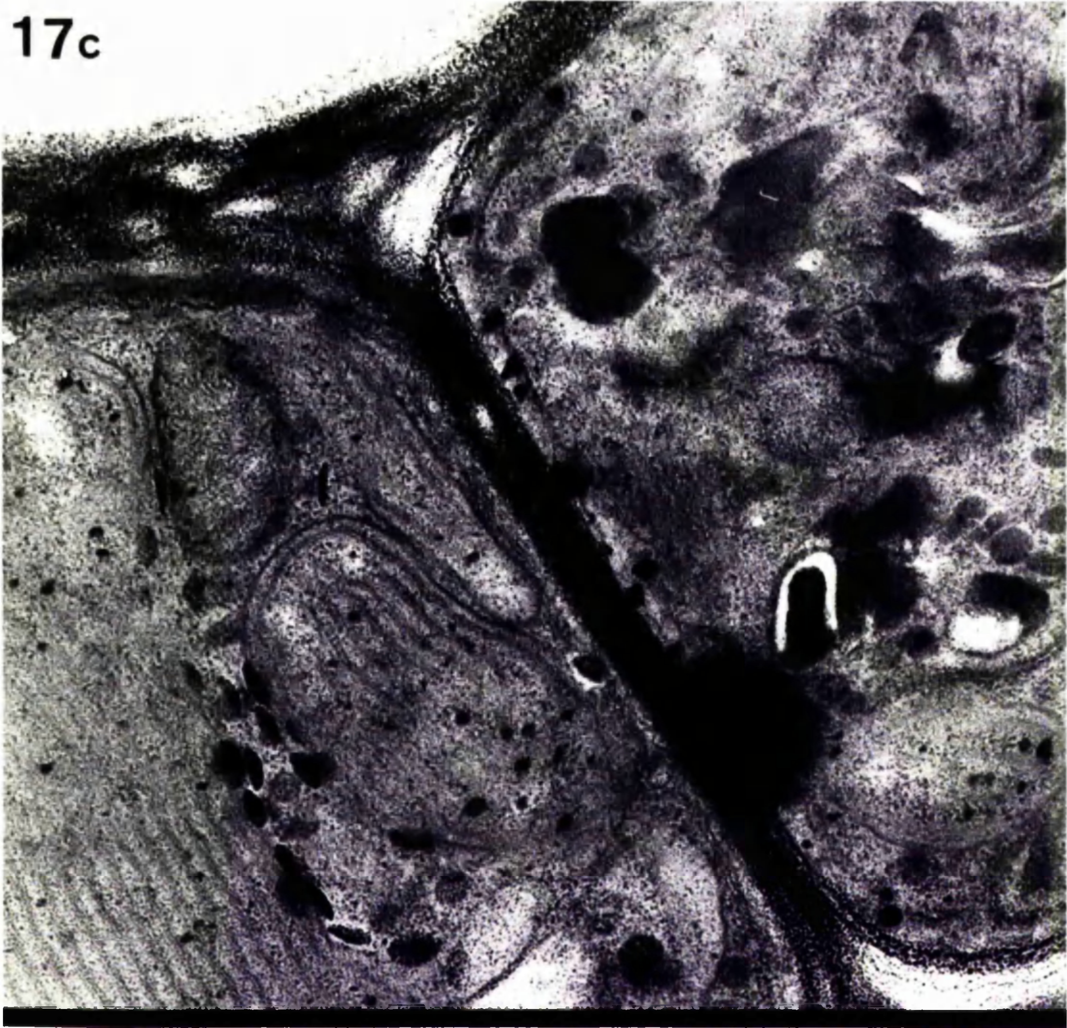

16 b

Figure 4.1.17a-d The cell wall structure of of *A. virgatula*. Note the high electron density of material in the cell wall. d shows the cell wall of the sporangium with evidence of two cell walls of an old sporangium. (magnification a,d x7000, b x18200, c x27500)

16c



17c



17d

The cell wall of Endophytic *Audouinella* species 2 showed the presence of two layers, the outer one more electron dense than the inner (Fig. 4.1.11a). The outer layer corresponds to the region of carboxylated polysaccharide as shown by the staining technique. The inner layer, however, appears to be composed of two regions the outer one more evenly granular in appearance and the inner of a more loosely packed almost thread like material. The nature of these components was not established. The plug cap also shows multilayered construction (Fig. 4.1.11b).

The cell wall and the cell junctions of Endophytic *Audouinella* species 3 are similarly constructed of a layer of low electron density with thin outer layers more electron dense (Figs. 4.1.12a-c). Cross section showed corrugations in this layer (Fig. 4.1.12d). The staining methods showed that the cell wall contains two different layers of different natures, carboxylated polysaccharide in the outer layer and sulphated polysaccharide in the inner layer. The detached structures on the outside shown in Figs. 4.1.12c,d are probably those giving the positive reaction with Ruthenium red.

The cell wall of Endozoic *Audouinella* species 1 showed a two layered structure with a high electron dense outline on the outer side which is most likely to be that reacting with Ruthenium red -the only positive reaction obtained with the histochemical tests (Figs. 4.1.13a-c).

The cell wall of Endozoic *Audouinella* species 2 showed two different layers, a dark (high electron dense) outer and a light (low electron dense) in the inner side (Fig. 4.1.14a). The cell junctions region showed a line of material of high electron density which may contain a mixture of carboxylated and sulphated polysaccharide according to results obtained from the staining reactions, and these may be contained in the wide diffuse layer shown (Fig. 4.1.14b).

The cell wall of *Audouinella asparagopsis* showed two layers, the outer one more electron dense and the inner one less electron dense (Figs. 4.1.15a-c). The outer layer corresponds to the only positive reaction region of sulphated polysaccharide shown by

the staining technique. However, cross section (Fig. 4.1.15c) showed no corrugations as shown by endophytic *Audouinella* species 3 (Fig. 4.1.12d).

The cell wall of *Audouinella newtonii* showed some layering but with regions of less evidence of differences in electron density (Figs. 4.1.16a,b). However, it showed corrugated layers of different electron density regions (Fig. 4.1.16c).

The nature of the layout of the fibrillar layers in these isolates were not clear even under high magnification (x54600).

The cell wall of *Audouinella virgatula* showed two layers. The outer one is very thin and less electron dense and the inner one thicker and of more electron density (Fig. 4.1.17a-d). Of particular interest is the appearance of the sporangium section where multilayers of high electron density can be seen which shows the evidence of sporangium proliferation (Fig. 4.1.17d). However, the inner layer appeared more darker than any other above species investigated. Histochemical techniques were not applied to this species as its cultures were lost when the growth chambers were accidentally switched off during the course of this study.

Discussion

The delicate nature of these *Audouinella* isolates appeared an obstacle which prevents us from a close look into the details of these plants under high magnifications especially when using tools such as scanning electron microscopy where material has to be subjected to different chemicals and treatments. The material was sometimes lost during these processes, or broken or deformed. The latter appeared to be more significant and may lead to misleading intrepretations when looking for any differences among these species which may considered characters against which proposed specific or even generic delimitation in this family can be tested. In this study it was found that the striated cell surfaces of these plants proposed by Garbary (1978b) cannot be used as a character for speciation as striated surfaces were not present in all samples of the same

species when these samples were treated separately. Moreover, in the same sample it was possible to find cells with striated and severely wrinkled surfaces and others with smooth surface. However striations and wrinklings were common in the erect systems with more delicate cells. These irregularities in the surface of these isolates appeared to be due to shrinkages caused by the processing procedure and the variable results can be due to a variety of reasons -of which relative cell age may be the most important. The cell junctions in endozoic *Audouinella* sp.2 showed that it was more tolerant of these shrinkages and these regions appeared as rings joining the cells, and this may be due to different supporting materials present in these parts. Striated cell walls were also observed by **TEM** in cross sections of endophytic *Audouinella* sp. 3. However, these corrugations were seen in some samples whilst they were absent in others. This may be due again to uneven fixation procedures and the condition of the sample, and tends to confirm the uncertainties obtained by the **SEM** examinations. These suggestion are supported by observation made by Garbary (1978b) where he found that in some samples only apical cells exhibited wrinkled surfaces. However, Garbary (1979c) used different methods and solutions for processing his material in his study, so that variation in results may be contributed in part to use of different fixatives.

Exobiotic species appeared to be more tolerant to treatment and thus showed less cell wall wrinkles, and this may be due to the thicker nature of the cell wall. This suggestion is supported by the appearance of the cell wall of *Audouinella virgatula* under **TEM** and the irregularities in cell wall surfaces of endobiotic species investigated in this study. Garbary (1978b) suggested that **TEM** studies must accompany **SEM** studies for confirmation of results.

Staining techniques showed variable results. These cell junctions of endozoic *Audouinella* sp. 2 appear to be composed of cellulose, mixed sulphated and carboxylated polysaccharides, pectic and phenolic materials. These rings were also seen in endophytic *Audouinella* species 1 which was also showed the presence of sulphated polysaccharides

in the cell junction regions. Whilst cell wall of *Audouinella asparagopsis* and endophytic *Audouinella* species 2 and 3 showed positive reaction only to Alcian blue and Alcian yellow, *Audouinella infestans*, *A. newtonii* and endophytic *Audouinella* species 2 showed positive reaction to Chlorazol Black E, and endophytic *Audouinella* species 2 and 3 and endozoic *Audouinella* species 1 showed positive reaction to Ruthenium red. These results cannot be interpreted easily because of inconsistency of these reactions to all reagents which are expected to give the same results, i,e. to detect the same chemical compound in the cell wall. These variable results obtained by histochemical methods may in part due to the thinness of the walls so making detection of various 'layers' difficult.

The cell wall micrographs show, as expected, the different layers that cannot be detected by light microscope, and show that in some instances the fine structure can be related to histochemical observations. In addition they revealed some differences in the nature of these layers with non fibrillar components of differing electron densities tending to predominate and variations in the embedded fibrillar material where visible. They also showed the typical multilayered cap of the septal plug or 'pit connection' of the Rhodophyta, although it was not possible to have sections of the pit connection region in all species .

However, these results have indicated that there may be differences in wall chemistry between species, some features of which can be equated to their fine structure. Hence this is an aspect of *Audouinella* species which warrants further detailed exploration and could yield information of taxonomic value.

4.2. Phycoerythrin pigments in *Audouinella* isolates.

Introduction

Phycoerythrins and phycocyanins are well known features of red algal pigment

arrays. They are proteinaceous and water soluble, so distinct from chlorophylls and carotenoids. Phycoerythrins can be classified according to their origins and their light absorbance. In red algae, three types of phycoerythrins are present, R- , B- , and b-phycoerythrins (Ragan 1981). R- phycoerythrin is found in the class Florideophyceae with absorption maxima at 565, 540, and 509 nm, b- phycoerythrin is found in the class Bangiophyceae with absorption maxima at 565 and 545 nm, and B- phycoerythrin is a phycoerythrin common in both Florideophyceae and Bangiophyceae with absorption maxima at 565, 545 and 495 nm (Ragan 1981). Glazer *et al* (1982) identified three R-phycoerythrins, type I (with absorption maxima of 566 > 543 > 497 nm) , II (with absorption maxima of 566 > 551 > 496 nm) , and R (with absorption maxima of 567 > 539 < 496) and two of B-phycoerythrins, type I (with absorption maxima of 542 > 567 > 502 nm) and II (with absorption maxima of 566 ~ 528 > 500nm). The absorption spectra of these phycoerythrin types depend on the ratio of the tetrapyrroles (phycoerythrobilin and phycourobilin) attached to each phycoerythrin.

Phycoerythrins are attached to photosynthetic lamella in submicroscopic phycobilisomes and can be isolated by suspension of the cells in phosphate buffer (pH 7) for disruption and then adding a detergent (Triton X-100) for their release from the membrane. On the other hand, they also can be isolated by suspending the cells in distilled water for some time, but in this method the protein may be partially dissociated or denatured at such very low ionic strengths, and may be degraded by proteases of the alga itself, or by proteases introduced through microbial contamination (Glazer *et al.* 1982). Earlier studies have shown the presence of phycoerythrin pigments of both R- and B- types in *Audouinella* species (Boney and White 1968; Boney 1972; Glazer *et al.* 1982). Glazer *et al.* (1982) investigated 31 *Audouinella* isolates grown in culture under different temperatures and light regimes together with numerous other red algae. They found that these pigments do not appear to be useful at familial, ordinal and class level in the taxonomy of the red algae they investigated, but they appear to be of

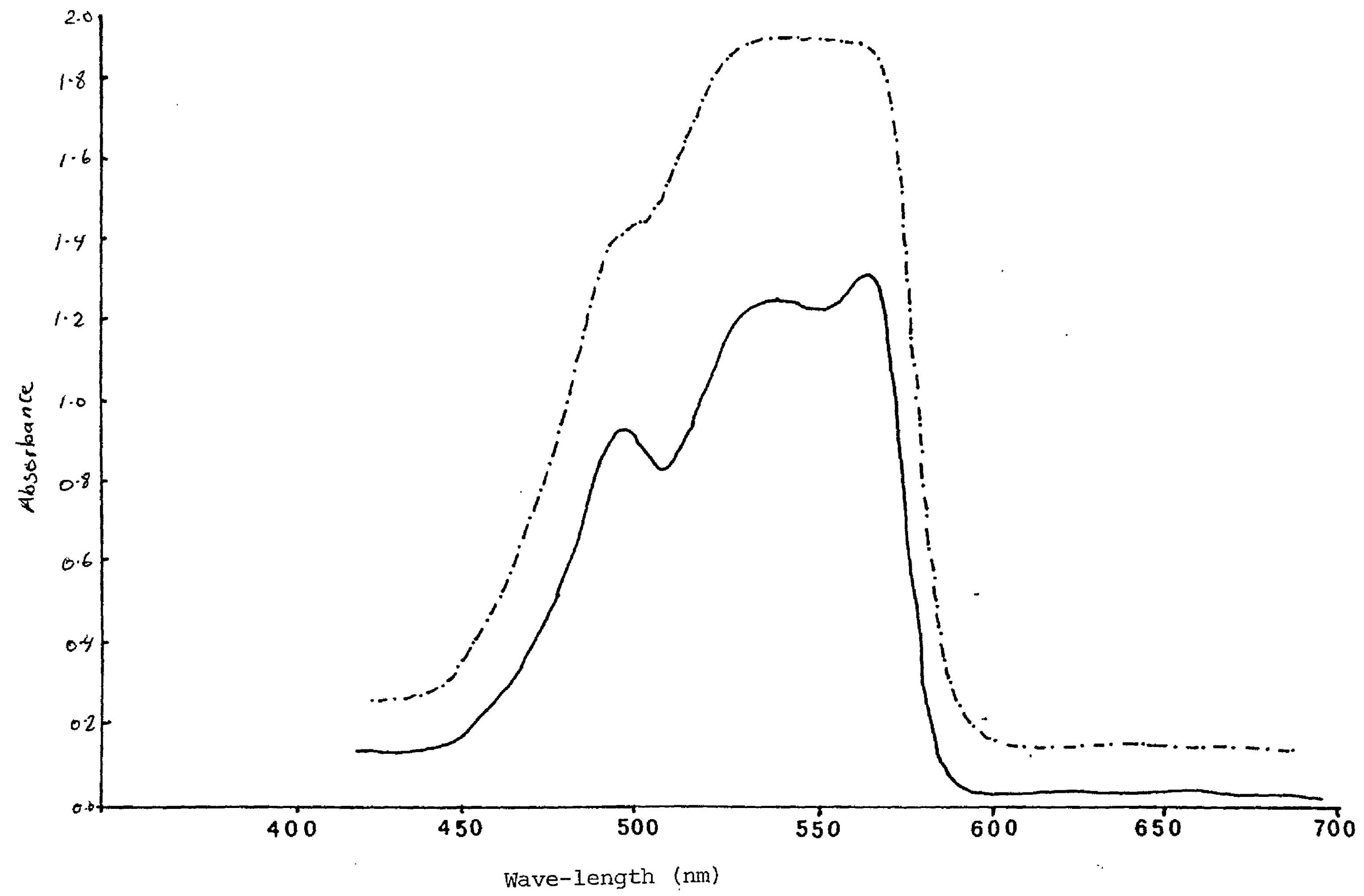
limited value in discriminating taxonomic groupings at the generic and specific levels. They stated that *Audouinella* species can be separated into two groups with B- and R-phycoerythrin types. Hirose *et al* (1969) proposed the division of Nemaliales into two groups according to the characteristics of phycoerythrins contained. Thus an investigation of the phycoerythrins of the *Audouinella* species under study seemed worthwile.

Results:

Table 4.2 shows maximum absorbance peaks recorded from Figs. 4.2.1-4.2.4 of *Audouinella* species investigated in this study and Table 4.3 shows results obtained from this study and from Glazer's *et al.* (1982) study.

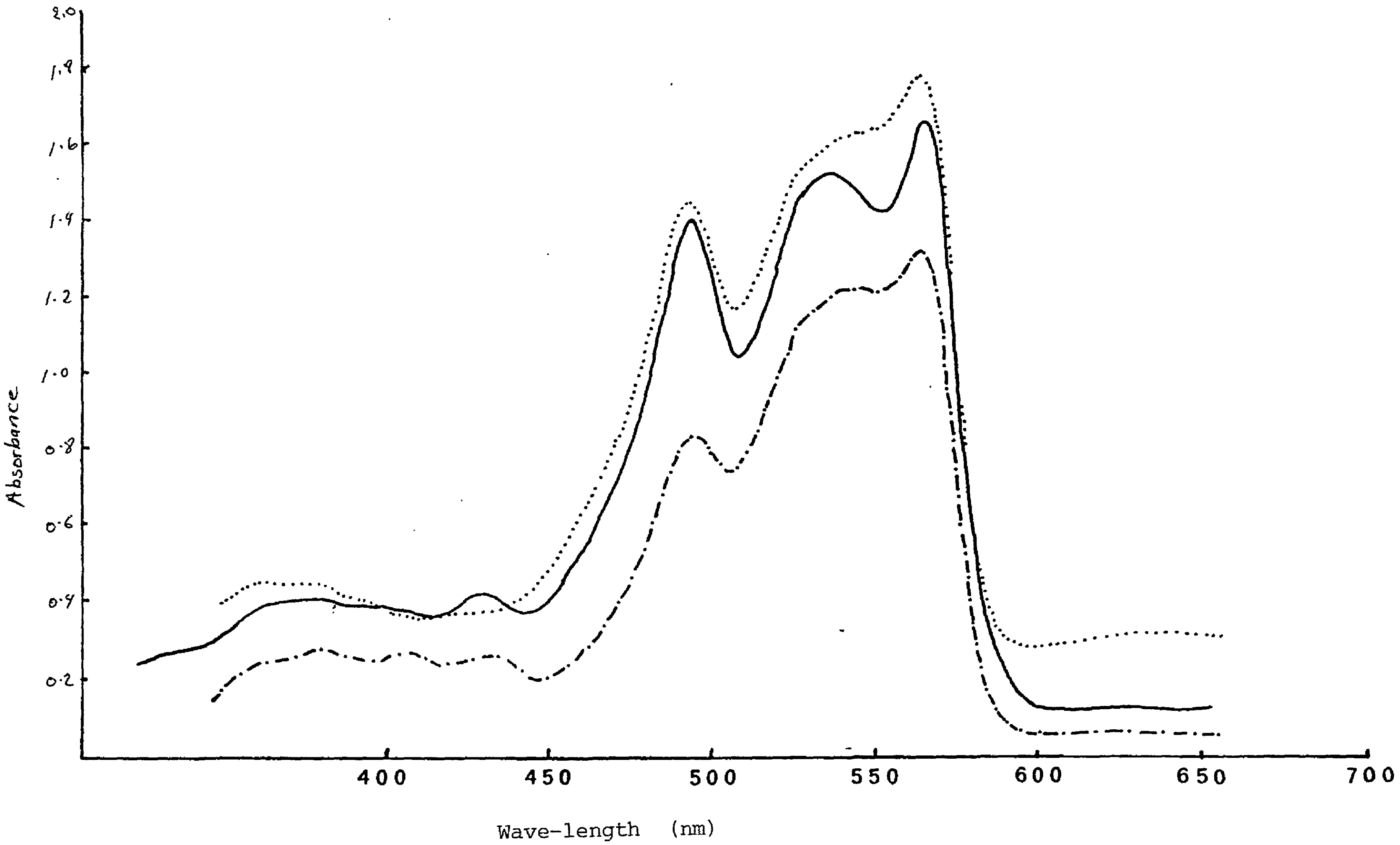
All samples produced similar traces for absorption spectra with the two peaks and a shoulder typical of R- and B- phycoerythrin with the exceptions of *Audouinella efflorescens* and endozoic *Audouinella* sp. 2 which both showed a broad peak rather than the typical curves observed in the other species. The variant obtained with *A. efflorescens* phycoerythrin has a peak lying at 500 nm, suggesting a variant of B-1 phycoerythrin. *Audouinella infestans*, endophytic *Audouinella* 1 and 3 all showed the presence of R-phycoerythrin and endophytic *Audouinella* 2 and endozoic *Audouinella* 1 and 2 showed R-1 phycoerythrin type. Glazer *et al* (1982) found that R-phycoerythrin is the commonest among *Audouinella* species and they were able to identify the R-phycoerythrin type I and II and B-phycoerythrin type I in these *Audouinella* species. Accordingly they concluded that these phycoerythrins could be used as a taxonomic marker at the species level. Table 4.3 shows that six different isolates from different localities of *Audouinella purpurea* contained the same R-phycoerythrin, on the other hand, four different sexual isolates of *Audouinella pectinata* showed some differences, three Washington isolates containing B-phycoerythrin and an isolate from France contained R-phycoerythrin.

Fig. 4.2.1 shows maximum absorbance peaks obtained with phycoerythrins of *Audouinella efflorescens* (_._) and *A. infestans* (__).



Absorbance
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
400
450
500
550
600
650
700
Wave-length (nm)

4.2.2 shows maximum absorbance peaks obtained with phycoerythrins of endophytic *Audouinella* species. 1 (.....), sp. 2 (_._) and sp.3 (__).



Absorbance
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
400
450
500
550
600
650
700
Wave-length (nm)

4.2.3 shows maximum absorbance peaks obtained with phycoerythrins of endozoic *Audouinella* species 1 (__) and sp. 2 (_._).

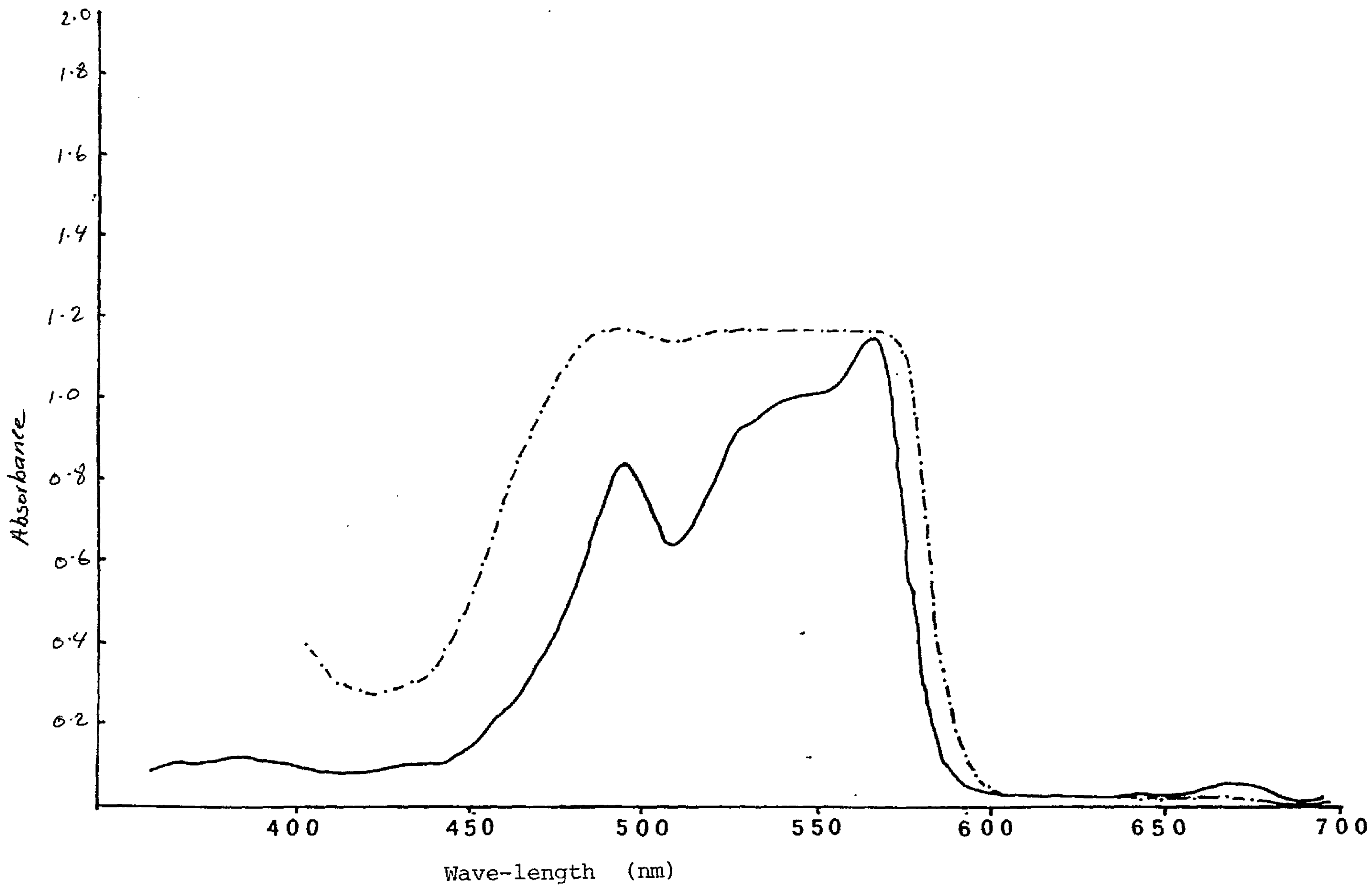
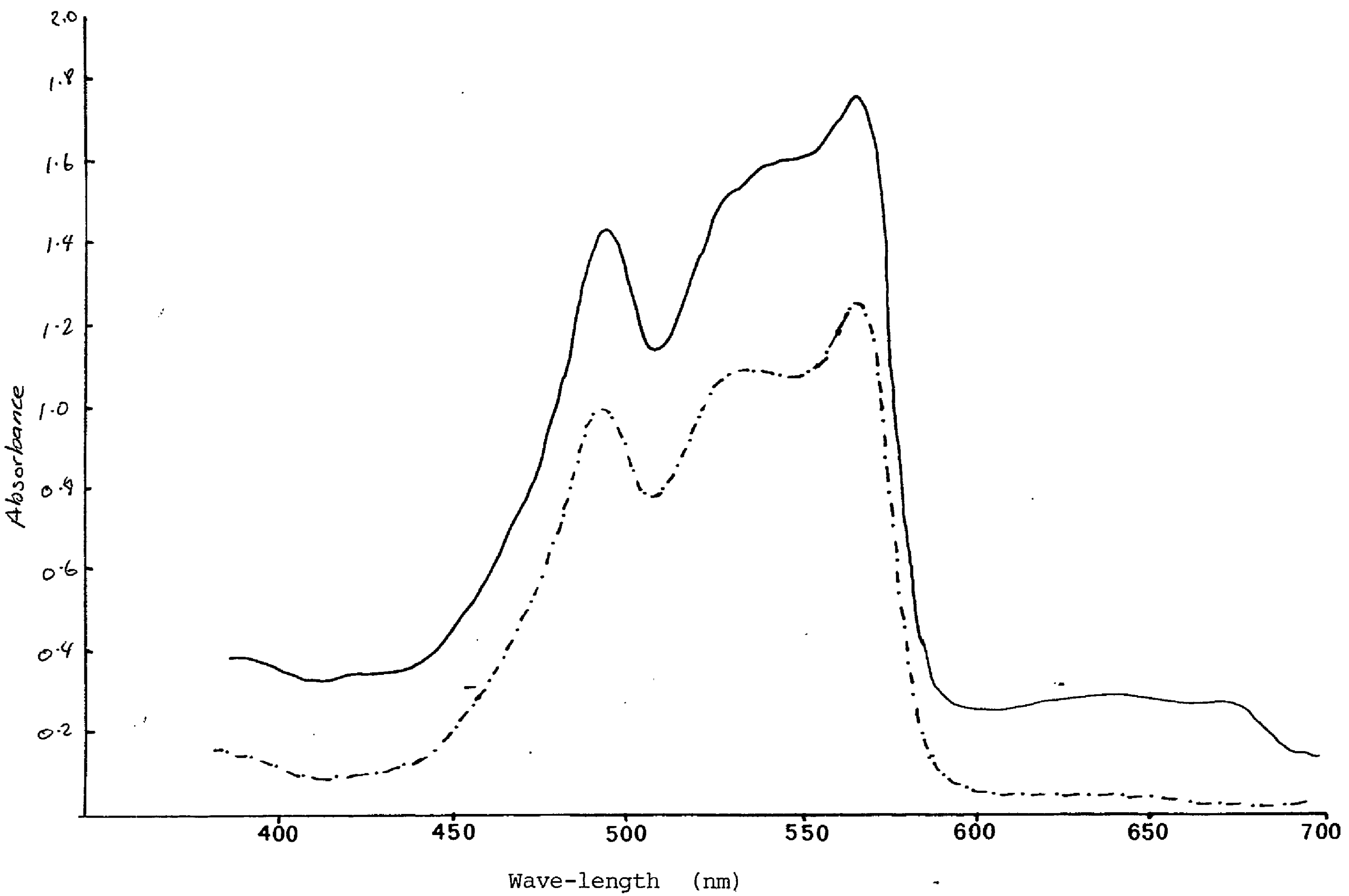

Absorbance
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
400
450
500
550
600
650
700
Wave-length (nm)

Fig. 4.2.4 shows maximum absorbance peaks of phycoerythrins obtained from fresh (__) and old (_._) tufts of endophytic *Audouinella* species 1.



2.0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
Absorbance
400
450
500
550
600
650
700
Wave-length (nm)

Table **4.2** The absorption spectra of *Audouinella* phycoerythrins

Species	Absorption	spectrum	maxima (mμ)
Audouinella efflorescens	563	541	500
Audouinella infestans	563	538	494
Endophytic *Audouinella* 1	564	532	495
Endophytic *Audouinella* 2	564	541	495
Endophytic *Audouinella* 3	564	538	494
Endozoic *Audouinella* 1	564	546	495
Endozoic *Audouinella* 2	568	543	493

Table **4.3** The absorption spectra and type of phycoerythrins in *Audouinella* species

species	Absorption spectra (mμ)	Phycoerythrin type
Audouinella. arcuata 482*	565, 539, 499	R
A. bonnemaisoniae 1671*	566, 543, 498	B-I
A. concrescens 603*	563, 550, 499	R-II
A. densa 483*	565, 538, 500	R
A. endophytica 423*	566, 543, 497	R-I
A. floridula 925*	564, 546, 498	R-I
A. floridula 1777*	563, 548, 479	R-II
A. membranacea 737*	565, 548, 500	R-II
A. pectinata 304*	564, 544, 498	R-I
A. pectinata 1659*	565, 545, 498	R-I
A. pectinata 1670*	564, 545, 500	R-I
A. pectinata 1686*	564, 540, 501	R-I
A. proskauri 487*	565, 544, 499	R-I
A. purpurea 591*	567, 542, 497	R-I
A. purpurea 655*	567, 542, 498	R-I
A. purpurea 1694*	567, 543, 498	R-I
A. purpurea 1695*	567, 545, 497	R-I
A. purpurea 1696*	567, 543, 498	R-I
A. purpurea 1699*	566, 542, 498	R-I
A. rhizoidea 547*	567, 540, 498	R
A. saviana 1553*	566, 545, 498	R-I
A. variable 716*	564, 540, 497	R
A. variable 1677*	566, 545, 498	R-I
A. sp. 679*	567, 542, 502	B-I
A. sp. 681*	566, 545, 500	B-I
A. sp. 1519*	566, 542, 502	B-I
A. sp. 1520*	566, 541, 499	B-I
A. sp. 1551*	566, 547, 498	R-II
A. sp. 1667*	566, 546, 498	R-I
A. sp. 1680*	566, 543, 498	R-I
A. sp. 2227*	566, 539, 498	R
Audouinella efflorescens	563 541 500	B-I
Audouinella infestans	563 538 494	R
Endophytic *Audouinella* 1	564 532 495	R
Endophytic *Audouinella* 2	564 541 495	R-I
Endophytic *Audouinella* 3	564 538 494	R
Endozoic *Audouinella* 1	564 546 495	R
Endozoic *Audouinella* 2	568 543 493	R-1

* Glazer *et al.* (1982).

No differences were obtained with the absorption spectra from different samples of similar age of the same species (as in endophytic *Audouinella* sp. 3) nor from samples of different age (as with endophytic *Audouinella* sp. 1).

The amount of phycoerythrin obtained from different samples of the same species were different and were not related to the biomass of the sample, but to the colour of the material. Thus endophytic *Audouinella* sp. 3 produced a smaller amount of phycoerythrin because of its brownish colour, especially when grown in higher light intensities. However, different species of algae are known to increase or decrease their quantities of these pigments as an adaptation to light-limiting conditions (Ramus *et al.* 1976).

Discussion

The fact that red algae can adapt to light-limiting conditions by increasing the total phycobiliprotein content or by specifically producing the phycobiliprotein type capable of absorbing the prevalent available wavelengths was demonstrated by the endophytic *Audouinella* species 3, when this alga was left under different light intensities.

The presence of both R- and B-phycoerythrins in *Audouinella* species under study suggests their affinities with the Bangiophyceae and Florideophyceae and makes the use of these pigments as taxonomic characters very limited. These observations thus lend support to the views of Boney and White (1968); van Der Valde (1973) and Glazer *et al* (1982). However, the phycoerythrin type and other morphological features may show some significance in their taxonomy especially when these bear some resemblance, as the case of *A. pectinata* from France, which Glazer *et al.* (1982) suggested represents a distinct species from the plants of the same name from Washington. Those *Audouinella* species with common features such as 'host' organism origin can be separated using the phycoerythrin type as in the case of endophytic *Audouinella* 1 and 2 which were both isolated from *Delesseria* but were found to contain phycoerythrin

types R and R-1 respectively. Grouping of these species according to the phycoerythrin they contained was suggested by Glazer *et al.* (1982). Among these groups it is possible to find some differences in the nature of peaks obtained such as the broad peak (flat peak) found in *Audouinella efflorescens* and endozoic *Audouinella* species 2.

Similar observations were reported by Boney and White (1968) for *Audouinella endophytica* (=*Acrochaetium endophyticum*). The presence of these broad peaks is likely to be due to presence of some other modification of phycoerythrin types, as those reported by Hirose *et al.* (1969). Glazer *et al.* (1977) related the presence and absence of peaks or shoulders to the structure of the phycoerythrin type. Gabrielson and Garbary (1987) concluded that this *Audouinella* complex is not monophyletic due to different phycoerythrins present in this group and suggested that studies on phycoerythrin types linked with the ultrastructure of pit plugs may prove useful from a taxonomic point of view.

The age of these *Audouinella* species appeared to have no effect on the type of phycoerythrin produced by these plants as different samples of different age of endophytic *Audouinella* species 1 showed the same peaks consequently the same phycoerythrin type.

4.3. Calorific values of some *Audouinella* species.

Introduction:

The study of cell wall components and cell volumes and contents of *Audouinella* species showed some differences in their wall constituents and cell contents. It was decided to study the calorific values of these species, or the energy stored in the biomass in the form of oxidizable chemical compounds in cell walls and cell contents. This is possible if the oxidizable material can be converted into heat and expressed in energy units (calories and kilocalories). Trophic dynamics studies have led to the development of different calorimetric methods of measuring such values. Paine (1971) stated that

there are at least four procedures that could permit evaluations of an organism's heat content.

The most favoured and most practical is that using the bomb calorimeter. In this procedure the oxidizable material can be combusted in a chamber of limited volume in the presence of pressurized oxygen. Different types of bombs have been developed, adiabatic and non-adiabatic, macro-, semi-micro- and micro-oxygen bombs. Selection of any of these calorimeters is determined by the amount of material available. The Phillipson's Micro Bomb Calorimeter was the most suitable one for these *Audouinella* species, as it combusts samples of 5 to 100 mg dry weight, and only small quantities of the *Audouinella* species were available for measurements. Measurements of calorific values of algae have been made in the past, but they were mainly for their selection and preference as food for marine grazers. Paine and Vadas (1969) investigated the calorific values of 72 marine algal species. These plants were collected from different localities with different environmental conditions. They found that seasonal factors have little effect on the calorific values, but factors such as phyletic affinity, water purity and depth of immersion were significant.

In this study all the *Audouinella* species investigated were of the same age and grown under the same conditions. The objective, as stated earlier, was to determine whether a measure of calorific values would be an indication of taxonomic affinities, viz., whether related taxa would contain similar quantities of oxidizable organic matter of similar chemical natures.

Results:

Figs. 4.3.2- 4.3.10 present the firing curves of the *Audouinella* species used, and Table 4.4 shows the calorific values obtained. The dry weights of the material used varied between 5.07 - 18.78 mg. The carrier filler was avoided in the smallest sample since this sample is still in this bomb's capacity range.

However, during the combustion of these *Audouinella* samples some of these species (viz., endophytic *Audouinella* sp. 2, and endozoic *Audouinella* sp. 1 and 2) did not ignite after the initial drying process. This procedure was repeated three times, and each time they were found not to ignite. After they were dried for a longer periods (10 days in the vacuum refrigerator), the combustion was successfully achieved.

Table 4.4 lists the *Audouinella* species in order of low to high ash free content. In terms of ash free content these species can be placed in 4 groups.

Group 1. *Audouinella infestans* and endophytic *Audouinella* species 1 have the lowest and similar ash free contents among these *Audouinella* species under study. They produced different calorific values, and probably contain different biochemical (oxidizable) materials.

Group 2. *A. asparagopsis* and *A. newtonii* both have similar ash free contents and calorific values. Whether they have similar oxidizable materials or not is not known.

Group 3. Endophytic *Audouinella* species 2 and 3 and endozoic *Audouinella* species 1 and 2. These species have ash free dry weights of similar orders of magnitude. Of these the two endophytes would seem to have similar types of oxidizable materials as they produced similar calorific values, but they are possibly distinct from the two endozoic species.

Group 4. *A. efflorescens* stands apart as the one species with highest quantities of combustible material, but with calorific values no higher than with the species of Groups 1 and 2.

Table 4.5 show calorific values and ash free contents for different species of marine red algae as given in Paine and Vadas (1969). The ash free content was as low as 17% for *Lithothamnion* sp. (3.94 calories per mg.) and as high as 91% for *Bangia fuscopurpurea* (4.97 calories per mg.). In the main these are red algae with more complex thalli and with tissue specialization, and so tend to give much higher calorific values than the 'range' shown with *Audouinella* species (4.26 - 2.73 calories per mg.).

Fig. 4.3.1 Firing curve obtained from combustion of benzoic acid sample used for calibration of the micro bomb and determination of calorific values of *Audouinella* species (refer to labelling already described in Materials and Methods Fig. 2.5).

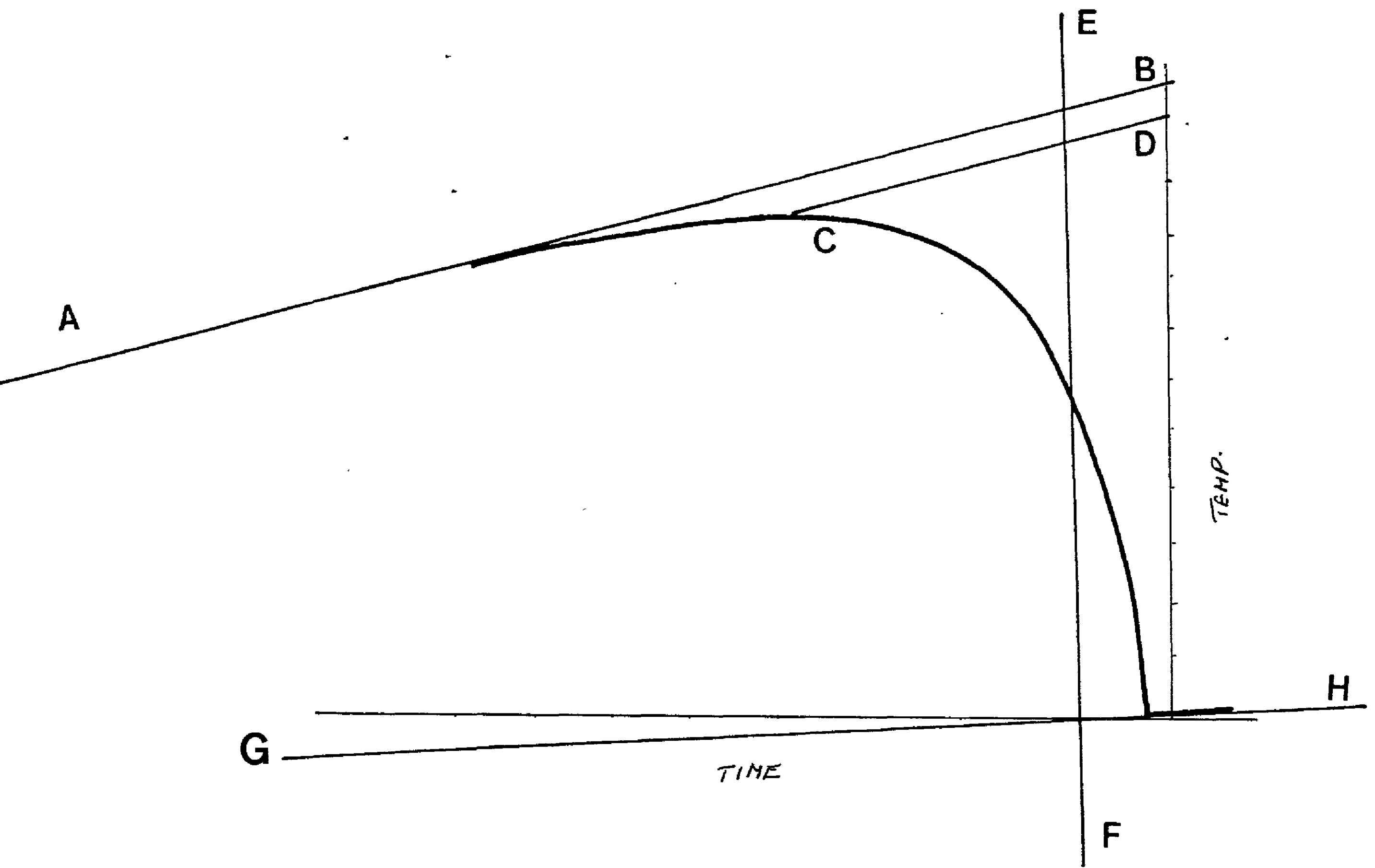
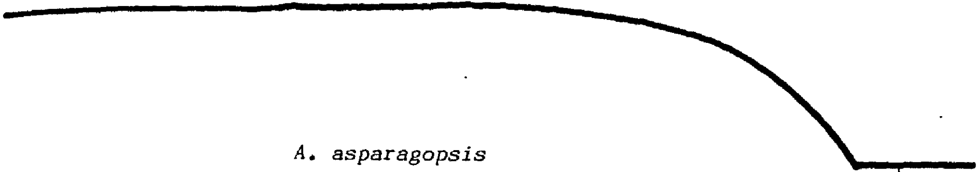
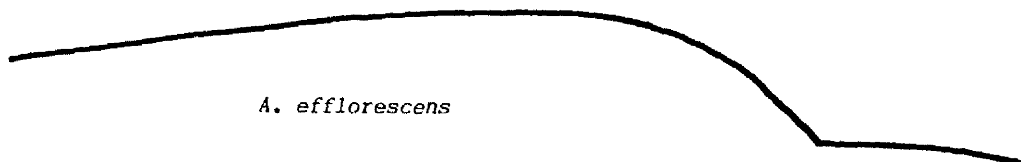

E
B
D
C
A
TEMP.
H
G
TIME
F

Fig. 4.3.2 Firing curve obtained from combustion of *Audouinella asparagopsis* sample (Dry weight 11.7 mg.).

