An ecophysiological study on the moss hydrogoniuh fontanum from the Asir mountains, Saudi Arabia

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AN ECOPHYSIOLOGICAL STUDY ON THE MOSS *HYDROGONIUM FONTANUM* FROM THE ASIR MOUNTAINS, SAUDI ARABIA

By

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

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ABSTRACT

The thesis describes a study on the ecophysiology of the moss *Hydrogonium fontanum* (C. Müll.) Jaeg., the dominant plant at a waterfall in Saudi Arabia. The influence of environmental variables and water stress on the growth, stress metabolite accumulation and phosphatase activities of the moss was studied in laboratory axenic culture along with observations and experiments conducted in the field. The variables chosen for growth experiments were light flux, flooding, nutrient concentrations and water stress. For phosphatase activities, the influence of temperature, pH, ions, water stress were studied. Differences were found in phosphatase activities for rhizoids, protonema and leafy shoots of the moss and, therefore, the phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activities of these fractions were also investigated.

H. fontanum was originally collected from the tufa-depositing waterfall (Water chemistry = 44 mg l^{-1} Na, 44 mg l^{-1} Ca). High Na and Ca had significant positive effect on yield of the protonema under the laboratory conditions. Low light intensity (10 μmol photon m^{-2} s^{-1}) decreased the yield, but high light intensity (90 μmol photon m^{-2} s^{-1}) increased the yield of the protonema.

The moss showed no response to water stress in respect to proline accumulation. Protein content decreased significantly over 48 h with increase in water stress. The influence of water stress was greater in terms of dry weight and chlorophyll content changes in protonema than in leafy shoots.

The protonema was capable of using various organic P substrates as sources of phosphorus and showed both PMEase and PDEase activities. PMEase and PDEase activities were detectable in all moss fractions (rhizoids, protonema, leafy shoots). Laboratory grown material showed higher activities than field grown material. Rhizoids produced the highest PMEase and PDEase activities among the moss fractions. Some leafy shoots collected from the field had low phosphorus content with high phosphatase activities, while others had high phosphorus content with low phosphatase activities.

Changes in phosphatase activities in batch culture were studied in relation to growth rate. PMEase activity was first evident when cellular P was 1.15% with low activity (0.117 μmol pNP mg d. wt^{-1} h^{-1}) and PDEase appeared 4 days later when cellular P was 0.54%. The activities increased up to day 12 after which the activities maintained this level.

The optimum temperatures, measured over a period of 1 h, for PMEase and PDEase activities were 60 °C and 65 °C with pH optima of 5.5-6.0 and 6.4-6.8, respectively. Of the six ions tested, Ca, Zn and P had significant inhibitory effects on the activities at the highest concentration used (10 mM).

Drying the moss decreases PMEase and PDEase activities by about 23% and 21% (5-d) and 3.7 and 2.8 times (3 months), respectively. Water stress (PEG treatment) also reduced significantly the activities of PMEase and PDEase with a greater effect on the activity of the latter.

A brief comparison in PMEase activity using two different substrates p-nitrophenyl phosphate (pNPP) and 4-methylumbelliferyl phosphate (4-MUP) was made to investigate the pH optima and time course. PMEase activity measured using 250 μM 4-MUP was about 60% of that measured using the same concentration of pNPP.

ABBREVIATIONS

°c	degrees Celsius
g	gramme
mg	milligramme
μg	microgramme
d. wt	dry weight
l	litre
ml	millilitre
μl	microlitre
m	metre
cm	centimetre
mm	millimetre
μm	micrometre
nm	nanometre
d	day
h	hour
min	minute
s	second
M	molar
mM	millimolar
μM	micromolar
μmol	micromolar
P	phosphorus
P_i	inorganic phosphate
PMEase	phosphomonoesterase
PDEase	phosphodiesterase
pNPP	p-nitrophenyl phosphate
bis-pNPP	bis (p-nitrophenyl) phosphate
4-MUP	4-methylumbelliferyl phosphate
K_m	Michaelis-Menten constant
V_{max}	maximum rate
AMeP	2-amino-2-methyl-1-propanol
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
DMG	3,3-dimethylglutaric acid
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
HEPES	N-2-hydroxymethylpiperazine-N'-2-ethanesulphonic acid
TES	N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid
Tris	tris (hydrpxymethyl)-aminomethane
n	number of samples
P	probability
S.E.	standard error
FW	fresh weight
Chl	chlorophyll
Chl *a*	chlorophyll *a*
Chl *b*	chlorophyll *b*
PEG	polyethylene glycol
TCA	trichloroacetic acid
V/V	Volume per volume

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1 INTRODUCTION

1.1 General introduction

In spite of their small size, mosses are widely known because of their gregarious habit, forming green carpets in wet or moist conditions. In addition to that, their brown capsules, which are borne on the gametophytes, also give them unique characteristics. Spores, when released from these capsules, germinate to form green filaments called protonema, which then form the leafy shoots (gametophytes) (Richardson, 1981). There are over 15,000 species of moss with varying size and structure worldwide, which range from small plants of less than half a millimetre high *(Ephemevum)* to about 70 cm (*Dawsonia*) (Richardson, 1981). These gametophyte stages also have rhizoids, which help in attachment to the substratum.

1.2 Ecology of aquatic bryophytes

Bryophytes are widespread in aquatic environments, particularly flowing waters, and in some are the dominant aquatic plants. Numerous mosses and liverworts species are wholly aquatic (Glime, 1981 & Watson, 1981). Some are predominantly aquatic, while others, such as *Brachythecium* and *Bryum* are found in both terestrial and aquatic environments. Welch (1960) and Crum and Anderson (1981) have found bryophytes in a large variety of freshwater habitats from small springs and headwater streams to ponds and large lakes.

The distribution of bryophytes in relation to other macrophytes within rivers has been studied extensively. Some authors (e.g. Sirjola, 1969; Holmes & Whitton, 1977b, 1981) have observed a zonation of macrophyte vegetation in rivers, with a dominance of bryophytes in the upper zone, where the substratum consists of large rocks and current velocity is high.

Several major rivers of north-east England (Holmes & Whitton, 1975a, 1977a, b, 1981), the river Wye on the England - Wales border (Merry *et al.*, 1981) and the river Etherow (Harding *et al.*, 1981) have been studied. In these, some liverwort and moss species are common in the upper reaches, other species were found further downstream, and some species were found along a great proportion of the river's length.