Fig. 4.3.3 Firing curve obtained from combustion of *A. efflorescens* sample (Dry weight 7.65 mg.).

Fig. 4.3.4 Firing curve obtained from combustion of *A. infestans* sample (Dry weight 8.62 mg.).


A. asparagopsis


A. efflorescens

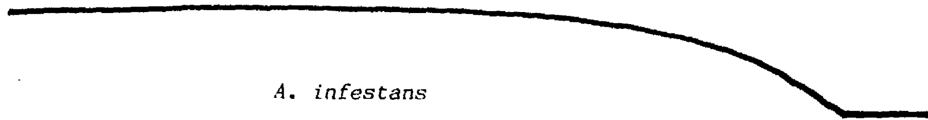
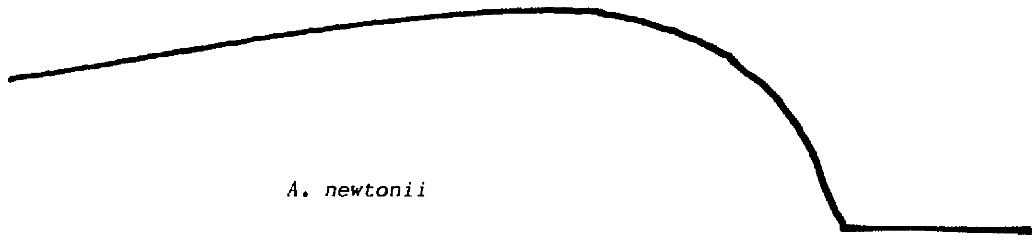
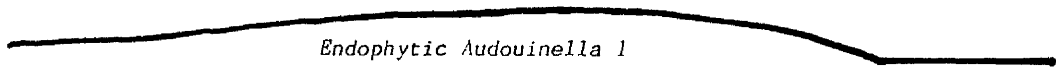

A. infestans

Fig. 4.3.5 Firing curve obtained from combustion of *A. newtonii* sample (Dry weight 16.3 mg.).

Fig. 4.3.6 Firing curve obtained from combustion of Endophytic *Audouinella* species 1 sample (Dry weight 5.07 mg.).

Fig. 4.3.7 Firing curve obtained from combustion of Endophytic *Audouinella* 2 sample (Dry weight 13.66 mg.).


A. newtonii


Endophytic Audouinella 1

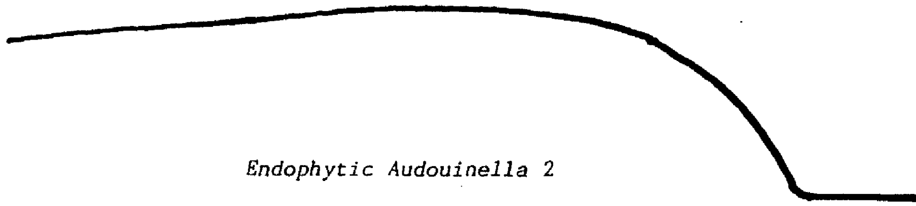
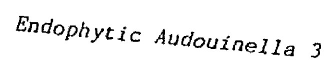
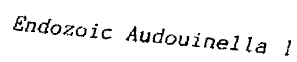

Endophytic Audouinella 2

Fig. 4.3.8 Firing curve obtained from combustion of Endophytic *Audouinella* species 3 sample (Dry weight 18.78 mg.).

Fig. 4.3.9 Firing curve obtained from combustion of Endozoic *Audouinella* species 1 sample (Dry weight 9.69 mg.).

Fig. 4.3.10 Firing curve obtained from combustion of Endozoic *Audouinella* species 2 sample (Dry weight 13.66 mg.).


Endophytic Audouinella 3


Endozoic Audouinella 1

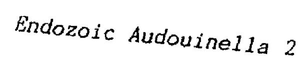

Endozoic Audouinella 2

Table 4.4 Calorific values of some *Audouinella* species

Audouinella species	Dry weight (mg)	Ash weight* (mg)	Ash weight* %	Ash free content** dry weight (mg)	Ash free content** dry weight %	cal. mg^{-1}
Endophytic *Audouinella* 1	5.07	2.33	46.0	2.74	54.0	3.48
A. infestans	8.62	3.72	43.6	4.9	56.4	4.26
A. asparagopsis	11.70	3.98	34.0	7.72	66.0	3.86
A. newtonii	16.3	5.1	31.3	11.2	68.7	4.06
Endophytic *Audouinella* 3	18.78	4.44	23.7	14.34	76.3	3.28
Endozoic *Audouinella* 1	9.69	2.04	21.0	7.65	79.0	2.73
Endophytic *Audouinella* 2	13.54	2.62	20.0	10.92	80.0	3.25
Endozoic *Audouinella* 2	13.66	2.59	19.0	11.07	81.0	3.03
A. efflorescens	7.65	1.06	16.0	6.44	84.0	4.04

*weight of residue after combustion

** weight of combusted material

Table **4.5** Calorific values of some marine red algae *

Species	Ash-free content %	cal. ash-free mg.$^{-1}$ dry weight
Bangia fuscopurpurea	91.0	4.97
Porphyra nereocystis	84.0	4.87
Porphyra perforata	90.0	4.79
Gelidium cartilaginium	88.0	5.00
Dilsea californica	73.0	4.84
Constantinea simplex	67.0	4.53
Lithothamnion sp.	17.0	3.94
Bossiella sp.	22.0	3.29
Corallina vancouveriensis	25.0	4.12
Calliarthron sp.	19.0	3.42
Endocladia muricata	90.0	4.56
Grateloupia doryphora	81.0	4.75
Prionitis lyallii	81.0	4.78
Callophyllis flabellulata	62.0	4.63
Erythrophyllum delesserioides	69.0	4.64
Schizymenia pacifica	66.0	4.47
Opuntiella californica	72.0	4.64
Gigartina corymbifera	70.0	4.36
Gigarina papillata	78.0	4.33
Iridaea sp.	75.0	3.89
Halosaccion glandiforme	88.0	4.68
Rhodymenia palmata	62.0	4.81
Anthithamnion subulatum	73.0	5.04
Microcladia coulteri	83.0	4.72
Ptilota filicina	71.0	4.78
Membranoptera sp.	78.0	4.74
Delesseria decipiens	68.0	5.44
Delesseria decipiens	72.0	4.90
Polyneura latissima	79.0	4.62
Polyneura latissima	79.0	3.85
Cryptopleura violacea	80.0	4.08
Polysiphonia brodiae	70.0	5.00
Polysiphonia sp.	68.0	4.88
Pterosiphonia bipinnata	69.0	5.30
Pterosiphonia sp.	69.0	4.80
Laurencia spectabilis	62.0	4.66
Rhodomela larix	69.0	4.94
Odonthallia floccosa	69.0	4.71

* Paine and Vadas (1969).

Table **4.6** Calorific values of some marine filamentous algae

Species	Ash free content %	cal. ash free mg.$^{-1}$ dry weight
Chlorophyta		
Cladophora sp. *	41.0	5.17
Spongomorpha sp. *	65.0	4.74
Phaeophyta		
Ectocarpus dimorphus *	75.0	5.09
Ralfsia sp. *	75.0	4.16
Elachista fucicola *	76.0	5.16
Rhodophyta		
Antithamnion subulatum *	73.0	5.04
Audouinella asparagopsis	66.0	3.86
A. efflorescens	84.0	4.05
A. infestans	56.4	4.26
A. newtonii	68.7	4.06
Endophytic *Audouinella* 1	54.0	3.48
" " 2	80.0	3.27
" " 3	76.0	3.28
Endozoic *Audouinella* 1	79.0	2.73
" " 2	81.0	3.03
Bangia fuscopurpurea *	91.0	4.97
Polysiphonia brodiae *	70.0	5.00
Polysiphonia sp. *	68.0	4.88
Pterosiphonia bipinnata *	69.0	5.30
Pterosiphonia sp. *	69.0	4.80

* Indicates data quoted from Paine and Vadas (1969).

Table 4.6 shows a comparison between different filamentous green, brown and red algal species obtained from the data of Paine and Vadas (1969) and from this study. The filamentous green algae produced higher calorific values than the *Audouinella* species. *Cladophora* and *Spongomorpha* have larger cells, thicker walls, and the filaments are more densely branched in *Cladophora*, and have different biochemical make-ups i.e, cellulose prominent in their cell walls. *Cladophora* sp., for example, has a lower ash free content (41%) than any *Audouinella* species but it produced the second highst calorific value among the filamentous algae. *Spongomorpha* sp. has an ash free content fairly close to, but lower than, *A. asparagopsis* and *A. newtonii* but again produced a calorific value higher than any *Audouinella* species under study. Filamentous brown algae showed similarities in their ash free contents and calorific values but they showed higher calorific values compared with *Audouinella* species of the same ash free content (e.g. Endophytic *Audouinella* sp. 3). These big differences suggest that known biochemical differences between the red and brown algae are reflected in the calorific values of their oxidizable substances. Although *Audouinella* species showed high ash free contents they showed lower calorific values (mean 3.55 cal./mg.) among filamentous red algae (mean value 4.84 cal./mg.). The two *Pterosiphonia* species had the ash free contents (69.0%) similar to that of *Audouinella newtonii* but these two different species of Pterosiphonia registered different calorific values (5.3 and 4.8 calories per mg.) and *Audouinella newtonil* showed a substantial lower calorific value (4.06 cal/mg.). These differences again are due to the relative complexity of their thalli and oxidizable components present in these more complex but basically filamentous red algae when compared with *Audouinella* species. *Antithamnion subulatum* is of branched filamentous form with large cells, and has an ash free content (73%) very close to ash free content of endophytic *Audouinella* species 3 (76%) but gave a higher calorific value than this *Audouinella* species. *Polysiphonia* species with branched corticated filaments showed similarities in their ash free content but gave higher calorific values compared with *A. asparagopsis* and *A. newtonii. Bangia fuscopurpurea* is similar to

the *Audouinella* species in its filamentous habit, but has a much higher content of combustible material and a higher calorific value in consequence, and the two membranaceous *Porphyra* species (Table 4.5) similarly have high ash free contents and corresponding calorific values. Only *Audouinella efflorescens* has an ash free content close to these, but a much lower calorific value. *Bangia* and *Porphyra* species are generally smaller celled with thicker walls, and these features, with differences in their biochemical materials, could explain the higher energy values of their constituent substances.

Discussion

The amount of heat from the combustion of the *Audouinella* species samples when converted into calories showed that there are differences between them. These differences are due to the body composition and to the combustible material present in each sample. Paine and Vadas (1969) related the differences they obtained to additional growth factors and immersion. Since all culture conditions under which the *Audouinella* species were grown were the same these results tend to confirm the variations observed in the cell wall studies. These variations such as presence of fibrillar material, and presence or absence of detectable carboxylated and sulphated polysaccharides could make important contributions to the calorific values, as with endozoic *Audouinella* species 1, which showed no signs of the presence of such materials by biochemical examination, and was found to have the lowest calorific value. In contrast, *A. infestans* also showed no signs of such materials but it has the highest calorific value. Low calorific values may be due to incomplete combustion of the material in endozoic *Audouinella* species 1 as it was found to require a longer drying process for its initial ignition. However, this suggestion is not supported by the results of the percent of the ash present in each sample. The nature of the combusted material is more likely to be the major contributor to these variations as the results showed that endozoic *Audouinella* species 1,

endophytic *Audouinella* species 2 and endozoic *Audouinella* species 2 with almost the same percentages (79, 80 and 81% respectively) of ash free contents gave different calorific values (2.73, 3.25 and 3.03 respectively). Similar results were also obtained by Paine and Vadas (1969) where samples of two species of the red alga (*Pterosiphonia*) with the same ash free content (69%) produced different calorific values, 5.3 calories per mg. with *P. pipinnata* and 4.8 calories per mg. with another *Pterosiphonia* species.

In terms of the cell wall structure it appears that the thinner the cell wall the lower ash free content produced as in the case of *Audouinella infestans* but the higher calorific value might be linked with a wall layer of low electron density and the quantities of non-fibrillar material present in the cell wall as was 'the case with *A. infestans*. Endophytic *Audouinella* species 1 also showed a similar cell wall construction but produced a higher calorific value in relation to the low ash free content found in this species. *Audouinella newtonii* and endophytic *Audouinella* species 2 had thicker walls with larger amounts of material of high electron density than the other species and produced lower calorific value in relation to their ash free contents. However, the thickness of the cell wall appears to contribute the major ash free content in each species. Endophytic *Audouinella* 3 and endozoic *Audouinella* species 1 and 2 relatively showed no differences in the cell wall construction by transmission electron microscopy and the staining technique showed the presence of carboxylated and sulphated polysaccharides in the cell wall of endophytic *Audouinella* species 3 and in the cell junctions of endozoic *Audouinella* species 2. These compounds would have contributed to the calorific value of these two species. Those cell wall features contributing to the calorific value of the endozoic *Audouinella* species 1 remain unknown as no clear results were obtained from histochemical studies. The cell wall thicknesses in these species were estimated from electron micrographs of the same magnification. Of the comparative data which are available (Table 4.5) those for

Bangia fuscopurpurea and the *Porphyra* species are of greater interest, since some species of *Audouinella* have been considered to show affinities with some members of the class Bangiophyceae. The results of the present study would suggest some differences in the biochemical values of the genera as reflected in the calorific values of their constituent materials.

The evidence from measurements of calorific values suggest that the biochemical natures of *Audouinella* species might be different from other filamentous red algae and possibly from one another. A combined study of cell wall structures and compositions, in association with other biochemical measurements, with measures of calorific values, might yield information of value in assessing relationships among these algae.

The grouping of these *Audouinella* species shown in Table 4.4 are only for sorting out purposes and do not necessarily suggest similarities in the species as species such as *A. asparagopsis* and *A. newtonii* have distinct morphological features. Endophytic *Audouinella* 2 and 3 also appear to be distinct species in terms of morphological features and hosts (*Delesseria* and *Polysiphonia* respectively).

4.4. Nuclei; their sizes in relation to cell volumes

Introduction:

The number of nuclei in each cell, their shape, size and arrangement in the cell vary considerably in the Rhodophyta. The position in the Florideophyceae is very complex. Apical cells are either uninucleate or multinucleate, the segments formed by their division having at the moment of formation the same nuclear characteristics as the apical cell from which they have been produced. In some genera where the apical cell is uninucleate the cells of the mature thallus remain uninucleate throughout the life of the plant whereas in other genera there is an increase in the number of nuclei as a consequence of nuclear division without any corresponding cell division. In those genera where secondary pit connections are formed nuclei are transferred between cells during the formation of these structures (Dixon 1966).

Exo- and endobiotic *Audouinella* species under study showed, as described earlier, morphological differences. These differences such as cell sizes, modes of growth, branching, chloroplast form do not always present obvious taxonomic criterion, and are not easily used to distinguish between the species. Whilst the cell contents viewed by light microscopy are used in taxonomic studies, and chromosome numbers measured in life history studies, few indications of nuclear volumes in relation to cell volumes are available. The diameter of the nuclei in red algae were found to be in average about 3 µm and showed considerable variation between species and also in different tissues or parts or cells of the same species, with further variation according to the time of the year at which the material was examined and the conditions under which the plant was growing (Dixon 1973). Magne (1978) stated that data on nuclei studies are very fragmentary and frequently inconsistent, but he suggested its re-assessment especially for red algae where the nuclei are small sized. Whittick (1987) investigated the relationship between cell volume, nuclear volume, and **DNA** levels in two species of Ceramiaceae (*Scagelia pylaisaei* (Mont.) Wynne and *Callithamnion corymbosum*

(Whitt.) S. F. Gray). He found that the nuclear volume and **DNA** levels are proportional to cell volumes. With such wide variations in cell size in the taxa under examination an investigation of this feature was thought worthwhile.

Observations:

Table 4.7 shows the nucleus and cell volumes of the *Audouinella* investigated. Both nuclear and cell volumes were wide ranging e.g. from 2.04 μm^3 in endophytic *Audouinella* species 4 to 119.43 μm^3 in endozoic *Audouinella* species 2, for nuclei and from 362.96 μm^3 in *A. infestans* to 5029.94 μm^3 in endozoic *Audouinella* species 2 for cells. Results indicate that in some taxa (*Audouinella efflorescens, A. infestans, A. virgatula*, endophytic *Audouinella* species 3, endozoic *Audouinella* species 1 and 2), irrespective of the range of nuclear or cell volume, the ratios are similar, suggesting that nuclear volumes remain proportional to cell volumes. For others (*A. secundata*, endophytic *Audouinella* species 2 and 4) then would appear to be variable relationships between nuclear and cell volume.

In addition to cell and nucleus sizes, the position of the nuclei in the cell were found to be different from one species to another. The nuclei in *A. virgatula* tended to be at the base of a cell, while in *A. infestans, A. efflorescens, A. secundata* , endophytic *Audouinella* sp. 2 and 3 and endozoic *Audouinella* sp. 1 tended to be in the middle in the first 2 species and laterally in the middle in the other 4. Nuclei of the endozoic *Audouinella* sp. 2 tended to be in the top third of the cell. The shapes of the nuclei were also varied. They were spherical, oval or irregular as in *A. efflorescens* which showed two condensed semispherical spots at the ends of each nucleus. This phenomenon made the measurement of nuclei diameters very difficult. However, all cells of the *Audouinella* species under study were uninucleate.

Table 4.7 Nuclei and cell volumes of exo- and endobiotic *Audouinella* species.

Audouinella species	Nucleus vol. range μm^3	cell vol. range μm^3	Ratio of N.vol/C.vol.	Mean
A. efflorescens	14.44	1040.6-	0.013	0.010
		1924.2	0.007	
A. infestans	1.78-	255.25-	0.060	0.07
	4.18	500.0	0.080	
A. secundata	11.40-	525.6	0.021	0.018
	27	2135.3	0.012	
A. virgatula	11.40-	767.6	0.015	0.013
	27	2348.0	0.011	
Endophytic	4.20-	1256.6-	0.030	0.023
Audouinella sp. 2	65.4	4071.5	0.016	
Endophytic	14.01-	1558.2-	0.009	0.0085
Audouinella sp. 3	33.5	3801.3	0.008	
Endophytic	0.88-	274.9-	0.032	0.0046
Audouinella sp. 4	4.2	692.7	0.060	
Endozoic	47.7-	1963.5-	0.020	0.021
Audouinella sp. 1	113.1	5043.8	0.022	
Endozoic	113.1-	4086.4-	0.027	0.0275
Audouinella sp. 2	179.6	6333.5	0.028	

Discussion:

The fact that vegetative forms of *Audouinella* species can some times arise in culture which bear no resemblance to known existing taxa led to further investigation of characters other than external features, such as nuclei volumes and cell wall constituents, in these *Audouinella* isolates under study.

Growth of isolates under similar culture conditions allow comparison between the nuclei of these species as Dixon (1973) reported that the size of the nuclei could be affected by the growth conditions under which the alga is growing.

As stated above nuclear volumes in relation to cell volumes seem not to have been investigated or used before in comparative studies with *Audouinella* species, In this study, the ratio of the nuclei to cell volumes showed little significance as the nuclei volumes were found to be directly proportional to the cell volumes. The ratios, however, appear to be typical for a particular species of red algae as it was the case with *Audouinella* species in this study and with Ceramiaceae species as reported by Whittick (1987). On the other hand, the investigation revealed some significant differences among these *Audouinella* isolates, such as the position of the nuclei.

The position of the nuclei was found to be consistent in every single species which may be of some taxonomical significance especially between species which very closely resemble each other e.g. *Audouinella secundata* and *A. virgatula* which showed relatively similar cell and nuclei volume, similar nuclei to cell volume ratios and share the same supporting organism as epiphytes on *Porphyra umbilicalis*. Therefore, the nuclei positions in addition to other morphological features may provide us with some further mean of delimitation of taxa. The significance of the relationships between nuclei volumes and cell volumes requires also some detailed studies of chromosome numbers and size and **DNA** contents -a specialized aspect which could not be investigated in the present work.

5. Monospore release in *Audouinella* species

Introduction

Monospores are apomictic reproductive structures in *Audouinella*. They are produced inside sporangia, which can be found, as stated earlier, singly or in clusters in different *Audouinella* species. They are the commonest modes of reproduction in the genus, and on occasions the spores are produced in large numbers. They are naked at the time of release.

The release of spores in *Audouinella* has been described by Rosenvinge (1909); Boney (1967); White (1968); and White and Boney (1969). However, these authors described either rupture of the sporangia or the post-release movement of the released spores on substrata. The movement of these spores was simply described as amoeboid. Its duration varied from a few minutes to several hours. In *Audouinella endophytica* (=*Acrochaetium endophyticum*) the amoeboid movement of a monospore lasted for 10 minutes after settlement on the substratum (White 1968; White and Boney 1969).

The release of reproductive structures in general in red algae had received less attention and few detailed studies have been carried out other than by Nakazawa (1958); Boney (1967 and 1978); Fetter and Neushul (1981). Because of the lack of detailed study Dixon (1973) stated that, the spores may be released either by a regular splitting of the wall, or by a more irregular breakdown of the wall, or by the detachment of a lid from the apex of the sporangium. In earlier study, Boney (1967) precisely described the monospore release in *Audouinella virgatula* which was effected mainly by an apical tear accompanied by momentary spore distortion, and sometimes with a distinct 'blow-out' of the sporangium apex.

In the present study two types of post-release behaviour were observed. One when spores were released and quickly settled, and the other when spores were released and remained floating. In the first cell shape changes lasted minutes and in the second lasted hours (occasionally) and most of the times days. The spores (of all epiphytic

Audouinella species) in the last situation remained floating and appear to be attached to bead-like thread of mucilaginous material. On many occasions these spores were seen having less chances to settle, to attach to the substratum, and to start germinating. On many occasions they were found to burst when they are transferred to a fresh medium. This phenomenon did not occur in the case of freshly released spores.

In this study, however, the theme was to investigate the release process rather than spore settlement. The spore release process as described by Boney (1967) is so rapid (2-3 seconds) (Fig. 5.1) that any changes in the shape of sporangia and monospores are not readily detectable by observation under light microscopy.

Present day methods make the detection of cell shapes changes ..etc. more easily possible. In this study, release of monospores of three epiphytic *Audouinella* species, (viz. *A. secundata*, *A. virgatula* and *A. seiriolana*), were recorded on a Videocassette tape recorder, with a view to determining whether there were differences in modes of spore release between the species and to obtain a better picture of the mechanisms and stages of the spore release process.

Results:

The rapidity of the spore release process is such that it is impossible to select a monosporangium in which the releasing process was imminent. The usual procedure was to concentrate observations on a group of monosporangia which, by their denser colour and appearance, were judged as being 'mature'. From such groups it was on occasions possible to detect the spore release process and to obtain a taped record. All told some two hours of video tape recordings were made over several weeks. It was found that young mature tufts of these plants were the most suitable for observation. They were found to release their spores quickly, and did not require any addition of fresh medium to the slide under the microscope to avoid desiccation. Old tufts and those young and immature, readily taken out of the growth chambers once before, were found to take a

longer time to release their spores, and more often not to release them at all.

All *Audouinella* species under study showed the same mode of spore release. However, the process was found to be slightly different for a released spore to another in terms of speed. In the first series (Figs. 5.2a-v) the stages are typical of the majority of releases observed. The mature monosporangium first became pointed at the top end due to localized accumulation of clear material, probably mucilage (Fig. 5.3b). The next visible change was an initial rupture in the apical region of the sporangium wall (Fig. 5.2b) and this was accompanied by the exudation of a small 'bleb' of mucilage (Fig. 5.2c). The apical tear then rapidly enlarged with the release of more mucilage (Fig. 5.2d-e), and the spore mass then moved upwards rapidly and out through the opening now formed (Fig. 5.2f-m), enlarging the torn opening of the apical region in its passage and itself becoming deformed in the process (Fig. 5.2f-q). At the same time the sporangial cavity below the spore became filled with a viscid, swirling and expanding mass of (it is presumed) mucilaginous material, and this expansion would seem to be the motive force pushing the spore out through the relatively small space available and causing its subsequent deformation. The spore gradually moved upwards and out of the sporangium (Figs. 5.2j-u). With the escape of the spore some exudation of this mucilage took place, and may have contributed to the primary mucilage sheath surrounding the spore. The final ejection of the newly rounded spore was achieved when the edges of the apical rupture 'snapped' back together by their inherent elasticity (Fig. 5m-u). The timing of the process is variable. The second series of photographs (Figs. 5.3a-l) shows that the phasing of the process described above cannot always be followed in detail -the initial 'bleb' formation may be too rapid, and only the release of the first mass of mucilage is then detectable.

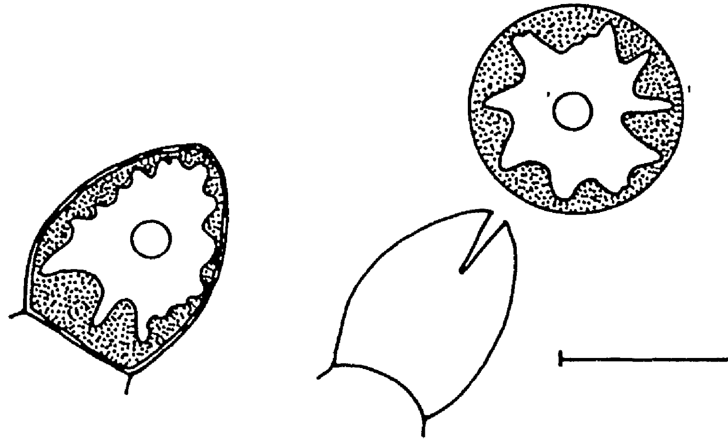
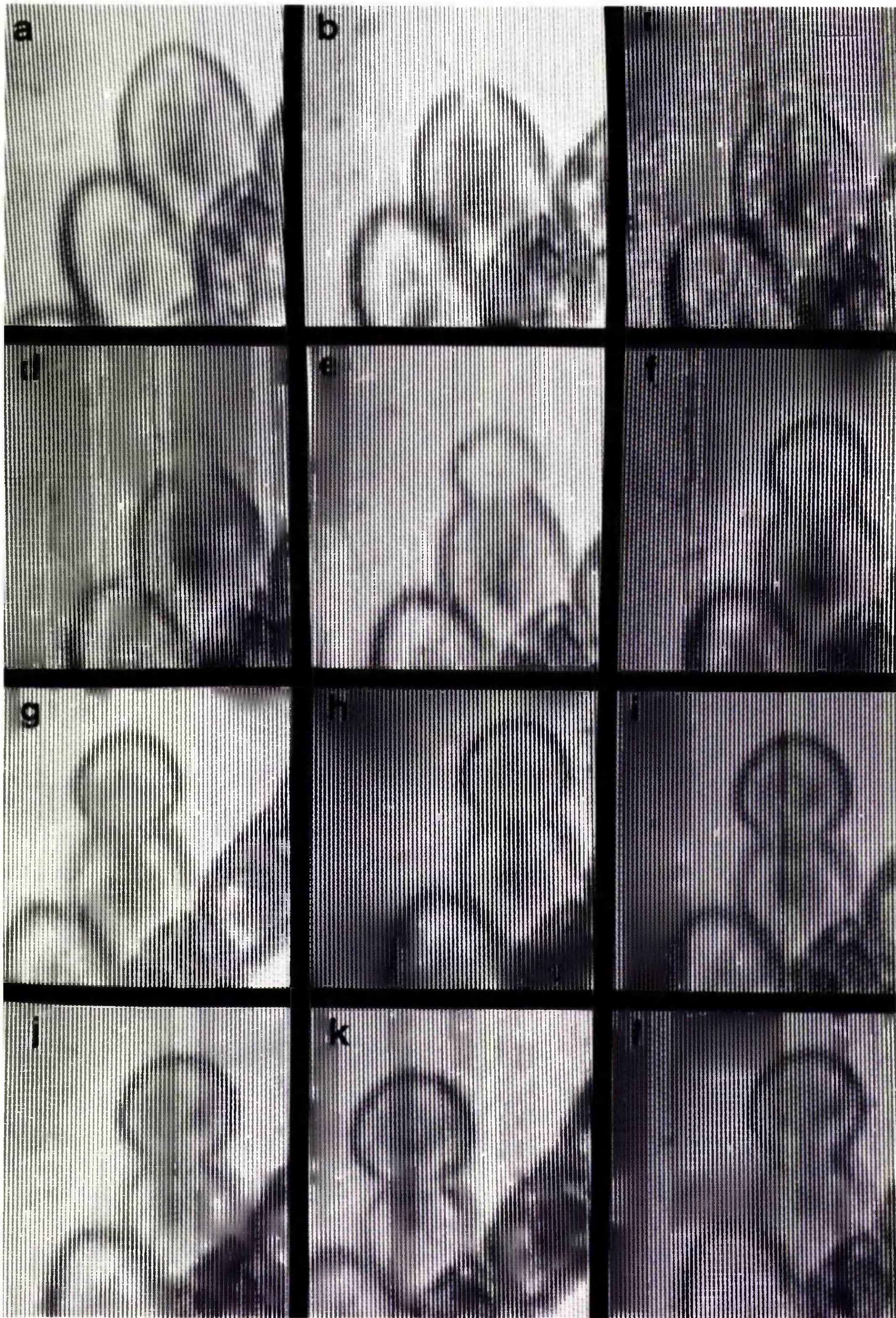


Fig. 5.1 Monospore before and after release -the sequence as would be seen by light microscopy (Scale measure = 10 µm).

Fig. 5.2**a-v.** stages of monospore release. **a.** mature monosporangium, a pale region (probably mucilage) can just be seen below the slightly pointed apex. **b.** rupture of the apex. **c-e.** release of the apical mucilage and enlargement of the wall rupture. **f-l.** gradual emergence of the monospore with some deformation, the expanding mucilage not yet visible. **m.** withdrawal of the edges of the sporangium for the final push and release of the monospore. **n-t.** gradual forced ejection of the monospore by the expanding mucilage below the spore. **u.** 'snapping' together of torn region of wall and final 'thrust' in space. **v.** released spore and empty sporangium.



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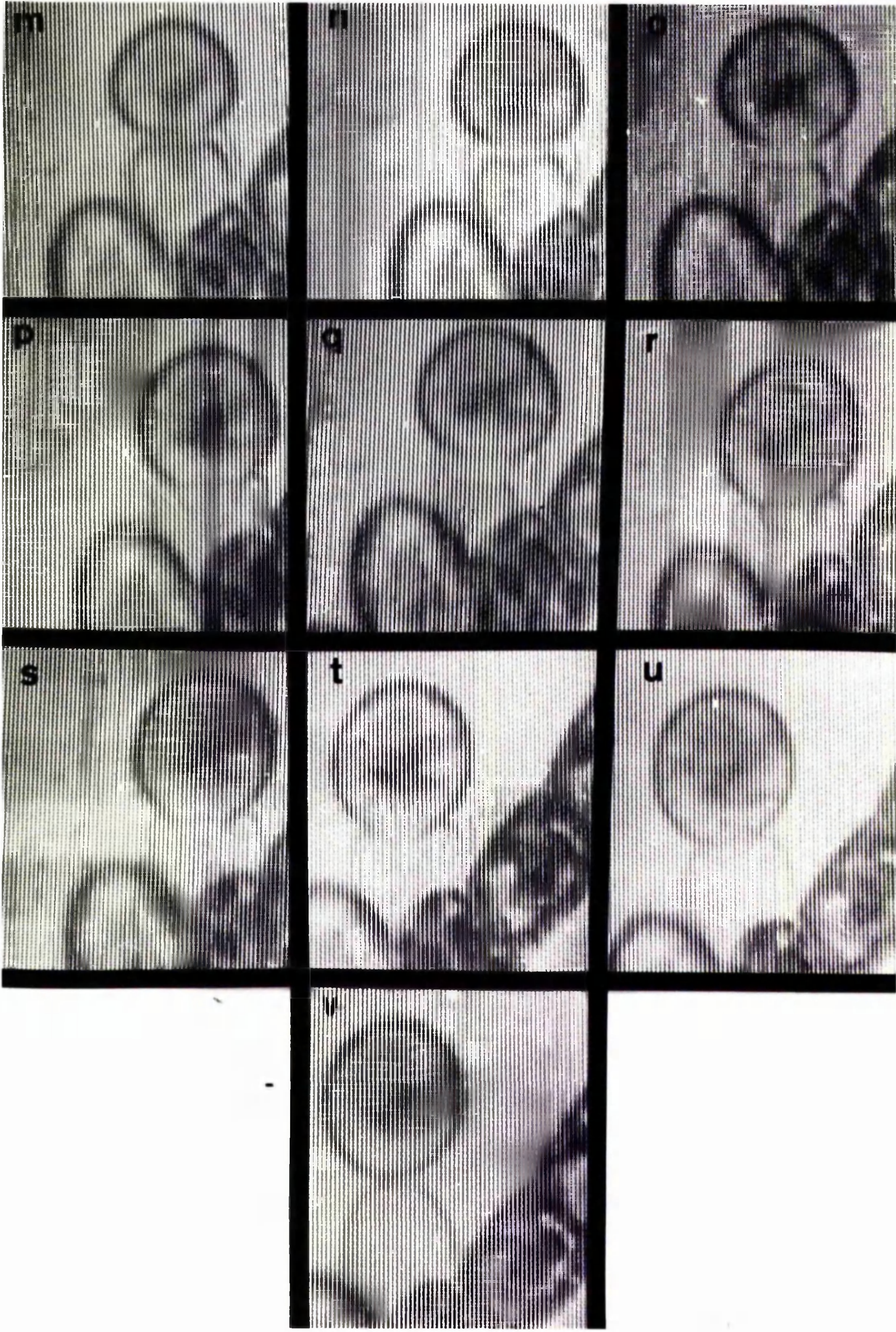
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Fig. 5.3a-l. stages of monospore release. **a.** mature monosporangium. **b.** pointed monosporangium. **c.** release of apical mucilage. **d.** emergence of the monospore under pressure of expanding mucilage -note mucilage accumulation. **e-k.** gradual release of the monospore caused by force of expanding mucilage. **l.** final release of the monospore after 'snapped' closure of torn wall region.

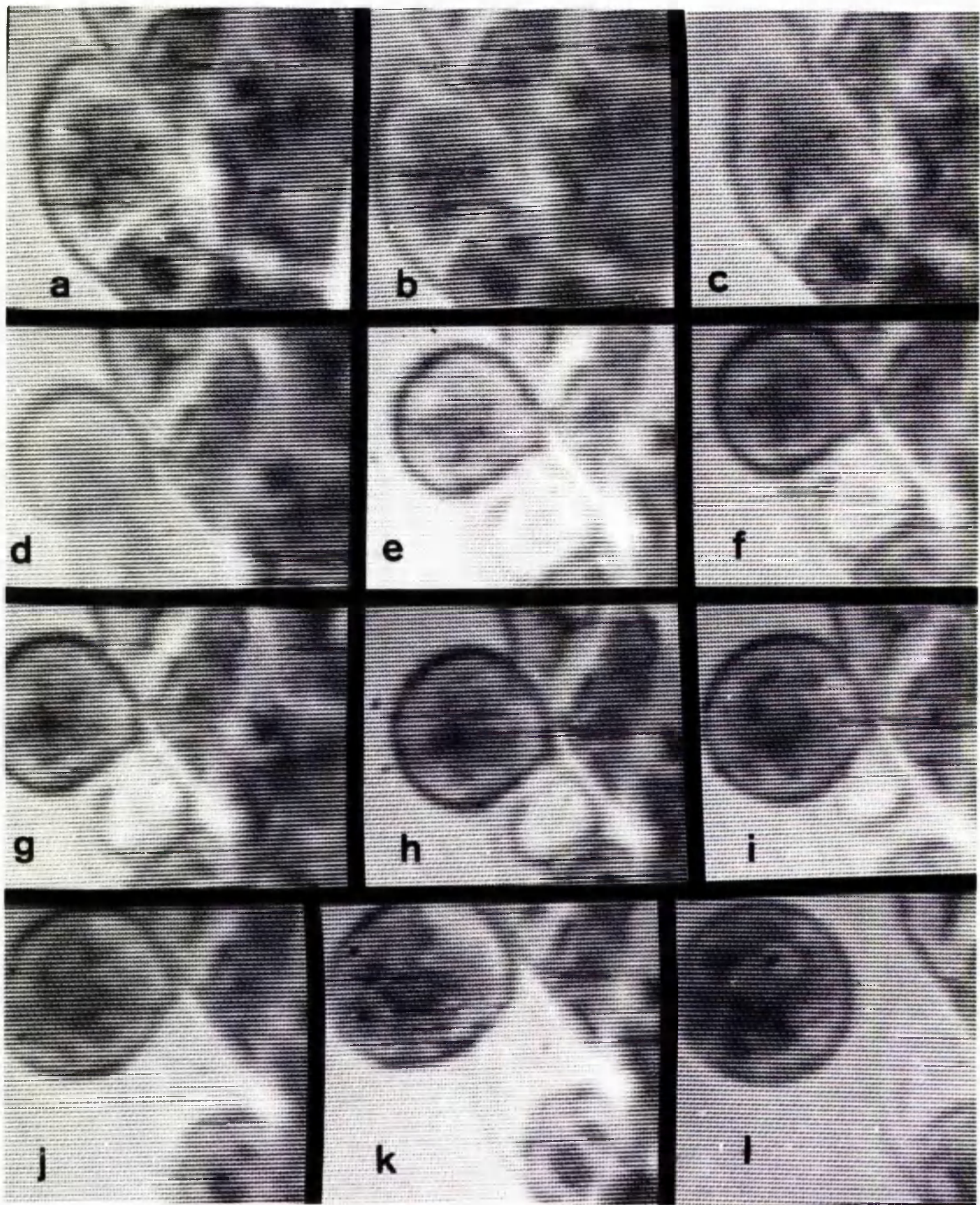
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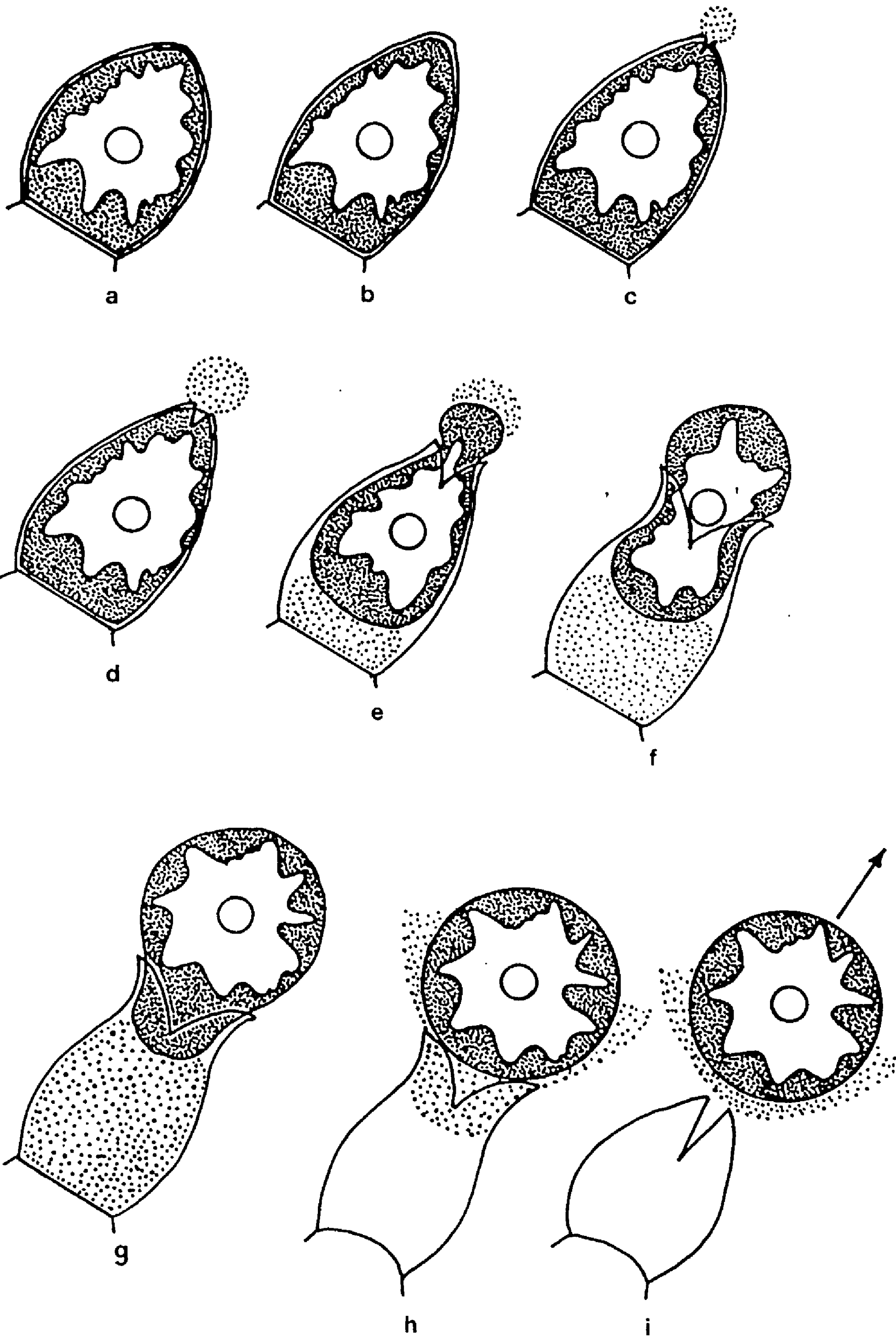
Fig. 5.4a-i. Diagrammatic representation of stages of monospore release. **a.** mature sporangium. **b.** pointed sporangium. **c.** rupture of the monosporangium apex and mucilage release. **d.** enlargement of the rupture and more mucilage released. **e.** start of spore emergence duė to swelling of basal mucilage which causes the enlargement of the wall rupture. **f.** more mucilage released in the sporangium and subsequent spore emergence. **g.** the mucilage final stage of ejecting the spore. **h.** sporangium wall starts to close its torn apex and pushes out the spore and mucilage. **i.** released spore and empty (slightly contracted) sporangium with torn region at maximum closure. (The body of the spore is shown in dark stipple, the mucilage in light stipple, and the chloroplasts shown unshaded).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The force of the spore ejection apparently causes the floating movement as some spores were ejected and travelled for some distance from the sporangia. Other spores were found to settle very quickly after their release. Fig. 5.4a-i is a diagrammatic summary of the process most frequently observed as shown in Fig. 5.2a-v. Fig. 5.5a-j is a variation of the usual method in which a more explosive release of the spore over the same time results in less deformation but a greater splitting of the sporangium wall. The role of pressure of expanding mucilage in the ejection process is seen when a release process is viewed in optical section when pressure of the expanding mucilage on its lower surface induces a concave depression, and that this is eventually lost when the release takes place (Fig. 5.6a-e). For the most part the spores released were healthy, and only occasionally did spore disruption take place (Fig. 5.7a-e). Both types of spores were surrounded with a layer of mucilage, but the unhealthy spores burst shortly after their release and the mucilage appeared to become watery and diffuse in the medium and eventually the remains of the spores disappeared.

Sudden changes in culture conditions (transformation from growth chamber temperature to room temperature; change in light intensity; and introduction of fresh medium) were apparently the main factors inducing release of the spores. Table 5.1 shows the number of spores released by six average sized 'mature' tufts of *Audouinella virgatula*, i.e, each tuft with 15-20 erect filaments of 20 to 25 cells per erect filament, over a period of 28 days. These tufts were grown under 12 hours day light at 12 $\mu E\ m^{-2}\ s^{-1}$ and at a temperature of 18^0 C. The tufts were transferred daily to fresh media (**ESW**) in small petri dishes and the spores released in each day immediately counted. The number of spores released in the first two days was high and dropped down dramatically over the third and fourth days and then spore output, although variable, remained low after the initial 'surge' until days 10-12 when for the most part the rates of spore production increased and remained high for the rest of the experimental period. It is clear that the proliferation must have taken place and the 300-400 cells per plant

were all capable of producing a monosporangium. On the other hand, Knaggs (1967) stated that tetraspore production under field and culture conditions for *Audouinella purpurea* (=*Rhodochorton purpureaum*) requires a certain level of energy. He also pointed out that the production of sporangia will be delayed considerably if the initial nutrient concentration was high so that the conditions governing tetraspore formation are different. Certain of the endobiotc isolates under study were found not to produce spores when fresh medium was added after short periods of time (2-3 weeks). Other factors such as the pressure caused by the coverslips appear to have little effect. There was, however, no evidence that spore release was associated with any particular time of day.

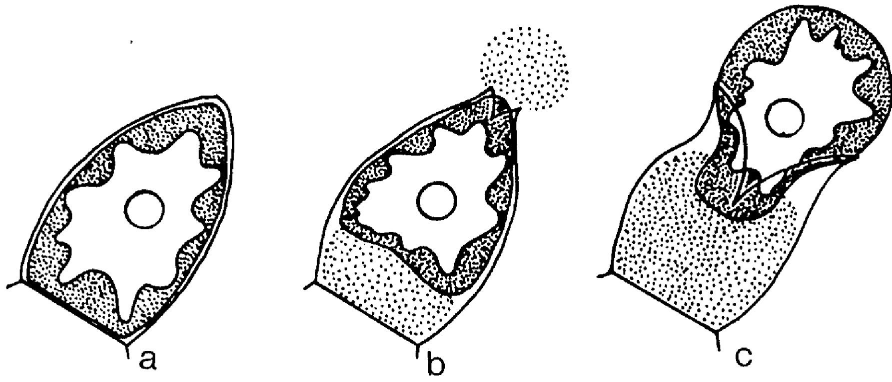
Timing of spore release was measured by time lapse and the number of 'frames' in replaying the video tape. It ranged from 1 to 5 (average 2.88) seconds for healthy spores and up to seven seconds for unhealthy (abnormal) spores.

However, as stated earlier, the start point when the sporangium starts to change its form from oval or subspherical to a conical shape, was not easily detectable. Thus the first rupture of the sporangium wall and the release of the spore only could be timed with certainty in this study.

Discussion

Monosporangia are modified cells in which the contents become spores without any preceding division of nuclei or contents. The released spores will thus bear the genetical data of the parent plant, and if produced in large numbers - as with exoblotic species - will be a highly effective means of propagation at little expense to the plant. Endobiotic species, on the other hand, more rarely produce monosporangia - possibly an influence of their prolonged vegetative state in the 'host' organism. The spore release process, however, is the same as with exobiotic species.

Fig. 5.5**a-j**. Diagrammatic of a more rapid process of monospore release. **a**. mature and pointed sporangium. **b**. rupture of the sporangium apex and mucilage release. **c**. emergence of the monospore caused by force of expanding mucilage force. **d-f**. gradual release of the spore caused by the snapped torn edges of the sporangium apex. **g-j**. closure of the torn sporangium apex.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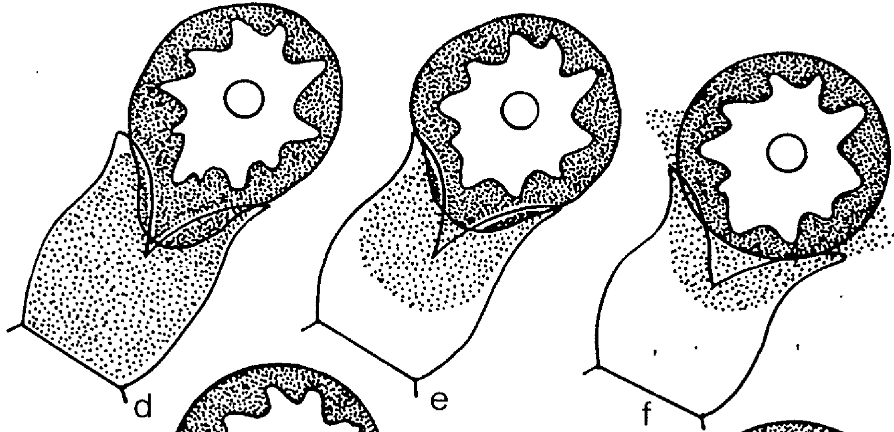
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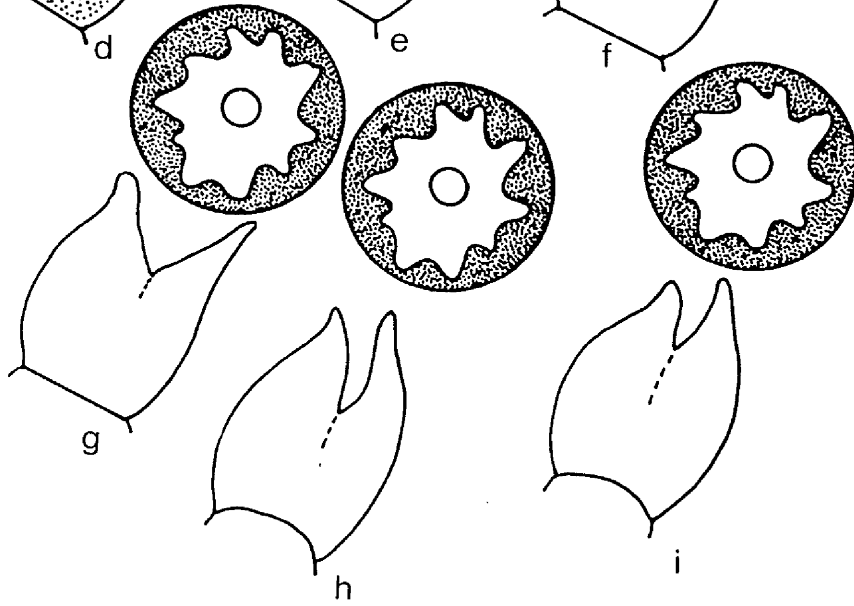
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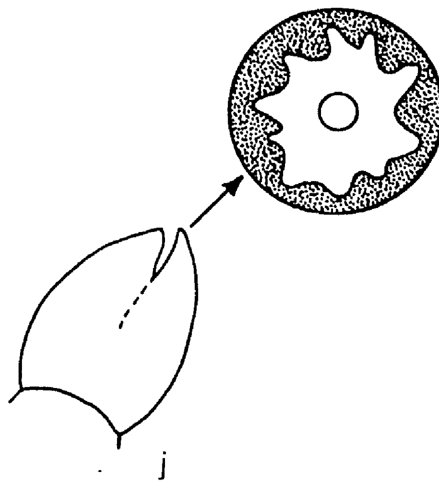
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Fig. 5.6**a-e.** Diagrammatic representation of stages of spore release showing the deformation of the monospore caused by the mucilage force. (as viewed in 'optical section'). **a,b.** apical rupture and mucilage 'bleb'. **c,d.** forced ejection of spore by expanding mucilage causing concave depression in basal region of spore. **e.** final stage of spore ejection.

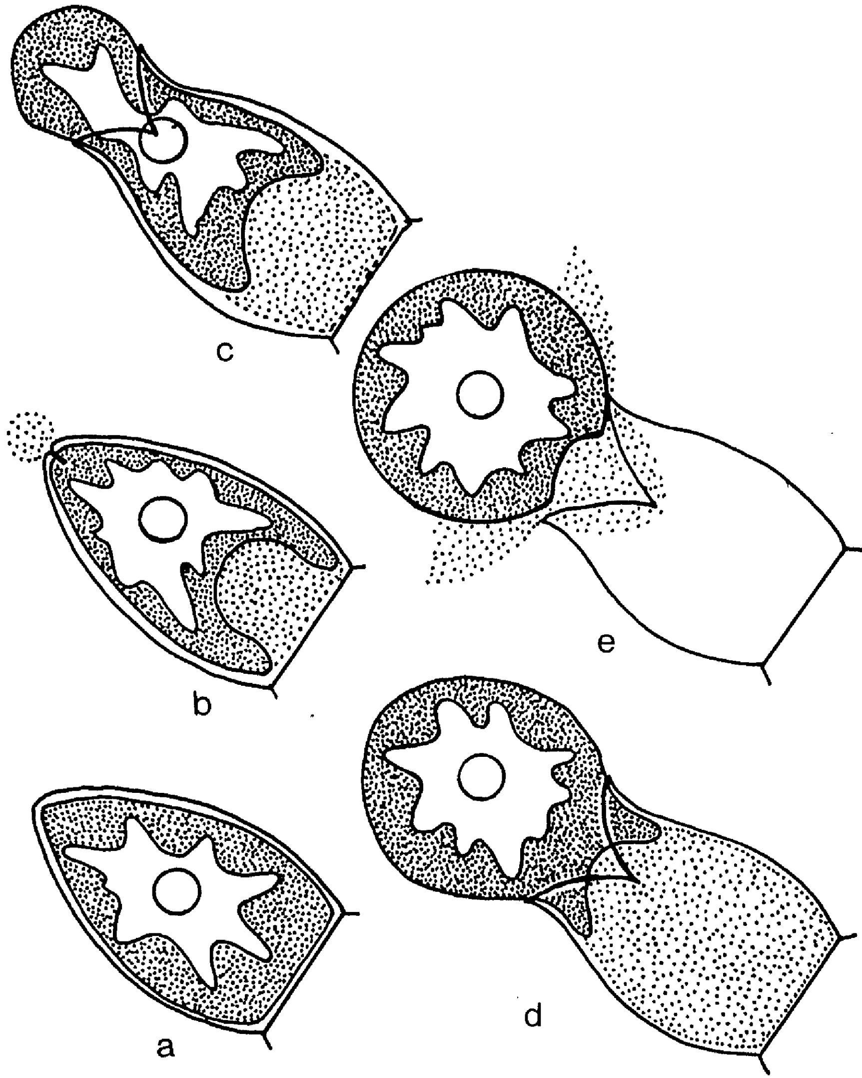
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Fig. 5.7**a-e**. diagrammatic stages of a released unhealthy monospore. note the formation of the vacuoles and enlargement of the monospore (**d-e**).

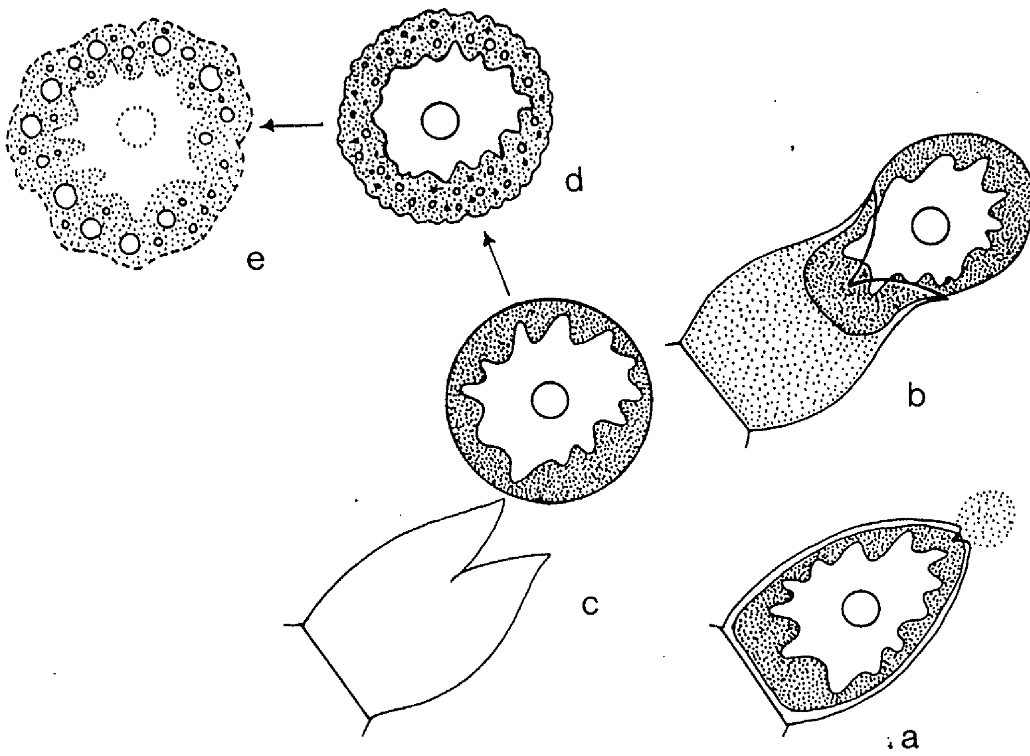
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Table 5.1 spore production and release in *Audouinella virgatula*

Day	spores released by individual tufts						Total
	1	2	3	4	5	6	
2	153	274	524	173	694	384	2302
3	166	208	192	106	297	270	1339
4	30	118	94	60	226	62	590
5	46	100	52	66	189	106	559
6	52	135	126	40	170	100	623
7	22	128	69	120	190	182	711
8	50	148	14	40	220	90	562
9	15	187	257	50	118	362	989
10	25	152	15	206	98	90	586
11	146	165	65	213	80	288	957
12	107	190	137	350	817	377	1248
13	55	391	210	618	340	378	1992
14	340	402	140	816	247	490	2435
15	163	316	254	912	234	468	2357
16	312	293	320	607	274	523	2329
17	173	265	304	655	260	506	2153
18	221	247	204	700	294	406	2272
19	249	170	260	521	259	354	1813
20	305	62	317	702	169	456	2011
21	293	146	370	606	260	482	2137
22	360	274	305	650	303	370	2262
23	300	290	203	440	300	385	1918
24	261	391	538	457	371	395	2313
25	284	302	261	812	335	373	2367
26	190	217	240	296	214	408	1565
27	158	155	128	257	139	410	1237
28	90	260	266	490	523	665	2224
Total	4556	5936	5865	109	6885	9380	43605

Non motile spores produced in monosporangia need some forcible release process to escape into the surrounding medium, after which they are passively dispersed. The release of spores is thought to be affected by sudden changes in nutrient concentration in the medium (Gibson & Whitton 1987). However, spore release is a process which occurs very quickly but does not happen all at once as was first thought. It follows very delicate and deliberate sequences to ensure the survival of the released mature spore. This was clear from the process recorded and described above, where the spore deformation took place to accommodate the initial very small wall rupture, and the protective material (the mucilage) which assist in producing the apical break in the wall is then released first easing the way for the spore, and later surrounding the spore to ensure its protection from the new surrounding environment and enhancing the attachment which is a relevant stage for spore germination. Fetter and Neushul (1981) observed a similar phenomenon with released spermatia of a red alga. They found however that the spermatium was not at the head of the mucilage strand. On the other hand, a spore mucilage sheath may well have a buoyancy potential, so prolonging the pelagic phase of the spore (Boney 1975).

The final ejection of the spore by force of the mucilage expanding and the snapping together of the edges of the ruptured sporangium causes the the spore to be ejected from the original tuft. This ensures space for attachment and growth of the new sporeling away from the original tuft. This also reduces the intercompetition between the germinated spores which was seen in cases where overcrowded spores, under culture conditions, remained in an early germinating stages and few of them continued to grew when transferred to a fresh medium. The speed of the release process was found to be as previously estimated and reported in literature (2-3 seconds) by Boney (1967). However, the time occasionally exceeds what was reported (5-7) seconds. What causes this increase in the releasing time is unknown.

As already stated, monosporangia are the simplest modes of spore production in the

red algae, and in some cases with endobiotic *Audouinella* species the only known method of propagation. Whilst their simplicity of form may suggest their primitive nature, it is clear that their release mechanism is far from simple, involving a phased bipolar release of mucilage from the spore. This process is one in which the first mucilage production is below the sporangium apex, the swelling of this causing the initial rupture of the wall. The second phase occurs at the opposite pole of the spore, in which an extensive release of mucilage and its swelling gives the propulsive force which ejects the spore, the underlying cell wall being the non moving basal 'plate' which ensures that the upward 'thrust' of the expanding mucilage pushes the spore against the opening already made in the apex of the sporangium wall. It is well known that once the site of a monosporangium is established, the wall of a discharged sporangium is quickly filled by an ingrowth from the subtending cell, and a second functional sporangium is obtained by a process of proliferation. This can be repeated over and over again (up to 14 times according to Boney 1967), each time the spore having to be ejected through several 'layers' of torn sporangium apices - but just as effectively as on the first occasion. The measurement of spore production shows the high spore productivity possible when a fertile plant is supplied with sufficient nutrients under suitable conditions of light and temperature. Almost every filament cell in the plants used bore a sporangium. Over the 28 days and whilst there was some further filament growth, most of the energy was put into monospore formation and release resulting in 43605 spores from the six 'mature' plants used. The results indicate that the proliferation process is an extremely rapid one, since on occasions more spores were obtained in a day than the total number of cells in the filaments. In addition to its efficient release process, the results indicate that a fertile *Audouinella* plant, under the right environmental conditions, can become an effective spore producing 'machine'. *Audouinella* species have evolved an effective means of propagation with their monosporangia - so effective that it is not unknown for monosporangia still to be produced on a plant with tetrasporangia or sexual structures.

6. Adaptation of exo- and endobiotic *Audouinella* species to substrata

Audouinella species are well known from a wide variety of habitats involving different substrata (Rosenvinge 1909; Drew 1928; Woelkerling 1970, 1971, 1983). Their morphology is very often thought to be influenced by the substratum they live in or on. Boney (1975) found that these plants can modify their growth as a result of adaptation to different substrata. Host specificity of the endobiotic species was first suggested by Rosenvinge (1909), and became incorporated into audouinelloid systematics (e.g Drew 1928; Papenfuss 1945; 1947). Therefore, many new species were named according to their 'host' organism. However, this taxonomic criterion became unrealistic as many species were reported from' different hosts (Woelkerling 1970 ; 1971). In the present study host specificity and substratum invasion were investigated with the *Audouinella* isolates under investigation. Of particular interest was the fact that certain of the endobiotic isolates, which showed no structural differentiation in the 'host' organism, developed prostrate and erect systems when grown in culture on certain substrata. This would suggest a morphological potential not indicated by their relative simplicity of form when growing within the 'host' tissue. A detailed structural study may provide us with more information that may be relevant for speciation of these plants. Light microscopy may be of limited value for the study of these organisms and attaching systems in relation to their substrata. Scanning electron microscopy (**SEM**) was used as a means of more closely examining this relationship.

6.a. Cross infection studies

Host specificity in the genus *Audouinella* (as described above) is considered as a diagnostic character in the speciation of this genus. Aziz (1965) suggested a parasitic relationship between these algae and their 'hosts'. Parasitism in *Audouinella* has not yet been investigated. Such studies would require special techniques and much care,

such as preparation of axenic (bacteria free) cultures. In addition reinfection of the 'host' organism is not all the time possible. However, many of the known endobiotic species of *Audouinella* have been successfully isolated and grown free of their 'hosts' in enriched media, which makes the parasitic relationship unlikely. Parasitism in red algae in general and the taxonomic importance of the supposed parasites is still in a confused state (Evans *et al.* 1978).

Introducing these endobiotic *Audouinella* species to different hosts (plants or animals), is known as cross infection. This process indicates whether or not they are host specific and whether they are strictly endozoic or endophytic.

On the other hand, where any individual species succeeds in invading the 'host', this will encourage closer observation. A comparison with culture conditions and those in nature will be possible, and may help reveal the identity of the species as they are very similar to each other when they are grown free of their host.

Any such penetration is an invasion of a substratum which may be organic (plant cell walls and chitin) or inorganic (calcareous substrata). Agar is a well known cell wall derivative obtained from red algae, especially genera of the order Gelidiales. -the agarophytes. Agar is composed of agarose (a linear polymer of D- galactose and anhydro- L- galactose) and agaropectin, a sulphated polysaccharide. Since endophytic *Audouinella* species are often found in cell walls of red algae, and sulphated galactose compounds are common wall components, an investigation of the penetration of agar by *Audouinella* species was also carried out.

Some attempts of relating the available 'living space' of endophytic filaments to their cell sizes were made by measuring the depth of the outer "cuticle" in which the filaments were growing. Measurements of 'living space' and cell dimensions of endophytes were made from slides of material from the west coast of USA prepared by Professor A. D. Boney (1969), and from diagrams in White (1968) and Drew (1928).

Results:

Invasion of plant tissue

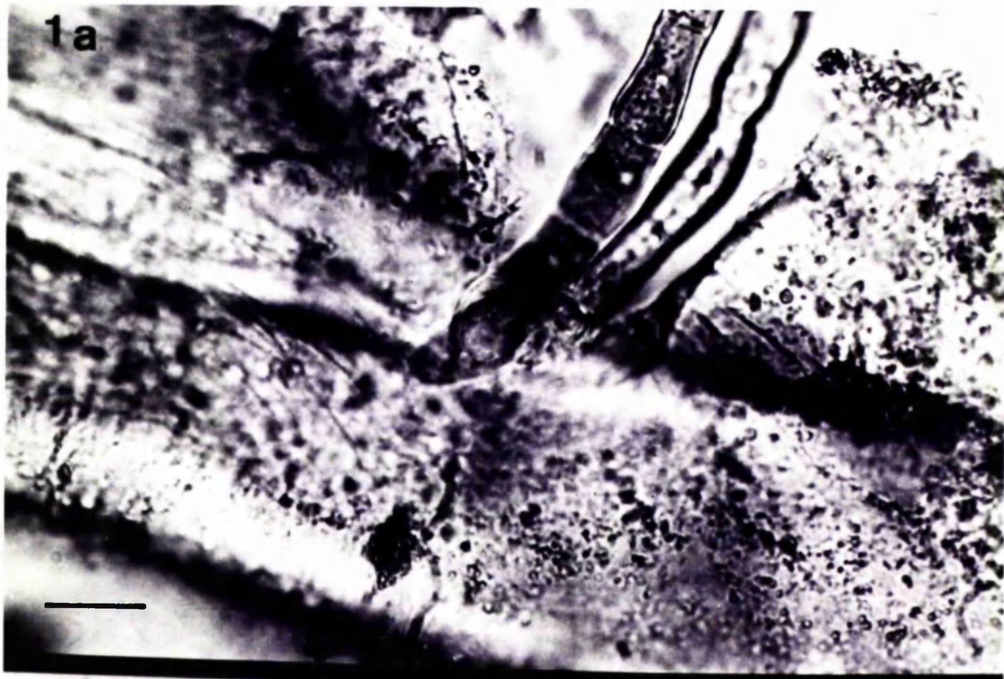
Invasion of the plant substrata did not occur by any of the experimental *Audouinella* species. However, epiphytism was common in all species. Endobiotic *Audouinella* species (namely *Audouinella infestans*, endophytic *Audouinella* 1 and 2 and endozoic *Audouinella* 1 and 2) were found to grow on the surface of the *Fucus* germlings for longer periods even when they were transferred to larger petri dishes and fresh medium was added frequently, these epiphytic growths appear to be well attached to the plant surface. Close examination showed no signs of penetration of the 'host'. Similar observations were obtained with other 'hosts' (viz. *Ceramium* and *Polysiphonia*).

Invasion of *Obelia*

Endozoic *Audouinella* species 2 was found to grew inside the dried *Obelia geniculata.* However, it was found that this alga penetrates the 'host' at the polyp operture (Fig. 6.1a,b). This suggests that under the conditions of experiment infection of the 'host' requires a suitable penetration point and the endozoid failed to penetrate the chitinous wall of the hydroid under the experimental conditions used.

Penetration of the perisarc was seen in only one sample of *Obelia*. The morphology of the filamentous growth was not very different from those growing free of their host except that the filaments within the *Obelia* appeared to be formed of long filaments without any lateral branches. The unchanged morphology appeared to be due to the space available in the *Obelia* perisarc. Presence of any internal material may give less living space for the internal filaments and this in turn causes the flattening and deformation of the algal filaments, as may also be seen in nature.

Figs. 6.1a-b. Invasion of *Obelia* perisarc by endozoic *Audouinella* sp. through an aperture. (bar. a=25μm b = 50 μm).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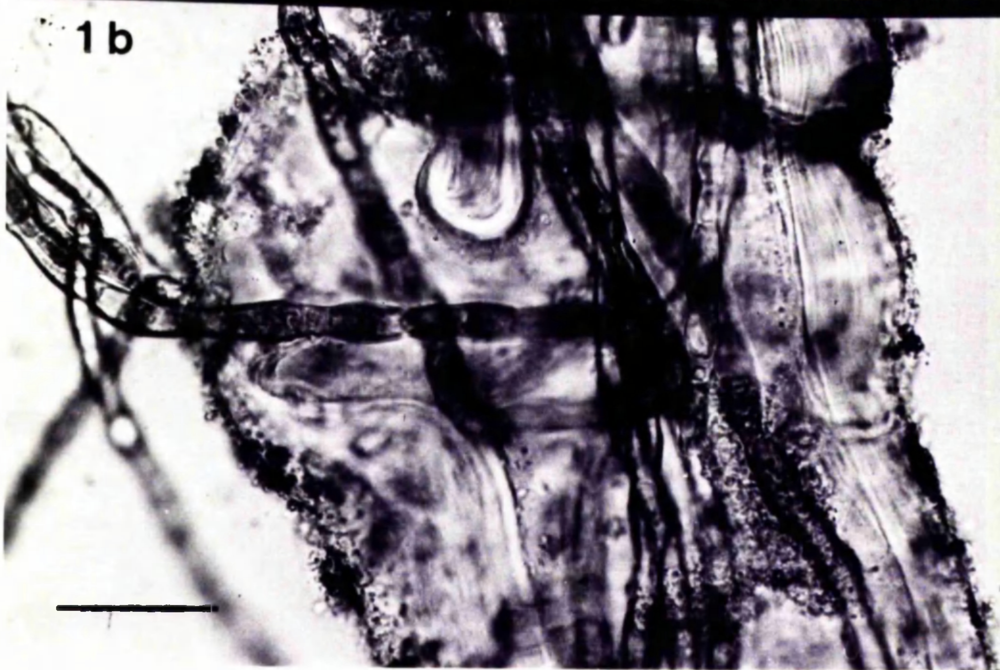
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6.b. Invasion of chitin by *Audouinella* species

Chitin is a nitrogenous polysaccharide comprising the principle structural material in the exoskeleton of Arthropods and of the perisarc of Coelenterate animals. The ability of *Audouinella* species to penetrate this material would be suggestive of an endozoic nature. On the other hand invasion of chitin may result in morphological changes in their cells and chloroplasts which can occur in nature, since cell and chloroplast morphology are widely used in classification of these plants.

As mentioned above, it was found that an entry point was apparently required for endozoic *Audouinella* species 2 to infect hydroid perisarcs of *Obelia geniculata.* The invasion of chitin by different life forms of *Audouinella* (epiphytic, endophytic, and endozoic) was examined, together with their modes of penetration where they occurred.

Observations showed that penetration was achieved by filament fragments of all endobiotic *Audouinella* species examined (namely *A. efflorescens*, *A. infestans*, endophytic *Audouinella* species 1,2 and 3, and endozoic *Audouinella* species 1 and 2 (Figs. 6.2-6.8). *Audouinella virgatula* (an epiphytic species) showed no signs of penetration, but was found to attach itself to the chitin by the basal system. It tended to inhabit the uneven surfaces where attachment was more easily made and protection was possibly provided (Fig. 6.9).

Spore penetration was found to be quicker than that of filaments. Spore penetration could be of two types, immediate penetration after attachment, or germination occurred on the surface of the chitin and the penetration took place after formation of few cells or even branches. Figure 6.10 show the penetration obtained by a spore of the endozoic *Audouinella* species 2.

Spores were obtainable only from a few isolates. Immediate penetration was seen in cases of *Audouinella infestans*, endophytic *Audouinella* species 2, and endozoic *Audouinella* species 2. The late penetration was seen with endophytic *Audouinella* species 1 and endozoic *Audouinella* species 1. Figure 6.12 show the penetration

obtained by the rounded cells of *Audouinella infestans* which have the same appearance as ordinary spores.

The penetration process by spores (i.e, the stages of germination and penetration) could not be extensively recorded either by light microscopy or by **SEM**, because of poor light penetration in chitin in the first case and due to the gold coating in the latter when the spore and the mucilage appeared as an indistinguishable mass on the chitin surface. However, It was observed that endozoic *Audouinella* species 2 the original spore became refilled by a chloroplast after the migration of the original contents during the formation of the first cell which invaded the chitin substratum. This phenomenon of chloroplast migration was confirmed in other observations made on this species when its spores were cultured in plastic petri dishes (Figs. 6.11a-c).

Penetration of chitin by filaments was observed with all endophytic and endozoic *Audouinella* species (viz. *A. infestans*, endophytic *Audouinella* species 1, 2, and 3 and endozoic *Audouinella* species 1 and 2). As expected it was found to take longer than the penetration by spores. This is probably due to the greater ability of spores to penetrate or because the spores possessed sufficient mucilage to secure themselves to chitin, thus facilitating early penetration. Filament fragments were more easily detached because of their lack of adhesive material, so that fewer filaments achieved penetration.

Filament penetration was often found to take place via the edges of the chitin and occasionally on the surface. This is due to the ability of these plants to penetrate between chitin layers which are present more frequently on the edges rather on the surface. **SEM** observations revealed that where penetration occurred on the surface the penetration were achieved through groove-like irregularities in the chitin (Figs. 6.3-8). With the fixation and other processing necessary these groove-like regions assume the form of cracks, possibly due to the weakness induced by invading filaments. Cell morphology was completely changed inside the chitin with all species. The cells of these species (namely *Audouinella efflorescens*, *Audouinella infestans* and endozoic *Audouinella* species 1 and 2) became irregularly outlined and flattened (Figs. 6.13-

15). Due to difficulties mentioned above concerning photographing these plants inside the chitin, diagrammatic representations of these flattened cells were made (Figs. 6.16-19). Late penetrants showed the ability of regeneration from that part growing inside the chitin more than the early penetrants. It was found that endophytic *Audouinella* species 1 and endozoic *Audouinella* species 1 produced erect filaments on both sides of the chitin after penetration. This observation suggests the different functions of different parts of these isolates.

6.c. Calcium carbonate invasion by *Audouinella* species

Preliminary observations showed that all the species under study (viz. *Audouinella infestans*, endophytic *Audouinella* species 1, 2, and 3 and endozoic *Audouinella* species 1 and 2) had the ability to grew attached to the calcareous surface provided that they have enough time to settle and attach to the substrate (shell fragments or aragonite). Observations by **SEM** showed that attachment may take place by the basal system as in *Audouinella virgatula* by flattening of the attaching filament cells, or by penetrating the irregularities in the calcareous substrata.

However, it was found that the superficial growths on the aragonite were lost during the processing procedure for **SEM** observations. This suggests that these plants require rough surfaces to attach or penetrate. This suggestion was supported by other observations when these plants were found to be absent from the inner surface of mollusc shells which was much smoother and with less surface irregularities than the outer surface.

Figures 6.20-24 show how these endobiotic species (namely *Audouinella infestans*, endophytic *Audouinella* species 1 and 3 and endozoic *Audouinella* species 1 and 2) invade the mullosc shells through the already existing irregularities in the surface and occasionally filaments grew out from these localized regions of penetration.

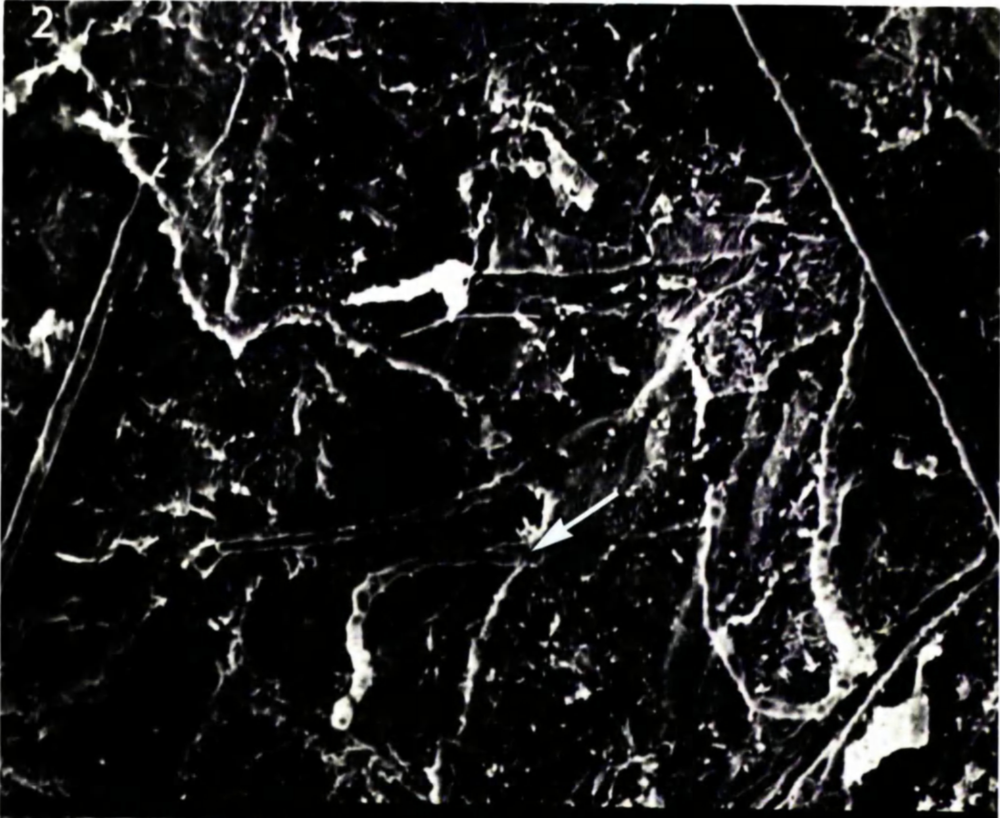
Audouinella virgatula (an exobiotic species) was found not to penetrate the shells

or the chitin substrata but was found to grew in groove-like regions on the surface (Fig. 6.25).

Under culture conditions these exo- and endobiotic *Audouinella* species were found to produce aragonite crystals at a localized attaching points in the petri dishes and occasionally these deposits were formed around the filaments at attaching points (Figs. 6.26 & 27). Whether these deposits are culture induced as Boney (1984) suggested or it is a natural phenomenon in these *Audouinella* species, as it is common in some other genera in the Nemaliales, is not established yet.

Fig. 6.2. SEM photomicrograph showing the penetration of *Audouinella efflorescens* into the chitin through the surface irregularities and between layers of chitin. The outline of the invading filament can be seen and the arrow indicates the entry point of this filament.

Fig. 6.3. SEM photomicrograph showing the chitin penetration by *Audouinella infestans* through a groove-like region, note the rounded cells in the hole area and the elongate cells forming the erect system emerging from the irregular surface.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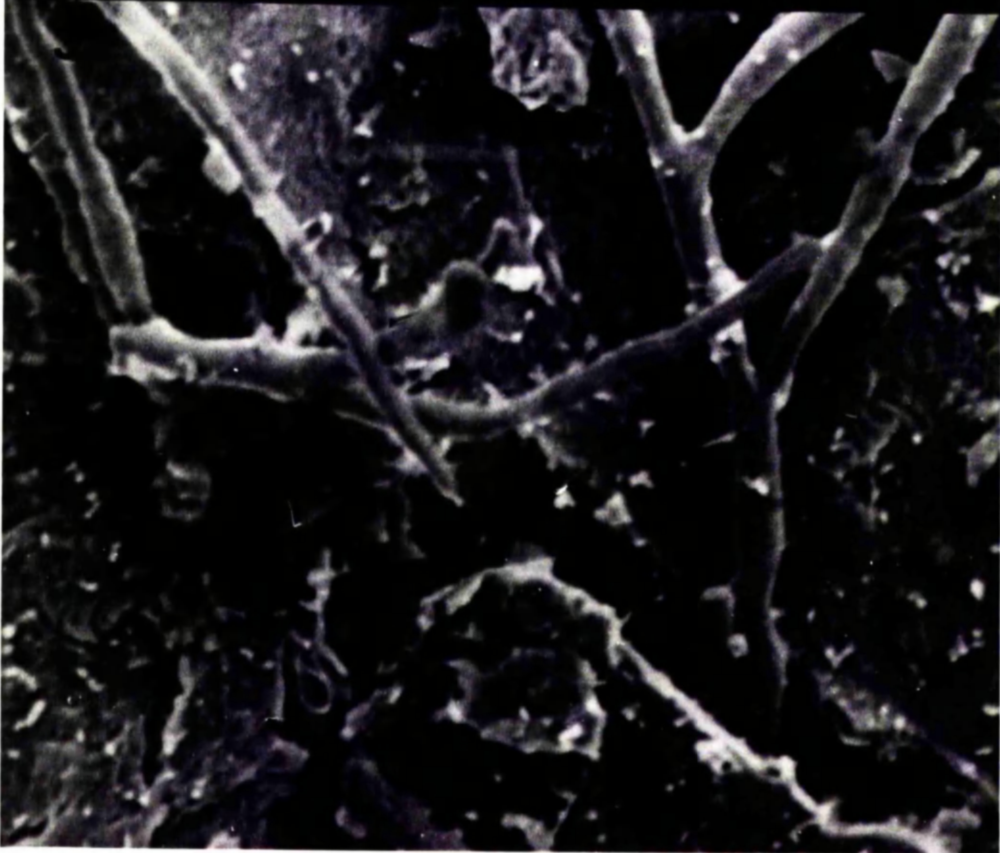

Fig. 6.4. SEM photomicrograph showing the chitin penetration by endophytic *Audouinella* sp. 1, note the emerging filaments from these cracks in the surface of the chitin, and a lateral branch (arrowed) penetrating the chitin.

Fig. 6.5. SEM photomicrograph showing chitin penetration by endophytic *Audouinella* sp.2 through uneven surfaces, note the rounded cells in the prostrate system and the elongated cell in the erect system.

Fig. 6.6. SEM photomicrograph showing chitin penetration by endophytic *Audouinella* sp. 3, the invading lateral filaments growing towards the chitin where entry points are available.

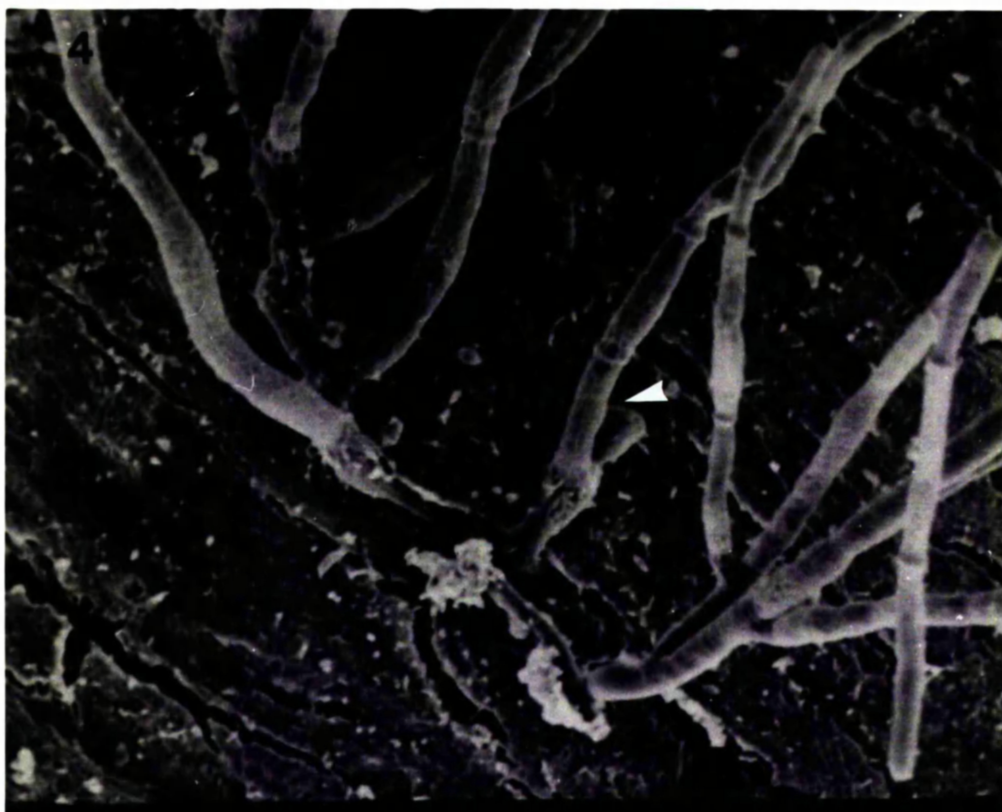
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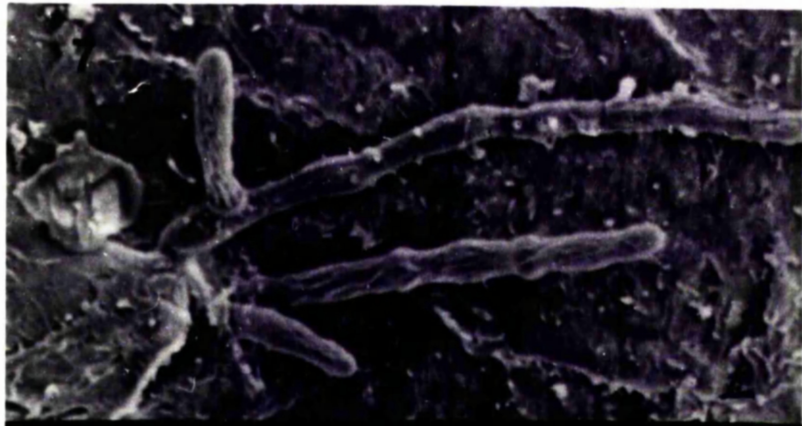
Fig. 6.7. SEM photomicrograph showing endozoic *Audouinella* sp. 1 emerging from filaments within the chitin.

Fig. 6.8. SEM photomicrograph showing endozoic *Audouinella* sp. 2 filament penetrating between layers of chitin.

Fig. 6.9 *Audouinella virgatula* growing in a groove-like area in chitin surface.