Gimingham and Birse (1957) have also observed aquatic bryophytes along streamside rocks and cliffs. In lakes, some bryophyte species have been observed to great depths (Light & Heywood, 1973; Light 1975; Priddle, 1980a). Most early records of deep-water bryophytes were made on Swedish lakes and these were reviewed by Persson (1942). Several records of deep-water mosses in lakes in America were published during the 1930s, for example Williams (1930) reported *Drepanocladus fluitans* and *Fontinalis antipyretica* at 19 m in Crystal Lake, Wisconsin; Juday (1934) observed these two species and *Chiloscyphus rivularis* at 20 m in the same lake. Light and Smith (1976) further reported a number of mosses in deep-waters from the highest Scottish lochs.

Light and Heywood (1973) reported mosses growing in deep-water in Antarctic lakes. The underwater observation team found mosses growing in 6 out of the 12 lakes surveyed and recognized two distinct communities, one consisting of *Amblystegium* species and a second community consisted of *Drepanocladus* sp. cf. *aduncus* and *Calliergon sarmentosum*.

Some characteristic morphological changes can occur in relation to environmental conditions. Flooding of some mosses results in marked morphological changes. Light and Heywood (1973) observed that in one of the lakes under study in Signy Island an increase in stem length with depth, ranging from 10-20 cm at 5 m to 30-40 cm at 10 m. Priddle (1979) has reported that there is a significant difference of leaf size and shape

of the two aquatic moss species *Calliergon sarmentosum* and *Drepanocladus* cf *aduncus* from that of their terrestial counterparts. Seppelt and Selkirk (1984) have found that in the Antarctica (near the Australian Casey Station) naturally-etiolated shoots of *Bryum argenteum* have leaves, which become longer and narrower and whose cell size increases notably, when compared to normal leaves of the same taxon.

1.3 Morphology and growth form

1.31 Gametophyte

In spite of the generally small size of the gametophyte, understanding of the structure has been made possible through the use of electron microscopy. In this context Schofield (1981) has made a complete review of the moss gametophyte. In all mosses the gametophyte consists of two main parts; stem and leaves. A third less pronounced component is the rhizoid system which resembles closely the protonema in structure and is developed to a varying extent (Watson, 1964 & Schofield, 1981).

1.311 Leaf

The leaves always have a single layer of cells. They are generally unistratose except in the costa. However, in the mosses *Sciaromium* and *Scouleria* the leaf margin is multistratose (Schofield, 1981). The cells of the costa in many mosses are more elongated than those of the body of the leaf which are usually quadrate and isodiametric.

In the species *Rhizofabronia* the margin is ciliate, while in *Tayloria laciniata* the margin is elongated into the teeth like protection. In *Mnium* and *Bryum* species the margin of the leaves are thickened.

Apices of the leaves range from acute to acuminate. In some species the apex is extended as a long colourless awn. The cells that are at the

angles of the leaf where it joins the stem, are often different in shape and colour. In some species like *Hypnum cupressiforme* they are smaller than other parts of the leaf.

In most of the mosses the first formed leaves of the shoots are borne in three ranks, but when the shoot elongates and more leaves are formed this three-ranked arrangement can be lost. However, leaves borne in three regular rows are common. In some mosses e.g. in *Paludella* and *Drepanophyllum*, the leaves are arranged in five rows. In other mosses the leaves are in three regular rows (Schofield & Hébant, 1984).

1.312 Rhizoids

In most mosses, rhizoids are formed on the stem and in some species they are also formed on the leaf base as seen in *Tortula princeps* and *Scouteria aquatica*. In *Drepanocladus*, *Plagiothecium*, and *Calliergon*, rhizoids are formed near the leaf tip (Crundwell, 1979). In some mosses (pleurocarp) rhizoids are abundant and they penetrate into the substratum for anchorage. Odu (1978a, b) reported that mosses which grow on hard surfaces produce more rhizoids than when they grow among vascular plants. However, the acrocarpous mosses have rhizoids in contact with the substratum whether it is soil, bark, wood or rock.

Microscopic examination of rhizoids showed that in the vast majority of mosses they are uniseriate, multicellular, branched filaments with oblique cross walls. The cell walls are smooth, but sometimes markedly papillose (Hirohama & Iwatsuki, 1980).

1.313 Protonema

Protonema grow from the spores under illumination and moist conditions. In many moss species two cell types can be identified in the

branching filamentous protonemal stage. First is the chloronema produced from spore germination or tissue regeneration (Ashton *et al.*, 1979) containing many protoplasts and cross walls. The second is caulonema, formed after a few days of growth containing few chloroplasts and having diagonal cross walls. Each cell in these filaments has the ability to produce branches. Under ideal conditions these branches grow and form chloronemal filaments, which has more or less the same feature of the primary chloronema, called secondary chloronema or form buds that ultimately become gametophores (Ashton & Cove, 1977; Cove *et al.*, 1978, 1980; Bhatla, 1982; Knoop, 1984).

1.314 Gemmae

Gemmae are specialized multicellular units of vegetative reproduction in certain mosses and liverworts. They may take various forms, from being disc-or plate-like to being filamentous or heart shaped. When separated from the parent they develop into new individuals identical to the parent. Production of gemmae is common in many mosses and these gemmae occur in the gametophores. The gemmae can form on the apex of the leaf, on rhizoids, shoots or leaf axils (Dhingra & Chopra, 1985). Whitehouse (1980) reported that in pleurocarpous mosses gemmae production became more abundant under particular conditions such as high humidity or in shaded sites. In *Hookeria lucens* and *Plagiothecium laetum* numerous gemmae are developed when they grow submerged, while the gemmae are absent in better-drained sites (Schofield, 1981). In culture the production of gemmae on protonema is widely observed and known in many mosses e.g. *Amblystegium serpens* (Selkirk, 1981), *Bryum riparium* (Whitehouse, 1980) and *Trematodon brevicalyx* (Dhingira & Chopra, 1983).

1.4 Growth-form

Growth-form of mosses has been classified by Gimingham and Robinson (1950), Horikawa and Ando (1952). It is based upon microenvironmental conditions, particularly moisture, at the time of growth. The studies of British mosses by Gimimgham and Robinson (1950) led to five growth-forms being recognized.

(1) Cushions, where the shoots arise to give a cushion-like appearance. They are most common in well drained habitats where relative humidity is low (*Bryum capillare*).

(2) Turfs; the main shoots are erect and have branches similar to, and parallel to the main stem, and form a continuous turf. They are not restricted to particular environments (*Barbula cylindrica*).