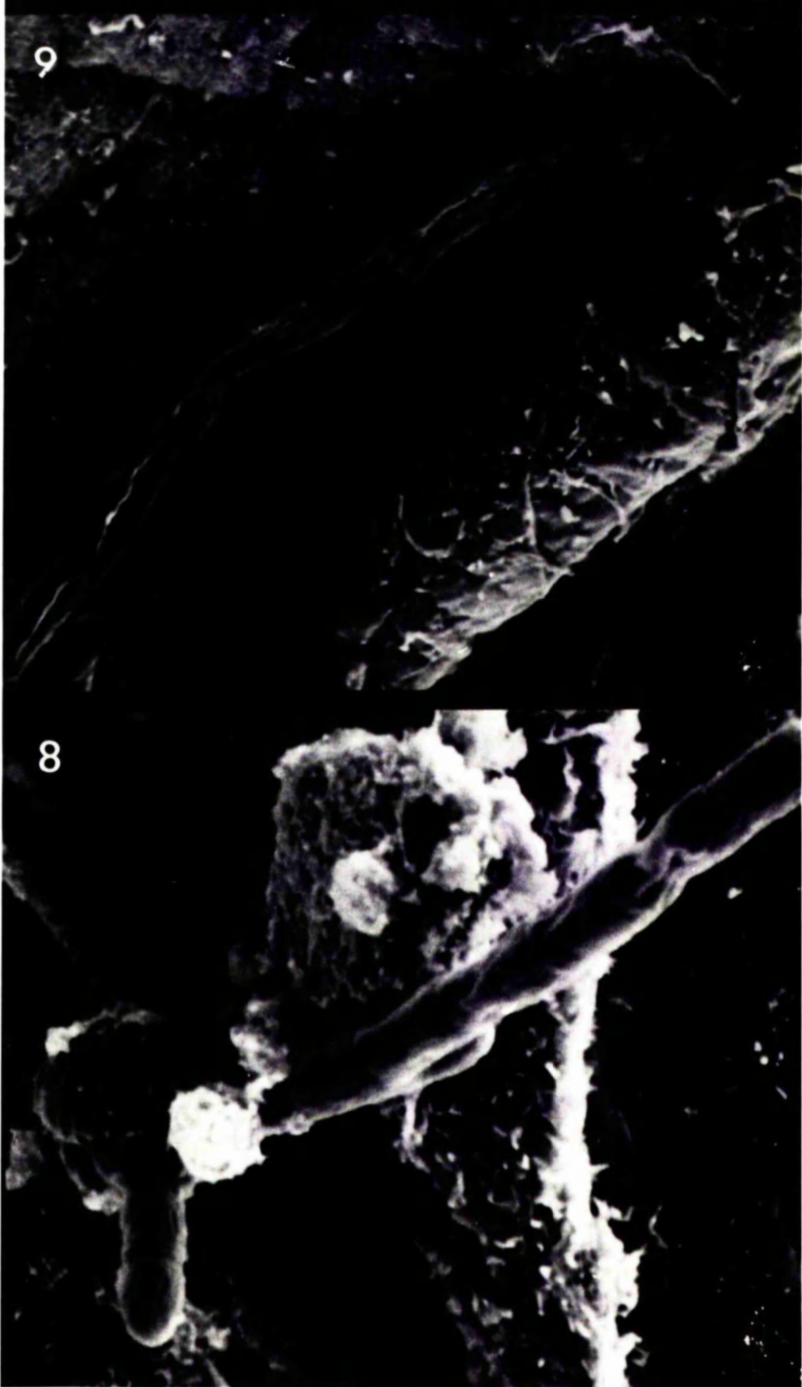

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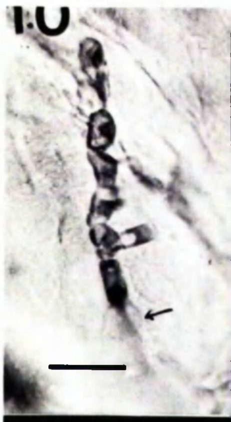
Fig. 6.10. Light microscopic photograph showing the penetration of the germinated spores (arrowed) of endozoic *Audouinella* species 2 in the chitin. (bar = 50 μm).

Fig. 6.11a-c. Show the inconsistency of migration of spore content of endozoic *Audouinella* sp. 2 during first stages of germination. (bar=50μm)

Figs. 6.12-15. Light microscopic photographs showing the irregular forms of cells of *Audouinella efflorescens*, *A. infestans* and endozoic *Audouinella* spp. 1 and 2 respectively when they grow inside chitin. Note the visible germinated rounded cell of *Audouinella infestans* (Fig. 6.12). (bar = 50μm).

Fig. 6.20. SEM photomicrograph showing penetration of mollusc shell by *Audouinella infestans* erect filaments through surface irregularities.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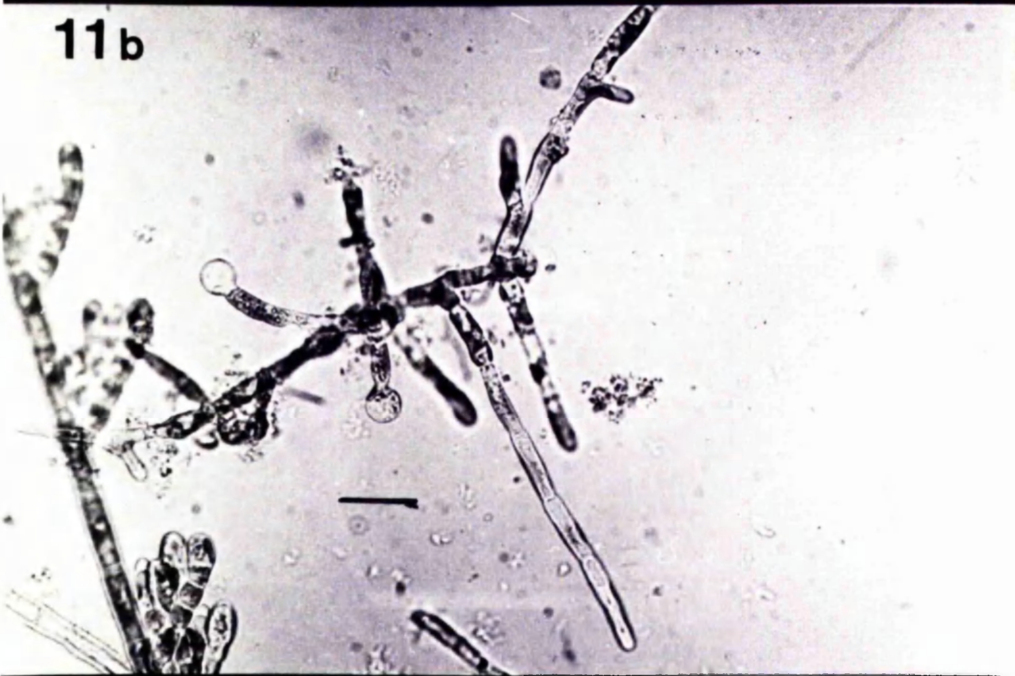
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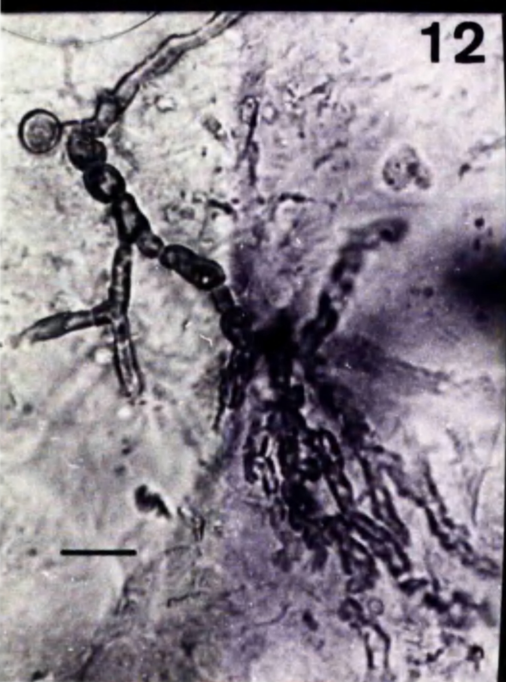
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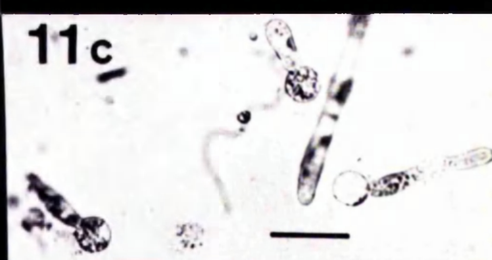
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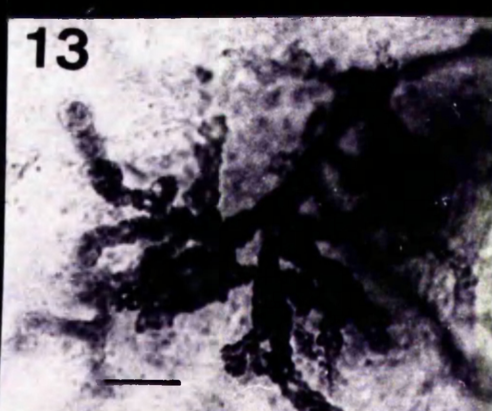
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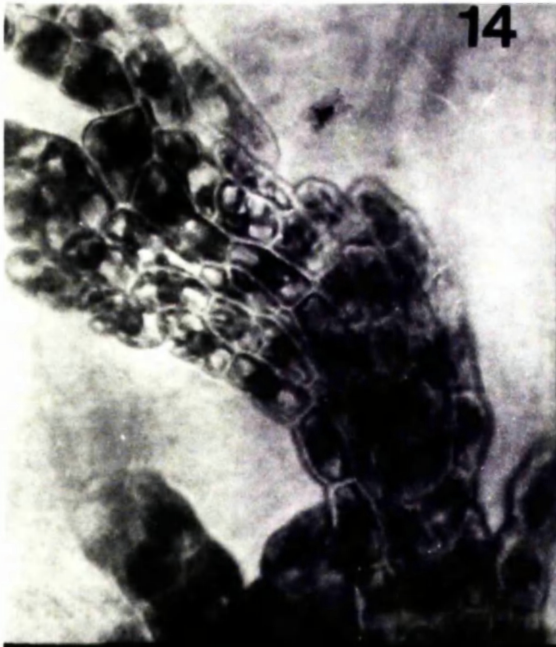
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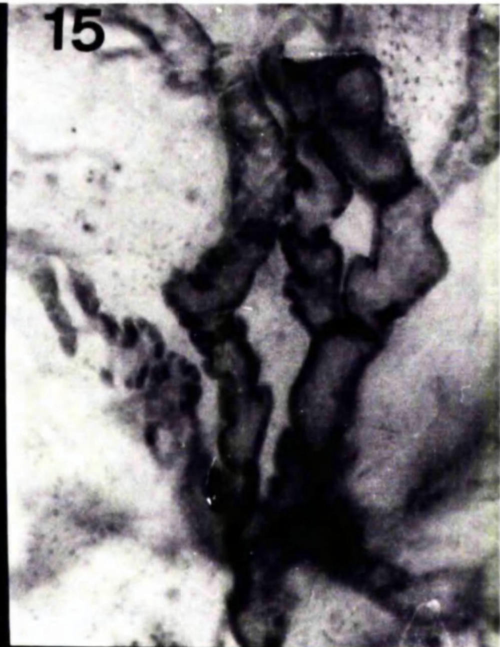
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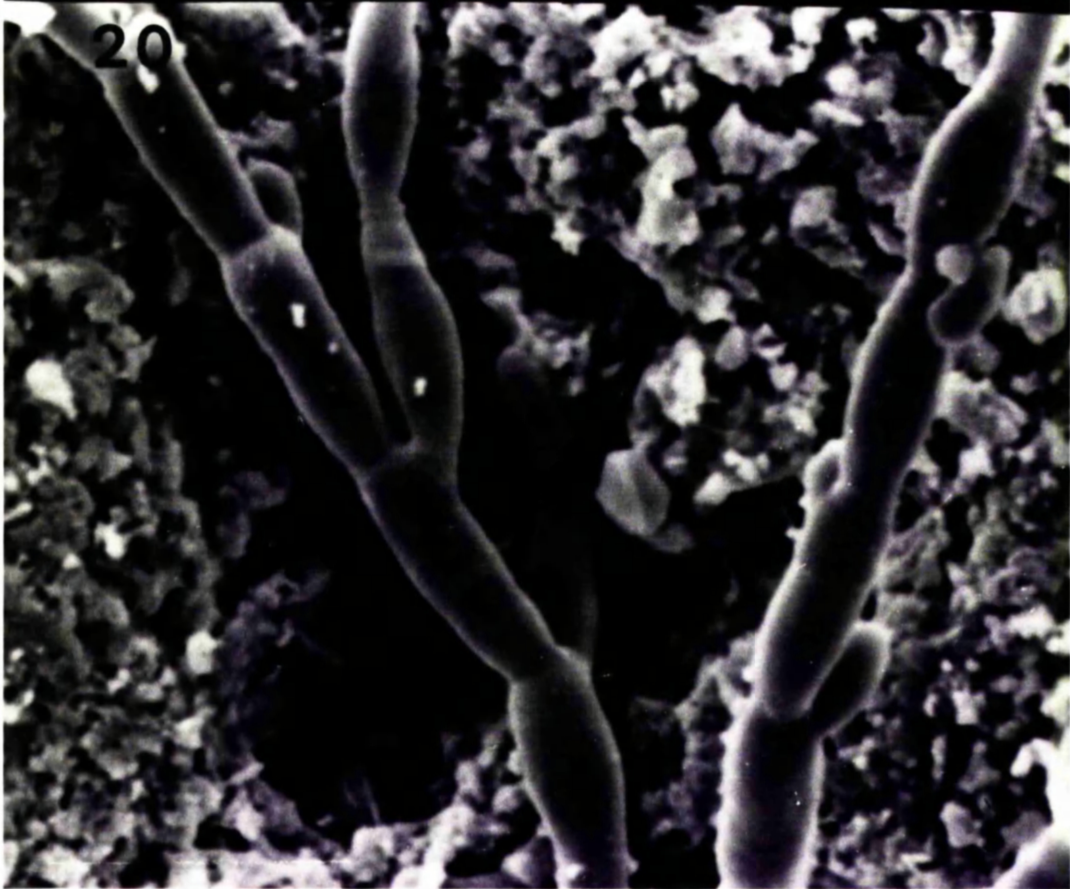
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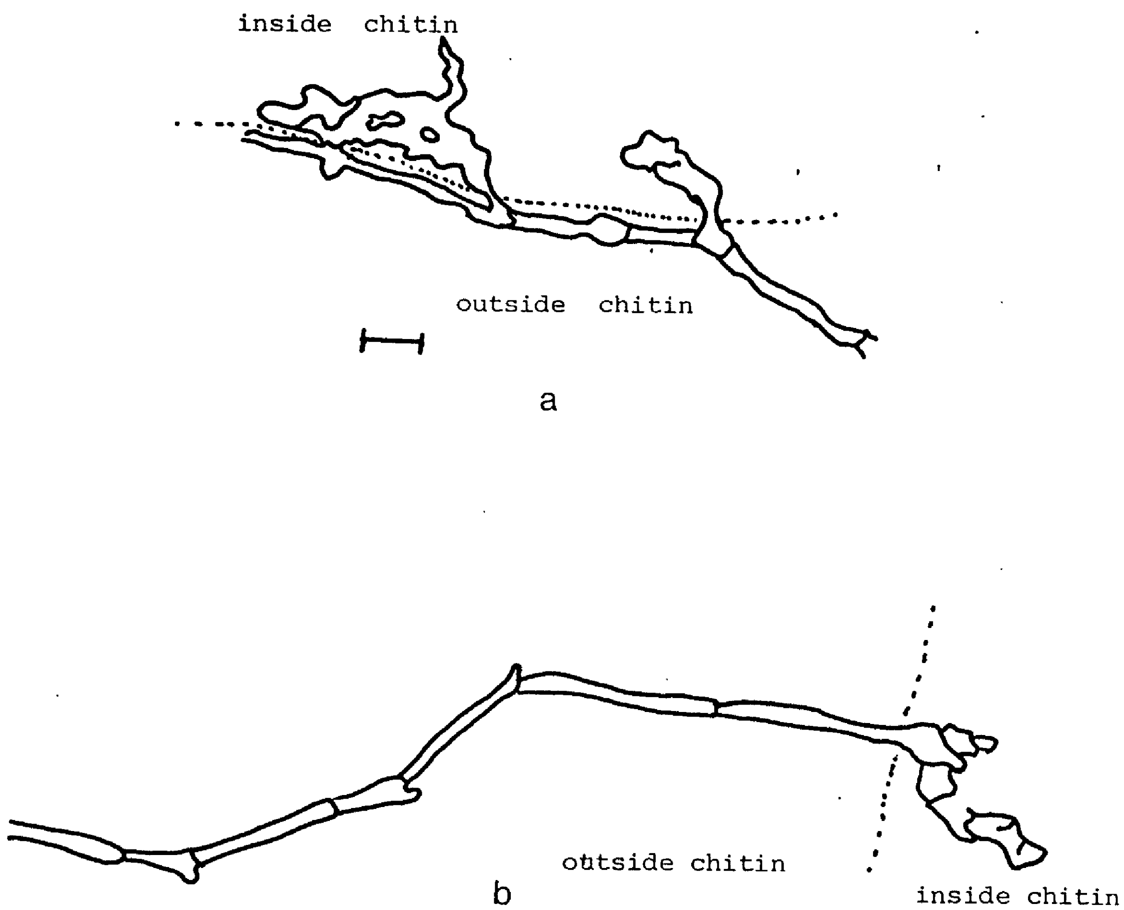


Fig. 6.16a,b. Diagrammatic presentation of morphological changes of the cells of *Audouinella efflorescens* inside the chitin. (bar = 25 μm).

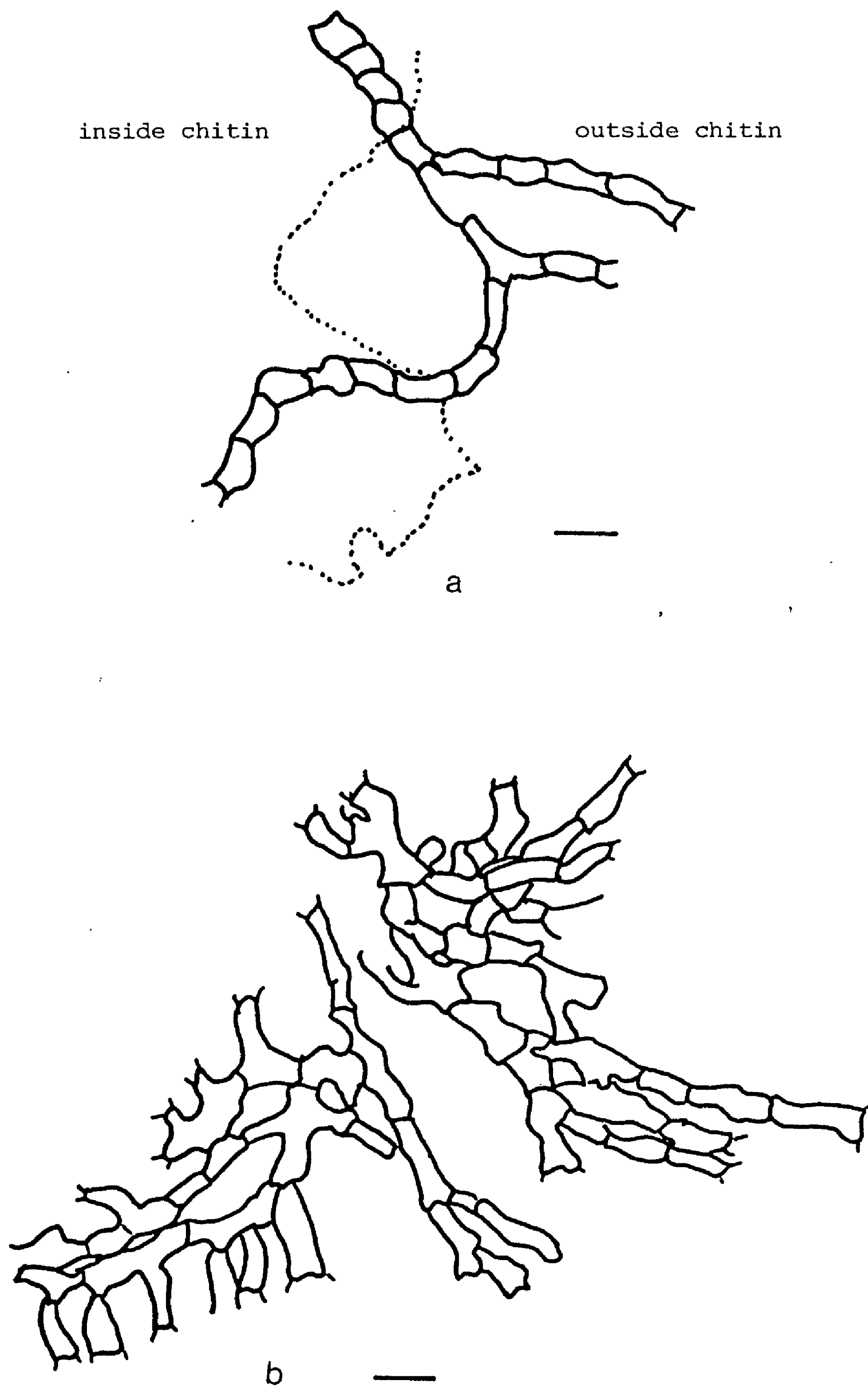


Fig. 6.17a,b Diagrammatic presentation of morphological changes of the cells of endophytic *Audouinella* species 1 inside the chitin. (bar = 25 μm).

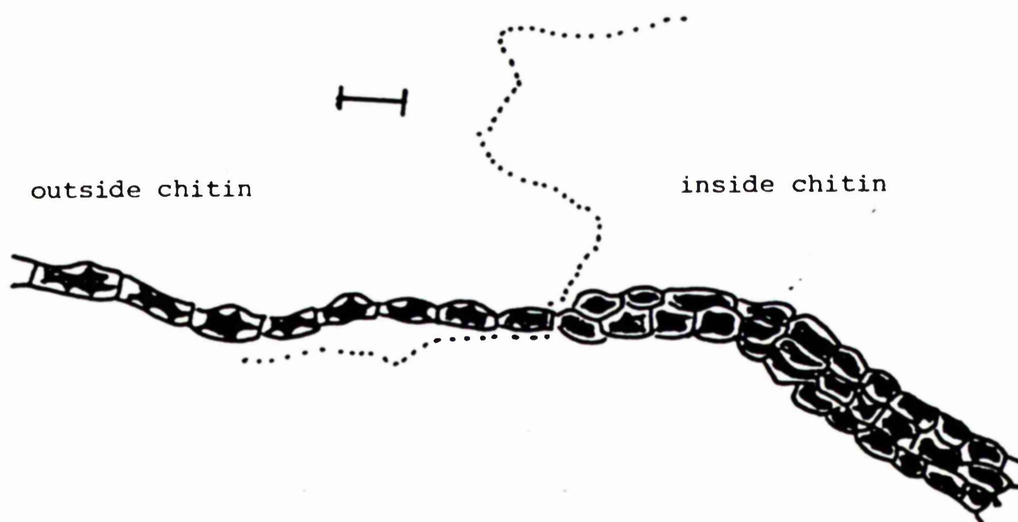


Fig. 6.18 Diagrammatic presentation of morphological changes of the cells of endophytic *Audouinella* species 3 inside the chitin. (bar = 25 μm).

Fig. 6.19 Diagrammatic presentation of morphological changes of the cells of endozoic *Audouinella* 2 inside the chitin. (bar = 10 μm).

Fig. 6.21. SEM photomicrograph showing the penetration of mollusc shell by endophytic *Audouinella* sp. 1 filaments through surface irregularities. Note the thick rings at cell junctions.

Fig. 6.22. SEM photograph showing the penetration of mollusc shell by endophytic *Audouinella* sp. 3 filaments through a surface depression. Note the difference between erect and attaching systems cells and the thick rings at cell junctions.

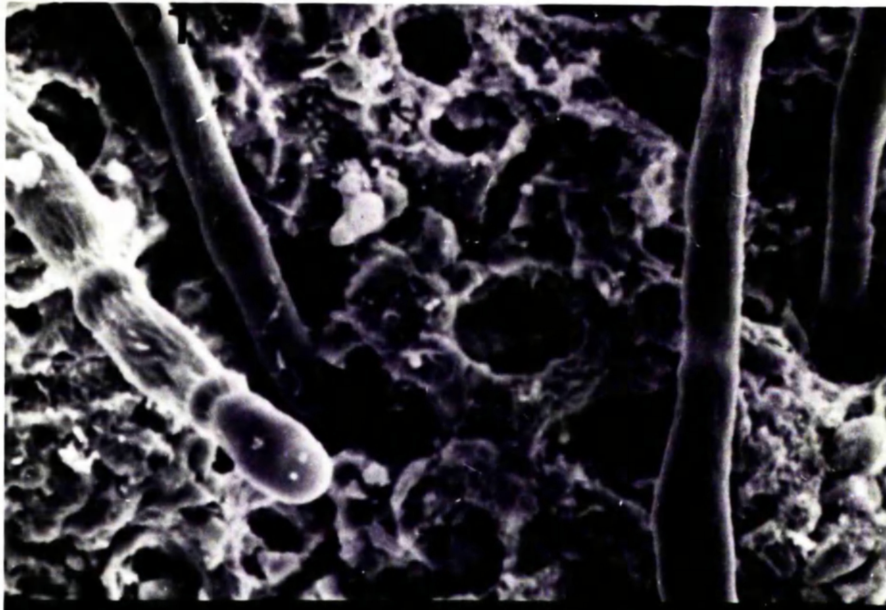
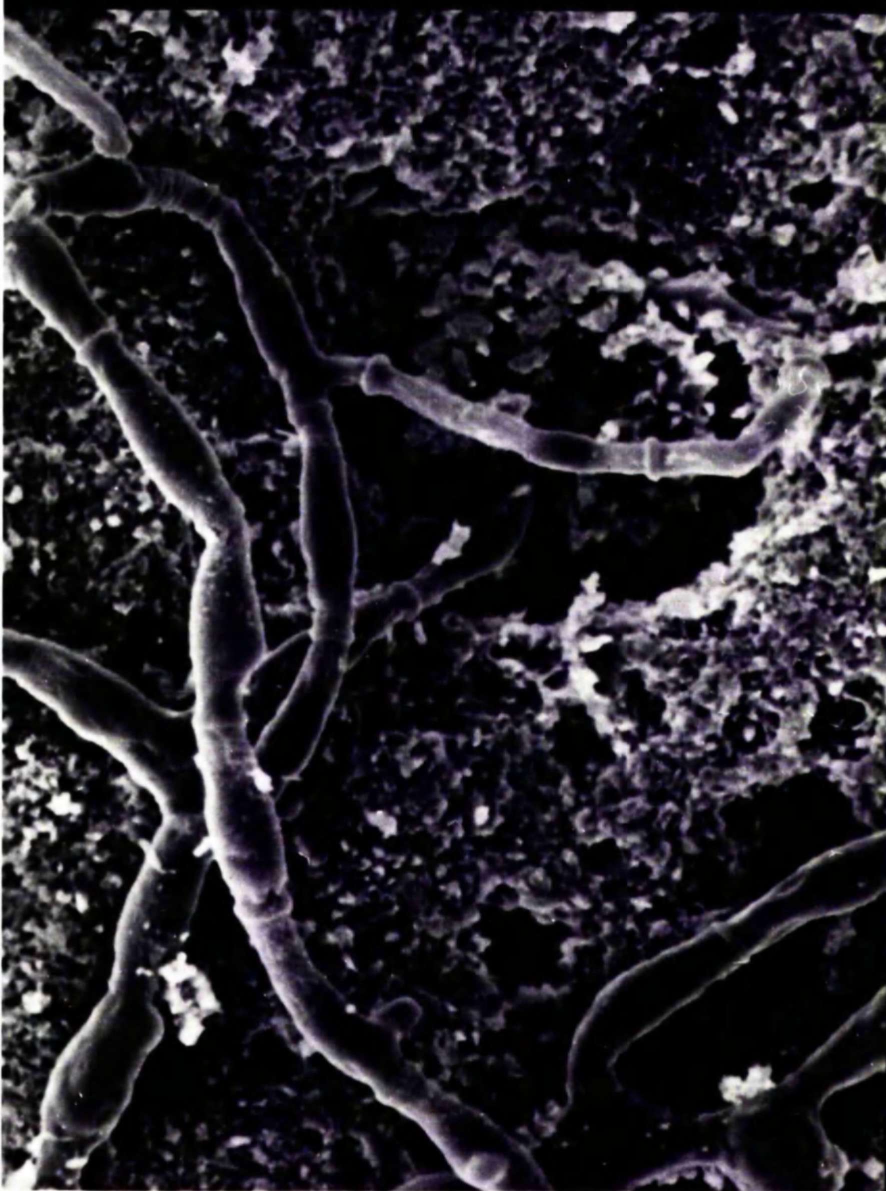
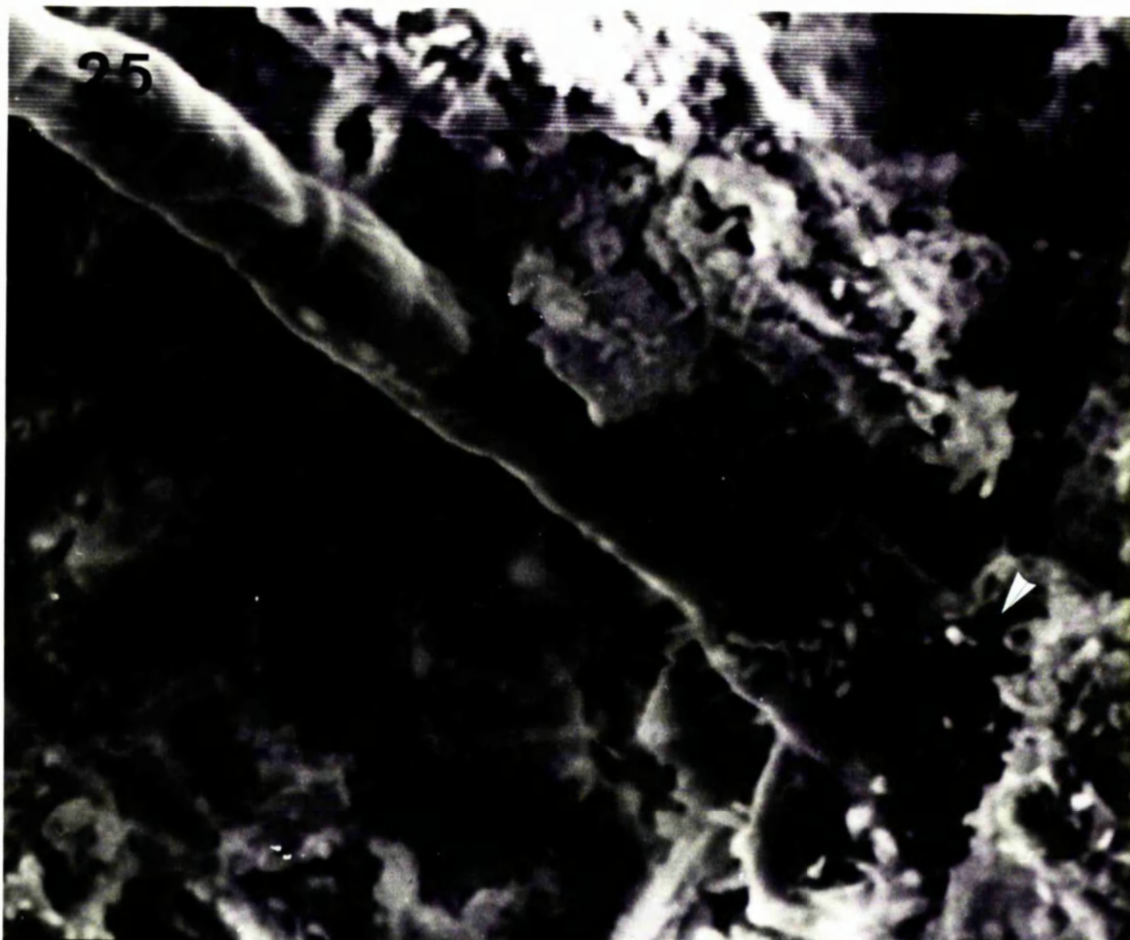



Fig. 6.23. SEM photograph showing the penetration of mollusc shell by a lateral filament (arrowed) of endozoic *Audouinella* sp. 1 .

Fig. 6.24. SEM photograph showing two filaments of endozoic *Audouinella* sp. 2 penetrating the same localized region in the mollusc shell.

Fig. 6.25. *Audouinella virgatula* growing in a protected area on the mollusc shell. -note the prostrate system (arrowed).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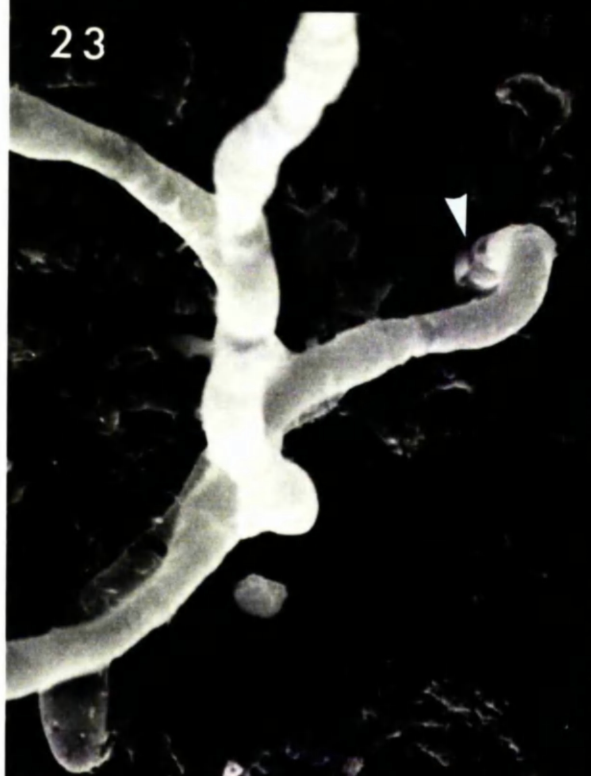
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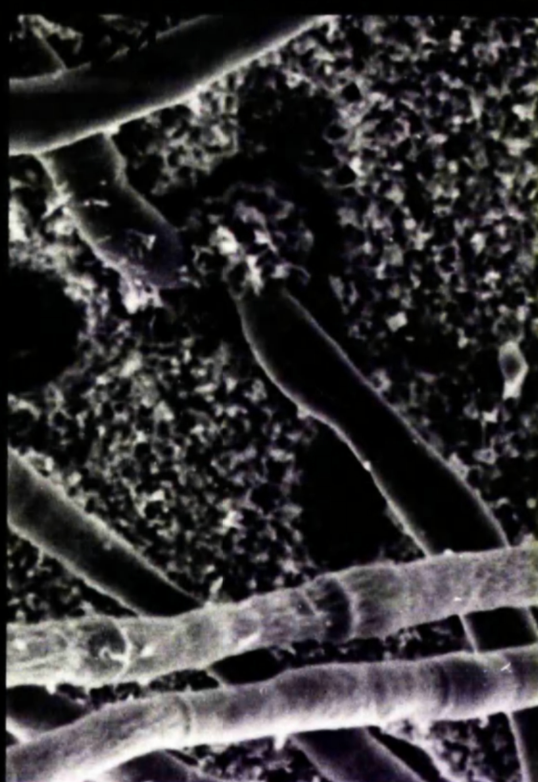
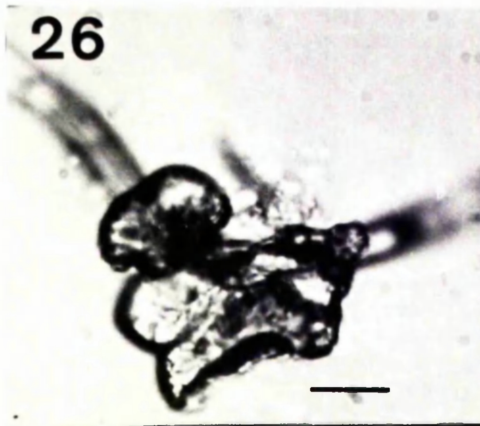


Figs. 6.26-27. Aragonite-like formed around the filaments of an endobiotic *Audouinella* species under culture conditions. (bar = 25 and 50 μm respectively).

Fig. 6.28 a-g. Endophytic *Audouinella* sp. 3 growing inside the agar. a,b showing the long straight lateral branches; c-g showing short spiral lateral branches. d,f showing the swellings at the end of the branches (arrowed). (bar = 50 μm).

Figs. 6.31-34. *Audouinella infestans* filaments showing signs of growth inside plastic base of a Falcon tissue culture vessel. (bar = 50 μm).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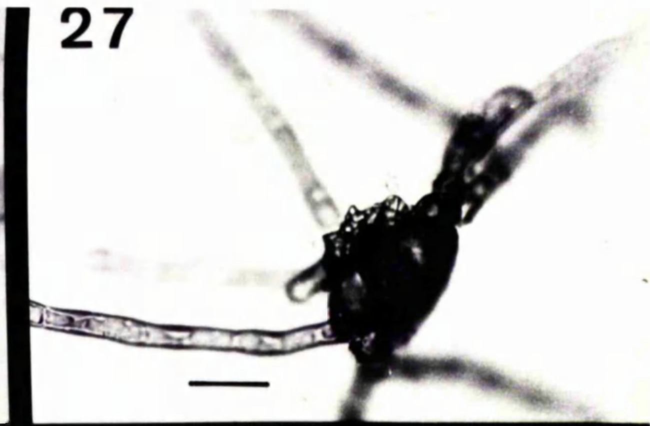
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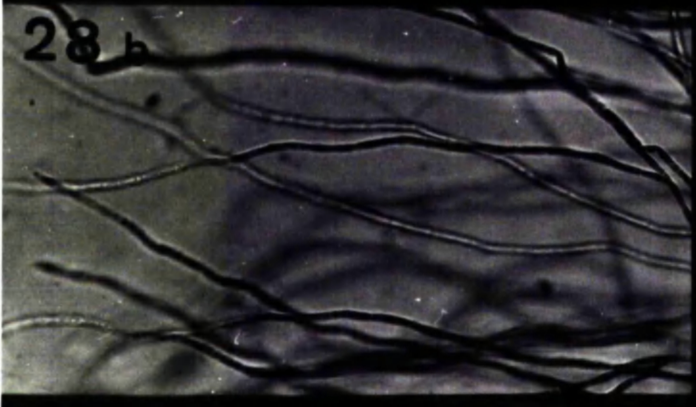
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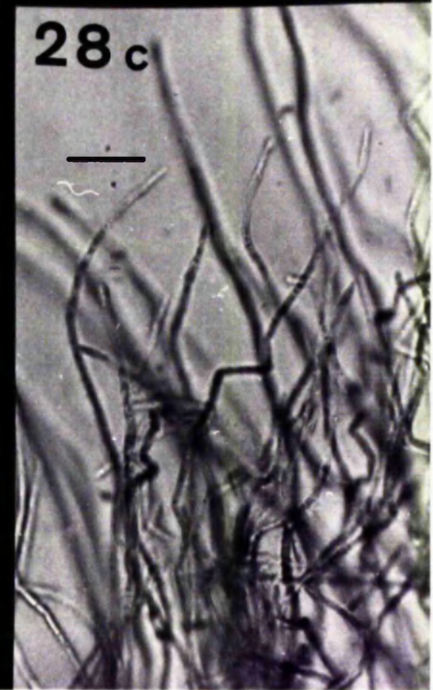
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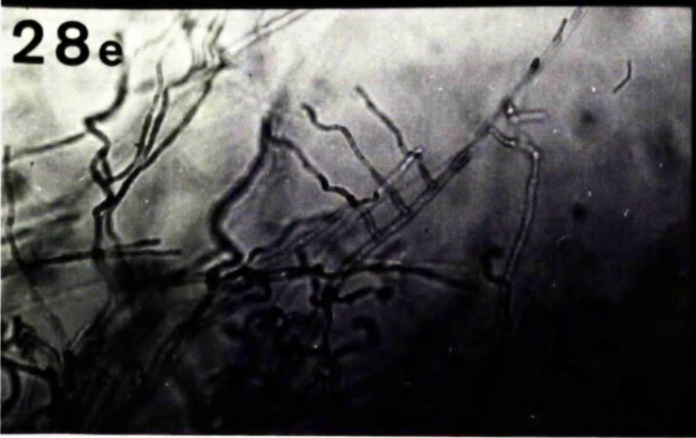
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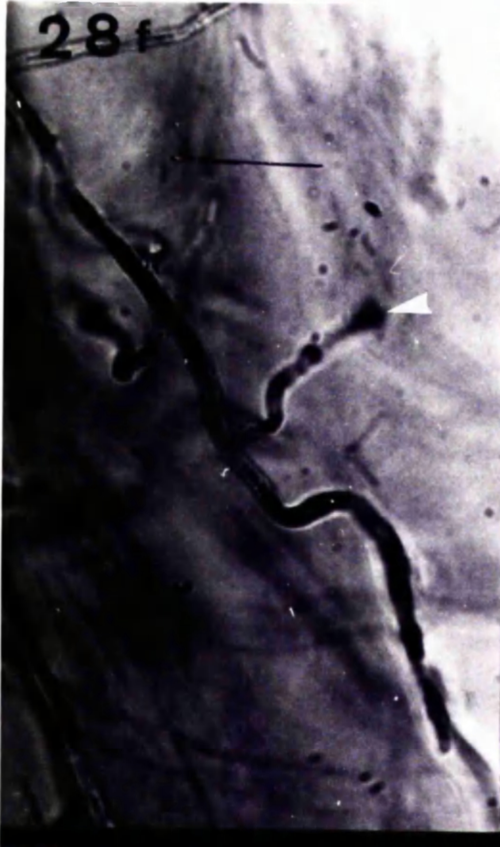
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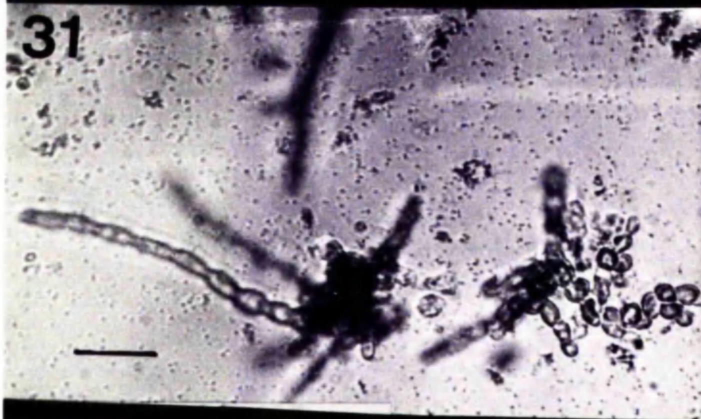
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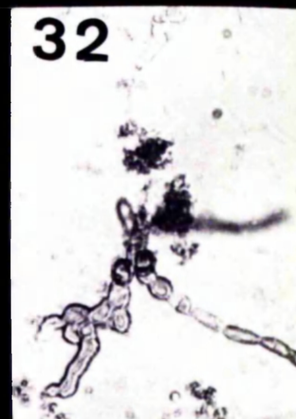
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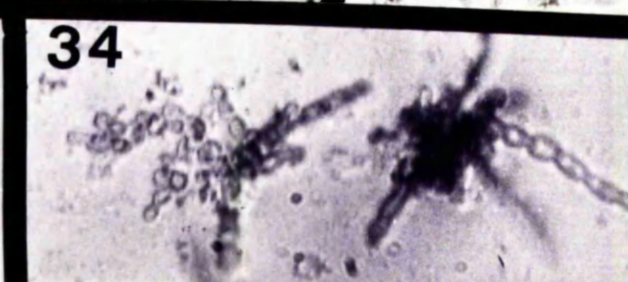
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6.d. Filament growth in agar

Endophytic *Audouinella* species 3 was found to grow in 1% agar in enriched sea water. Its filaments were found to spread into the agar producing lateral branches with very elongate colourless cells at the tips, whilst cells of the main branch and those distant from the tips were distinguished by their larger size and the presence of large chloroplasts. The tips of the main branches appeared to have a colourless swellings with small terminal 'blebs'. These small swellings suggest that these filaments 'digest' their way through the agar creating these spaces at the end of the main branches which give the 'bleb' like appearance.