(3) Dendroid; the main shoots are erect, but the stem apex is terminated by a cluster of branches, forming miniature 'trees', dendroid forms are in sites where relative humidity is very high (*Mnium undulatum*).

(4) Mats; in which the stems creep over the substratum and are frequently attached to it by rhizoids. They are not environmentally restricted (*Brachythecium rivulare*).

(5) Wefts, in which the creeping shoots form a very loose interwoven carpet, and rhizoids, when present, are generally confined to basal portions of the main stem and they are least common on rock surfaces (*Hylocomium splendens*).

An additional growth-form recognized by Horikawa and Ando (1952) is the pendulous forms which are restricted to rock or bark in areas of high humidity. The base of the main stem is attached to the substratum and the main shoot hangs downward in an epiphytic habitat (*Neckera douglasii*).

1.5 Drought concept

I.51 General introduction

The ability of mosses to survive in varied conditions where environmental factors such as water stress (drought), light intensity and flooding can influence their growth and development has been of great interest to several workers. The structural adaptation of mosses to different environmental conditions has been studied by Proctor (1979) and the degree of plasticity in adapting to different climates by Longton (1979).

Proctor (1981) and Richardson (1981) have carried out an extensive discussion of the literature on the physiological behaviour of mosses. A lot of attention has been paid to the response of mosses to desiccation (drought) and the way desiccation induces damage. Rapid desiccation causes more damage than slow drying (Schonbeck & Bewley, 1981a; Moore *et al.*, 1982; Penny & Bayfield, 1982). The degree of damage caused by desiccation is seasonal (Dilks & Proctor, 1976). The continuous drying of mosses is more damaging than drying interspersed with moist periods (Schonbeck & Bewley, 1981b). When dried mosses are hydrated, subsquent desiccation is not followed by an immediate effect on metabolism (Busby & Whitfield, 1978). These authors also observed that considerable water loss may occur before any damage is apparent.

Hinshirs and Proctor (1971) and Smith and Molesworth (1973) have reported that the recovery of lower plants, including some mosses, from drought is accompanied by an enhanced respiratory burst. The authors are of the opinion that these plants have evolved physiological adaptations, which allow suspension of metabolism during drought periods, with a resumption when water is once more available.

Many mosses tolerate severe desiccation without irreversible changes of viability. A number of studies have been made on this phenomenon: Hinshiri and Proctor (1971), Lee and Stewart (1971), Gwózdz and Bewley (1975), Dilks and Proctor (1974, 1979) and Gupta (1977). These show that after rehydration the moss gametophytes are able to recover most of their metabolic activities.

Many publications have been made on a wide range of bryophyte species and their responses to desiccation of varying duration and severity. There are variations in their ability to survive desiccation, which range from very extremely tolerant species like *Tortula ruralis* (Bewley, 1973) to very low tolerance ones e.g. *Plagiochila spinulosa* (Dilks & Proctor, 1974). Although many mosses do not have structural adaptations to regulate water loss, they do process physiological adaptations, which allow them to suspend their metabolism during the drought periods as seen in *T. ruralis* (Bewley, 1973). Lee and Stewart (1971) have reported that in the two races of *Acrocladium cuspidatum* and *Hypnum cupressiforme* (wet and dry race) desiccation always resulted in steady decline in photosynthesis, but the decline was slower in drought-tolerant species than in drought-sensitive ones. They stated that the mosses which tolerate drought conditions were faster in recovery from desiccation than the drought-sensitive species. Willis (1964) and Lee and Stewart (1971) found that in some mosses which are drought tolerant the increases in the rate of respiration occur within hours of rehydration and then fall to near normal levels. Stiles (1960) demonstated that this enhanced respiration upon rehydration appears to be a general feature of droughted plants.

The extent of damage to the photosynthetic and respiration organelles during drying and re-hydration has been investigated by several workers (Dilks & Proctor, 1974, 1979; Gupta, 1977; DiNola *et al.*, 1983).

1.52 Water contents and drought stress

Water is a major factor controlling growth and distribution of mosses. The periodic hydration and desiccation to which many bryophyte species are subjected (Anderson, 1974) and the differences between species in tolerance of and recovery from desiccation (Dilks and Proctor (1974) suggest that a study of relationships between water status and net assimilation could provide an insight into the ecological tolerance limits of many moss species.

Gupta (1978) demonstrated that the initial rate of water loss could provide an indication of the desiccation resistance. Hinshiri and Proctor (1971), Lee and Stewart (1971) and Peterson and Mayo (1975) have reported that the net assimilation rate decreased with decreasing water content until a very low water content was reached at which no gas exchange was detected. Dilks and Proctor (1979) stated that the response of photosynthesis in relation to water content of bryophytes of dry habitats (e.g. *Tortula intermedia*, *Camptothecium lutescens*) show an optimum, with photosynthesis declining again at high water content, whereas respiration may be stimulated by water stress, but is unaffected by high water contents.

Measurement of the relationship between net assimilation and water content, however, provides information which is not readily comparable from one species to another since the same value for water content may reflect considerable differences in physiological status in different species (Anderson & Bordeau, 1955).

1.53 Drought stress and chlorophyll content

The percentage reduction of chlorophyll following desiccation is a good measure of desiccation resistance or injury in many brophytes (mosses)

(Gupta, 1978). The study provides a clear indication that one of the reasons for the susceptibility of photosynthesis to desiccation is the damage caused to the major photosynthetic pigments chlorophyll *a* and *b* by the treatment. The relative humidity (RH) has an important role in that the damage to pigments at 0% RH is greater than at 50% and 96% RH. The author also found that severe desiccation decreased the ratio of chlorophyll *a* to *b* and that the demage to chlorophyll *a* was greater than that to chlorophyll *b*.

1.54 Drought stress and protein content

Bewley (1972) reported that polyribosomes level decreased during desiccation of the moss *Tortula ruralis*. A rapid resumption of protein synthesis on the conserved polyribosomes was shown after re-hydration. On the other hand Hsiao (1970) and Nir *et al*. (1970) found that water stress in higher plants showed an irreversible change in polyribosomes integrity which is in contrast to those of mosses.

Desiccation-tolerant mosses lose their capability for protein synthesis on drying (Bewley, 1972, 1973). Gwózdz *et al*. (1974) reported that in *Tortula ruralis* the extent of loss of polysomes is influenced by the speed at which desiccation occurs. The slow water loss causes complete depletion of polysomes, whereas rapid water loss results in the retention of nearly 50% of the polysomes in the dried state. This loss of polysomes during desiccation is the consequence of ribosomal run-off associated with a failure of the reinitiation process (Bewley, 1979 & Dhindsa & Bewley, 1976, 1977). Gwózdz and Bewley (1975) and Tucker and Bewley (1976) have found that in the dried condition, *T. ruralis* (terrestrial moss) contains potentially active ribosomes and cytoplasmic rRNA and mRNA. Such studies show the effects of severe water stress on the synthesis of protein complex in tolerant mosses.

The speed at which desiccation is administered quite markedly affects the rate at which protein synthesis recovers. Even though rapid-dried mosses contain significant amount of polysomes (Gwozdz *et al.*, 1974) low-dried mosses resume protein synthesis at a faster rate (Gwozdz *et al.*, 1974). Studies by Bewley and Thorpe (1974), Bewley *et al.*, (1978) and Dhindsa & Bewley (1977) have shown that rapid desiccation is more damaging to cellular integrity. Loss of protein synthesis may be an effect of this disruption. Further studies by Oliver and Bewley (1984) demonstrated that newly synthesized RNA, i.e. that which is synthesized on rehydration following desiccation, is quickly processed into ribosomal sub-units, ribosomes, and polysomes. However, it takes longer for the new RNA components to be processed and utilised in protein synthesis after rapid-drying than it does after slow-drying.

Several aspects of metabolism are affected by water deficit, including inhibition of protein synthesis and changes in amino acid metabolism (Barnett & Nylor, 1966). Inhibition of protein synthesis and hydrolysis of existing protein result in profound changes in the concentrations of free amino acids in the tissue (Barnett & Naylor, 1966; Routley, 1966)

Proline accumulation as a result of drought stress appears to be a general phenomenon in higher plants. The ability for proline accumulation, however, differs between different tissues of plants (Singh *et al.*, 1973) and is strongly influenced by previous exposure to drought stress and genotype (Singh *et al.*, 1973). Stewart *et al.* (1966) stated that proline accumulation in the drought stressed plants resulted from an inhibition of protein and polysaccharide synthesis and a consequent channelling of amino acid and carbohydrate metabolism into the synthesis of proline.

Although proline is normally a minor component of the pool of free amino acids in glycophytes, it has been observed to accumulate under conditions of water stress. Thus Barnett and Naylor (1966) found that in a water stressed plant (Bermuda grass) there was a rapid increase in free proline, which accumulated to a level of 1.2 mg g^{-1} dry weight. Similar observations have been made for other species including Ladino clover (Routley, 1966), broad bean (Stewart *et al.*, 1966) and barley (Singh *et al.*, 1972). Singh *et al.* (1972) found that barley varieties differed in their capacity to accumulate proline under stress; resistant varieties accumulating higher levels of proline under water stress than non-resistant varieties.

Although some workers e.g. Singh *et al.* (1972, 1973) and Stewart and Lee (1974) reported positive correlations between proline accumulation and stress tolerance or adaptation to stress, the results of others such as Hanson *et al.* (1979, 1980) and Ferreira *et al.* (1979) indicate a negative correlation between the two characteristics. Proctor (1990) stated that when compared with vascular plants, mosses subjected to water stress show little change in pools of solutes such as proline, carbohydrates or organic acids.

Dhindsa and Matowe (1981) found that great increases occurred in superoxide dismutase (SOD) and catalase activities and decrease in lipid peroxidation in *Tortula ruralis* during slow drying. In contrast, the enzyme activities remained unchanged on rapid drying in *T. ruralis* and remained at low levels during either slow or rapid drying in *Cratoneuron filicinum*.

1.6 Phosphorus occurence and forms in the environment

Phosphorus is the eleventh most abundant element in nature, its average concentration in the environment being estimated as 0.1% by weight, and is thus classed as a trace element. P is classed as a macronutrient, but its content in plants is much less than that of N, K or Ca. P as a limiting element, however, is more important to plant growth than calcium and probably more important than potassium. Phosphorus generally occurs in nature in the oxidized form, either as phosphates or organic P compounds.

Phosphate can be divided into:

(1) Orthophosphates which are generated from the weathering of rocks or from biological metabolism or degradation.

(2) Polyphosphates (chain phosphates) which are produced by biological activity.

(3) Metaphosphates (ring phosphates) which also are produced by biological activity.

(4) Ultraphosphates (branched ring phosphates).

Orthophosphates and polyphosphates are frequently introduced into waters by man (Broberg & Persson, 1988).

Rigler (1973) categorized, the analytical define of phosphorus fractions, as follows

Tot P	total phosphorus
PP	particulate phosphorus 0.45 μm
SP	soluble phosphorus < 0.45 μm
SRP	soluble reactive phosphorus
SUP	soluble unreactive phosphorus

Particulate P (colloidal P) is derived from five sources:

1. Cells of plants, bacteria and animals.
2. Weathering products such as primary or secondary minerals.

3. Direct precipitation of inorganic P or adsorption into other precipitates.
4. Degradation and fragmentation of cells, providing organic detritus.
5. Flocculation of organic macromolecules, resulting in larger sized aggregates.

Particulate organic phosphorus in aquatic ecosystems dominates total organic P, and has been comprehensively reviewed by Broberg and Persson (1988).

Particulate organic P is the major constituent of organic P forms, it is, however, not readily available to plants. Dissolved organic P (DOP) can be regarded as the more important fraction because of its availability to the biota and its rapid turn-over.

Some pools of DOP do not undergo rapid hydrolysis by phosphatases, and these compounds may constitute a major part of the DOP pool. One such pool of DOP compounds are the nucleotides or polynucleotides. Six DOP fractions were obtained from sea water, three of which were identified as nucleotides or polynucleotides (Philips, 1964). Broberg and Persson (1988) reported that up to 4.2% of the total P in bogs was assigned to nucleic acid. Minear (1972) observed that up to 50% of high molecular weight DOP excreted from organisms was DNA or its fragments.

Hino (1989) found that 65% of DOP in lake waters have apparent molecular weights between 300-10,000 daltons. Less than 10% of the DOP estimated higher molecules greater than 10000 daltons. Addition of phosphodiesterases alone did not show P_i release from high molecular weight DOP compounds. Although a combination of phosphodiesterases and phosphomonoesterases increased the amount of P_i released by 30% when compared to release of P_i phosphomonoesterases alone.