Other endobiotic *Audouinella* species (viz. *A. infestans,* endophytic *Audouinella* 1 and 2 and endozoic *Audouinella* species 1 and 2) were found to invade the agar in the same way of the endophytic *Audouinella* 3. It was not possible in all cases to get a clear view of how these plants invade the agar because of the limitation in the photographing unit available and the thickness of the agar. Attempts were made to get thin sections of agar containing these growth forms. These thin sections were put on glass slides and then examined and photographed. Fig. 6.28a-f show different morphological features of the endophytic *Audouinella* species 3 growing inside the agar. These features include the long straight and short spiral lateral branches, and the small swellings formed at the tips of the filaments. Penetration into agar by these *Audouinella* species was also measured by a simple experiment as shown in Fig. 6.29 and summerized in Table 6.1. The cultures were carried out in a tissue culture multi-well of 3.5 ml per well, filled with 1% agar in enriched sea water and kept under temperature of 10-15^{0}C and irradiance of 22.4 $\mu E\ m^{-2}\ s^{-1}$, with 4 replicates of each *Audouinella* species used. The penetration rate of these species was measured and it was found that endozoic *Audouinella* species 2 has the highest capacity of penetration than any of the other species. This appears to be due to the large cell size of this species, as it was found that the species with relatively large cells (viz. endophytic *Audouinella* species 1 and 3 and

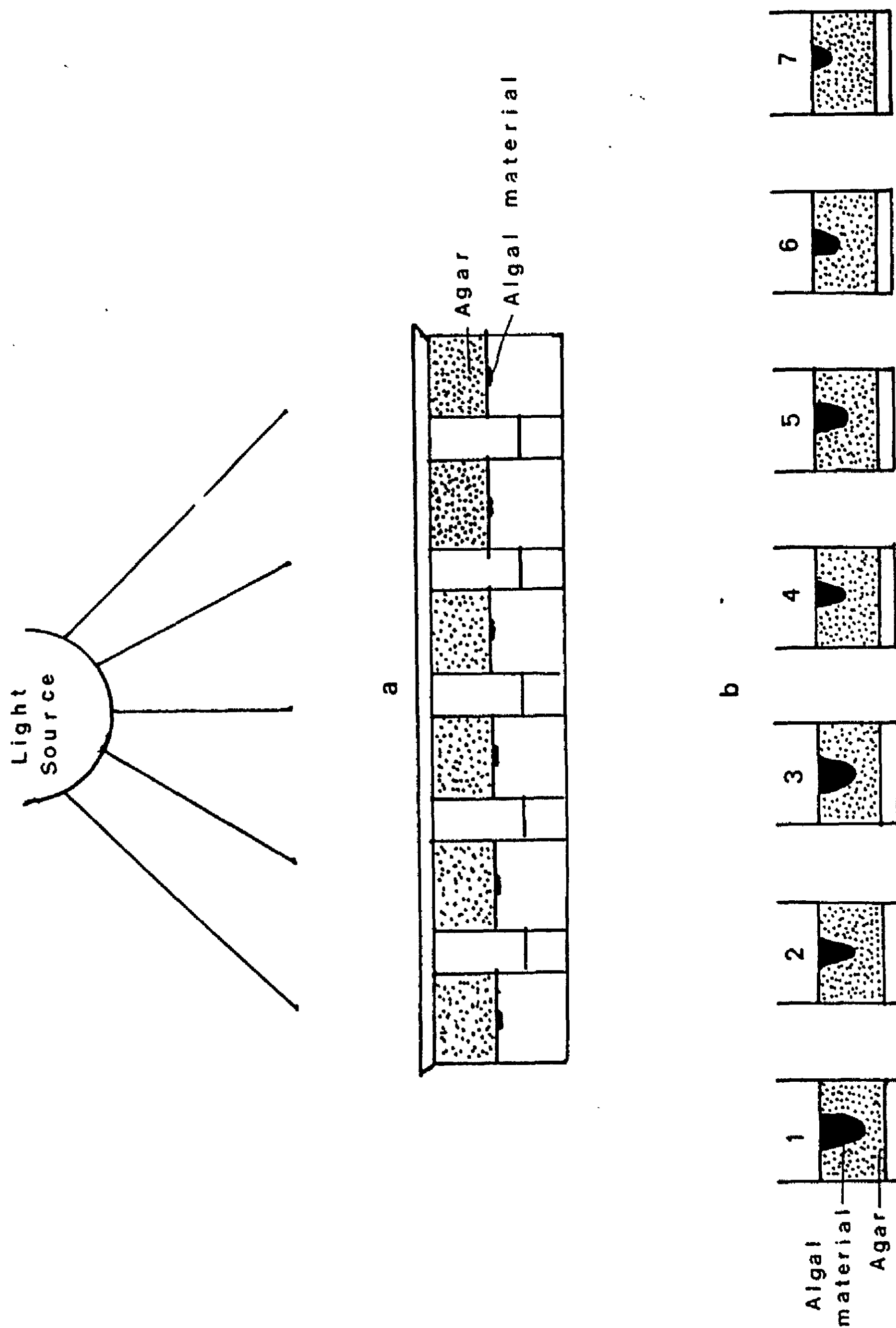


Fig. 29.a,b Diagrammatic presentation of the penetration of *Audouinella* species into agar material. a. The culture vessel containing algal material placed on the top of the agar. b. representation of the different penetration depths obtained by different *Audouinella* species.

endozoic *Audouinella* species 1) have a relatively higher penetration rate than these species with relatively small cell size (viz. *Audouinella asparagopsis*, *A. infestans* and endophytic *Audouinella* species 2) (Table 6.1).

6.e. The 'living' space concept

Cells of endophytes live in the outer "cuticle" of the 'host' in a situation of flux of radiant energy and of nutrients, carbon dioxide etc. being taken up by 'host' cells. Each cell of the endobiont can be envisaged as living in a diffusion gradient which is usually cylindrical inside this available 'living space' within the 'cuticle' region.

The 'living space' studies showed that there is a significant relation between this space available and the cell dimensions (Table 6.2). It was found that where the space was relatively large, such as in case of *Delesseria* sp., the number of the endophyte filaments increased. Boney (unpublished data, Fig. 6.30) found that an endophytic *Audouinella* identified as *Audouinella chylocladiae* (Batt.) Dixon, growing endophytically in the red alga *Stenogramme interrupta* (C.Ag.) Mont. collected from different localities off the San Juan Islands, Washington State U.S.A, demonstrated remarkable changes in its cell sizes due to the living space available in the host. In the outer cortex area where the cells of the host are very close to the surface, the cells of the endophyte were much smaller than those in the inner cortex where cells of the host are larger and with thicker cell walls which allowed the endophyte to find its way between them and enlarge its cells. This enlargement would be significant in the life of these endophytes in terms of nutrient and light absorption as the flux of these two essential components decreases from the surface area towards the inner part of the 'host'. The colour of the endophytic *Audouinella chylocladiae* was found to get darker and bluish in the inner parts of the 'host' tissue and the chloroplast form to change.

Table **6.1** Penetration of *Audouinella* species into agar

Audouinella species	Penetration depth (mm)
Endozoic *Audouinella* sp. 2	7.5
Endophytic *Audouinella* sp. 1	6.0
Endophytic *Audouinella* sp. 3	6.0
Endophytic *Audouinella* sp. 2	5.0
Endozoic *Audouinella* sp. 1	5.0
Audouinella asparagopsis	4.0
Audouinella infestans	3.5

Table **6.2** `Living space` in relation to cell dimentions of endophytes.

Host spp.	Living space diameter "depth" (µm)	Endophyte cell length (µm)	cell diameter (µm)	L. S. diameter to C. D. ratio
Rhodoptilum sp.*	7.5	5.5	2.125	3.5
Gracilaria sp.*	4.75	4.75	1.75	2.7
Botryocladia sp.*	3.75	7.0	1.5	2.5
Stenogramme sp.*	3.17	9.25	1.5	2.1
Delesseria sp.*	13.25	7.5	2.7	4.9
Ceramium ruprum **	8.0	6.9	2.1	3.8
Gastroclonium sp.**	15.0	7.5	3.0	5.0
Dasya sp.**	18.0	6.0	3.0	6.0
Polysiphonia elongata ***	13.0	7.2	3.6	3.75
Heterosiphonia plumusa ***	23.0	11.4	4.1	5.6
Delesseria sanguinea	11.7	24.5	3.75	3.12

* measurements made from slides prepared by A. D. Boney

** measurements made from drawings of Drew (1928)

***measurements made from drawings of White (1968)

(1) Measured as width of "cuticle" representing the diameter of the cylindrical `living space`, and the length of the endophyte cell.

(2) Cell measurements given as mean size (cells within the `host` are of uniform size).

Table 6. 2 continued

volume of living space μm^3 (1)	cell surface area μm^2 (2)	S. A to Living space V ratio
242.95	36.7	0.12
84.17	26.11	0.31
77.31	32.98	0.42
73.00	43.59	0.60
1034.14	63.62	0.06
346.8	45.52	0.13
132.5	70.68	0.53
1526.8	56.55	0.03
955.67	81.43	0.08
4736.4	146.8	0.03
2634.07	288.63	0.10

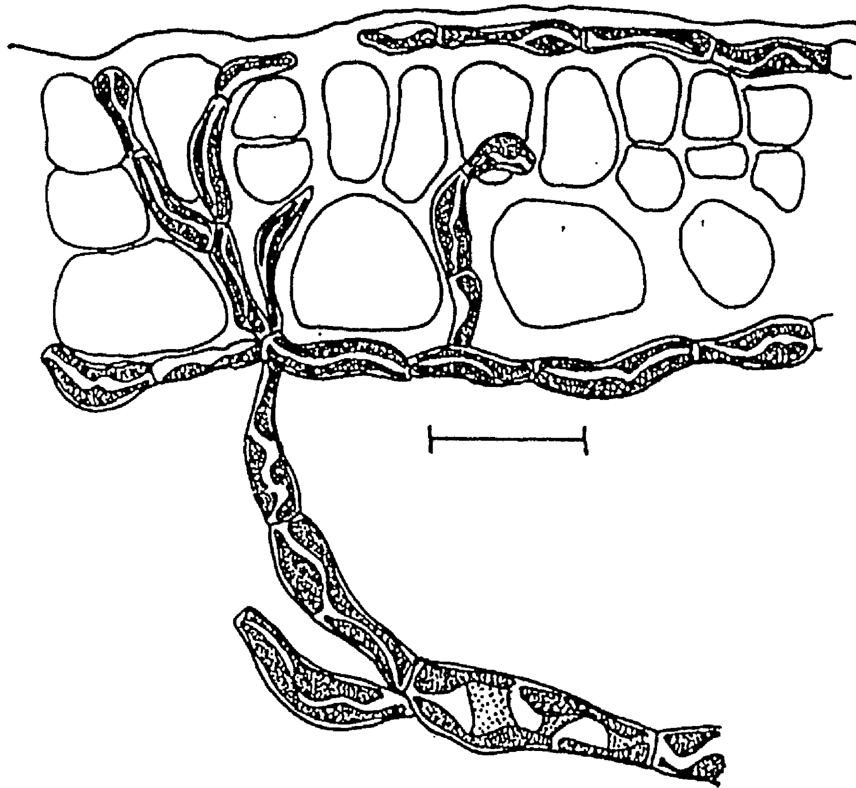


Fig. 30. Endophytic *Audouinella* species cells in the 'host' plant *Stenogramme interrupta*.(bar = 10 μm).

Of particular interest is the relation between the surface area of the cells of endophytes and the 'living space' as the endobiotic cells are either long and thin or short and of wide diameter enabling a diffusion gradient between the 'living space' and the endophyte cells. If the ratio is a low one then the volume of 'cuticle' living space relative to the cell is high. The tendency then for cells to be longer and thinner - and often for more than one endophyte filament to be present. If the ratio is a high one then the volume of the 'cuticle' living space is low - and the endophyte cells are larger- The diffusion gradient here would be a much steeper one than the above because the 'host' cells are closer to the plant surface. In such circumstances one tends to find larger celled endophytes but usually single filaments (not several as with *Delesseria* species). It was found that the ratio between the surface area of endophytes cell and the 'living space' volume decreases as the length of the cells (i.e, the ratio between cell length and cell diameter) decreases (Table 6.2).

Table 6.2 summarizes data on the relationship between available cell living space and the absorptive cell surface area. A low value to the ratio indicates a large 'living space' relative to the cell absorptive surface area. The lowest ratios were (0.06-0.3) were obtained with endophytic cells in *Dasya, Heterosiphonia* (*Audouinella endophytica*), *Delesseria* sp., with the endophyte cells 2-2.8 times as long as broad. Ratios of 0.08-0.13 were obtained with the endophytic cells in *Polysiphonia elongata, Delesseria sanguinea* (endophytic *Audouinella* species 1), *Rhodoptilum* sp. and *Ceramium rubrum*. Of these, the most elongated cells were these with endophytic *Audouinella* species 1 in *Delesseria sanguinea*, and noticeably in regions where the 'cuticle' is thicker more than one endophytic filament was found. The highest ratios of 0.31-0.6 were obtained with endophytic cells in *Gracilaria* sp., *Botryocladia* sp., *Gastroclonium* sp. and *Stenogramme* sp.. These 'hosts' supply less living space, and the endophyte cells are larger and thicker. The endophyte in *Stenogramme* species is a different case -the long thin cells in the cuticle are continuous with larger cells deeper

in the 'host' thallus.

6.f Possible invasion of the plastic base of a Falcon tissue culture dish by *Audouinella infestans*

Audouinella infestans when cultured in falcon tissue culture petri dishes was found to develop some irregularities in the cell morphology and at different level of focussing similar to these irregularities shown by these cells when they were growing inside chitin (Figs. 6.31-33). These observations suggest that *Audouinella infestans* may invade such material or may have effected entry by some small surface irregularity and then penetrated between the layers of plastic material.

6.g. Erect and prostrate system morphology; observations by scanning electron microscopy

Epiphytic forms of *Audouinella* often have well defined prostrate and erect systems. Endophytic and endozoic forms when grown free from their 'host' organism often develop a prostrate system or an attaching system, but do not show such differentiation within the 'host' tissue.

An attaching system has an obviously important role in the life of epiphytic or epilithic species. It may also be an important feature in species definition. Dixon and Irvine (1977) used the morphology of the attaching (prostrate) systems to distinguish between *Audouinella* species (i.e. epiphytic, endophytic, or endozoic forms).

Observations

Audouinella virgatula. As already described this alga has a distinct multicellular basal system and a filamentous erect system. The attachment to the substrata is best seen only in areas with a relatively rough surface, and takes a longer time on smooth surfaces. These epiphytes appear to devote much energy to attachment on

smooth surfaces and the vegetative growth is consequently reduced. The Figs. 6.34, 6.35 and 6.36 show that two individuals of the same age on different substrata produced different numbers of erect filaments, i.e, two filaments on mollusc shell, one filament on rough glass slide, and a short-one filament on smooth glass slide. On the other hand, the attaching systems sizes were different on different substrata. Larger systems were formed on smooth surfaces. These morphological adaptations explain the smaller tufts of these plants found in nature, where mechanical interactions will take place. Tufts on a smooth surface were completely lost during processing for **SEM**, whereas plants of the same age on glass slides with a rough surface subjected to high speed rotating machine still remained attached to the substratum.

A. infestans. This alga showed a little distinction between the attaching and erect system on rough substrata (i.e, mollusc shells and chitin). However, on these surfaces some of attaching system developed with angular and elongate cells.

This alga apparently relies on irregularities in the substratum and first establishes itself in grooves of the mollusc shells or between layers of chitin (Figs. 6.3 & 6.20).

Endophytic *Audouinella* species 1. This alga, under **SEM** examination, showed no attaching system as this alga penetrated the substratum. The erect system was dichotomously or irregularly branched. Cells were cylindrical and occasionally with large rings at cell junctions (Figs. 6.4 & 6.21).

Endophytic *Audouinella* species 2. This alga, under **SEM** examinations, showed more rounded cells in the prostrate system than in the erect system, with occasional long cylindrical cells present (Fig. 6.5 & 6.37).

As this alga grew in the form of compacted tufts, observations by **SEM** were very difficult and the attaching area could hardly be seen. However, to achieve this, tufts on substrata were partially lifted to expose the attaching areas, consequently most of the plants were lost in processing.

Endophytic *Audouinella* species 3. This alga showed a clear attaching system

composed of irregularly branched filaments with broad irregularly shaped cells. The erect system is filamentous growing at right angles from the attaching system, with barrel shaped cells which under **SEM** showed thick rings at the ends (Fig. 6.22). This would suggest different wall structures with the two systems.

Endozoic *Audouinella* species 1. The prostrate and erect systems in this alga were not distinct under **SEM** examination. Observations showed that, all parts of the plant have the same form of cells. It appears that this alga establishes itself inside the substrate material then grows afterwards as the material showed growing areas with more than one filament coming from one point (Fig. 6.7).

Endozoic *Audouinella* species 2. This alga has an attaching system composed of a filament oppositely branched. Under **SEM** examination, cells were cylindrical to irregular shaped. However, cylindrical cells only are present in the erect system (Figs. 6.8 & 6.24).

Discussion

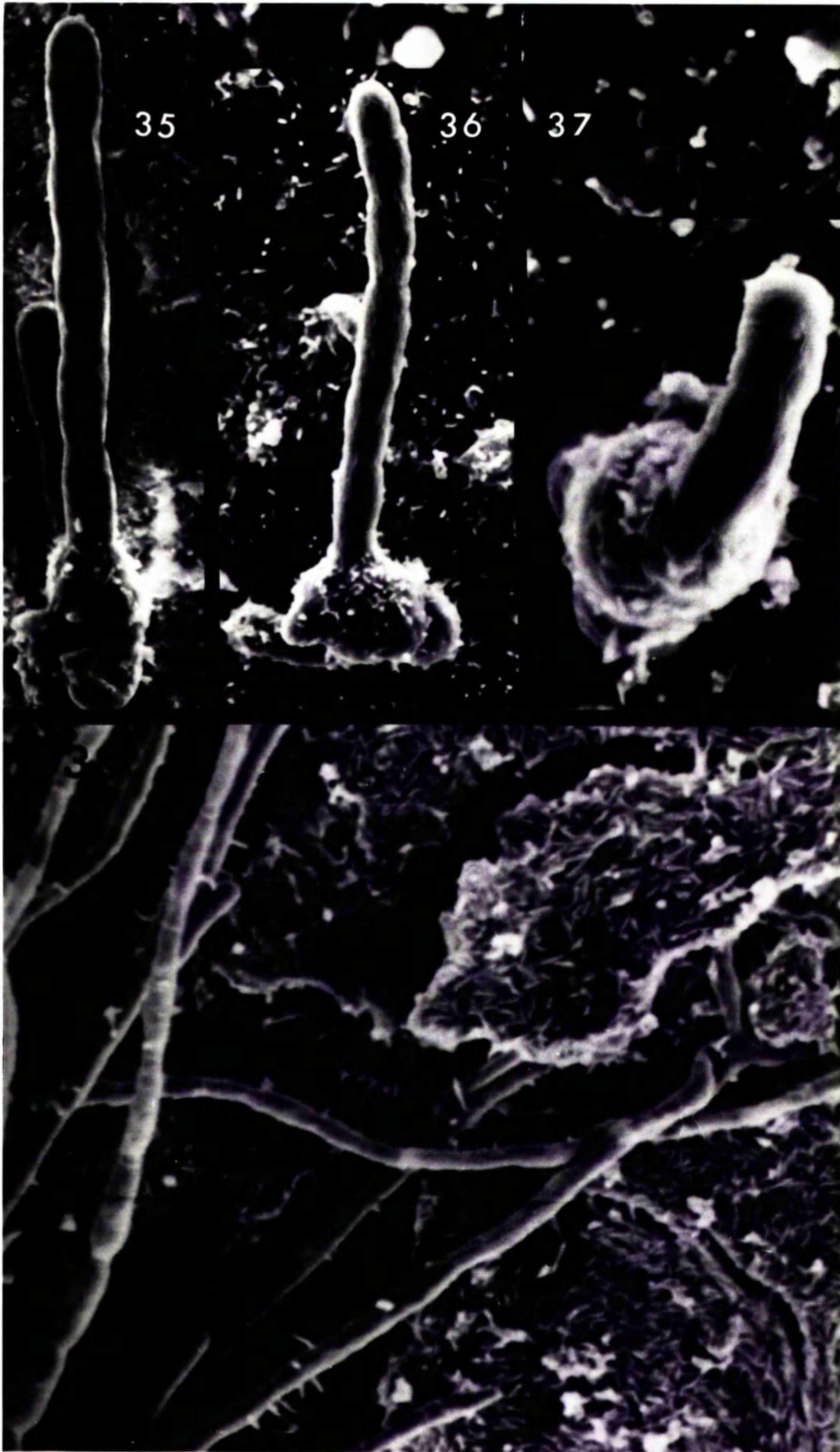
It is evident from these studies on substrata invasions and adaptations that these *Audouinella* species under study can invade chitin, calcium carbonate and agar substratum provided that they have sufficient contacting time for their establishment either externally or internally. Other studies have shown the same phenomenon (White and Boney 1969; 1970; Boney 1980). However, invasion of living plant material did not occur, although epiphytism on *Fucus spirales* did occur under culture conditions. Whether recognition and preference (Boney 1980), or the nature of the plant's surface prevented the endophyte from invading the host tissue is not yet known. Tam *et al.* (1987) also failed to obtain reinvasion with *Audouinella porphyrae* and *A. vaga* in *Porphyra* and *Pterosiphonia* species (the original host of these *Audouinella* species respectively). It was found in the present study that penetration of the chitin and the calcareous material took place through superficial irregularities in the substrata. Boney (1980) suggested that a number of extracellular reactions due to the

Fig. 6.35. *Audouinella virgatula* growing on mollusc shell.

Fig. 6.36. *A. virgatula* growing on rough glass surface.

Fig. 6.37. *A. virgatula* growing on smooth glass surface.

Fig. 6.38. Elongated cells of the erect filaments of endophytic *Audouinella* sp. 2.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presence of exoenzymes and extracellular products may take place to break down the algal polysaccharides and the chitin or to decompose the calcium carbonate. The results of the present investigation suggest that endobiotic species isolated from plant 'hosts' are capable of penetrating substrata of likely animal association. e.g, chitin and calcium carbonate. Observations also suggest that the filaments actually penetrate the agar gel, and the small terminal 'blebs' observed suggest localized region of agar dissolution. This would not be unexpected since endophyte filaments tend to be most often found in red algal hosts, with wall substances of a similar nature to agar. If the filaments are 'digesting' their way through the agar, would the slender colourless cells be responsible for producing the enzymes ?

Host specificity is unlikely to be significant in this genus, as thought for a long time, as different hosts were found to be infected with the same species and reinfection of the principal 'host' did not take place at all times. However, red algae appeared to be the preferred 'host' plants of endophytic *Audouinella* species. Boney (1980) found that 60 of the host plants of these *Audouinella* endophytes reported in the literature were red algae and especially species from the order Nemaliales. The selection of these particular hosts may take place because the endophytes occupy the same niche (as suggested by Boney 1980), or because these 'host' plants provide these endophytes with a suitable 'living space'. The 'living space' concept was first brought into discussion by Boney (1975; 1977; 1978; 1982). It is evident that these endobiotic *Audouinella* species can regulate their size and presence according to the 'living space' available. Table 6.1 shows that there is a correlation between the space available within different hosts and the sizes of the endophyte cells. Moreover, the number of the filaments per space area was found to increase as the space increases. The relation between these endobiotic *Audouinella* species and the 'host' organism is not known. Certainly these `hosts` provide the endobiotics with protection and the habitats of the endophytes are in regions of energy and nutrient flux. Whether they gain any advantage from the extracellular products of the 'host' is not known.

Audouinella species have been found to invade animals with calcareous shells and live within the calcareous substrata and are classified as endozoic *Audouinella* species. Boney (1975) isolated a red filamentous alga provisionally identified as *Acrochaetium alcyonideae* var. *cylindricum*. Other species were listed by Boney and White (1967) and Woelkerling (1971, 1973).

7. Ecological strategies in *Audouinella* species

During isolation of and experimentation on exo- and endobiotic *Audouinella* species, some observations were made regarding their ecological strategies, e.g. processes which become apparent under certain culture conditions, and may prove of significance in the life of the plant in nature if duplicated. These strategies include hair formation, certain modes of sporulation, regeneration and adaptations to different environmental factors.

7.a. Hair formation in *Audouinella* species

Introduction

Hairs are a colourless elongate cells. Their formation is thought to be caused by environmental factors. Very few studies have been carried out to investigate the formation of hairs in *Audouinella* species. West (1972) found that hair formation in *A. proskaueri* (=*Acrochaetium proskaueri*) was induced by high light intensity. In other red algae, (*Hypnea musciformis*, *Gracilaria* spp. ,*Agardhiella subulata* and *Ceramium rubrum*), Whoriskey & De Boer (in Lobban *et al.* 1985) found that hairs were induced by moderate agitation of the water and low nitrogen concentration. Gibson and Whitton (1986) reported that phosphorus and nitrogen deficiencies caused both the formation and controlled the size of hairs in some fresh water Chaetophorales. They also found that *Stigeoclonium* populations in nature without hairs were able to produce hairs in culture. In this study, during the establishment of *Audouinella* isolates, some individual tufts of *Audouinella virgatula* developed these hairs under culture conditions. On the other hand, no hairs were seen in this species when freshly collected from nature. These observations encouraged the investigation of this phenomenon in some species of *Audouinella*.

Results:

Both *Audouinella secundata* and *A. virgatula* in media without added phosphate were found to die at an early stage, especially at high light intensities and high temperatures. Under low light intensities and low temperatures they were found to live longer but stayed in the early developmental stages (i.e, the first prostrate system cells), and their colour was pale. Their cells were of small size and with contracted chloroplasts (Fig. 7.1a-c). These plants were found to grew normally when they were transferred to normal **ESW** at a higher light intensity and higher temperature (26.6 $\mu E\ m^{-2}\ s^{-1}$ and 15^{0} C).

The cultures without nitrate addition were found to behave differently. *Audouinella virgatula* was found to produce hairs after 10-15 days under a higher light intensity (26.6 $\mu E\ m^{-2}\ s^{-1}$) and high temperature (15^{0} C) conditions (Fig. 7.2), while the cultures under lower light intensity (2.6 $\mu E\ m^{-2}\ s^{-1}$) and high temperature (15^{0} C), lower light intensity and lower temperature (10^{0} C), and a higher light intensity and low temperature were found not to produce hairs under these conditions even when they were left for long periods of up to three months. Cultures of *Audouinella virgatula* without added nitrate nitrogen were found to be pale and to die when they were subjected to higher light intensity (60 $\mu E\ m^{-2}\ s^{-1}$) and low temperature for long periods. *Audouinella secundata* cultures, on the other hand, were found to produce hairs when nitrate was not added to the medium, and when they were subjected to low light and low temperature over a long time (55 days) (Fig. 7.3). Other conditions, such as lack of added nitrate under high light intensity and high temperature,and lack of added phosphate, appear to have no effect on hair formation, but were found to cause colour changes (i.e, from dark red to pale red), and death of these plants occurred after long periods (4-6 weeks). Spore production in both *Audouinella* species was seen to take place under conditions where hairs were formed. Both spore and hair production appeared to be simultaneous on occasions (Fig. 7.4).

Fig. 7.1a-c *Audouinella* species with small cell sizes and contracted chloroplasts after being grown in **ESW** without added phosphate under low temperature and low light intensity. (bar = 25 µm)

Fig. 7.2 Hair formation in *Audouinella virgatula* in medium without added nitrate and under a high light intensity and high temperature. (bar = 50 µm)

Fig. 7.3 Hair formation in *Audouinella secundata* grown in medium without nitrate and low light and temperature conditions. (bar=50µm)

Fig. 7.4 Simultaneous hair and spore formation in *Audouinella virgatula*. (bar = 50 µm).

1a

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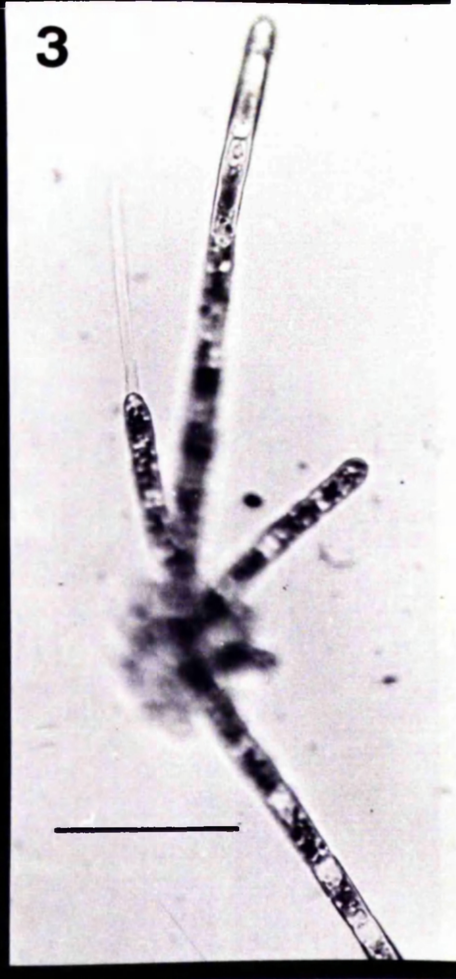
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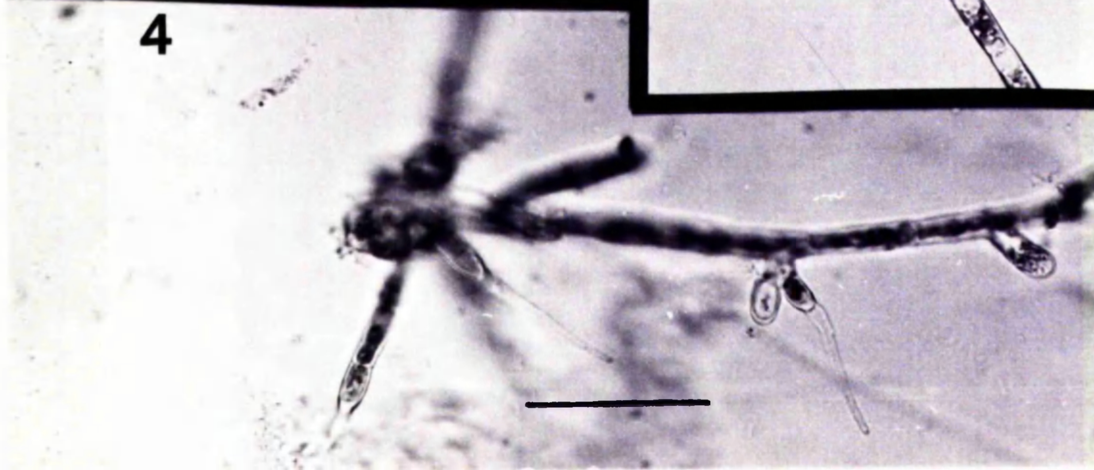
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7.b. Some features of sporulation in *Audouinella* species

Under normal conditions *Audouinella* species produce and release monospores (p. 14) during and over a period of time which considerably differs from one species to another.

Epiphytic species (viz. *Audouinella secundata*, *A. seiriolana* and *A. virgatula*), under unfavourable conditions such as unchanged medium and unchanged light and temperature conditions, were found to produce a very small number of erect filaments (c. 1-4 filaments per tuft). These filaments were usually terminated by a sporangium (Fig. 7.5). The spores remained unreleased until the conditions improved. As soon as the conditions were improved the spores were released, germinated and grew producing normal tufts. Cultures of *A. secundata* were left for more than a year under low light intensity in the same medium with both terminal and lateral sporangia which were found not to release their spores until they were transferred to a fresh medium and the light intensity increased. In *A. secundata* all spores were found to be released and no *in situ* germination had occurred. These released spores grew into normal plants under normal culture conditions.

Released spores under unfavourable conditions (especially high light intensity, low temperatures and unchanged medium), were found to form a single layered prostrate system when they became attached to a substratum with *A. secundata*, or to produce a massive ball of cells when they remain floating in *A. virgatula*.

Spores of *A. virgatula* were found to germinate *in situ* when there was exhaustion of the nutrients in the medium. On other occasions when mature tufts of this alga were broken and transferred to a fresh medium, small branches were found to release viable spores after 21 days, or to have *in situ* germinated spores.

Audouinella newtonii, an epiphytic species first described by Garbary (1978c), was found to behave differently from the above epiphytic *Audouinella* species. Although it has all the features of epiphytic *Audouinella* (discoid prostrate system and erect

filaments) this alga was found to be easily detached by a simple mechanical action. Detachment of this alga induced vegetative growth,in which larger free floating tufts were formed and more monospores released. It lost the pattern of its usual branching and consequently it appeared as an endobiotic species in lacking any differentiation into prostrate and erect systems (Fig. 7.6). Spore production was induced by breaking the tufts, but in this alga tufts continued to grew vegetatively if not broken up (as described later).

Sporulation in *A. infestans* and endophytic *Audouinella* species 2 was induced by disturbance and breaking of the filamentous tufts. It was found that when tufts of these plants were broken or disturbed old monospores were released in 4 days. and more new sporangia were formed. When tufts of these plants were broken two types of reproductive structures were seen in cultures, ordinary monospores produced in sporangia and small rounded cells formed in the prostrate system. These latter cells when they were broken off from the original tuft grew vegetatively. They differed from the germinated monospores in that where the contents of the original spore migrate to the newly formed cell (first cell), in the case of these rounded cells no cell contents were seen to migrate (Fig. 7.7). Undisturbed tufts, on the other hand, take longer to release their spores. On most occasions these plants continue to grew vegetatively and produce less spores. However, single filaments of *A. infestans* when they are broken off the original tuft were found to grew vegetatively and to produce spores simultaneously (Fig. 7.8).

Disturbance and breaking of tufts did not always result in the production of spores. In the other endophytic and endozoic *Audouinella* species studied it had no effect on spore production.

Endozoic *Audouinella* species 1&2 were found to produce two types of spores on two different types of vegetative structure. Large tufts with long filaments and small tufts with short filaments. Cells of these two types of filaments were different in shape and

size (Fig. 7.9). When the large tufts were broken and placed in fresh media, the small pieces of filaments continued to grow vegetatively and produce the same form of tuft as those from which they were separated. On the other hand, if the large tufts were left undisturbed for long periods (usually months) they produced filamentous outgrowths which had the appearance of epiphytes rather than outgrowths from the original filaments (Fig. 7.10). These small filamentous outgrowths were a few cells in length, and when they were fully grown formed a ball-like mass of a delicate cells. They produced monospores inside single spherical sessile monosporangia (Fig. 7.11). This type of spore when released produced a long filament with cells similar to the filaments of the large tufts on germination, and these filaments later produced a large number of normal monosporangia. These monosporangia were produced on short lateral branches (1-2 cell long) (Fig. 7.12). These spores when they released germinated and produced filaments similar to the large tufts.

These delicate ball-like tufts are easily broken into small pieces when they are touched or disturbed. Every part of them will grew vegetatively into long filaments with the same features of the original large tuft and start producing a large number of spores.

This phenomenon explains the production of monospores once a year as described earlier, as these plants have the potential to reproduce in nature by propagation if grown free from the 'host' unless they were kept undisturbed for long period. At the start of this study, these plants were not given the chance to form these two types of tufts because of their being repeatedly broken into small pieces and transferred to fresh medium in subculturing.

7.c Regeneration in *Audouinella* species

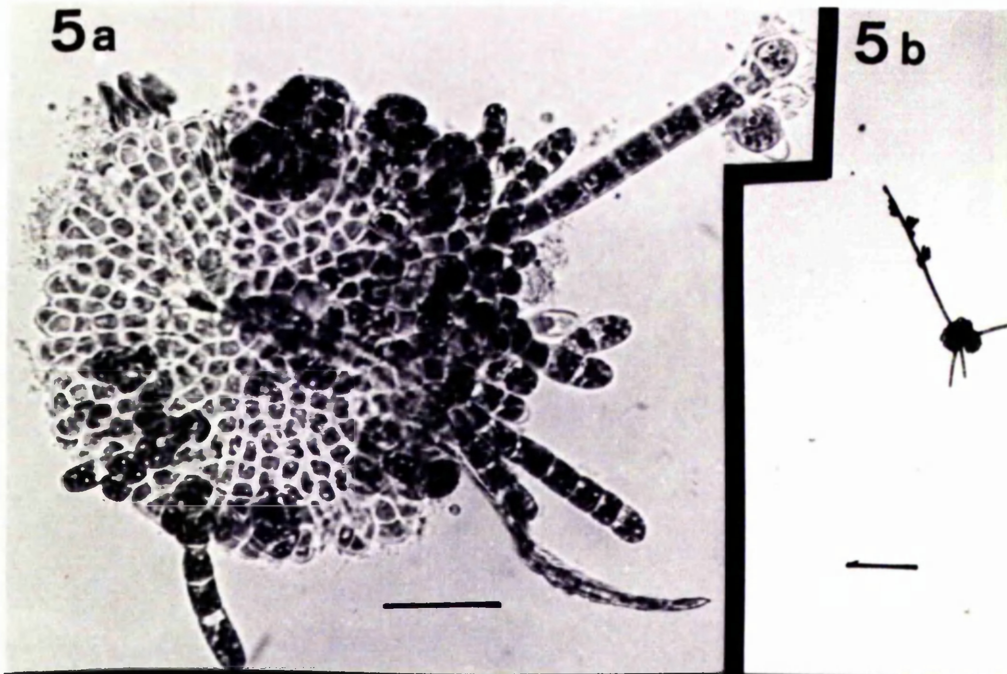
Introduction:

The capacity of seaweed fragments to regenerate new erect and basal systems is a widespread phenomenon. In macroscopic red algae with a multiaxial thallus structure,

Fig. 7.5a,b Tufts of *Audouinella virgatula* with 1-4 erect filaments, note the one which has the terminal sporangium. (bar: a=25μm and b=100μm)

Fig. 7.6 Tuft of *Audouinella newtonii* undifferentiated into erect and prostrate system. (bar = 25 μm)

Fig. 7.7 a-b a. rounded cells b. A germinated rounded cell from *Audouinella infestans* with the contents of the original cell not migrating to the newly formed vegetative cell. (bar = 25 μm).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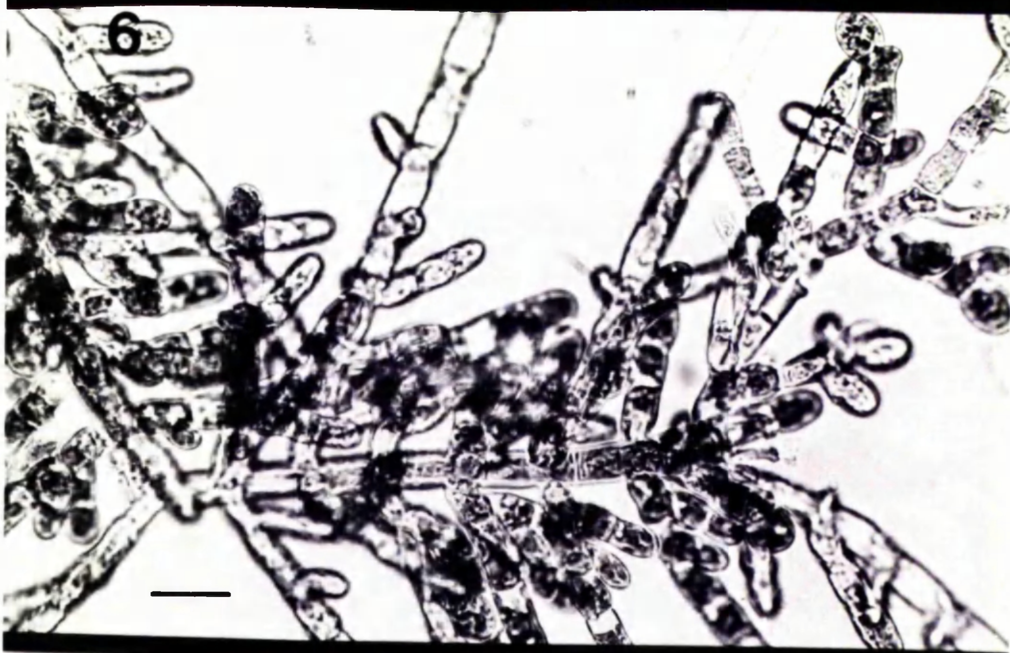
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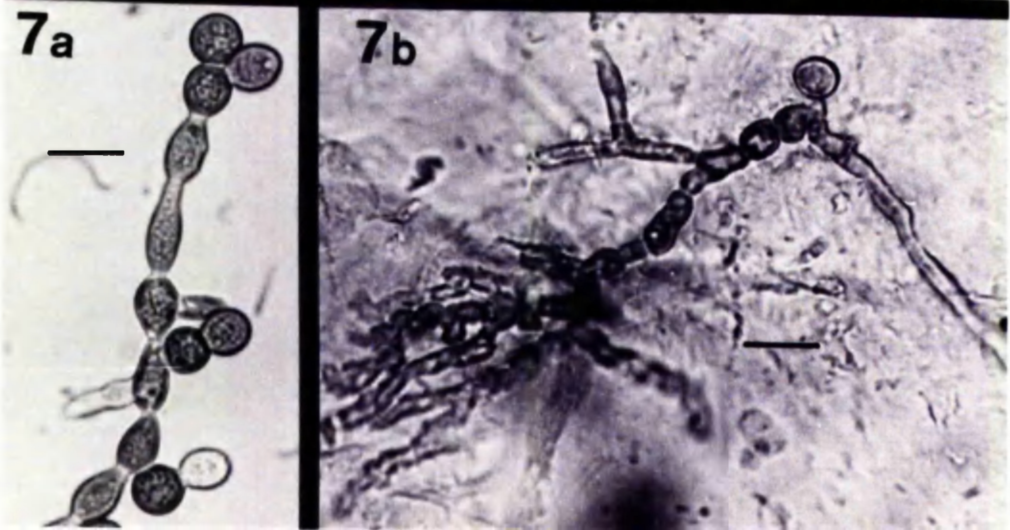
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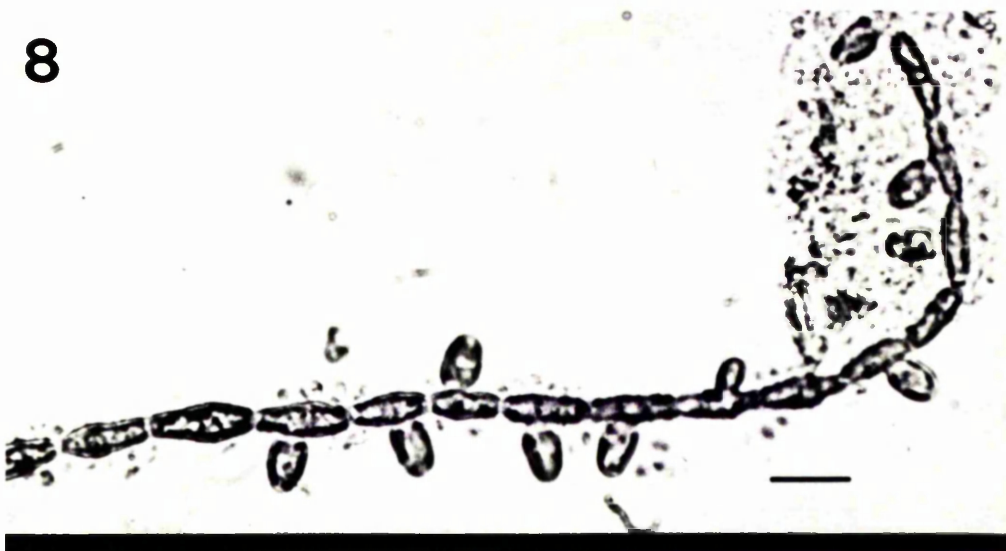
Fig. 7.8 Simultaneous spore production and vegetative growth in *Audouinella infestans*. (bar = 25 µm)