1.7 Biologically available phosphorus

HPO_4^{2-} and $H_2PO_4^-$ are the forms of P which are the most commonly utilized by living organisms. They are the ionic forms of phosphorus which predominate and are present in relation to the pH. Many organisms have two uptake systems for phosphate, one being "diffusive" and the other "rapid" which occurs when the internal P concentration is low, and is presumably growth limiting (Ducet *et al.*, 1977) Energy is required for these two systems, but Whitton (1967) described colonies of *Nostoc* which appeared to take up P passively when the external concentration was very low.

Many different substrates can be utilized as P sources by algae and cyanobacteria (blue-green algae) which are capable of phosphatase activity (Ihlenfeldt & Gibson, 1975; Livingstone, *et al.*, 1983; Al-Mousawi, 1984; Mahasneh, *et al.*, 1990; Whitton, *el al.*, 1990, 1991; Islam & Whitton 1992). Broberg (1985) stated that the availability of different phosphorus compounds to algae is dependent on the algae enzyme pool, the phosphorus status of the algae, the orthophosphate uptake rate, the nature of the P compounds and environmental conditions.

1.8 Phosphatases

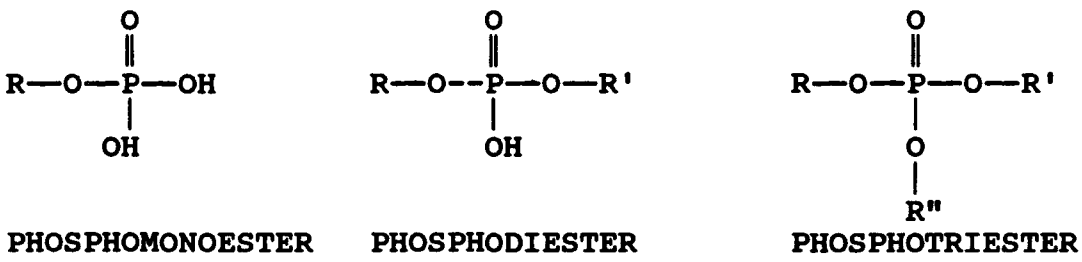
Phosphatases are enzymes which promote the degradation of complex phosphorus compounds into orthophosphate and an organic moiety. Phosphatases are believed to have an essential role and function in the nutrient dynamics of the aquatic environment (Jansson *et al.*, 1988).

1.81 Concept of the phosphatase

The term phosphatase is most often used for the enzymes which catalyse the hydrolysis of esters and anhydrides of phosphoric acid and is used synonymously with phosphomono-esterases abbreviated as PMEase which are a

group of enzymes which catalyze the hydrolysis of rich variety of phosphomonoesters (PMEase). Similar but functionally different enzymes are the phosphodiesterases or nucleases (PDEase) which are able to hydrolyze a wide range of nucleotides. PDEases are distinguished into two categories on their ability to hydrolyze 3′ and 5′ nucleotides. Phosphodiesterase I hydrolyzes nucleic acids to nucleocide 5′-phosphates and phosphodiesterase II hydrolysis nucleic acids to nucleocides 3′-phosphates (Kelly *et al.*, 1975). General formulae of different types of phosphate esters are shown in Fig. 1.1.

Fig. 1.1 General formulae of phosphate esters



McComb *et al.* (1979) suggested that phosphatases may be involved in metabolic processes other than hydrolysis, for example in transport of substances across membranes and synthesis of new organic phosphates.

The breakdown of phosphomonoesters by PMEase is the most common catalytic breakdown. The reaction mechanism is divided into four steps (McComb *et al.*, 1979).

(1) Non-covalent binding of the substrate to the enzyme (EH).

(2) Alcohol release from the complex and P_i becomes covalently bound to the enzyme forming a phosphoryl-enzyme compound.

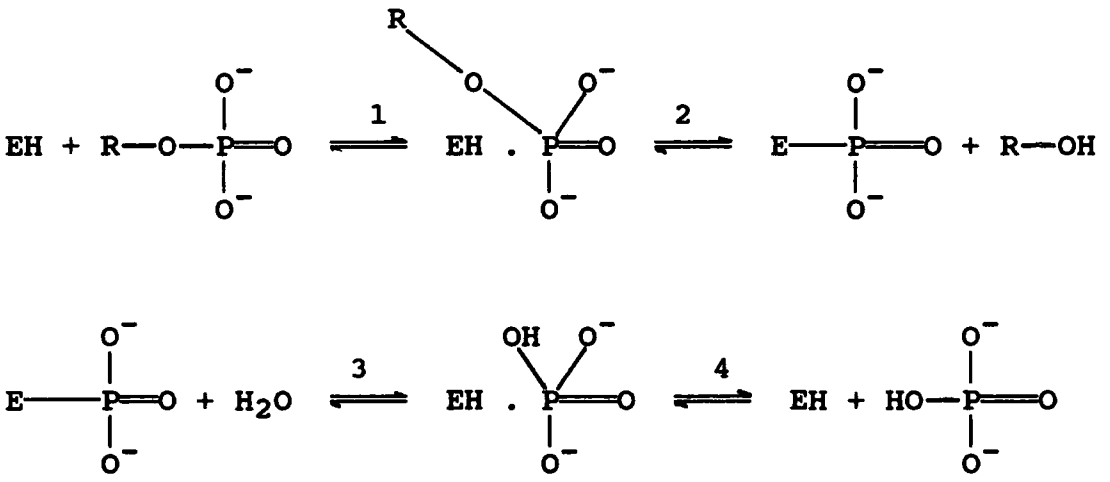
(3) Conversion of the phosphoryl-enzyme compound, through uptake of water, to a non-covalent complex.

(4) Release of P_i and regeneration of free enzyme.

Any of the steps 2-3 can be rate-limiting for the overall reaction (McComb *et al.*, 1979).

Fig. (1.2) shows the reaction schemes for all the enzymes involved in the catalyzed removal of P from phosphate esters, as described by McComb *et al.* (1979). The phosphatase activity will mainly depend on the type and concentration of the substrate and the enzyme. Other factors which have effect on phosphatase activity are temperature, ionic strength, pH and metal ions (McComb *et al.*, 1979).

Fig. 1.2 Reaction scheme for the enzyme catalyzed P of phosphate esters as described by McComb *et al.* (1979).



1.82 Sources of phosphatases

Phosphatases have been observed in bacteria, algae, fungi, bryophytes and higher plants (Siuda, 1984; Press & Lee, 1983).

1.821 Bacterial phosphatase activity

Bacteria are one of the most important contributors to environmental phosphatase activity. Most of the investigations on PMEase structure and function have been done on *E. coli* (McComb *et al.*, 1979). PMEase also has been seen in bacterial strains, which however isolated from lake waters (Jones, 1972a; Chrost *et al.*, 1984; Halemejko & Chrost, 1984). Early studies on aquatic bacterial phosphatases were demonstrated with marine species (Thompson & Macleod, 1974a, b; Hassan & Pratt, 1977) where it has been shown that marine bacteria appear to have PMEase located in the periplasmic space.

1.822 Cyanobacterial and algal phosphatase activity

Phosphatase activity has been found in all major groups and numerous species of Cyanobacteria (blue-green algae) and algae, though not universal (Healey, 1982). Phosphatase activity has been frequently demonstrated in cultured Cyanobacteria and algae (Kuenzler, 1965; Kuenzler & Perras, 1965; Healey, 1973; Doonan & Jensen, 1980; Flynn *et al.*, 1986; Grainger *et al.*, 1989; Whitton *et al.*, 1990, 1991; Islam & Whitton, 1992).

Phosphatase activity has been located on the cell surface and in cell membranes (Brandes and Elston, 1956; Kuenzler and Perras, 1965; Moller *et al*, 1975; Flynn *et al.*, 1986). The release of extracellular enzymes in algal cultures has been reported (Healey, 1973; Wynne, 1981; Siuda, 1984; Grainger *et al.*, 1989; Whitton *et al.*, 1990, 1991; Islam & Whitton, 1992). Phosphatase activities in aquatic environments have been attributed to algae and were detected in phosphorus deficiency (Jansson *et al.*, 1988).

1.823 Phosphatase activity in higher plants

It has been reported that most higher plants, while rich in non-specific and specific acid phosphatases, do not contain any appreciable

alkaline phosphatase activities (McComb *et al.*, 1979; Lee, 1988; Slatter, 1989; Gabbrielli et al., 1989). Acid phosphatase activity has been reported in a number of plant tissues including roots. This activity was localized in apical meristems and outer surface cells (Esterman & McLaren, 1961; McLaren & Gahan, 1970; Shaykh & Roberts, 1974).

Chang and Bandurski (1964) suggested that plant root phosphatases may be involved in the mineralization of organic phosphorus present in the soil or in added organic materials. Tarafdar and Claassen (1988) stated that the amount of P hydrolysed by clover root phosphatases surpassed the amount of P taken up by the plant by a factor of 20.

1.824 Phosphatase activity in mosses

Very little is known about phosphatase activity in bryophytes. The study made by Press and Lee (1983) showed that acid phosphatase activity was detectable in all 11 *Sphagnum* species tested from a range of habitats where the level of activity was found to be related to their phosphate nutrition status. The highest acid phosphatase activity was in the oligotrophic species with the lowest tissue phosphorus concentrations. Conversely, low acid phosphatase activity was reported in species from the more mesotrophic and eutrophic sites, with higher tissue phosphorus. They suggested that the enzyme in *Sphagnum* behaves similarly to that from higher plants in relation to the phosphate supply.

1.83 Acid phosphatase (AcPA) and effect of ions

Phosphatases typically have optimum hydrolyzing capacity at different pH values and hence the common separation into acid and alkaline phosphatases. Acid phosphatases are enzymes of wide specificity which cleave phosphate-ester bonds and thus play an important role in the mineralization of organic phosphate (P) in the environment, where their

activity may be correlated with low levels of free inorganic ions (P_i) (Spiers & McGill, 1979; Appiah & Thomase, 1982). Acid phosphatases have their optimum activity below pH 7.0, generally between pH 4.0 and 6.0; however that does not mean that they are totally inactive at, or do not tolerate, other pH values. These activities have been found as extracellular and cell-bound enzymes in some organisms (Siuda, 1984). In Lake Gårdsjön (acidified lake) (Jansson *et al.*, 1981) four different acid phosphatases, based on their molecular weights, were found. Møller *et al.* (1975), Wynne (1977), Schmitter and Jurkiewicz (1981), Siuda (1984) have found acid phosphatases within the cells (cellular) rather than in contact with the surrounding medium. Møller *et al.* (1975) detected acid phosphatase activity in the cytoplasmic fraction of marine diatoms; Wynne (1977) found acid phosphatase within cells of *Peridinium cinctum*. Jansson *et al.* (1988) suggested that acid phosphatase synthesis is generally not inhibited by P_i and that acid phosphatases are probably constitutive enzymes produced for internal P-metabolism.

Acid phosphatases are non-metallic enzymes, which are not activated by divalent cations, and are specifically inhibited by fluoride (Cembella *et al.*, 1984a). An investigation carried out by Juma and Tabatabai (1988) into inhibition of acid phosphatase in plants by metal ions revealed that Zn (II) and W(VI) had little effect on acid phosphatase activity in maize roots, but these ions inhibited activity in soybean roots by 25% and 62%, respectively. Hasegawa *et al.* (1976) observed that Hg (II), Cu (II), Fe (III), Zn (II) and Co (II) inhibited acid phosphatase activity of wheat roots by 100, 82, 82, 46, and 31%, respectively. Willett and Batey (1977) stated that in serpentine-tolerant strains of *Festuca rubra*, *in vivo* activity of these enzymes was differently affected by external Ca^{2+} concentrations, but not by Mg^{2+} or Ni^{2+}, which are considered among the factors responsible for serpentine toxicity (Proctor & Woodell, 1975)

Metal ions inhibit enzyme reactions by complexing the substrate, by combining with active groups of the enzyme or by reacting with the enzyme-substrate complex. The mode of inhibition also depends on the nature and size of ions (Alverez, 1962).

1.84 Temperature dependence

Phosphatases have Q_{10} values between 1.5 and 3.0 and temperature optima between 30 and 60°C, which is above the temperature of the natural environment (Jansson *et al.*, 1988). Huber and Kidby (1984) have made several investigations on natural waters and algal cultures, all data showed that the optimum temperatures were between 25-50°C. Grainger *et al.* (1989); Whitton *et al.* (1990, 1991); Islam and Whitton (1992) have tested the influence of temperature on phosphatase activities on some cyanobacteria (blue-green alga) species and found optimal temperatures for these species phosphatase activities were between 30-42 °C.