Fig. 7.9 Different morphological filaments formed by endozoic *Audouinella* species 2. (bar = 25 µm)

Fig. 7.10 Formation of secondary filaments in endozoic *Audouinella* species 2. (bar = 25 µm)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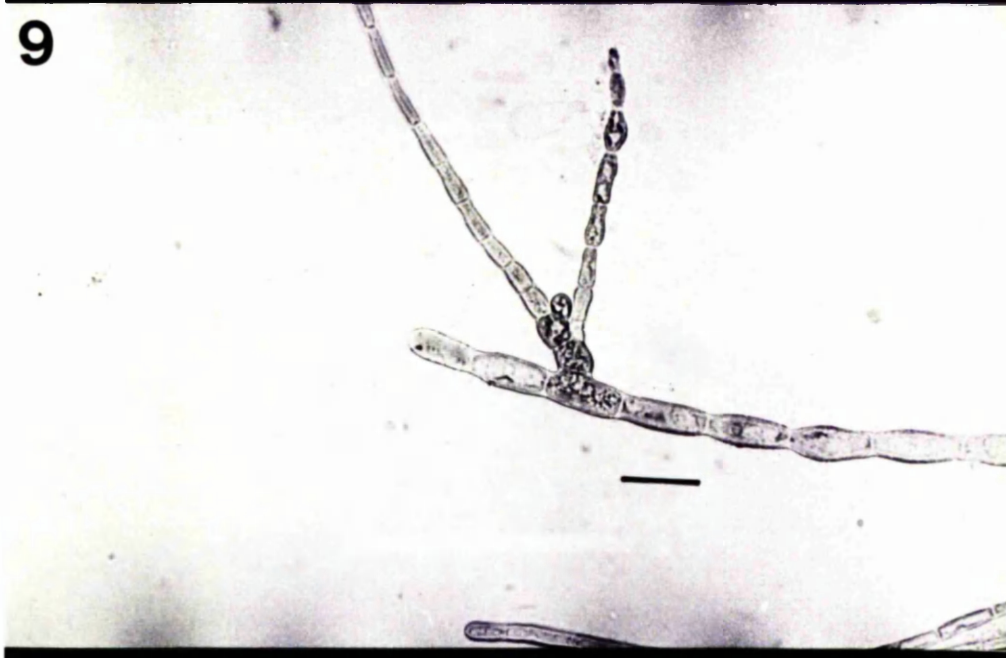
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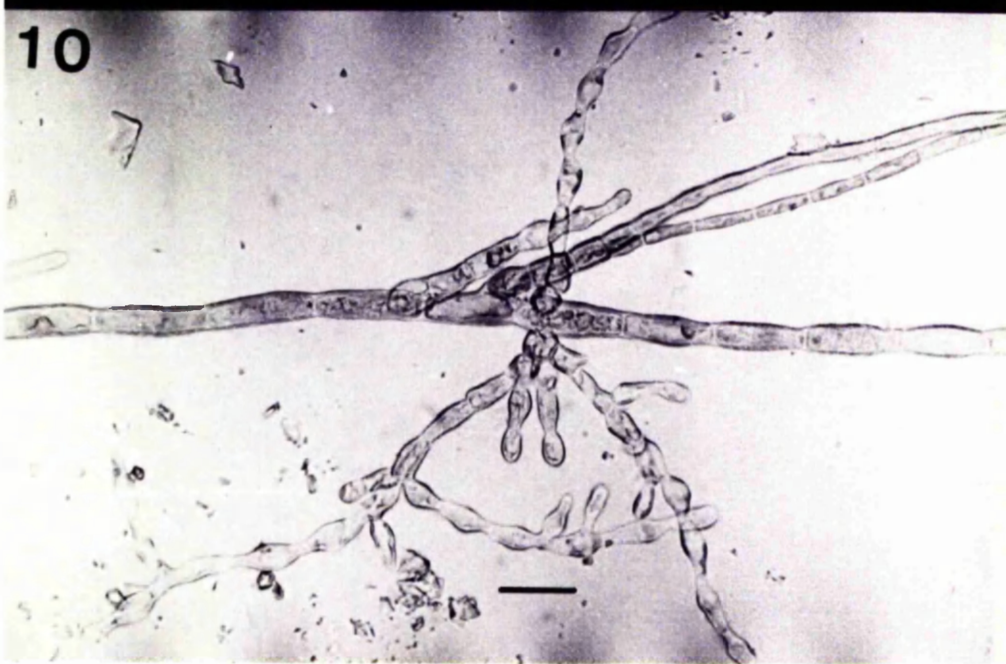
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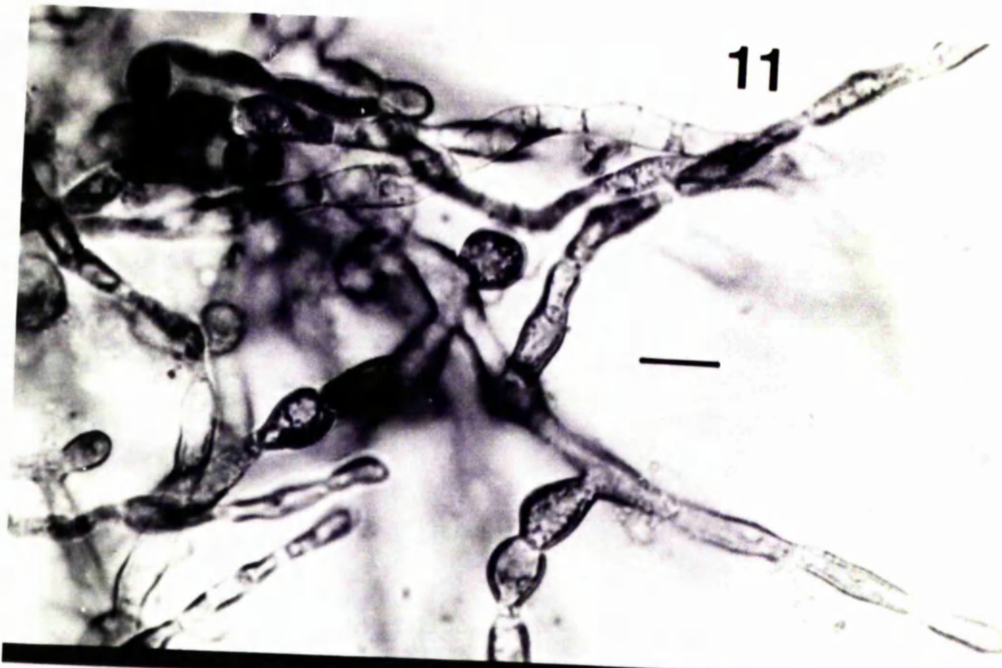
Fig. 7.11 Sessile sporangium produced by ball-like tufts in endozoic *Audouinella* species 2. (bar = 25 μm)

Fig. 7.12 Monosporangia formed by the primary tufts in endozoic *Audouinella* species 2. Note the short branches carrying the sporangia. (bar = 25 μm)

Fig. 7.13 Formation of erect branches after the prostrate system of *Audouinella virgatula* been brought to fresh medium and suitable growth conditions. (bar = 25 μm)



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two kinds of adventive shoots has been distinguished and named on the basis of their origin: those arising from medullary cells at cut surface ("de novo regeneration"); or those formed by renewed growth of lateral filaments which form the thallus cortex "proliferations" (Perrone and Felicini 1972, 1976).

The simplest kind of regeneration involves filamentous red alga having apical growth as demonstrated by *Audouinella purpurea* (=*Rhodochorton purpureum*). In this alga, single cells or fragments of two or three cells usually regenerated first a rhizoid, and later, from the opposite end, began to extend new photosynthetic cells and sometimes photosynthetic cells were produced from both sides as well as rhizoids (Pearlmutter and Vadas 1978).

The role of regeneration in the life of these plants is very important as these plants are subject to many mechanical actions which cause damage and break them into small pieces. On the other hand, some of these plants were found to produce a small number of reproductive structures which may take a long time to be produced.

In this study one method of subculturing the *Audouinella* isolates was to break tufts into small fragments and then provide them with suitable growth conditions. However, these isolates were observed not to have the same morphological features (with or without distinct erect and basal systems). Thus an investigation of their regeneration in culture seemed worthwile.

Results:

Epiphytic *Audouinella* species (*A. secundata, A. seiriolana* and *A. virgatula* were found not to regenerate from fragments of the filaments of their erect system. The fragments of *A. seiriolana* and *A. virgatula* remained alive until they released viable spores, or these spores germinated inside the sporangium, as in case of *A. virgatula*, and then developed into a normal filamentous tuft. Release of spores by broken erect system filaments may continue for long periods (up to three weeks in the case of *A.*

virgatula). *A. secundata* filaments did not release any spores when their tufts were broken. *A. newtonii*, as described earlier, was found to regenerate from broken filaments and spore production was enhanced by filament breakage.

However, it was found that with old exhausted tufts of filaments which had a few pale-coloured living cells, often these cells regained their colour when transferred to fresh media. Those cells which had lost their colour became red as well but whether they regenerated from fragments or were refilled by growth from neighbouring cells was not clear. The viable cells were usually seen at the tips of the erect branches, near the sporangia, and in the prostrate system.

The prostrate system is apparently the only perennial part of epiphytic *Audouinella* species. It was found that regeneration takes place from the prostrate system of all epiphytic species except *A. newtonii* where the prostrate system is very small and its regeneration could not be followed in this study. The prostrate system was found to be the last part to die when these plants were subjected to unfavourable conditions such as prolonged nutrient depletion, and when these exhausted plants, with prostrate system cells only surviving, were brought back to suitable growth conditions they were found to produce erect branches (Fig. 7.13). Moreover, they were found to produce more branches than when they grew continually under suitable conditions without any interruption. In this case it was found that every cell in the prostrate system can behave independently from the rest of the system and produce an erect branch. This phenomenon explains the occasional presence of large tufts in the cultures and the benefits gained by these plants from regeneration.

It was found that in *A. virgatula* the prostrate system may produce large or small numbers of erect branches, depending on the number of live cells remaining. If the dead cells are more numerous than the live cells, the prostrate system produces a very limited number of erect filaments (1-3). A surviving cell in the prostrate system produces a small cell mass before erect filament formation -similar to that formed in spore germination. One of these filaments usually matured earlier than the others and

started to produce sporangia (Fig. 7.14). In the other case where there were larger numbers of living cells in the prostrate system, a larger number of erect branches was produced and so larger tufts were formed and the vegetative growth was found to continue for longer periods before production of sporangia (Fig. 7.15).

Endobiotic *Audouinella* species were found to behave differently from the epiphytic species when they were broken into small fragments. *Audouinella efflorescens*, endophytic *Audouinella* species 1, and endozoic *Audouinella* 1 and 2 were found to produce colourless rhizoid-like cells. Whether these are true rhizoidal cells or damaged cells having lost their pigments is not clear. Later when the tufts were larger, these cells were found to disappear and apparently became filled by ingrowths from the neighbouring cells. These cells were thus different from those rhizoids formed by *Audouinella purpurea* (=*Rhodochorton purpureum*) described by Pearlmutter and Vadas (1978).

Audouinella infestans and endophytic *Audouinella* species 2 grown in culture have two distinct parts (erect system with elongate cells and prostrate system with rounded cells). These parts were found to regenerate differently. The erect system fragments of both species were found to grow vegetatively and to produce spores simultaneously while when fragments of the prostrate system fragments were found to grew vegetatively until they produced erect branches. These tufts produced from the prostrate system fragments, however, were found to behave as other vegetatively grown tufts, which produce small numbers of spores unless they are disturbed again. The erect system fragments of both species were often found to grew unattached until they reached a certain size heavy enough to settle on the substratum, whilst fragments of the prostrate system quickly became attached to the substratum. This phenomenon appears to be due to the ability of the cells of the prostrate system to achieve attachment, as single cells of the prostrate system where found to attach to the substratum and start to grew vegetatively (Fig. 7.7).

Audouinella efflorescens was found to be the most sensitive species of the isolates under study. In addition to its sensitivity to light intensities, it was found that transformation to a fresh medium after being for a long period in the same medium, and breaking of the filamentous tufts, caused the loss of pigments. However, when tufts of this species were transferred to a fresh medium and were not broken they initially lose their colour but after a while they regained their normal appearance.

Endophytic *Audouinella* species 3 was found to be more tolerant of environmental conditions such as desiccation. it was found to continue growing after having been subjected to gradual desiccation (by leaving the culturing petri dish uncovered) for a period of over than 48 hours, and it was found to grew on the edges of culture vessels with just part of the tuft touching the medium surface.

7.d Other observations

During this study, it was found that these *Audouinella* isolates developed some morphological and physiological adaptations to the growth conditions.

Audouinella virgatula was found under unfavourable conditions to produce either long tiny filaments bending away from the original tuft, or produced two different types of spores which developed into two different types of filamentous mass. One formed filamentous masses with the same morphological features as described earlier, and the other developed tiny creeping filaments (Figs. 7.16-17). In this case germinated spores very close to individual attached filaments tufts were found to be a mixture of young plants, normal and those with tiny creeping filaments. These last sporelings were found to develop into normal tufts when they were provided with suitable conditions (such as fresh **ESW** medium). Germinated spores of *Audouinella virgatula* were found to develop into two forms, one form when they were attached and the other when they remained floating. In the first case only the prostrate system developed, and if the conditions were not suitable for further development it remained in such a state until conditions

were improved. In the other case a massive ball of cells developed and remained drifting in the medium. When these two germination types were brought to suitable growing conditions such as fresh medium and suitable temperature and light intensity, they developed into larger tufts than normal (Fig. 7.15).

Endobiotic *Audouinella* species were found to lose their pigments and eventually die when they were subjected to high light intensity. However, they were found to have the ability to recover from these conditions and regain their colour if they were brought to lower light intensity before the death of all the cells.

In an experiment where cultures of endophytic *Audouinella* species 3 were kept under growth chamber and bench light conditions, equal fresh weights samples of these cultures were ground in equal volumes of 90% acetone and showed different absorption maxima when the extracts of these samples were subjected to spectrophotometeric measurements (Fig. 7.18). These results show how this species can adjust its pigment content to suit different light intensities.

Discussion:

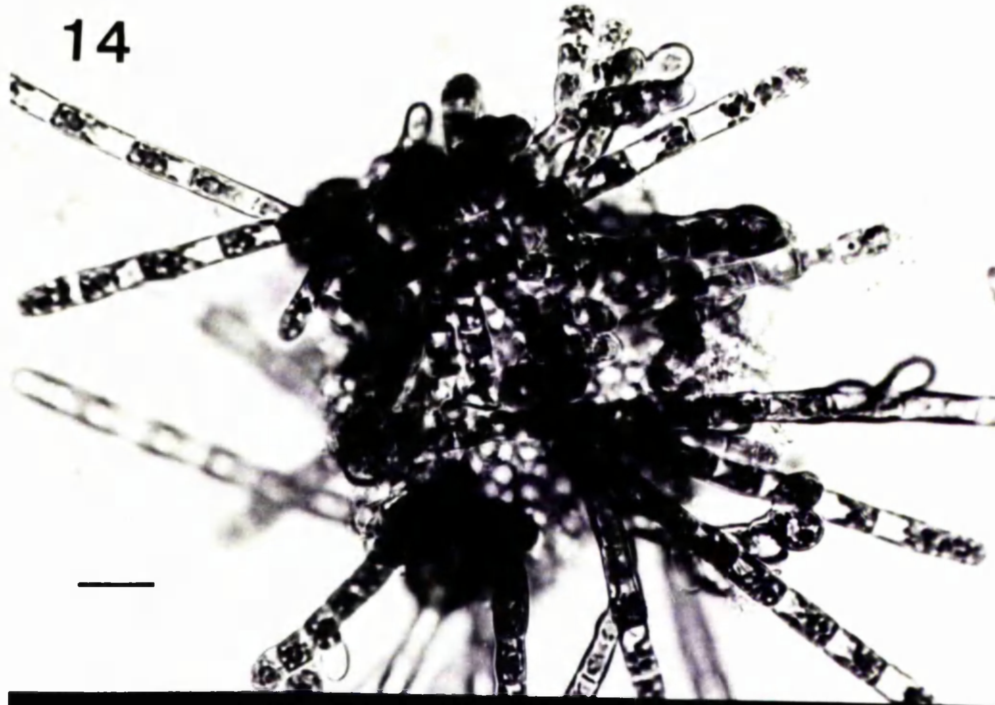
Nutrient role in life of algae is well investigated and is well known to play a limiting factor for the growth and reproduction of algae. However, limiting factors are different from one species to another (Tilman *et al.* 1982).

West (1972) concluded that hair formation is induced by high light intensity and excluded any nutrient effect. Other authors relate the hair formation to lack of nutrients, especially nitrate. Boney (1959) found that *Plumaria elegans* sporelings bearing hairs were produced under nutrient-poor conditions, and the hairs were shed when the sporelings were transferred to nutrient-rich conditions (cited by Norton et al. 1981). In this study, hairs were produced as a result of lack of added nitrate. The role of these hairs is not clear. They are thought to be formed for protection from high light

Fig. 7.14 Maturation of single filament in each new tuft formed by a prostrate system of *Audouinella virgatula*. (bar = 25 µm)

Fig. 7.15 Large tuft produced with an individual prostrate system of *Audouinella virgatula* with large number of viable cells. (bar = 25 µm)

Fig. 7. 16 Tiny long creeping filament produced by *Audouinella virgatula* under unfavourable conditions. (bar = 25 µm)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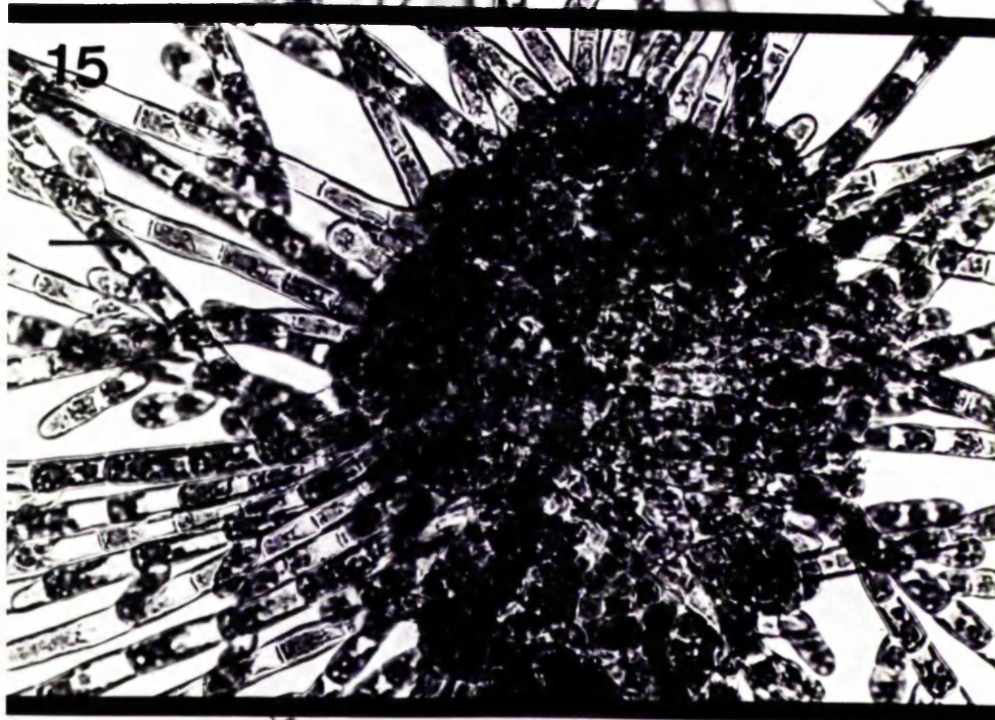
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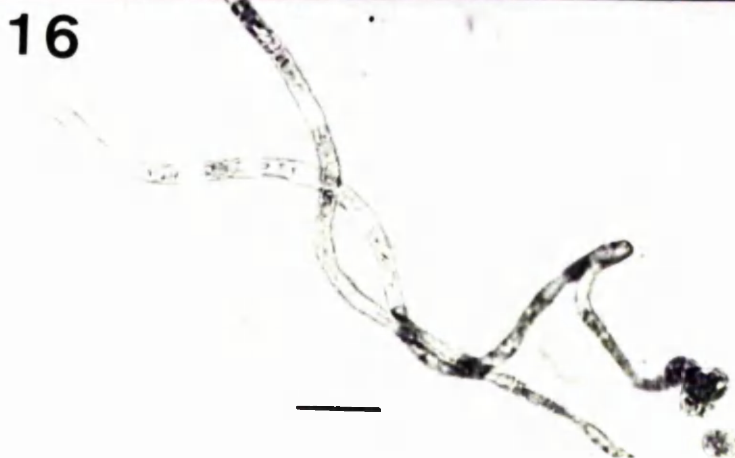
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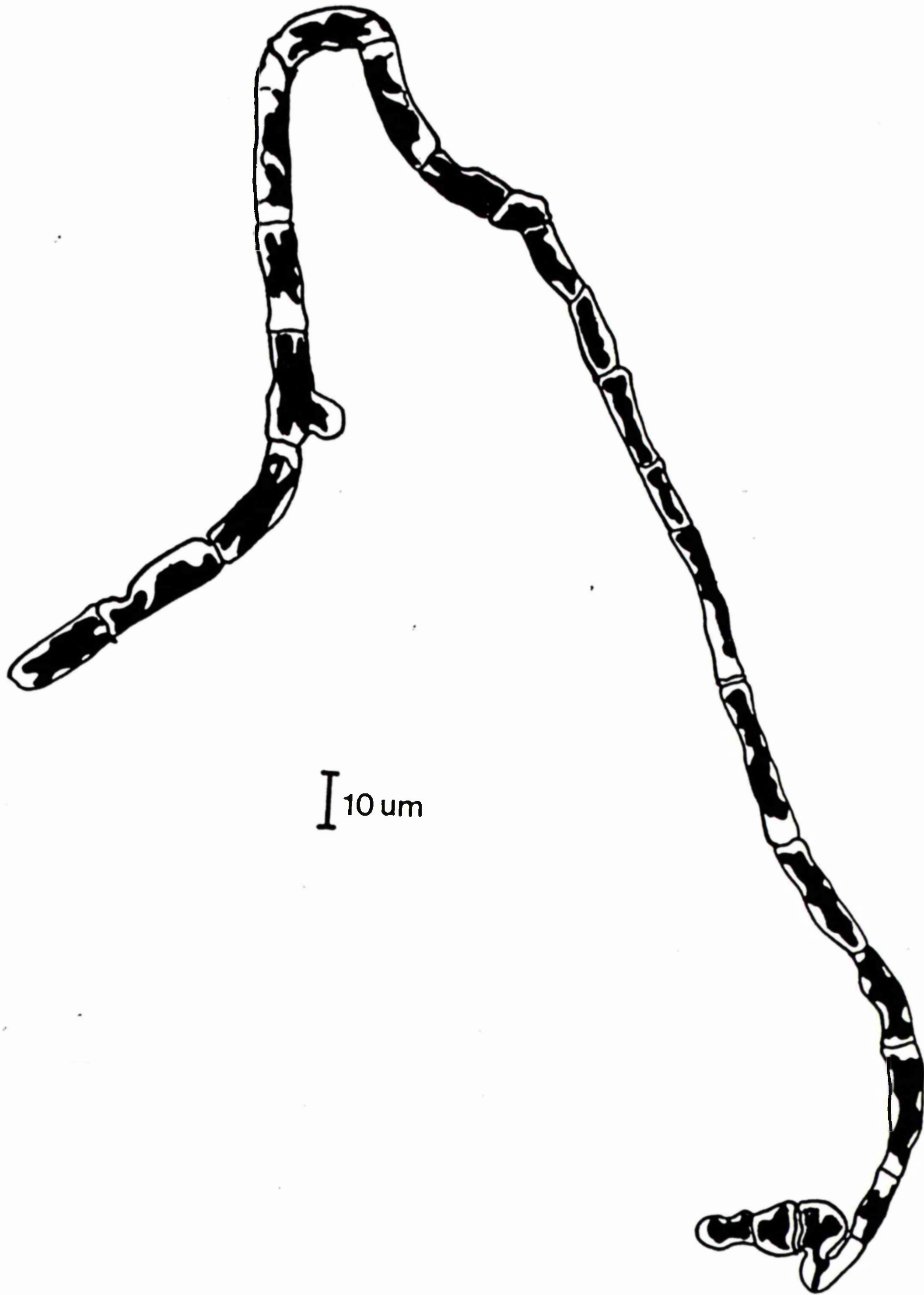


Fig. 7.17 Diagrammatic drawing of a tiny filament produced by *Audouinella virgatula*. The drawing made by use of drawing tube.

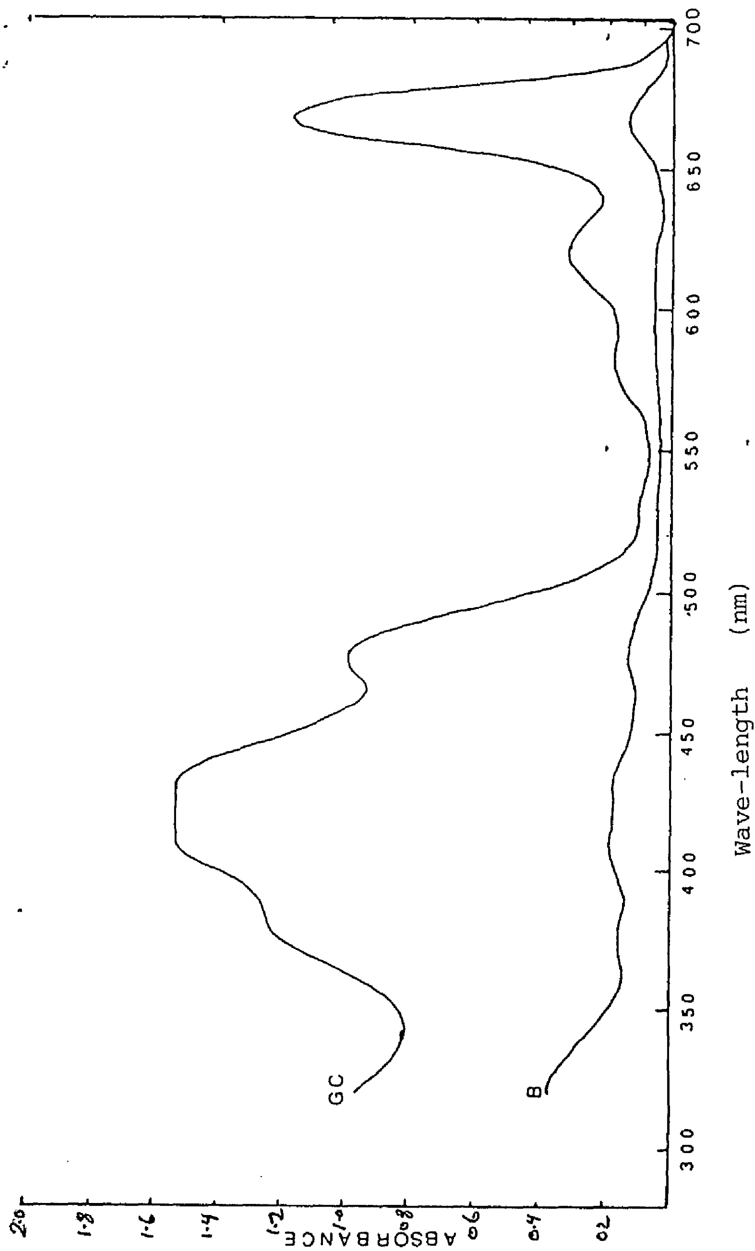


Fig. 7.18 Absorption maxima of pigments from two samples of endophytic *Audouinella* species 3 grown under growth chamber and bench conditions. B. Bench material. G.C. Growth chamber material.

intensity (Berthold 1882, cited by West 1972), or for the increase of the nutrient absorption surface area (Rosenvinge 1911 cited by West 1972; Gibor 1973; and Schonbeck and Norton 1979). As expected phosphorus (supplied as phosphate) is necessary for the growth of both *Audouinella* species.

When the temperature is lowered the kinetic uptake is slowed down. Therefore, the activity and the growth of these audouinelloid algae is slowed down and they live longer under conditions of low light intensity and low temperature, and they have been found to produce sporangia under these conditions. Meanwhile, these epiphytic species were found to produce very few erect branches and were not releasing their monospores. Moreover, many cells tended to die if these plants were kept for a long time in media lacking added phosphate and under low light intensity and low temperature except those cells which were very close to sporangia, cells near the prostrate system, and the cells of the prostrate system. Even these cells were observed to die at the last unless the conditions were improved. The fact that some cells can survive long periods of phosphate P depletion, especially those in or near the basal system, whilst most of the cells of the filaments are dead, indicates a valuable ecological feature -a 'seed' of cells which can enable regeneration of the erect filament to take place if conditions return to normal. -assuming that a suitable proportion of cells of the prostrate system survive.

In cultures where nitrate was not added each alga responded differently. *Audouinella virgatula* was found to produce hairs and monosporangia at early stages under high light intensity and high temperature (Fig. 7.4). The number of monosporangia produced under these conditions was much fewer than those produced under control conditions with normal nitrate enrichments. Spore release, on the other hand, did not take place in the N-free media. On most occasions spores under these conditions were found to show *in situ* germination with formation of 4 cells not more. Erect filament sizes were also found to be much smaller than those formed under control conditions.

Growth form and *in situ* germinations like those observed in this study were occasionally found to take place under unfavourable conditions, such as stagnation of the

medium and subjection to the same conditions for long periods.

Under low light intensity and high temperature (15^0 C) *Audouinella virgatula* plants were found to grew in cultures where nitrate was not added, but the tufts were not healthy, producing unhealthy spores which never grew to form a new generation.

Audouinella secundata was found to die at an early stage in cultures where nitrate was not added under high light intensity and high temperature and to grew under low light intensity and high temperature. The growth forms were much smaller than those obtained under control conditions. Under low light intensity and low temperature, these plants were found to grew very slowly and started to produce hairs and spores after 55 days. Cultures of *Audouinella secundata*, where nitrate was not added, were found to die under low temperatures and high light intensity before producing sporangia.

In this study, hair formation was found to terminate the vegetative growth and trigger the formation of sporangia. Knaggs (1967) stated that under culture conditions lower levels of nutrients are required for sporulation in *Audouinella purpurea* (=*Rhodochorton purpureum*). In this study it was found that fresh medium enhances proliferation of sporangia and increases spore production. This study supports the theory that hairs are formed to increase the absorptive surface to provide the organism with adequate energy for sporulation. West (1972) found that these hairs were formed in cultures where the growth is most rapid and they were most abundant on the actively growing plants. Sporangium formation either awaits improvement in conditions or, if formed the spores germinate within the sporangia (*in situ* germination) to produce the first prostrate cells which are more tolerant of unfavourable conditions.

The spore production can be the first reaction of these *Audouinella* isolates to unfavourable condition, as they were found to produce 1-4 erect filaments of which a single filament with some or single sporangia as it was the case of *Audouinella virgatula* or produce under nutrient depleted conditions a delicate form that can easily be broken in order to produce growth forms that can produce spores.

Regeneration is of limited value to exobiotic species, except *Audouinella newtonii*, as *Audouinella secundata*, *A. seiriolana* and *A. virgatula* were found not to produce any new vegetative structures from detached erect filaments apart from releasing viable spores in the first and second species or *in situ* germination in the third species. It was found that every viable cell in the prostrate system cells of exobiotic species has the ability to form a new individual plant and as these remain attached to the old prostrate system each individual ball-like prostrate system can clearly be seen as each is an independent individual. Endobiotic species were found to benefit from breaking down as every fragment can produce a new individual. Of particular interest was the potential regenerative of the rounded cells of the prostrate systems found in *Audouinella infestans* and endophytic *Audouinella* species 2. Each can develop independently and produce a new individual. This phenomenon appears to be very important in the life of these plants as these cells appeared to have the ability to attach themselves to a substratum much faster than the cells of the erect system. Other endobionts which have no such rounded cells showed their ability to adjust themselves when they grow free from their host. They were found to have the potential to regenerate from any part in the erect system which makes the regeneration a reliable method for production of new individuals. Their spores when they germinated produced tufts with attaching systems (a simple prostrate system which was not seen when they grew within the host). The attachment appeared to be of great importance in the life of these plants as they were found to grew into much larger tufts when they were attached than when they remained floating. The well developed (perennial) prostrate system of exobiotic species is both a specialized structure which serves for attachment and as a perennial source of new erect filaments as required -either seasonally, or after damage- perhaps by browsing molluscs. It retains its assimulating properties, and increases in surface area relative to the 'lost' of erect filaments. Erect filaments are assimilatory and reproductive in function, but seem to have lost their potential for individual regeneration when detached.

Then they tend to produce sporangia. Endobiotic forms grown free from their 'host' organisms may develop prostrate and erect system, in which all parts are capable of regenerative growth, or from unspecialized filaments but with the same properties.

8. The effect of daylength and night breaks on the growth and sporing of *Audouinella* species

Introduction:

Light is the most important factor affecting plants. Seaweeds, as a result of their wide range of habitats, are subjected to different light regimes during their life. Light intensity and duration along with other factors such as tidal movements, dissolved organic and inorganic compounds etc, controi the presence of the seaweeds in zonai vegetation. Red algae are shade plants. They have the ability to regulate their light-harvesting pigments under different light regimes (Dring 1981; Ramus 1983), and accordingly their growth is enhanced or inhibited by light. Kain (1987) found that the growth of *Plocamium cartilaginum* (L.) Dixon was inhibited by high as well as low irradiances. In the present study endophytic *Audouinella* species 3 was found to regulate its phycoerythrin and chlorophyll pigments when it was grown under different light regimes (**p. 183**). In the present investigation the epiphytic *Audouinella* species namely *A. secundata* and *A. virgatula* were found to form colonies on the edges or on lobed areas of *Porphyra umbilicalis*. This phenomenon may suggest a way of escaping the high light intensity. Jacobs *et al.* (1983) reported that the presence and frequency of *A. virgatula* on the edges of leaves of eelgrass *Zostera marina* L. is much greater than its presence on the faces of the leaves.

Morphogenesis in red algae is known to be caused by light quantity and quality (Murray and Dixon 1973; Mathieson and Burns 1975; Norton 1975; Calvert 1976).

Morphological changes of *A. secundata* were seen in this study during collection of these plants at different times of the year. It was found that tuft sizes were different i.e, tufts were getting smaller toward mid-summer. Other culture studies have shown morphological changes in *Audouinella* species. White and Boney (1969) found significant differences in the cell size of *Audouinella* species grown into media with

different salinities, and sporangia formation were not obtained under a short day (8 hours illumination) low temperature (5^0 C)regime. West (1972) found that 15^0 C and light intensity of 1500 lux are a favourable conditions for tetraspore production, branches development and hair formation in *Audouinella proskaueri.* Stegenga and Borsje (1977) obtained a maximal increase in the cell number of the main axis in *A. polyblastum* and *A. hallandicum* (=*Acrochaetium polybastum* and *Acrochaetium hallandicum* respectively). Garbary (1979b) reported a decrease in cell length and branching frequency in *Audouinella* species as a result of the effect of high temperature (23^0 C).

Life histories, on the other hand, were found to be affected by light regimes (Stegenga 1973, 1978; Abdel-Rahman 1982a, 1982b; Lee and Kurogi 1983). The effect or the control of some aspects of life cycle by the timing of light and darkness is what is known as photoperiodism.

Photoperiodism was first named by Graner and Allard in 1920 (Dring 1984). The phenomenon is well investigated in flowering plants. Algae are yet to be investigated in detail, although they can be easily used and controlled because of their small size and short life cycle. Photoperiodism, as defined by Terborgh and Thimann (1964) is a light induced phenomenon in which there is a continuation of the effect following transference to non inducing conditions after a set time, and there is sensitivity to short breaks in either the light or dark periods.

Photoperiodism was detected for the first time in algae by Kurogi (1959); Dring (1967); and Rentschler (1967). They found that the *Conchocelis*-phase of *Porphyra tenera* responds to day length in much the same way as flowering plants in terms of critical day length required to induce reproduction. Other red algal responses were listed from earlier studies by Guiry (1984), in which 13 species of different red algae were shown to have different responses in terms of growth and reproduction. Some species showed quantitative responses and others showed an absolute response. *Audouinella* species were found to produce different reproductive structures as a result

of day length effects (Abdel-Rahman 1982a, 1982b; Dring and West 1983; Lee and Kurogi 1983; and West 1968).

However, whether light duration or intensity is the main factor which algae respond to is still not clear as different algae respond to light intensity and duration in different ways. e.g. Stegenga (1978) stated that light intensity has little effect on sporangia formation in *Audouinella purpurea* (=*Rhodochorton purpureum*). Borsje (1973), on the other hand, found that tetraspore production is a genuine short day effect in *Audouinella virgatula* (=*Acrochaetium virgatulum*), but tetrasporangia were completely absent at 4^0 C. Moreover, one of the Dutch clones he studied produced no tetraspores under any conditions he tested. Short light breaks in the dark periods, on the other hand, were found to induce formation of tetraspores' in *Audouinella asparagopsis* (=*Acrochaetium asparagopsis*) (Abdel-Rahman 1982b).

From the above results reported in the literature, it appears that every algal species has its environmental requirements for a particular response, and most results seems to have been drawn from one generation (i.e, they did not carry the experiment again starting from the germinated reproductive structures produced under the previous experimental conditions) in each experiment. This is far from what is happening in nature.

In this study both day length and night breaks effects have been examined with some *Audouinella* species for as many generations as possible, i.e, the experiment was carried repeatedly from a germinated spore until the plant formed produced sporangia and viable spores.

Results

Audouinella secundata was found to respond to a short-day regime more positively than to a long-day one in terms of survival, growth and spore production. Under long-day regimes *A. secundata* first generation tufts were found to grew very fast, and to

produce and release monospores after 8 days (Fig. 8.1). The second generation plants were found to delay monospore production and release for long periods (undetected). *A. secundata* under a short-day regime, on the other hand, was found to produce and release monospores after 15 days but the release was delayed in the second generation for a further 2 days. This relatively short period was found to be convenient to carry the experiment for further generations under short-day regimes, while long-day experiments were abandoned.

Under short-day regimes *A. secundata* generations were found to differ in terms of the number of erect filaments per attaching system, the number of cells per erect filament, the cell dimensions and time required to produce and release monospores. The number of erect filaments formed in the first generation under an irradiance of 16 μE $m^{-2}s^{-1}$ and a temperature of 15^0 C were 4-9 (average 7) in the first generation, 13-16 (average 15) in the second generation, and were uncountable in the third generation being very short and compacted. The number of cells per erect filament decreased from the first through the second to the third generation, from 14, 9, to 5 cells per filament respectively. Cell dimensions decreased slightly from 12 to 10 μm in length and from 11 to 10 μm in diameter. The time required for production and release of monospores was increased from generation to another 15, 17, and >28 days for the first, second and third generation respectively. Reduction of irradiance from 16 to 10 μE $m^{-2}s^{-1}$ showed no effect neither on monospore production and release or on other morphological features described above.

As the results showed reductions of filament length and increase in the number of erect filaments, the spore production through generations was found to be well balanced among individual tufts of the same generation, as the numbers of juvenile sporelings attached to cover slips very close to the original tufts were found to be roughly the same. This method of spore counting is not all the time ideal as spores were found to travel for some distance from the tufts after release. However, counting of spores before their

release proved difficult as this technique required putting individual tufts under high magnification which causes damage to the tufts and the released spores were needed for the next generation experiment.

Audouinella virgatula was found to respond to both long and short-day regimes in experiments through three generations. The response was different through generations of the same day length regime. In experiments with quantum irradiance of 16 $\mu Em^{-2}s^{-1}$ * (Fig. 8.2) it was found that under long-day conditions the spores from the first generation of the long-day produced plants which reached the spore production stage but a similar first generation spore crop failed to grew under short-day regime at the same irradiance. Whilst spores from the long-day second generation grew and reached maturity, i.e, produced monosporangia and viable spores' under the short-day regime whilst spores of the same crop failed to grew beyond a few cells under continued long-day and eventually died.

Generations from the short-day, with the same irradiance as above, were found to continue growing normally under the same light regime. But germinated spores from these populations were found not to grew beyond a few cells under long-day regimes at the same irradiances.

Spore production and release in general was delayed by short-day regimes and through the succeeding generations of both long and short-days. It was found that it took 8 days under a long-day and 12 days under a short-day regime in the first generation, and was delayed for a further 4 and 5 days in the second generation under long and short-day regimes respectively.

Morphological changes of *Audouinella virgatula* such as tuft size, number of erect filaments and number of cells per erect filament, and cell size were also found to take place under long and short-day regimes. Tuft sizes of the first generation population decreased from long to short-day conditions as a result of decrease in the number of erect filaments per attached disc (12-9), but the number of cells per erect filament

showed no significant change (12-23 to 12-20 cells). Cell size, however, increased slightly under short-day regime (14-17 μm in length and 9-16.5 μm in diameter) while their size under the long-day regime was 10-14 μm in length 10-13 μm in diameter.

Reduction of irradiance to 10.0 μE $m^{-2}s^{-1}$ caused the delay of production and release of spores in *Audouinella virgatula* (Fig. 8.3). Spores were found to be produced after 14 days under the long-day regime and 21 days under the short-day regime. Since no spores were released under long-day regime in the second generation experiments were carried for short-day populations only.

The second generation, from the spore crop produced under short-day conditions with irradiance of 10 μE $m^{-2}s^{-1}$, was found to grew under both regimes of the same irradiance but tufts formed under the long-day regime were found to be larger than these formed under short-day regime. These tufts had more erect filaments (7-8) with short lateral branches, whilst under short-day regime not more than 3 erect filaments were formed and lateral branches were not as frequent as in the tufts formed under long-day regime. However, no spores were produced under long-day regimes in the second generation after 21 days, and the experiment was not carried out for any longer time to avoid any other factors intervening such as nutrient depletion in culture.

On the other hand, the second generation population under short-day regime with the same irradiance as above was found to form sporangia after 21 days but no spores were released in this period. Therefore, the experiments were not carried out for the third generation under these reduced light irradiances.

In night-break experiments no reproductive structures other than monospores were produced by any *Audouinella* species investigated in this study.