1.85 Phosphatase activity as a phosphorus deficiency indicator

Phosphatase activity has been used as a phosphorus deficiency indicator (Pettersson, 1980, 1985; Gage & Gorham, 1985). Aaronson and Patni (1976) demonstrated that secretion of acid phosphatase activity increased after the addition of glucose-1-phosphate and glucose-6-phosphate. Press and Lee (1983) found that acid phosphatase activity in 11 *Sphagnum* species was negatively correlated with tissue phosphorus concentration. Enzyme activity was reduced by phosphate enrichment and increased by phosphate starvation. Kuenzler and Perras (1965) and Fitzgerald and Nelson (1966) stated that P-limited marine and freshwater algae produced alkaline phosphatases and that production stopped when the algae were P-sufficient. These two papers were the basis for the use of

algal alkaline phosphatases as biological indicators of the P-status of the environment.

Phosphatase activity as a phosphorus deficiency indicator has been studied and tested in several cyanobacteria (blue-green alga) species and it has been found that alkaline phosphatases were increased when P was limiting (Grainger *et al.*, 1989; Whitton *et al.*, 1990, 1991; Islam & Whitton, 1992). Acid phosphatase activity may, like alkaline phosphatase activity, increase at low phosphate concentrations (Olsson, 1990). Aaronson and Patni (1976) found that both acid and alkaline phosphatases were repressed or inhibited by extracellular inorganic phosphate. The investigation by Kuenzler and Perras (1965), stated that only one of a sample of marine algal species showed increase in the acid phosphatase activity after increased phosphorus deficiency. Price (1962) reported that the synthesis of acid phosphatases by *Euglena gracilis* was repressed by phosphate. Acid phosphatase may thus have a role as a deficiency indicator, as does the activity of alkaline phosphatases. In general, however, acid phosphatase activity has been found to be less regulated by phosphorus concentration as compared to alkaline phosphatase activity (Kuenzler & Perras, 1965; Lien & Knutsen, 1973; Patni *et al.*, 1977; Wynne, 1981).

1.86 Substrate affinity

The ability for phosphatases to combine with and hydrolyze their substrates is given by the K_m value (Michaelis-Menten Constant). K_m is the substrate concentration when the reaction proceeds at half of its maximum speed. A low K_m means that the enzyme has higher affinity to the substrate and the opposite for high K_m. Therefore, the use of the Michaelis-Menten equation for a mixture of enzymes, which all hydrolyse the same substrates is incorrect from a theoretical standpoint. However, from

a practical standpoint, a group of enzymes can be characterized by calculating a Michaelis-Menten constant. K_m varies with substrate structure, indicating that the phosphatase activities are not substrate specific (Pettersson & Jansson, 1978).

Other factors which significantly influence K_m are pH and temperature. Usually the K_m values reported for acid and alkaline phosphatases are between 10^{-6} and 10^{-4} M. Pettersson (1980) found that the K_m of the phosphatases varied annually by an order of magnitude in Lake Erken, with the lowest values in situations of phosphorus deficiency. He concluded that phytoplankton adapted themselves to low phosphorous supply not only by increasing their enzyme production but also by producing enzymes with improved the ability to utilize low substrate concentrations.

1.9 The Asir mountains region

Intensive investigations for bryophyte communities have not been done, although useful studies by Vesey-Fitzgerald (1955) give a good background on the environment and vegetation. More recent studies (Frey and Kürschner, 1984; Kürschner, 1984; Pursell and Kürschner, 1987) have thrown more light on the bryophyte communities in the Asir Mountains. These studies describe the extent of bryophytes and also the identification of species.

1.10 Aims

Very little is known about Tanumah, the study area, in terms of the relationship between the vegetation and the environmental features. *Hydrogonium fontanum* grows in abundance in the waterfall and adjacent to it where it appears to be involved in tufa-deposition. The moss plays an important role in the ecology of this specific habitat.

The aims of the research project were:

1) To examine the morphology and growth stages of *H. fontanum*.

2) To quantify the influence of environmental factors, on the growth.

3) To study the ability of the moss to mobilize organic phosphate through phosphatase activity associated with its different developmental stages and to examine the influence of some environmental factors in this activity.

2. MATERIALS AND METHODS

2.1 Computing

Three computing systems were used for the study. Routine calculations were performed using the QUATTRO Pro spreadsheets programme on a Research Machines IBM PS/2 model 30 (Microsoft). Statistical analyses were carried out using SPSSX running on an AMDAHL 470/V8 Mainframe operating under the Michigan Terminal System. Graphic output was carried out using the SIGMA PLOT (4.1) suite of software.

2.2 Laboratory analytical methods

2.21 Light

Measurements of light were made with a Macam Q101 lightmeter (Macam Photometrics Ltd, Scotland). All incident light was measured as photosynthetically active radiation (PAR); the readings were recorded as photon flux density (μmol photon m^{-2} s^{-1}).

2.22 pH

pH measurements were carried out using an Ingold combination WTW E50 electrode and EIL meter (model 7050). The probe was calibrated with BDH standard buffer solutions, prepared in MilliQ water, immediately before measurement was taken; the pH of the buffers was arranged so that one was higher and one lower than the pH of the solution under investigation.

2.23 Mass determination

Mass was measured on an Oertling R51 balance to five decimal places. All references to weight refer to mass.

2.24 Colorimetric analysis

All large volumes (more than 300 μl) for colorimetric analysis were measured using a LKB Biochem Ultrospec 4050 Spectrophotometer. Glass cuvettes with a path length 1.0 cm were used for all readings between the visible and infra-red range of the spectrum.

The MCC Plate Reader was used for a large percentage of the colorometric analysis for phosphatase activity. Assays used pNPP and bis-pNPP as substrates, Absorbance Program 1 and Filter Code 1 (405 nm) were used.

2.25 Fluorimetric analyses

BAIRD-ATOMIC Fluoripoint Spectrofluorimeter was used for fluorescence measurement for phosphatase activity when using 4-MUP as a substrate. polystyrene cuvettes with a path length 1.0 cm were used for all fluorescence readings at wavelengths of 440 nm emission, 356 nm excitation.

2.3 Standard culturing techniques

2.31 Culture vessels

For liquid media the vessels used for culturing were 100-ml flasks (Pyrex glass). Solid media were prepared either in pre-sterilized disposable plastic petri dishes or deep glass containers (Pyrex storage dishes).

2.32 Cleaning of glassware and utensils

All the glassware was washed in tap water with 2% Decon (Decon Laboratories Ltd, England) detergent, then place in 4% HNO_3 solution for at least 12 h and then rinsed in distilled water six times prior to drying at 100°C. Silicon rubber stoppers (Sanko Plastics Co., Japan) used for

culture flasks were soaked in 2% Decon were rinsed in hot water and then rinsed six times in distilled water before use. All volumetric glassware was dried at room temperature; plastics were dried at 40°C.