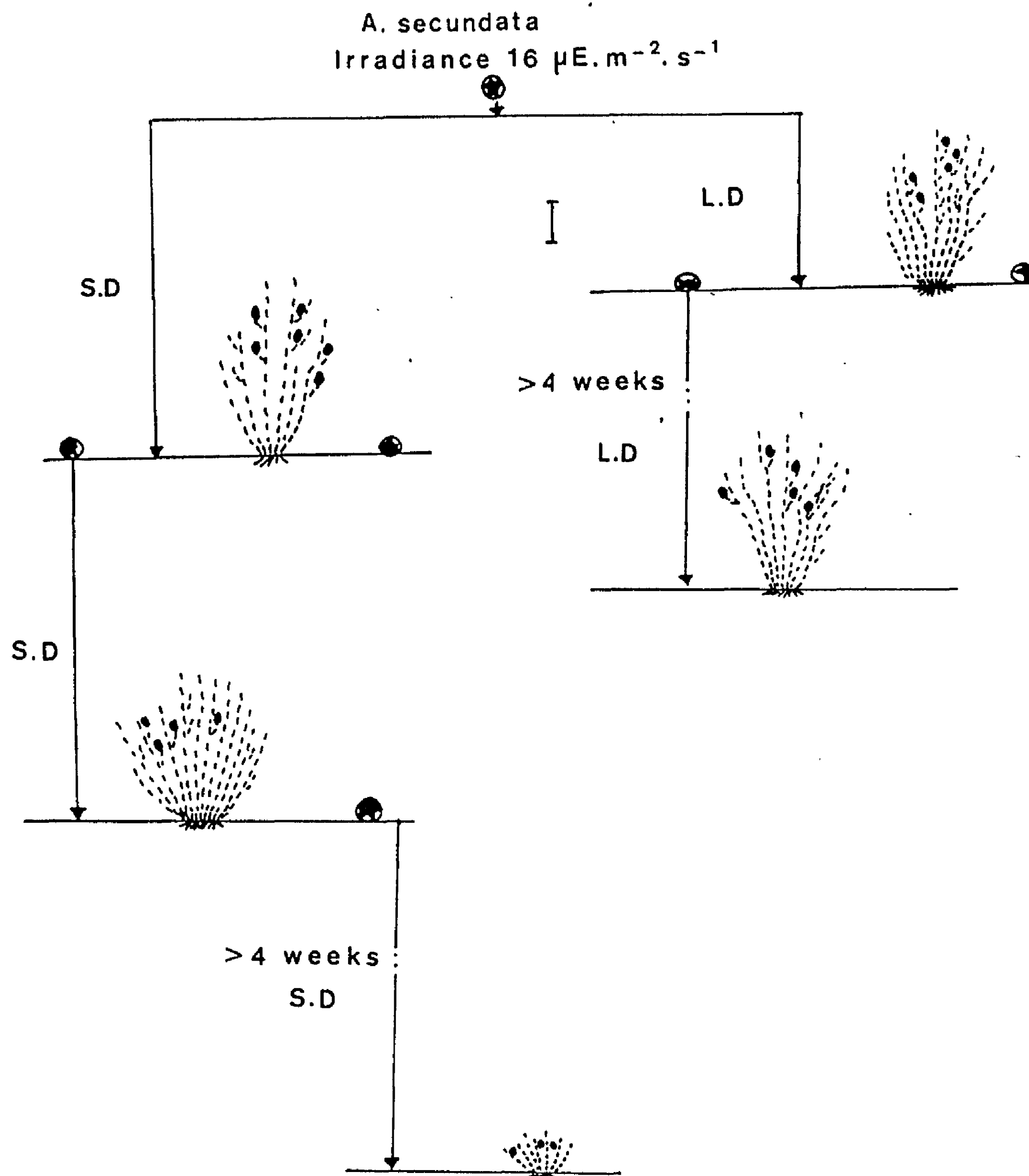


Fig. 8.1 Diagrammatic presentation of the effect of day length on the growth and spore production of *Audouinella secundata* under temperature of 15^0 C. (L.S. = Long day, S.D. = Short day, bar = 2 days. Irradiance = 16 $\mu Em^{-2} s^{-1}$).

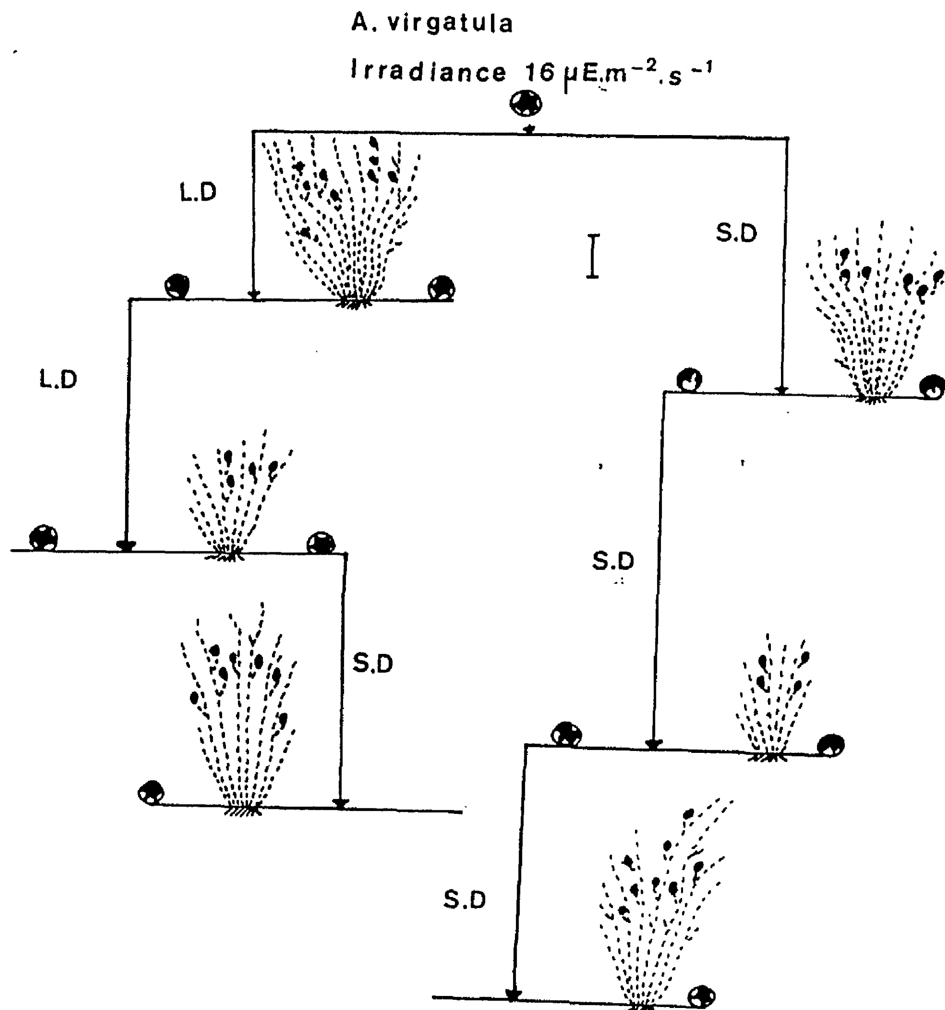


Fig. 8.2 Diagrammatic presentation of the effect of day length on the growth and spore production of *Audouinella virgatula* under temperature of 15^0 C. (L.D. = Long day, S.D. = Short day, bar = 2 days. Irradiance = 16 $\mu Em^{-2}\ s^{-1}$).

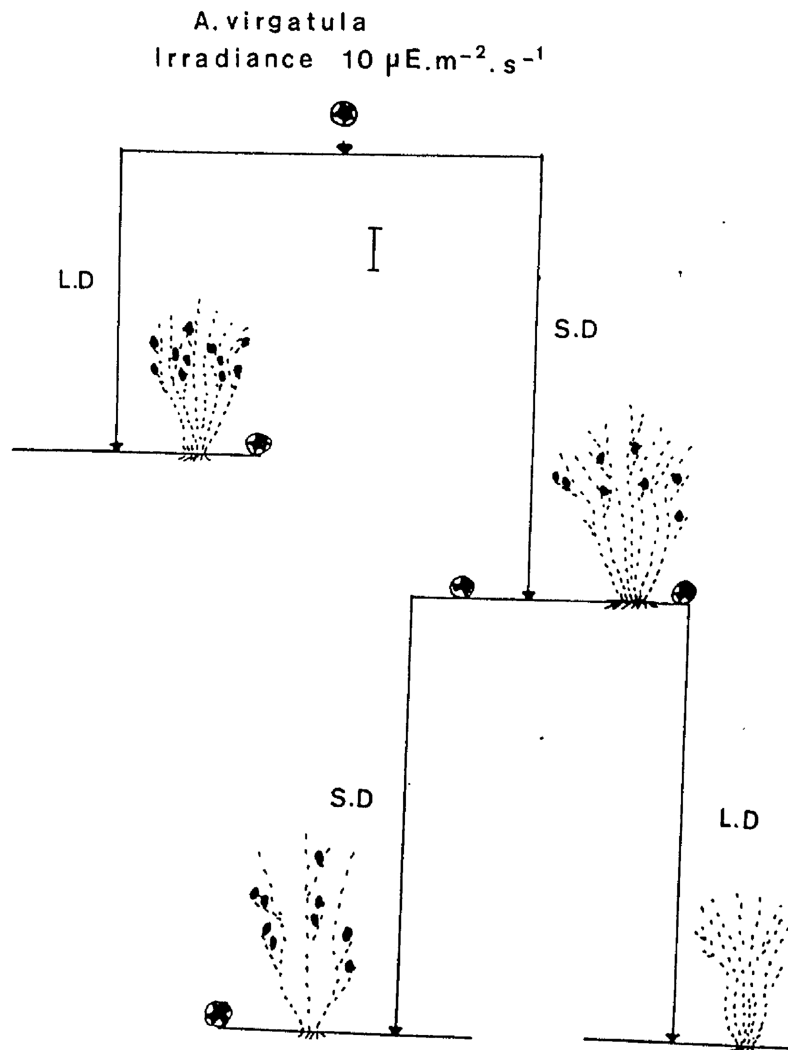


Fig. 8.3 Diagrammatic presentation of the effect of day length on the growth and spore production of *Audouinella virgatula* under temperature of 15^0 C. (L.D. = Long day, S.D. = Short day, bar = 2 days. Irradiance = 10 $\mu Em^{-2} s^{-1}$).

A. virgatula was found to grew very slowly and no spores were produced after 4-5 weeks under short night-breaks, 2 hours of light of irradiance of 60 $\mu E\ m^{-2}\ s^{-1}$ given after 14 hours of darkness, and to die in long periods of darkness, i.e, 8:40 light and dark cycle.

A. asparagopsis and *A. infestans* were found to produce and release spores in cultures under 8:40 light and dark cycle with two hour light-breaks after 14 hours of dark period, after 3-4 weeks under conditions of temperature of 15^0 C and quanta irradiances of 24 and 60 $\mu E\ m^{-2}s^{-1}$ and after 5-6 weeks under quanta irradiance of 14 $\mu E\ m^{-2}s^{-1}$.

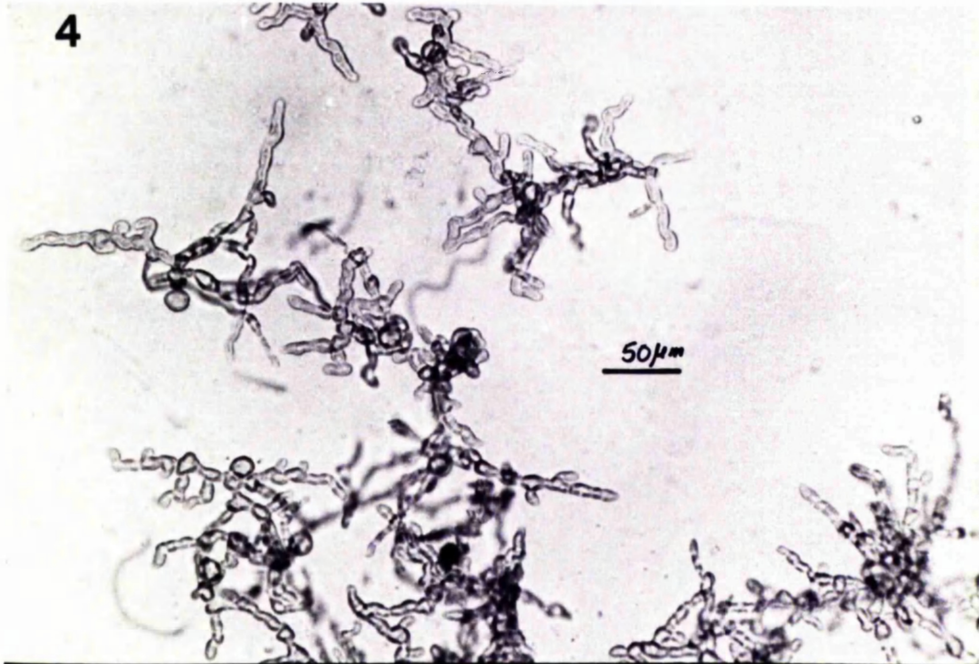
However, under long dark periods (8:40 Light and Dark cycle) without any night breaks, cultures were found to produce less spores. It was not possible to make counts of spores but more sporelings were seen in cultures of both species under short light-breaks than other cultures with an uninterrupted long dark periods (40 hours) of darkness (Figs. 8.4-5).

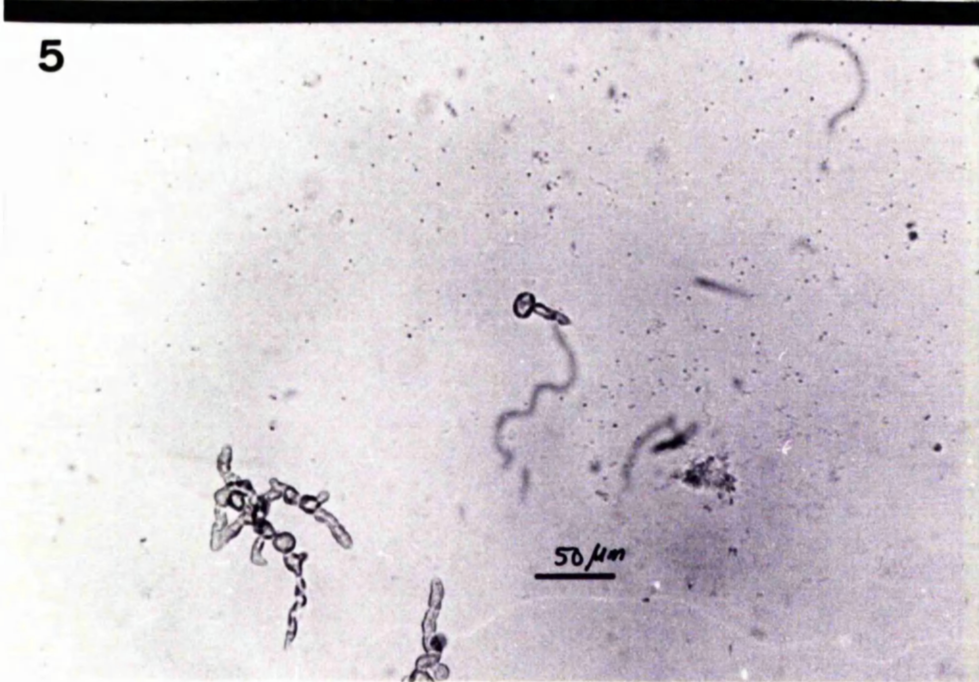
Discussion

Measurements of the growth rates of these exobiotic *Audouinella* species under the above experimental conditions was assessed by counting the number of erect filaments and number of cells per erect filament. These results show that the amount of irradiance (light intensity and duration) is important in the life of these plants. In the first part of these experiments the day length was more significant in the survival of successive generations as the second generation of *Audouinella secundata* under long day regime did not reach the stage of producing and releasing spores. On the other hand, spore release and production in different generations of both exobiotic species was delayed under short day regimes, but under the long day regime became favourable when irradiance was lowered, as with the second generation of *Audouinella virgatula* from a spore crop produced under short day regime of irradiance of 10 $\mu E\ m^{-2}\ s^{-1}$ was found to grew vegetatively under long day regime of the same low irradiance. This compares with the

Fig. 8.4 Spore production in *Audouinella asparagopsis* under long dark periods, with two hour night break under temperature of 15^0 C (note the number of germlings and their size).

Fig. 8.5 Spore production in *Audouinella asparagopsis* under long dark periods without any night breaks under temperature of 15^0 C(note the number of germlings and their size).


4
50μm


5
50μm

second generation of the same species from the spore crop produced under the short day regime at an irradiance of 16 $\mu E\ m^{-2}\ s^{-1}$, which failed to grew under long day regime of irradiance of 16 $\mu E\ m^{-2}\ s^{-1}$. Further evidence was that *Audouinella virgatula* grew vegetatively for 4-5 weeks under 8:40 Light and Dark cycle with irradiance of 60 $\mu E\ m^{-2}\ s^{-1}$ and being given 2 hours night breaks of the same irradiance after 14 hours of darkness. Thus the day length effect on *Audouinella virgatula* appeared to be photosynthetic rather than photoperiodic.

These responses are more likely to happen in nature as the day length increases or decreases continuously during the year, and more than one generation is unlikely to be produced and to be subjected to similar conditions or to sudden changes in conditions as these been used in the experimental conditions are less likely in nature. Temperature is known to play an important factor in spore production in the sea weeds. Whether it has any effect here on these plants could not be tested. This was simply because the facilities could not provide the variable temperature conditions at the time this work was carried out.

Tetrasporogenesis did not take place in any of these isolates under study and appears to be of genetical and geographical significance rather than solely due to other environmental factors such as light and nutrients. Thus the critical day length must be found if any response to the day length of these plants to be achieved. Dring and West (1983) found that critical day length correlates with the geographical origin of the isolate. In their study, which involved six geographical isolates of *Audouinella purpurea* (Lightf.) Woelkerling (=*Rhodochorton purpureum* (Lightf.) Rosenvinge, they found that these isolates produced tetrasporangia only in short days at 10^0 C, and the critical daylength increased with latitude of origin from 9.5 hours for an isolate from California to 14.5 hours for one from Antarctica. In this study nutrient depletion seems to have no effect as Knaggs (1967) suggested, as the plants were left on occasions in the same medium for periods up to 4 weeks under continuous light and at temperature

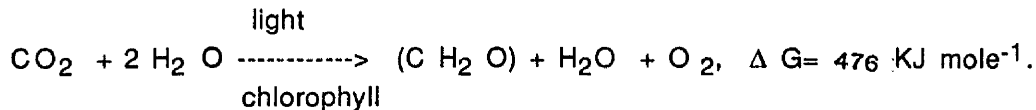
of 10^0 C, after which they were found to become bleached as a consequence of nutrient depletion.

9. Some observations on photosynthetic rates in *Audouinella* species

Introduction:

All higher plants and algae share the phenomenon of photosynthesis, in which light energy is utilized in the formation of carbohydrate molecules and stored in their cells.

The general equation of photosynthesis can be written as:



$$CO_2 + 2\,H_2O \xrightarrow[\text{chlorophyll}]{\text{light}} (CH_2O) + H_2O + O_2, \quad \Delta G = 476 \text{ KJ mole}^{-1}.$$

(CH_2O) represents stored carbohydrate. The reaction is powered by light, requiring 476 K $cal.mol^{-1}$ per hexose molecule produced, when CO_2 is reduced to CH_2O and water is oxidized to O_2 by dehydrogenation. The oxygen liberated in photosynthesis comes from the water rather than from the CO_2.

The light is absorbed by various classes of pigments of which chlorophyll a, carotenoids and phycoerythrins are the most important in the genus *Audouinella.* Algae in general are well known to be colour-coded i.e, their chlorophylls are masked by other pigments from which the diagnostic colour of each algal class is derived, such as brown, green and red. It is also known that these pigments can be adjusted by these plants to suite the environmental conditions, especially light intensity.

Most, but not all, of the chloroplast pigments are involved in the light reactions of photosynthesis, specifically the capture of photosynthetically active radiation (**PAR**). Seaweeds are obligate photolithotrophs wholly dependent upon ambient light energy for synthetic metabolism. In nature seaweeds can be found in different photic zones. Thus light reaching these plants is variable in quantity, quality, and duration. The ability of these plants to capture **PAR** is the means to growth, to reproduction, to physical adaptation and to biological interaction. The process of photosynthesis is more complicated than it appears from the outline equation above. The release of O_2 cannot be directly related to CO_2 fixed in the process. Other reactions are taking

place in the meantime such as respiration and photorespiration.

Algal material has been used in pioneering photosynthetic studies, *Spirogyra* sp. (Chlorophyta) was used by Engelmann (1883) to demonstrate that the chloroplast is the seat of photosynthesis and oxygen release in the cell by use of aerotatic bacteria (Hipkins 1985). Haxo and Blinks (1950) subjected thin bladed algal thalli (green, brown and red) to wavelength bands of similar energy values and at the same time measured photosynthetic output of oxygen by a polarographic method. They demonstrated in all cases close correlations between the action spectra and the absorption spectra of the thalli -indicating that all pigments were playing a role in the photosynthetic process. Emerson and Arnold (1932a and 1932b) used algal materials to establish the concept of photosynthetic units (**PSU**) '-the number of chlorophyll molecules essential for evolution of 1 mol. of oxygen. Ramus (1981) assumed, in the absence of conclusive data, that algal photosynthetic units (**PSU**) are adaptive to light. Detection of these adaptabilities can only be done by measuring the metabolic rates of different species under different light regimes.

Red algae are of special interest to phycologists because of their extreme vertical distribution in marine habitats. This phenomenon led to more investigations of different localities with different geographical characteristics. These investigations revealed that this was not true in all areas investigated, i.e, seaweeds other than the reds were present in some localities deeper than they were expected to be found, and their ratio to red algae was found to be much higher than previously thought (Larkum *et al.* 1976; Doty *et al.* 1974).

Red algae are known to inhabit shaded habitats more than other seaweeds. This is apparently due to the role of accessory pigments as wavelength antennae present in their photosynthetic apparatus, and their ability to adjust the quantities of these pigments under different light intensities. In a recent review Kirk (1986) related their success as being in part due to the thin blade-like structures of many sublittoral

algal plants. However, Kirk (1986) stated that it is not only the light which controls the distribution of algae but other factors such as temperature and the ability of the alga to adapt to a particular substrata must play a significant role. He added that chromatic adaptation is of less significance in distribution of algae in marine ecosystems as collective evidences for and against this concept are equally in balance. The evidence in favour of chromatic adaptation included the distribution of algal plants in clear and turbid waters, in which the classes represented were found not to be the same. The green algae were found to grow deep down in the clear waters while in coloured and turbid waters the green algae were much less abundant deep down as much light attenuation takes place and only blue-green or green light is available (see Kirk 1986). Levring's studies (1966, 1968) showed that the photosynthetic rates of red algae in turbid and clear waters indicated little change in adaptation with depth. The evidence put forward against chromatic adaptation comes from the depth distributions of algal plants in clear coastal waters such as in Australia, which show similar pattern of distribution in the more turbid northern European shores. The thick deeply coloured thalli can achieve maximum absorption irrespective of the type of pigment present in the thallus. Thus Kirk (1986) suggested that lower respiration and lower grazing susceptibility are likely to be significant factors in algal distribution. The irradiances which were found to saturate photosynthesis in various deep-water red algae (about 100 $\mu E\ m^{-2}\ s^{-1}$) were regarded as very low by Luning (1981). Red algae were found to be more sensitive to light than other algae and photosynthesis in red algae can be activated much faster than in other algae (Ramus 1981). On the other hand the ratio of different pigments which red algae can adjust such as phycoerythrobilin (**PEB**) and phycourobilin (**PUB**) appeared to be the most significant factor in the success of red algae in the marine habitats (Yu *et al.,* 1981 and Kirk, 1986). Phycoerythrin production in blue greens is known to be completely suppressed or reduced by red light and maximum rates of photosynthesis are obtained under green

light (Fay 1983).

Measurement of O_2 evolved in the photosynthetic process is widely used as a method of productivity measurement in the field and the laboratory, and is achieved by different methods, of which suspended light and dark bottles with the quantities of oxygen evolved measured by the Winkler method are used in the field and in the laboratory, and oxygen electrodes are mainly used in experimental microhabltats. These two methods both involve the enclosure of the alga or portion of it into a cell or vessel for a certain periods. These periods may some times be prolonged, and then errors and multifactorial effects are increased.

The oxygen electrode was found to be the most suitable means of measuring the photosynthetic activities of the *Audouinella* spp. (**see p. 37**) Their small biomasses available prevented accurate measurements being made with light and dark bottles and subsequent oxygen determination by the Winkler method. In nature the endobiotic species especially grow in densely shaded habitats within the 'host' organisms. Measurements of photosynthetic rates with *Audouinella* species, especially these isolated from endobiotic habitats, appear not to have been carried out before. This was thought to be an aspect of their biology well worth investigating. The problem of obtaining an adequate biomass in the right physiological condition for experiments sometimes limited both the species available for examination and replication of experiments.

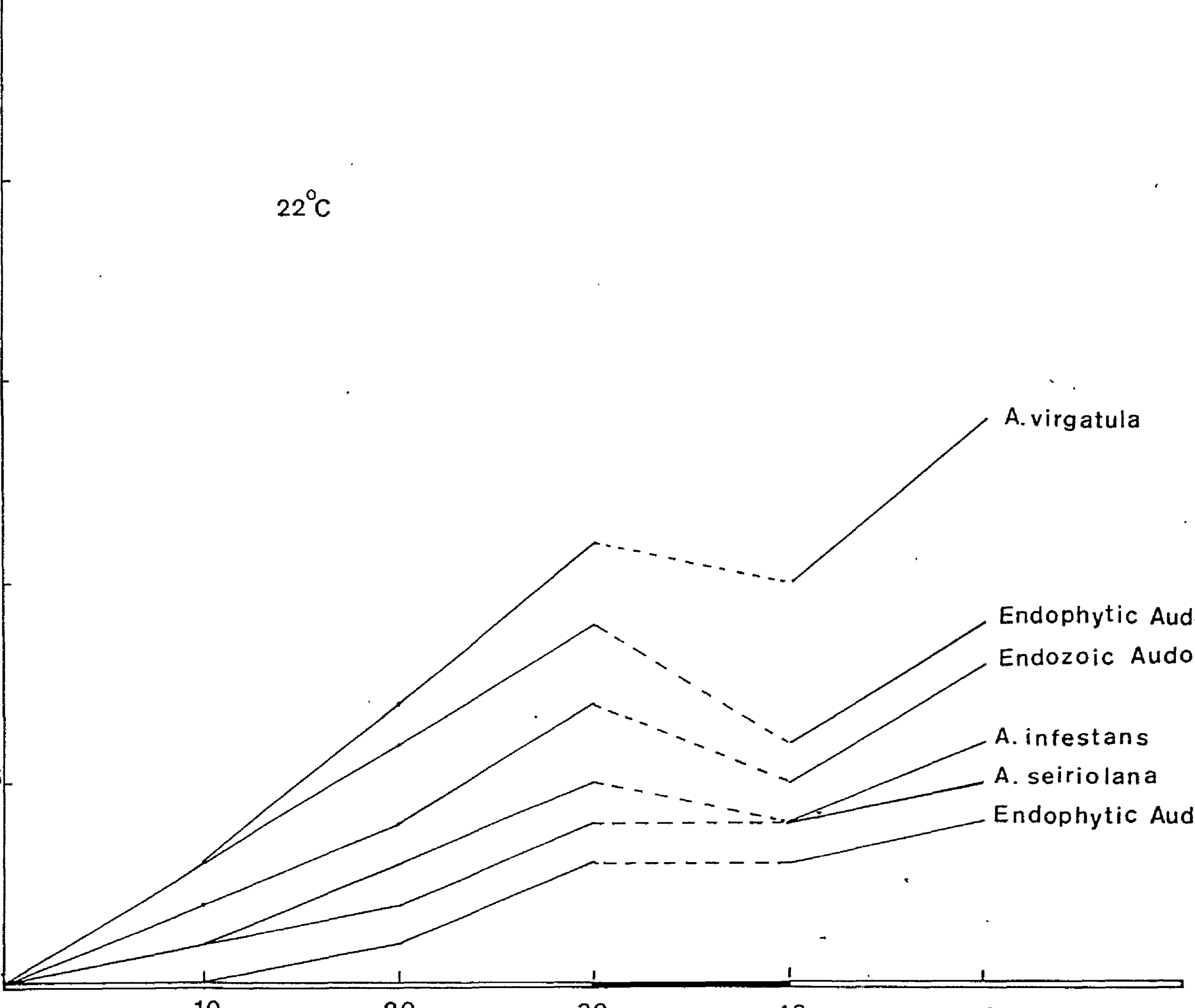
Results:

Fig. 9.1 shows that the *Audouinella* species immediately started to photosynthesize at different rates when they were illuminated at an irradiance of 141 $\mu E\ m^{-2}\ s^{-1}$ and at temperature of 22^0 C, except endophytic *Audouinella* sp. 2 which was found to start after a lag period of 10 minutes. The rate of photosynthesis with *A. virgatula* -an epiphytic species- was higher than that of the endobionts examined. When these plants were darkened, *A. virgatula*, *A. infestans*, endophytic *Audouinella* sp. 1 and

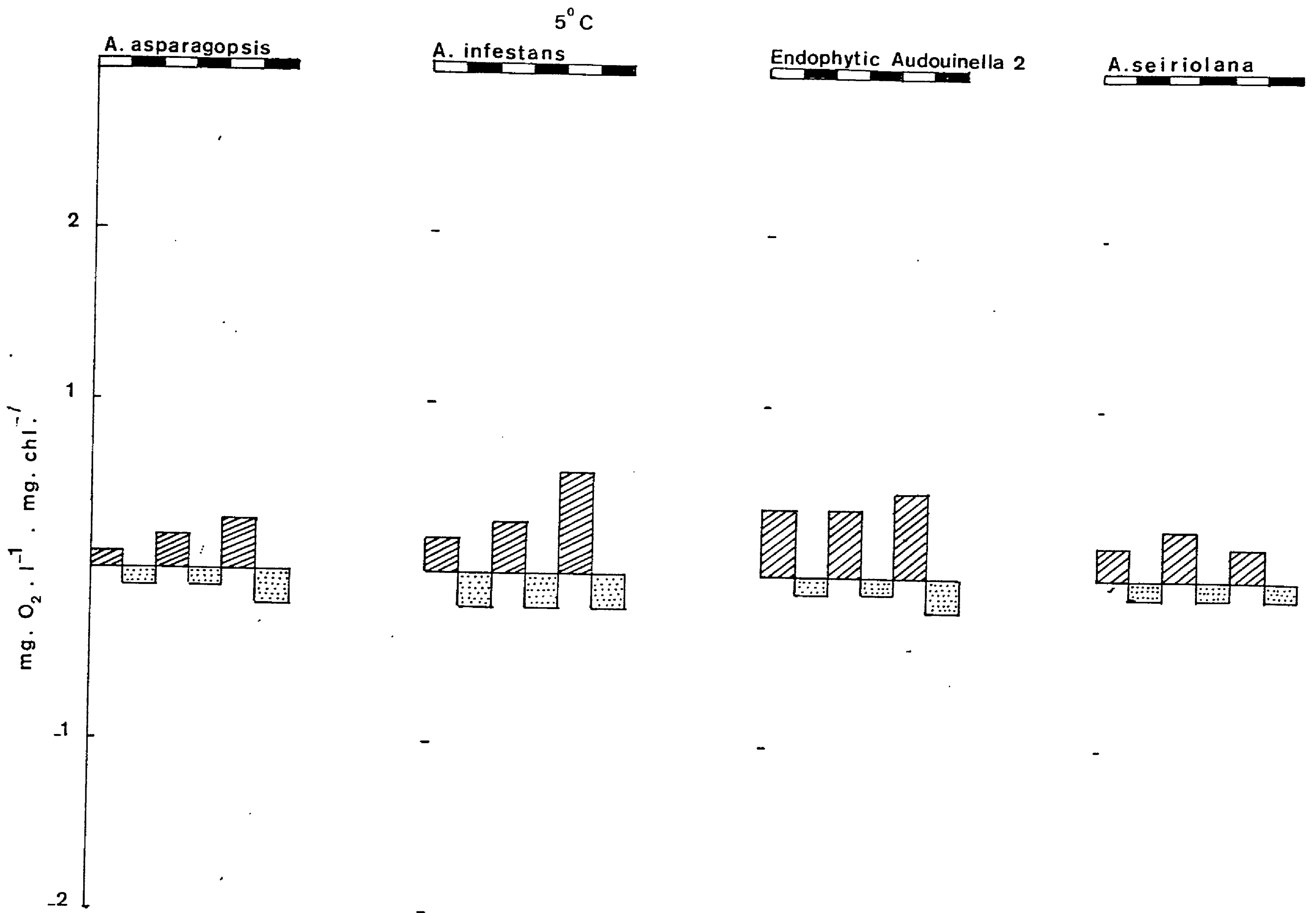
endozoic *Audouinella* sp. 2, all showed reductions in the O_2 content of the water, suggesting that respiratory activity was now being measured. This was less apparent with *Audouinella seiriolana* and endophytic *Audouinella* sp. 2. However, when all plants were re-illuminated ail started immediately to photosynthesize with their rates similar to that before the dark period.

Figs 9.2-4 summarize the results of experiments in which *Audouinella asparagopsis*, *A. infestans*, *A. seiriolana* and endophytic *Audouinella* sp. 2 were subjected, after being kept in the dark for 2-3 hours, to light and dark periods of 10 minutes each, with light irradiance at 141 µE m^{-2} s^{-1} during lighted periods and at temperatures of 5, 10 and 22^0 C. In these experiments *Audouinella asparagopsis* showed a little significant increase in oxygen release and steady respiration at temperatures of 5^0 and 10^0 C. It also showed similar rates of respiration at 22^0 C, and slightly higher oxygen production. *A. infestans* at 5^0 C showed what appears to be a gradual increase in oxygen release with each period of illumination, together with a steady respiration rate and showed fluctuations in the rates of both photosynthesis and respiration at 10^0 C. At 22^0 C rates of oxygen release and uptake, though variable, were higher, suggesting higher photosynthetic and respiratory rates. These fluctuations were accentuated by the lack of replication because of the relatively small biomass available. Photosynthetic rates with *A. seiriolana* indicated the production of similar quantities of oxygen at 10^0 C and 22^0 C, more than at 5^0 C, although the samples showed steady rate of respiration at 5 and 10^0 C, but showed a higher rate of respiration at 22^0 C. Endophytic *Audouinella* species 2 showed an increase in the photosynthetic activity as the temperature increased and the experiment progressed. However, as suggested above the respiration rate is only an assumption because of the obvious fall in oxygen level. *Audouinella infestans* appeared to be much more active than other *Audouinella* species.

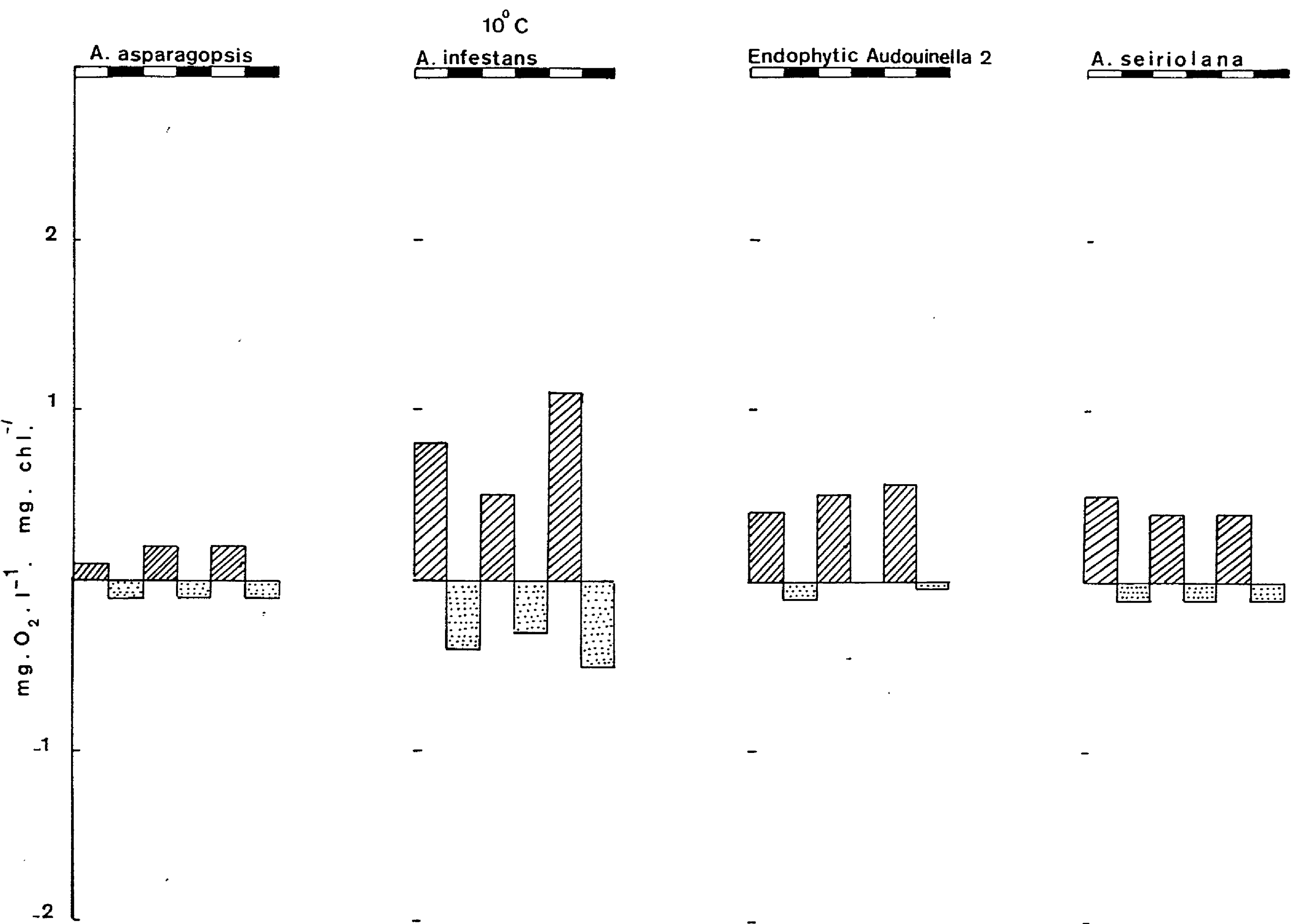
Fig. 9.1 shows that the *Audouinella* species immediately started to photosynthesize at different rates when they were illuminated at an irradiance of 141 $\mu E\ m^{-2}\ s^{-1}$ and at temperature of 22^0 C, except endophytic *Audouinella* sp. 2 which was found to start after a lag period of 10 minutes. (For all figures, zero on the vertical axis represents the equilibrium value obtained with the water in the electrode cell prior to introduction of the algal sample).


22°C
2.0
1.5
1.0
0.5
mg. O2. l-1. mg. chl.-1
10
20
30
40
50
Time in minutes
A.virgatula
Endophytic Audouinella 1
Endozoic Audouinella 2
A. infestans
A. seiriolana
Endophytic Audouinella 2

Figs 9.2 summarizes the results of experiments in which *Audouinella asparagopsis*, *A. infestans*, *A. seiriolana* and endophytic *Audouinella* sp. 2 were subjected, after being kept in the dark for 2-3 hours, to light and dark periods of 10 minutes each, with light irradiance at 141 $\mu E\ m^{-2}\ s^{-1}$ during lighted periods and at temperatures of 5,^{0}C.

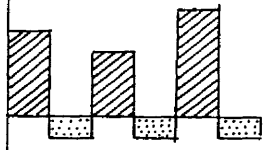

5° C
A. asparagopsis
A. infestans
Endophytic Audouinella 2
A.seiriolana
2
1
-1
-2
mg. O2 . l-1 . mg. chl.-1

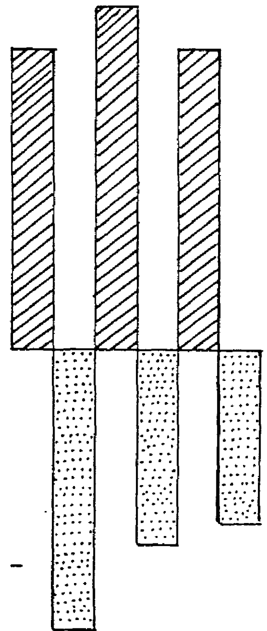
Figs 9.3 summarizes the results of experiments in which *Audouinella asparagopsis*, *A. infestans*, *A. seiriolana* and endophytic *Audouinella* sp. 2 were subjected, after being kept in the dark for 2-3 hours, to light and dark periods of 10 minutes each, with light irradiance at 141 $\mu E\ m^{-2}\ s^{-1}$ during lighted periods and at temperatures of 10^0 C.

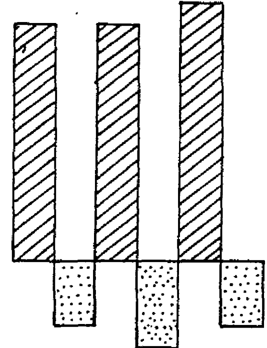

10°C
A. asparagopsis
A. infestans
Endophytic Audouinella 2
A. seiriolana
mg. O2 . l-1 . mg. chl.-1
2
1
-1
-2

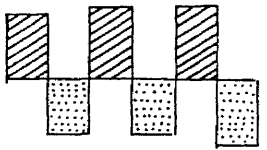
Figs 9.4 summarizes the results of experiments in which *Audouinella asparagopsis*, *A. infestans*, *A. seiriolana* and endophytic *Audouinella* sp. 2 were subjected, after being kept in the dark for 2-3 hours, to light and dark periods of 10 minutes each, with light irradiance at 141 $\mu E\ m^{-2}\ s^{-1}$ during lighted periods and at temperatures of 22^{0} C.

22°C

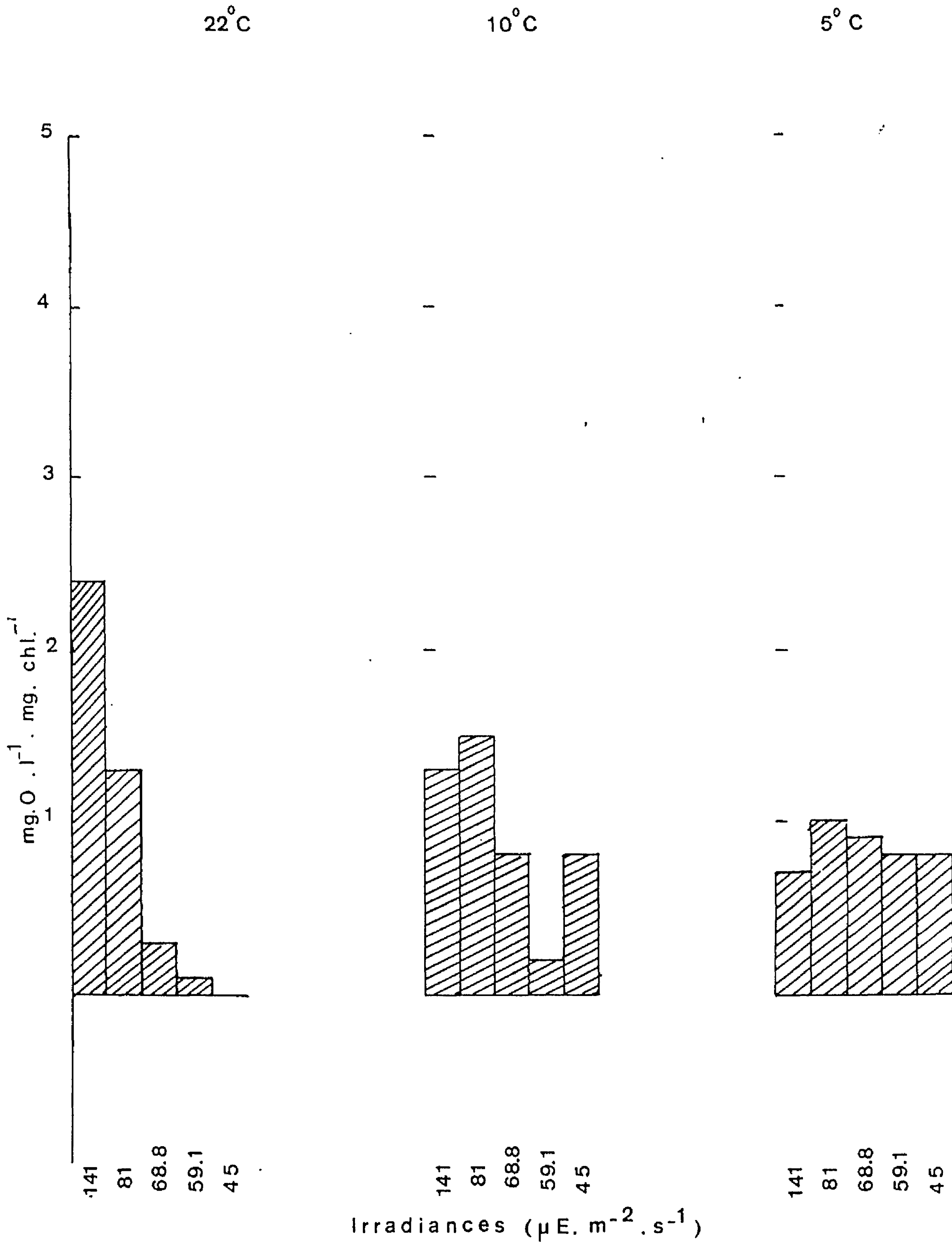




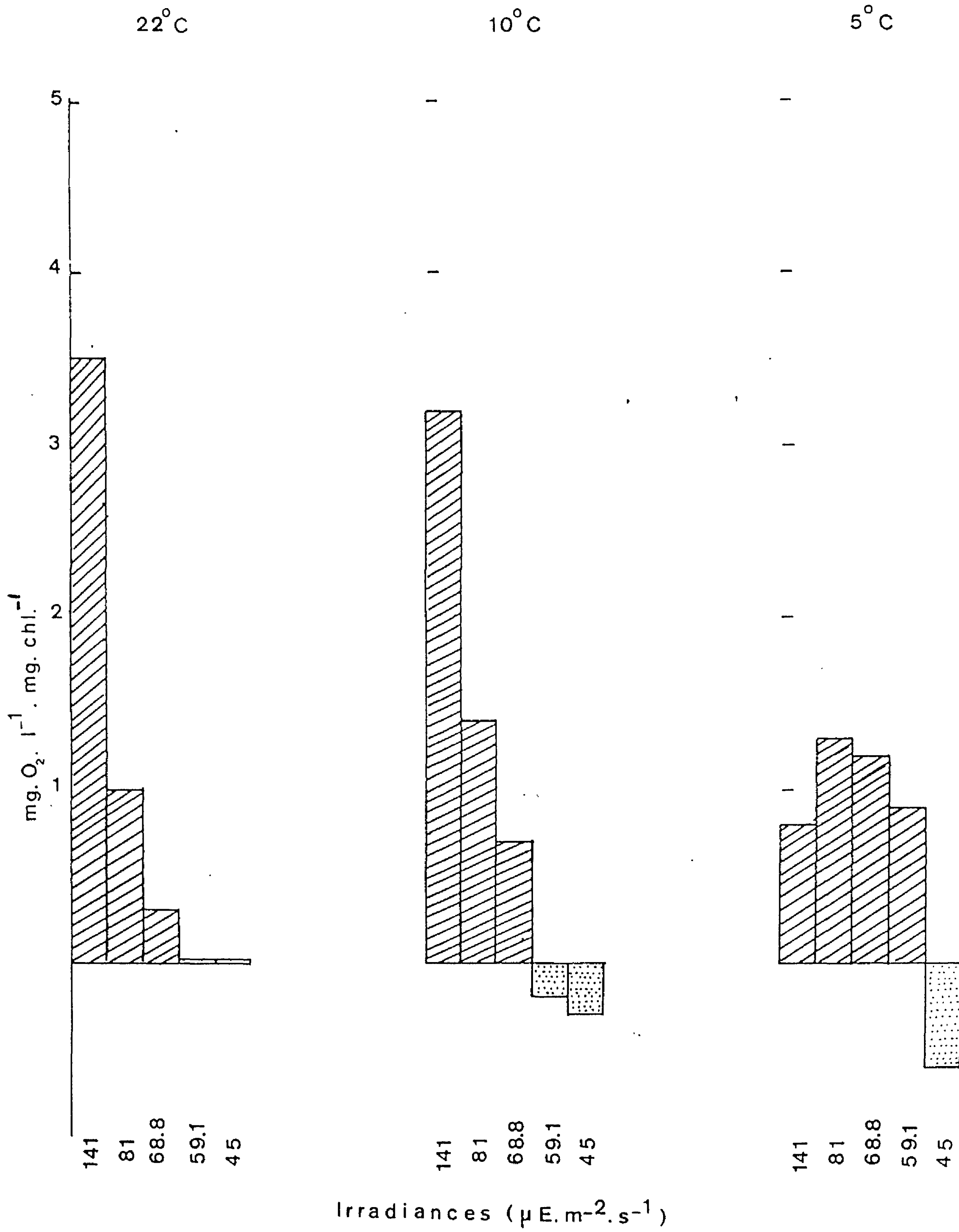




Figures 9.5 shows photosynthetic rates of *Audouinella asparagopsis* at different light intensities and at different temperatures.


A. asparagopsis
22°C
10°C
5° C
5
4
3
2
1
mg.O . l-1 . mg. chl.-1
141
81
68.8
59.1
45
Irradiances (μE. m-2 . s-1)

Figures 9.6. shows photosynthetic rates of *Audouinella infestans* at different light intensities and different temperatures.



A. infestans
22°C
10°C
5°C
5
4
3
2
1
mg. O2. l-1. mg. chl.-1
141
81
68.8
59.1
45
141
81
68.8
59.1
45
141
81
68.8
59.1
45
Irradiances (μE. m-2. s-1)

Figures 9.5-7. show photosynthetic rates of *Audouinella* species at different light intensities. The photosynthetic rate reductions in the three species (namely *Audouinella asparagopsis*, *A. infestans* and endophytic *Audouinella* species 2) occured at all three temperatures as the irradiance decreased from 141 to 45 $\mu E\ m^{-2}\ s^{-1}$. With measurements made after 30 minutes after each illumination period. *Audouinella asparagopsis* at 22^0 C showed a dramatic fall in the oxygen production when the light intensity decreased from 141 to 81 to 68.8 and then to 59.1 $\mu E\ m^{-2}\ s^{-1}$, and at 45 $\mu E\ m^{-2}\ s^{-1}$ was found to have reached a point where no oxygen release was measurable. At 10^0 C, *A. asparagopsis* showed variable photosynthetic rates as the light was reduced from 141 to 81 $\mu E\ m^{-2}\ s^{-1}$ and from 59.1 to 45 $\mu E\ m^{-2}\ s^{-1}$, while the photosynthetic rate dropped as the light was reduced from 81 to 59.1 $\mu E\ m^{-2}\ s^{-1}$. Light intensity appeared to have little effect on *A. asparagopsis* at 5^0 C. *Audouinella infestans* showed a decrease in the photosynthetic activity at all temperatures as the light was reduced except at temperature of 5^0 C where the photosynthesis rates remained similar. At 10^0 C and 22^0 C this alga appeared to favour the high light intensity and photosynthetic rates fell as the light intensity decreased. Endophytic *Audouinella* species 2 showed a reduction in the photosynthetic activity under all experimental temperatures as the light intensity was reduced, except at 10^0 C where light intensity levels of 141-68.8 $\mu E\ m^{-2}\ s^{-1}$ appeared to have no significant effect as similar oxygen production rates were obtained at these irradiances levels.

Figure 9.8 shows that the photosynthetic rates of *Audouinella* species under investigation were affected by long periods of darkness, where these plants were kept under total darkness in the growth chamber at 2-5^0 C for 7^+ days. These plants were subjected to unfiltered light of 141 $\mu E\ m^{-2}\ s^{-1}$ for 30 minutes then to light of different colours for 20 minutes each using water filters coloured with different dyes (described in chapter 2). Short dark periods (24-48 hours) appeared to have little effect on the photosynthetic rates.

After 24-48 hours of darkness, all *Audouinella* species (namely *A. asparagopsis*, *A. infestans*, *A. seiriolana* and endophytic *Audouinella* species 2) were found to respond to unfiltered light and produce more oxygen under this light than

under the other colour bands. However, in terms of individuals endophytic *Audouinella* 2 appeared to produce more oxygen that the other species when it was subjected to unfiltered light, and all other species responded differently. In respect to the different colours the *Audouinella* species under experimental showed that oxygen production was higher in blue, green, yellow than in orange and red light.

The longer dark period appeared to affect *A. seiriolana* most which showed a positive response only under the yellow light, whilst endophytic *Audouinella* species 2 showed a positive effect in terms of oxygen production consistency. Other reactions of the other species to light spectra were similar to those obtained after the short dark periods except endophytic *Audouinella* species 2 which showed more response to orange light than to the red light (R_1) and *A. infestans* showed more response to green light than to the blue light.

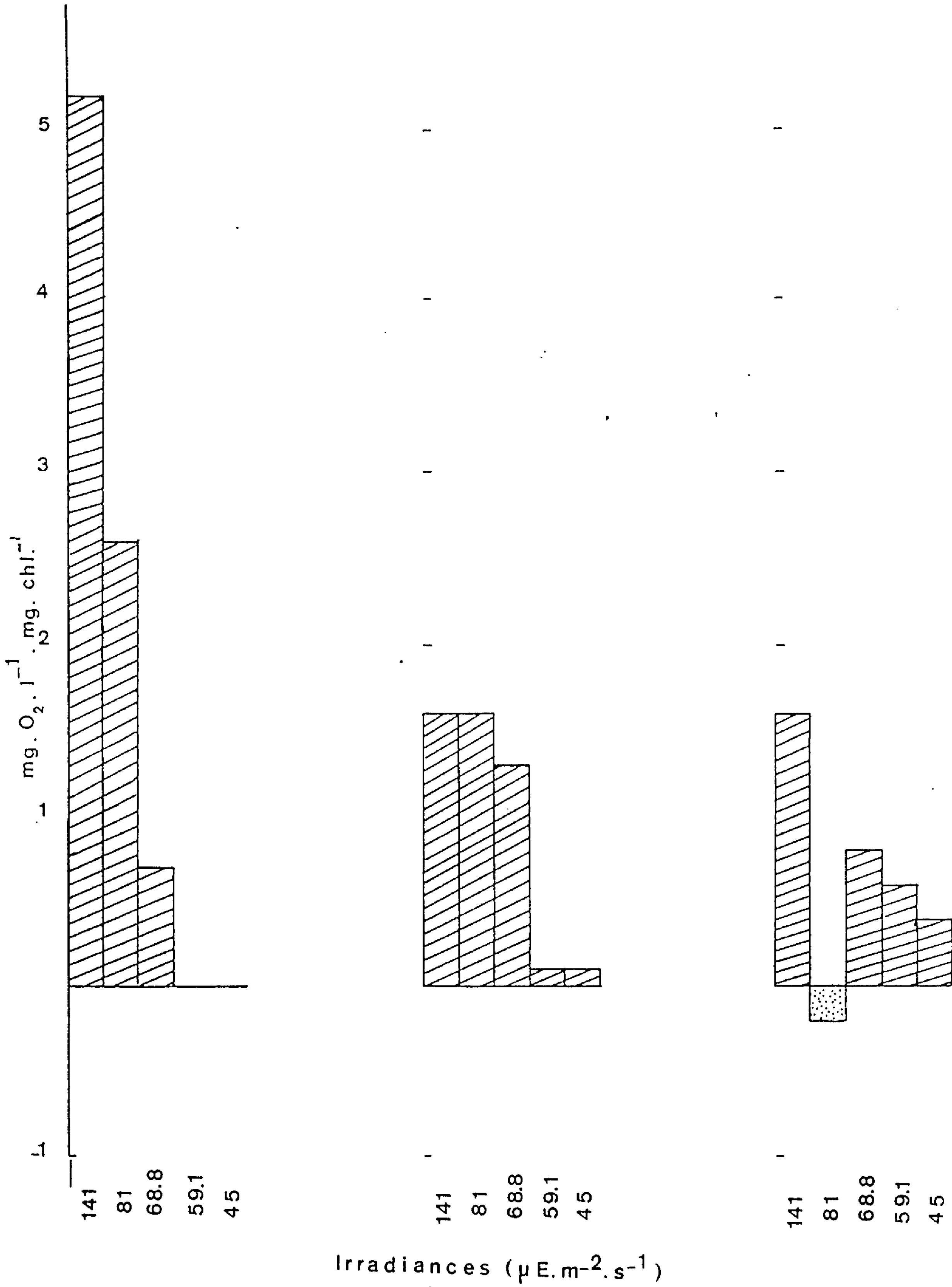
Fig. 9.9 shows the results obtained from measurements of the photosynthetic activity of *Audouinella* species when subjected to different light spectra at temperature of 22^0 C and after being kept in the growth chamber at a temperature of 15^0 C, and covered with blue, green, or red transparent plastic covers for 13 days.

Cultures of *Audouinella asparagopsis* kept under the blue light showed more oxygen production when was subjected to different light colours than when these cultures kept under green and red light. However the cultures kept under blue green light seemed to respond more to irradiation with blue and green light than to the other light colours.

Similar results were obtained with *A. infestans* except these cultures kept under the green light produced more oxygen when subjected to the yellow light.

Cultures of endophytic *Audouinella* sp. 2 kept under the red light showed no significant differences in oxygen yield in the range of colours. These cultures kept under blue and green light showed the maximum responses to these colours.

Figures 9.7. shows photosynthetic rates of Endophytic *Audouinella* species 2 at different light intensities and at different temperatures.



Endophytic Audouinella 2
22°C
10°C
5°C
mg. O2 . l-1 . mg. chl.-1
5
4
3
2
1
-1
141
81
68.8
59.1
45
Irradiances (μE.m-2.s-1)

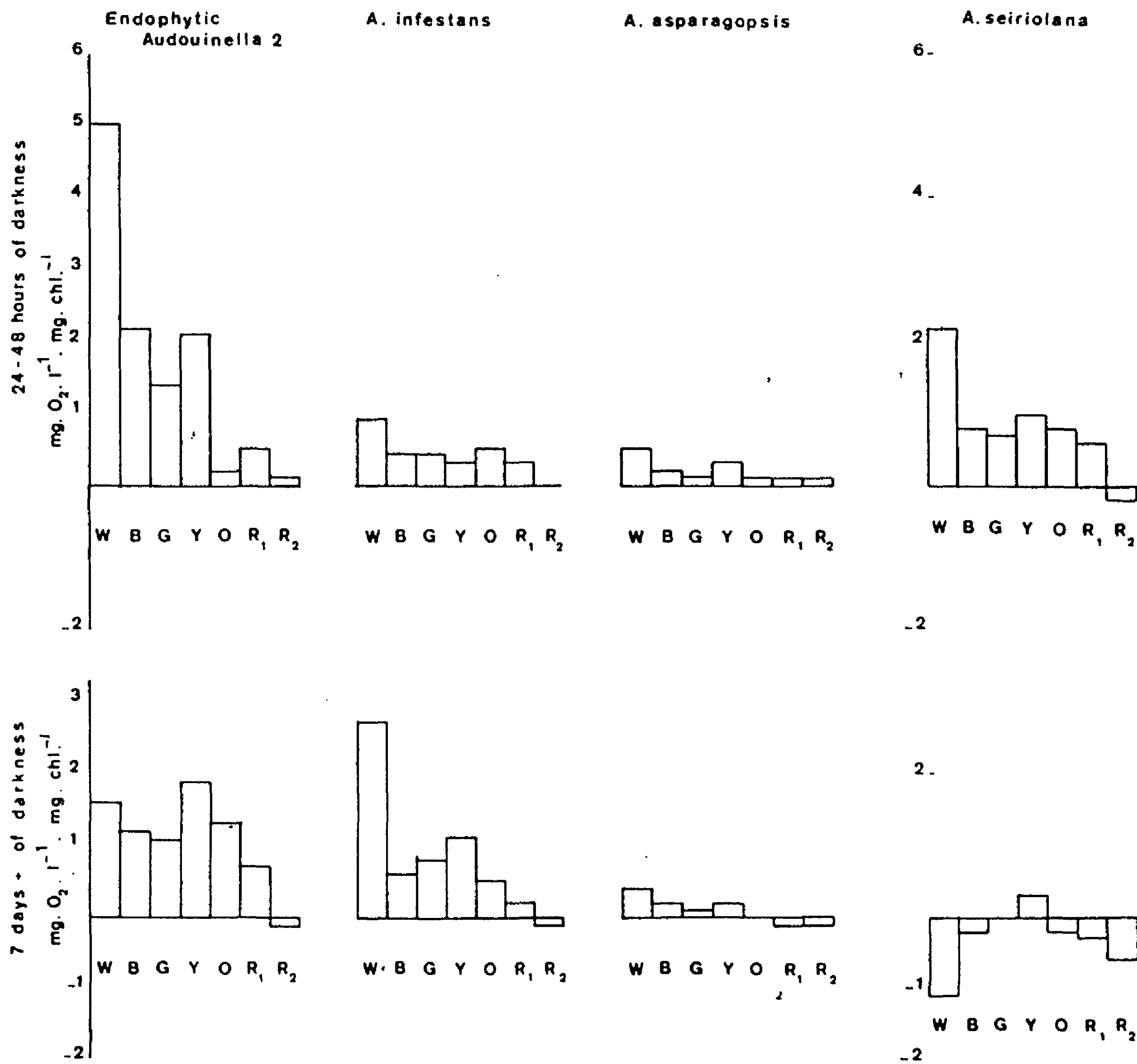
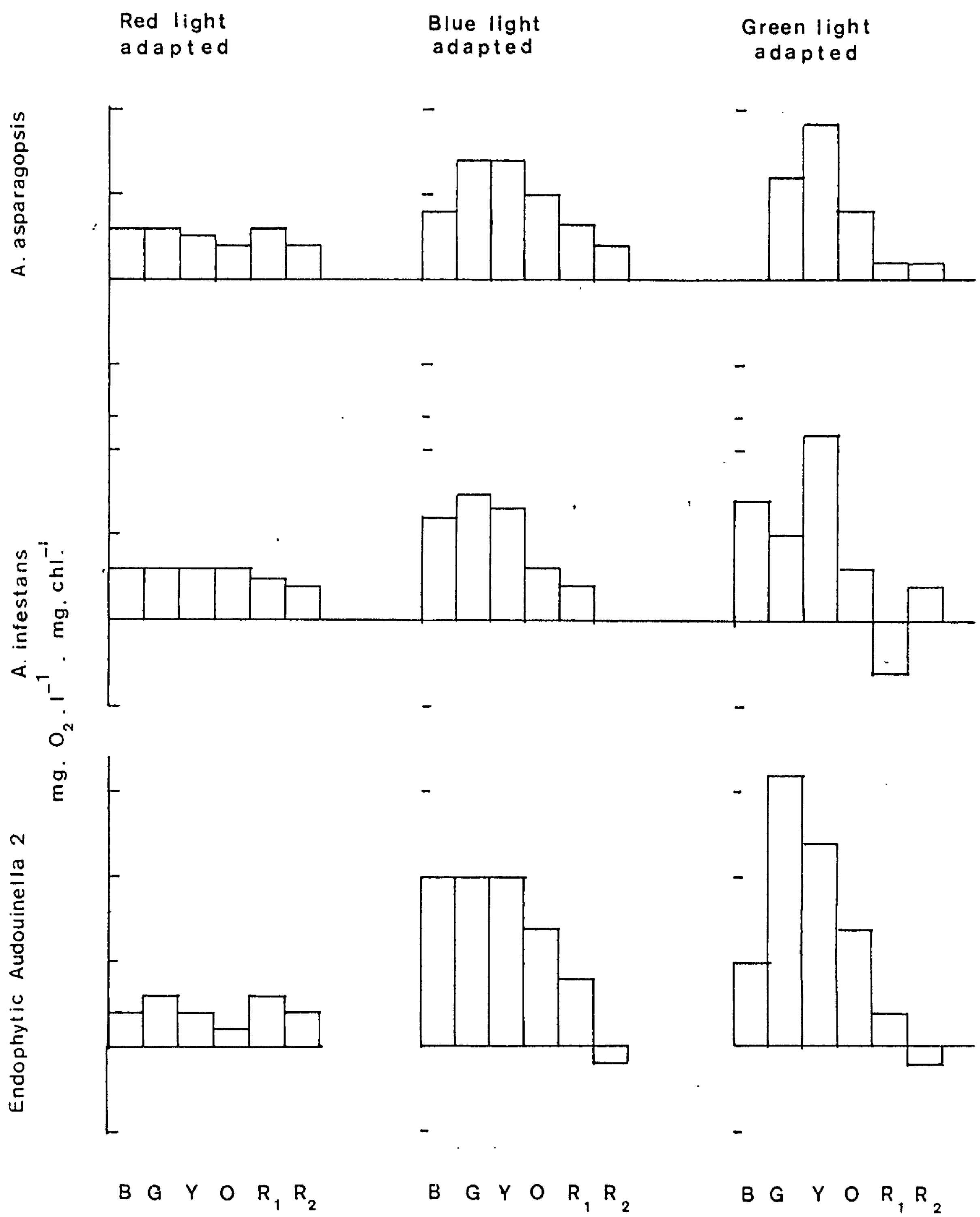


Figure 9.8 shows that the photosynthetic rates of *Audouinella* species under investigation were affected by long periods (7 days) of darkness at 2 ⁰C.

Fig. 9.9 shows the results obtained from measurements of the photosynthetic activity of *Audouinella* species when subjected to different light spectra at temperature of 22^0 C and after being kept in the growth chamber at a temperature of 15^0 C, and covered with blue, green, or red transparent plastic covers for 13 days.



Red light adapted
Blue light adapted
Green light adapted
A. asparagopsis
A. infestans
Endophytic Audouinella 2
mg. O2 . l-1 . mg. chl.-1
B G Y O R1 R2
B G Y O R1 R2
B G Y O R1 R2

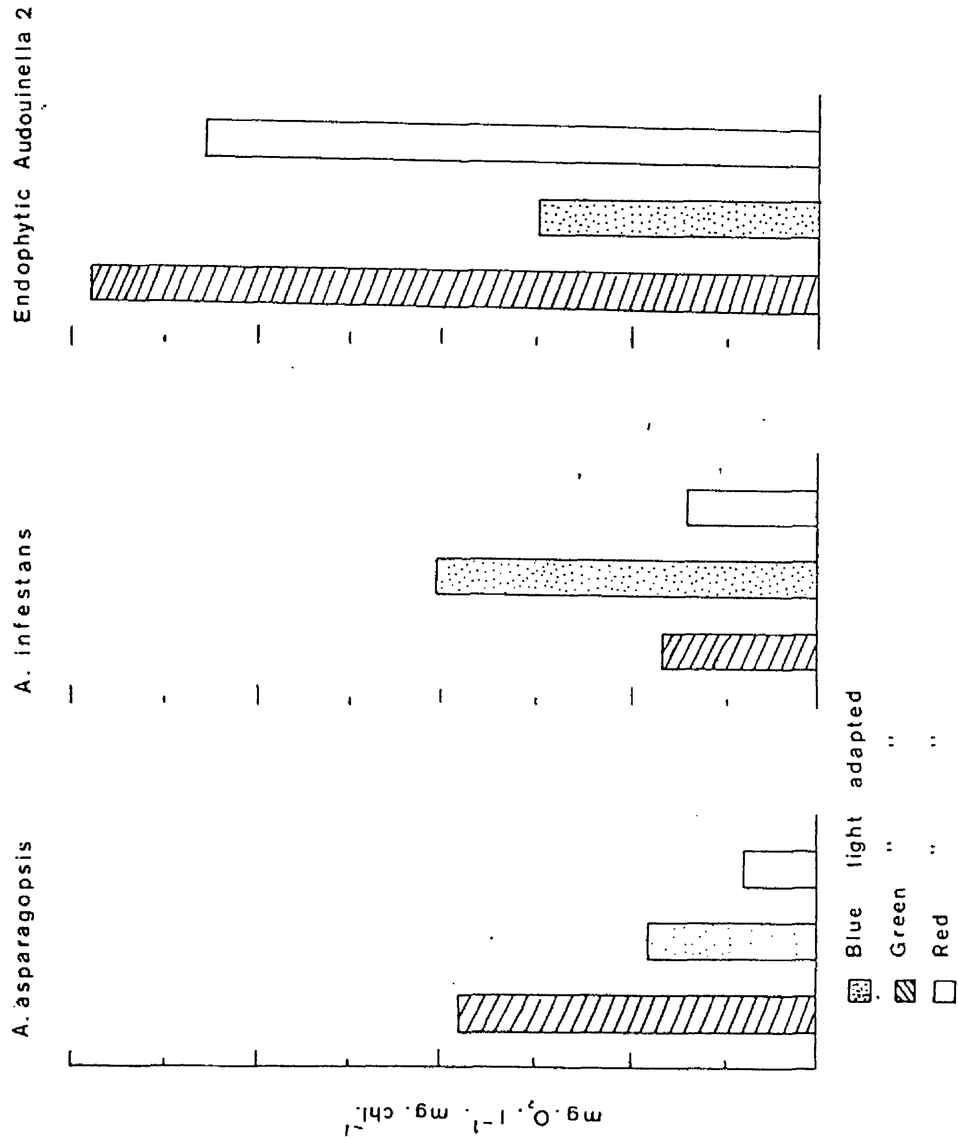


Fig. 9.10 shows results obtained from samples from the above colour adapted cultures when cultures kept as in 9.9 were subjected to unfiltered light for 30 minutes at temperature of 22^0 C.

Fig. 9.10 shows results obtained from samples from the above colour adapted cultures when these cultures where subjected to unfiltered light for 30 minutes at temperature of 22^0 C.

Green light adapted cultures of *A. asparagopsis* produced more oxygen than those from blue and red light. Blue light adapted cultures of *A. infestans* produced more oxygen than those from green and red light. Cultures of endophytic *Audouinella* species 2 from green and red light showed more oxygen production than the culture from blue light. Generally endophytic *Audouinella* species 2 produced more oxygen than the other species.

Discussion:

The wide range of habitats that *Audouinella* species inhabit would suggest that differences in photosynthetic rates might be found among these species. The epiphytic species were collected from the intertidal zone, and therefore might be expected to be adapted to higher light intensities than the endobiotic species which normally grow in more shaded habitats. Whilst the endobionts (especially these which live in the basal region of subtidal 'host' plants such as the endophytes from *Delesseria* species) might be expected to experience very dim light in nature which may just be enough to trigger the photosynthetic process. Therefore these plants may not require such high light intensities as the epiphytes. Noticeably in the experiments the epiphytic *Audouinella virgatula* showed a higher photosynthetic rate than the endobiont species. The photosynthetic rate of *A. virgatula* seemed to be unaffected by a short dark period.

The light spectrum may affect the endophytes more than the epiphytes as endobionts can inhabit various depths in sea water and it is known that red light is completely absorbed in first ten centimeters of surface water whilst the other components are reduced by attenuation with depth. However, the shorter the wave length the deeper it reaches and the higher energy it contains, as seen in the penetration of the

wavelengths in the blue and green regions. This study shows that for endophytic *Audouinella* species maximum photosynthesis is obtained in the blue-green spectral range although in the experiments the blue and green filters used produced lower light intensities values than the others. It may be questioned whether this represents chromatic adaptation as originally described, or whether the colour densities used were of the light intensities more favoured by the endophytes (a shade effect). However, in 'white' light of higher intensities the endophytes showed enhanced rates of photosynthesis, so that the above results may be some indication of chromatic adaptation.

The endobiotic species as expected were found more tolerant to dark periods, possibly because of their adaptation to the shaded habitat where their 'host' organism lives. On the other hand, thes endobionts are more "opportunist" in taking the advantage and immediately responding to any available increase in light intensity in the medium as they showed their instant photosynthetic activity.

As expected, temperature, appeared to affect the photosynthetic rates of these *Audouinella* species -an effect which was directly proportional to the temperature, i.e, the photosynthetic rates increases as the temperature increased. But not all the species were effected to the same degree which suggests that there may be physiological differences between different species, and may be linked with the variable responses shown under different light colours and intensities.

10. General discussion

Speciation in the genus Audouinella, as earlier stated, is in a complicated and confused state. This confusion is mainly due to the morphological characters used which tend to vary under different growth conditions. Thus a large number of species have been described in the literature (390 species up to 1980). Different authors have tried to reduce the number of species in this genus acknowledging the likely intraspecific variation e.g Garbary (1979b) reduced 170 entities to 112 species. Other authors e.g. Glazer *et al* (1982) were not sure of 8 of the *Audouinella* species they used in their study and therefore they gave numbers only to these isolates in order to prevent adding extra confusion to this matter. In this study a similar situation was met with and only 2 entities of the endobionts studied were attributed to known species (viz. *Audouinella efflorescens* and *A. infestans*). These were easily identified because of the unique appearance of the spiral chloroplast and the large cell size of *A. efflorescens* and the available descriptions of *A. infestans* from the British Isles in nature and in culture by other authors. The other entities isolated are certainly distinct species from each other because of the clear morphological differences between them observed and recorded in this study and obtained under similar culture conditions. However, since only a few cells were seen in nature it is not possible to compare the morphological features obtained under culture conditions with those recorded in the literature which been described for isolates from nature. Moreover, it is well known that these plants can modify their morphology under different environmental circumstances. Thus morphological features alone are not reliable taxonomical characters. Some features do stand out, such as the development of an attaching system in the endozoic *Audouinella* sp.2 a character which separates this species from the endozoic *Audouinella* species 1, which otherwise has very similar morphological features. Caution should be applied before adding new species to an already long and confusing list. Boney and White (1967) advised a cautious

approach in considering speciation in such simple life forms. Boney (1972) suggested that with endobionts a "Koch's Postulate" approach should be carried out for isolates before coming to any conclusions. In this approach if a bacterium was isolated from a diseased tissue and then grown in culture, to verify that the bacterium in culture was the one causing the diseased condition it was regarded essential that it should then be taken from its culture, re-injected into a suitable test organisms and see then if the identical disease conditions arose. But other authors (e.g. Tam *et al* 1987) did not consider this to be necessary when they synonymized their *Audouinella* species under investigation (see page 11).

Since culture conditions can be exactly replicated in different centres, perhaps one approach would be to correlate forms obtained in culture with these in nature from which they are derived. With present day systems of information storage and retrieval more rigorous correlations of growth forms would then be possible.

It is also evident that these isolates are distinct from each other according to the results obtained from histo- and biochemical studies. A combined observation carried out by **SEM** and **TEM** showed that certain irregularities in the cell surface can be seen in cross sections of the cell wall (e.g. endophytic *Audouinella* species 3) whilst other species showed these irregularities only in the sections in terms of the number of different layers of fibrillar and non fibrillar material and their thicknesses as shown in terms of different electron densities. The type of septal plugs found in these species confirms their affinity to the class Nemaliales. The staining techniques, calorific values and nuclei and cell volumes studies appeared to be of some importance in separation of these species. It was also revealed that these species can be put into two groups according to the phycoerythrins present, a large group with R-phycoerythrin and a small group of few species with B-phycoerythrin. These results support those obtained by Glazer *et al.* (1982). The presence of B-phycoerythrin in both Bangiaceae (Bangiophyceae) and in Acrochaetiaceae (Florideophyceae) suggests a close relationship and adds further

evidence to that for septal plugs as established by Bourne *et al.* (1970); Lee (1971); and in this present study. These results support the phylogenetic scheme for the Acrochaetiaceae proposed by Garbary (1979c), in which he assumed that the Bangiaceae is a primitive family in the Bangiophyceae and closely related to Acrochaetiaceae.

Other biochemical attributes could be useful with such a species complex as indicated by the bomb calorimetry results where only small biomasses obtainable under culture conditions were required. Calorimetric values of algal tissues have mainly been examined for their trophic values. With such simple forms as obtained in the present study, more marked differences in calorific values were obtained than with macroscopic red algae. The results obtained in this study suggest that a combination of calorific values with more "in depth" histo- and biochemical studies may be useful for classification of these plants, as the isolates showed mixtures of cell wall compounds such as carboxylated and sulphated polysaccharides, cellulose, pectic and phenolic compounds. Time was not available for a combined study of stained sections and electron microscope observations, but this approach, with measures of calorific values, might prove worthwhile.

The simple morphology and small size of these species appeared an advantage for their survival in marine environments. It was clear from observations made on the epiphytic *Audouinella* species (viz. *A. secundata*, *A. seiriolana* and *A. virgatula*) that under stress these species tend to keep their size to a single filament with an attaching system or even some times remain in the early stages of germination (i.e, few cells which form the prostrate system) until the conditions became suitable for their growth and they can remain in such juvenile forms for long periods. The small size as stated earlier reduces the chances of being broken or washed off from their supporting organism, as these epiphytes were found not to regenerate from the erect filaments and occasionally were found to require an attachment to a substratum for growth to continue. Thus, fragmentation is less beneficial to these epiphytic *Audouinella* species. However, it was found that free floating filament fragments of these epiphytic species may remain

healthy and alive and still produce monospores. The filament cells tend to die off after spore release. This process may contribute to their dispersal in the marine habitats. The mechanism of spore release showed that such simple life forms have evolved an effective spore discharge process, and that a large number of spores can be produced by sporangium proliferation by a single plant which may far exceed the number of vegetative cells in the erect filaments.

Occurrence of these epiphytic *Audouinella* species on non-perennial and relatively delicate and fragile supporting plants (viz. *Porphyra umbilicalis*, *Polysiphonia* and *Ceramium* species), provide them with a growing substrata and dispersal opportunities as these supporting plants are easily broken into small fragments with the whole epiphyte and carried away by water currents. Fragmentation is an effective means of vegetative propagation and dispersal in marine algae. Clokie and Boney (1980), using bottle brush impingers, collected living identifiable plant fragments of 50-60 species of marine algae over a 32 day period. These supporting plants, due to their elastic body structure, provide protection to the epiphytes by absorption of the mechanical actions created by the water currents and wave action in the marine habitats.

Fragility which is considered a disadvantage to some organisms, appears sometimes to be an advantage to certain endobiotic *Audouinella* species. Endozoic *Audouinella* 1 and 2 and endophytic *Audouinella* 1 and 3 were found to produce more delicate and fragile filamentous forms at the tips of old filaments when they were left for long periods undisturbed in the same medium. These filamentous forms were found to be broken easily even by a little disturbance. These delicate tufts of cells were found to have the capacity to produce new individual tufts with cells of normal size. These delicate filaments were obtained under stress conditions. To be effective in nature the endobiotic filaments would have to grew outside of the 'host' organism. This is known to happen with some species described as being 'partially' endophytic or endozoic. The vegetative cells from the attaching systems of *Audouinella infestans* and endophytic *Audouinella*

species 2 were found to attach much more quickly than those from the erect system. These cells were also found to germinate as ordinary spores. Thus the cells of the reduced forms of these endobiotic species are less specialized and can perform several functions -photosynthesis, attachment, or reproduction, depending on the occasion and environmental conditions.

Other functions of modified cells (namely hair-like cells), such as protection or increment of the surface uptake, are known in epiphytic *Audouinella* species. Species such as *A. secundata* and *A. virgatula* were found to produce a long colourless cells (hairs) when they were subjected to nutrient depletion. These hairs are occasionally described for other species in nature and presumably have the same functions of nutrient uptake or protection from light. The nature of these cells are not known. They are possibly vegetative cells which have lost their pigments (chlorophylls and other accessory pigments) because of the high light intensity. This assumption is supported by observations made on the endophytic *Audouinella* species 3 which was found to lose its brown-red colour under high light intensity and become almost colourless. When these colourless plants were returned to lower light intensity, they regained their colour gradually and simultaneously in all cells rather than cell by cell as been observed in other species where some intercalary cells lost their pigments due to damage or death and where recovery took place from adjacent cells as an inner growth which filled the dead cells. It was not possible to carry out any pigment extracts from these 'colourless' cells because they are not available in large quantities and could not be isolated, but they are known to have cytoplasm in them.

The flexibility of the life forms of the endobiotic species in their ability to invade and adapt to different substrata make the classification of these plants according to their 'hosts' doubtful. Endophytic and endozoic species were found to have the potential to invade both chitinous and calcareous materials. In addition, their cells shapes became irregularly outlined in the substrata which makes the measurement of the cell size very

difficult, and the shape then becomes an unreliable character in their taxonomy. Also the presence of prostrate systems in free living forms of endophytic *Audouinella* species 1 and endozoic *Audouinella* species 2 make their morphological appearance very different from life forms within the 'host'.

The life history concept seems applicable to only a few species of *Audouinella* (see p.10). In this study, day-length and night breaks experiments did not suggest a life history sequence of sporophyte and gametophyte phases with these isolates, which suggests that perhaps a sexual phase is not always obligatory.

Endobiotic *Audouinella* species are mainly found in nature in red algal 'hosts' in the holdfast region or occasionally in the branches. This is apparently due to their ability to digest the cell wall of these plants as results obtained in this study showed the ability of these plants to penetrate agar. The advantage of such a habitat is the flux of nutrients and light energy if 'host' cells are actively photosynthesizing in the dark, or in long periods of dull weather when radiant energy input to 'host' plants is markedly reduced the endophytes would seem to be in a state of 'suspended animation'. Above all these 'host' plants appear to provide the endobionts with protection to become a perennial source of spores or vegetative cells for new generations which can invade new hosts or alternatively can produce free living forms as has been shown in this study that almost all endobiotic isolates, except one which was found growing in *Polysiphonia elongata*, have produced free living forms. One question which remains unanswered is whether these endobiotic algae living in the basal regions of subtidal 'hosts' such as *Delesseria*, and so often shaded still further from the reduced Photosynthetic Active Radiation (PAR) available, might be capable of heterotrophic activities during the winter months.

Photosynthetic rate experiments showed that these plants, especially the endobionts, are 'photosynthetic opportunists', being able to commence active photosynthesis as soon as exposed to adequate light -a process just as quickly 'switched off' when this illuminance falls below a certain critical level. As expected, photosynthesis is

temperature dependent, and is very quickly 'switched on' after dark storage. In nature, these plants in their 'host' may thus take instant advantage of any short lived 'pulses' of illumination during winter seasons, whilst during periods when the levels of **PAR** are increased growth and may be sporulation take place, as the free living forms in the present work were found to show active vegetative growth during suitable growth conditions and only under unfavourable conditions, e.g. due to nutrient depletion when kept for long periods in the same medium, did these endobionts started to form special vegetative structures to help survive these stress conditions, by breaking off from the old tufts and commencing spore production.

To describe a number of apparently distinct species without giving them defined names leaves one open to the accusation of taxonomic incompleteness. But, for the reasons given earlier, it was thought inadvisable to create entities on culture based studies alone, when in nature only a few cells were viable of the "parent" organism, and these bore no resemblance to their "offspring" in culture. One of the major problems in the taxonomy of endobiotic *Audouinella* species is that so many entities have been established from the descriptions of a few cells in the 'host' organism. The research described in this thesis represents an attempt to break away from the hitherto entirely descriptive approaches to the taxonomy of *Audouinella*, and to examine methods which might lead to a more effective synthesis of the accumulating taxonomic data. Whilst some of the results do indicate worthwhile tries of investigation, other data have shown the marked degrees of morphological flexibility shown by their relatively simple life forms. These attributes point to the need for a more rigorous control of environmental conditions for culture studies in different centres to ensure that comparative studies can be made on a reliable basis. The general conclusions at the present time point to the *Audouinella* entities being regarded as the more simple (primitive?) forms of Florideophyceae. It is this feature which underlies the taxonomic problems of the taxa. Their simplicity of form and 'opportunist' physiological behaviour enable them to

exploit a variety of habitat conditions very effectively, but to the continuing confusion of the morphologically-biased taxonomists. Finally, an effective starting point would be a clear definition at generic level. Despite the attempts by Woelkerling (1971; 1973; 1983), Dixon and Irvine (1977) and Garbary (1979c), already an additional genus *Liagorophila* has been re-introduced (Lee *et al.* 1986), and two different generic names of what is probably the same species has appeared in literature at the same time *Audouinella alariae* (Lee and Kurogi1983; published in January) and *Chromastrum alariae* (Kuiper1983; published in May).

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Appendix 1

Culture Medium (Boney and Burrows's 1966)

Aged seawater from Firth of Clyde, filtered through a double layer filter paper (Whatman No.1) and then pasteurised by heating it at 73^0 C on two consecutive days. The formula of the medium is as follows: To 1 litre of the above seawater the following solutions were added.

50 ml of solution **A**,

which contained 4 gram. l^{-1}. Na_2NO_3.

2 ml of each of the following:

$1.47 g.l^{-1} MnSO_4.4H_2O$

$0.0023 g. l^{-1} CuSO_4.5H_2O$

$0.064 g. l^{-1} CoCl_2.6H_2O$

$0.005 g. l^{-1} LiCl.H_2O$

$0.23 g. l^{-1} NaMoO_4.2H_2O$

15 ml of solution **B**, which contained:

2.6 g. l^{-1} tetrasodium salt EDTA and 0.2 g.l^{-1} $FeSO_4.7H_2O$

1.5 ml of solution **C**, which contained:

1.5 g. l^{-1} $Na_2HPO_4.12H_2O$

2 ml of solution **D**, which contained:

4.98 g. $l^{-1} ZnSO_4.7H_2O$

Each of the above solutions was made up in distilled water, autoclaved separately and stored in a refrigerator until used.

To avoid precipitation in the seawater 50 ml of distilled water were added to each 950 ml of seawater before pasteurization.

Appendix 2

Gluteraldehyde-Cacodylate Fixative + Seawater

1 M	sodium cacodylate	10.7015g.	in 50 mls of distilled water.
1 M	sodium chloride	5.844g.	in 100 mls of distilled water.
1 M	calcium chloride	5.5493g.	in 50 mls of distilled water

Fixative

1 M sodium cacodylate	10 mls
25% Gluteraldehyde	8 mls
1 M sodium chloride (NaCl)	5 mls
1 M calcium chloride ($CaCl_2$)	0.05 ml
Filtered seawater	50 mls
Distilled water	make up to 100 mls

pH 7.6 Osmolarity 990

Buffer

1 M sodium cacodylate	10 mls
1 M sodium chloride (Na Cl)	20 mls
1 M calcium chloride (Ca Cl_2)	0.05 ml
Filtered seawater	50 mls
Distilled water	make up to 100 mls

pH 7.6 Osmolarity 980

Osmium tetroxide fixative

1 M sodium cacodylate	5 mls
4% osmium tetroxide (Os O_4)	12.5 mls
1 M sodium chloride (Na Cl)	7.5 mls
1 M calcium chloride (Ca Cl_2)	0.025 mls
Filtered seawater	25 mls

pH 7.6 Osmolarity before adding OsO_4 **1170**

Appendix 3 ...

Bomb calorimetry; the firing technique

1. Prepare dried samples in pellet maker and keep it in a desiccator.

2. Clamp bottom half of bomb in vice. Put 0.025 ml distilled water in bottom of the bomb.

3. Put top half of bomb in clamp at eye level. Using 4 cm of 0.15 mm platinum wire, connect firing wire to terminal and pull out to taut V-shape.

4. Put a known weight tray with a known weight of pellet onto sampler holder in the bomb and bend the V-shaped firing wire so that point is touching pellet (but not the tray) and holding it in position.

5. Screw top onto bottom and tighten until metal meets metal using correct spanner.

6. Fit quick release connection from oxygen cylinder, slowly fill to 25 atmospheres pressure.

7. Connect calorimeter to recorder, and switch on the recorder to establish room temperature trace (baseline).

8. Place bomb on thermocouple ring. Wait until steady trace, with a very slow increase or decrease, is accomplished.

9. Connect leads to bomb terminals and replace both covers.

10. Switch on calorimeter, check charge (35 volts). Switch to fire, return charge and then switch off calorimeter.

11. Observe rise time and peak on recorder. Wait until there is a linear cooling rate.

12. Switch off recorder.