2.33 Media

2.331 Stock solutions

All salts were BDH Analar grade stock prepared in MilliQ water and then kept in the refrigerator at 4.0°C until required. The stock solutions were renewed every 3 months.

2.332 Growth medium (Chu 10 G)

Chu 10 G medium was used as the nutrient growth medium modified (Table 2.4) from the original No 10 medium of Chu (1942). Modifications included increased concentrations of magnesium and calcium, with the addition of fluoride, in order to make the solutions comparable in composition and concentration to those occurring at the site (Whitton *et al.*, 1986).

To prepare one litre of medium, *ca* 500 ml of distilled water were taken in a beaker, with 0.6 g HEPES added as buffer. The pH dropped to *ca* 5.0 and this was adjusted to 7.6 by the gradual addition of 1.0 M NaOH. This buffered solution was transferred into a one litre volumetric flask and stock solutions were then added and the final volume was made up with distilled water. Trace elements were added to the medium as AC microelements (Kratz & Myers, 1955) with low Mn. EDTA was used as a chelating agent in order to prevent precipitation of iron in the medium.

2.333 Phosphatase assay medium

This was a further modification of Chu 10 G medium, where N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) was removed, KH_2PO_4 was replaced with KCl and the iron was halved.

Table (2.1) Concentration of mineral and salts in Chu 10 G and assay medium.

Salt	Chu 10 G		Assay Medium	
	mg l^{-1}	μM	mg l^{-1}	μM
$MgSO_4.7H_2O$	100.00	401.60	25.00	101.40
$Ca(NO_3).4H_2O$	86.40	365.90	0	0
$NaHCO_3$	15.85	188.60	15.85	188.60
$CaCl_2.2H_2O$	28.00	190.45	35.83	243.7
$FeCl_3.6H_2O$	2.42	8.97	1.21	4.45
Na_2F	0.05	1.20	0	0
Na_2-EDTA.$2H_2O$	3.18	8.97	1.67	4.45
KH_2PO_4	7.80	57.15	0	0
H_3BO_4	0.72	115.60	0.72	11.56
$MnCl_2.2H_2O$	0.045	2.28	0.04	2.28
Na_2MoO_4	0.007	0.02	0.007	0.02
$ZnSO_4.7H_2O$	0.056	0.19	0.056	0.19
$CuSO_4.5H_2O$	0.02	0.07	0.02	0.07
$CoSO_4.7H_2O$	0.01	0.03	0.01	0.03
$NiSO_4.7H_2O$	0.038	0.03	0.038	0.03
NaOH	*ca* 60.00	1500.00	0	0
HEPES	600.00	2517.00	0	0

Table (2.2) Elemental composition of media.

Element	Chu 10 D medium		assay medium	
	mg l^{-1}	μM	mg l^{-1}	μM
P	1.00	32.28	0	0
S	19.73	615.37	3.2607	101.70
Cl	14.45	398.06	20.43	562.80
Na	40.10	171.70	4.5432	197.60
N	10.26	708.25	0	0
K	2.2431	57.38	2.2431	57.38
Ca	22.30	556.40	9.7674	243.80
Mg	9.86	405.68	2.4645	101.40
Mn	0.0120	0.218	0.0120	0.218
Fe	0.5010	8.97	0.2505	4.48
Ni	0.0019	0.03	0.0019	0.03
Co	0.0022	0.03	0.002	0.03
Cu	0.005	0.07	0.005	0.07
Zn	0.0126	0.19	0.0126	0.19
B	0.1249	11.56	0.1249	11.56
Mo	0.0026	0.02	0.0026	0.02

2.34 Sterilization

The medium was sterilized immediately by autoclaving for 15 min at 120°C and 15 P.S.I. and then allowed to stand for at least 12 h prior to use to allow equilibration with the atmosphere. In the case of solid media, 1% (w/v) agar was added before autoclaving. After autoclaving and cooling to about 40°C, growth medium were poured into the pre-sterilized petri dishes in a laminar flow cabinet. When using the deep glass containers the agar was added to the container before autoclaving.

2.35 Maintenance and subculturing

Moss stock cultures were maintained either in 50 ml liquid medium incubated in the 25°C growth room under continuous light (*ca* 20 μmol photon m^{-2} s^{-1}) or on slope agar in test tubes, which were incubated in very low light in a cooled incubator at about 10°C. Subcultures to fresh medium were made after 3 months. Stocks for experimental purposes were maintained either in the growth room or in the shaker tank under continuous light of 85 μmol photon m^{-2} s^{-1}.

Subculturing for stock culture was done either by picking up small aliquots of the moss material with sterilized forceps or wire loops and inoculating to fresh medium under aseptic conditions using a laminar air flow cabinet.

2.351 Inoculation using moss shoots

Uniform inocula of the moss shoots were achieved by inoculating ten tips (1 cm) for each replicate of liquid and solid media. The size of the inoculum was determined at the start of the experiments by measuring dry weight and chlorophyll content to ensure that the inocula were uniform.

2.352 Inoculation using protonema

The filamentous protonema were homogenized in growth medium by taking it in a plastic syringe and passing through sterile needles (Gillette Surgical Ltd, U.K) several times. Then 1.0 ml of the homogenate was introduced to 25 ml of medium in a 100-ml conical flask. The protonema were transported by automatic pipette fitted with a sterile plastic tip.

2.36 Incubation

Experiments were carried out in batch culture under continuous light either in the growth room illuminated from above the cultures by white

fluorescent tubes or in the thermostatically controlled shaker tanks illuminated from below by warm white fluorescent tubes giving a light intensity between 85-100 μmol photon m^{-2} s^{-1} depending upon the position of the flasks in the tank. In order that each flask received an equal amount of light, positions were randomized every 12 h. Dark conditions were achieved by wrapping black painted flasks with two layers of aluminium foil and one layer of black polythene. A shaking mechanism moved the flasks through a horizontal distance of 40 mm, 60 times min^{-1}. Temperature was maintained at 25°C (based on the results of preliminary experiments carried out to determine the optimum growth temperature).

2.4 Isolation and purification of moss material

2.41 Chemical treatment

The moss shoots were dipped for a few seconds in different concentrations (0.5-5%) of sodium hypochlorite as a means of removal of surface contamination and then they were left in both fresh sterile liquid and solid media for a week prior to being examined under a microscope for contaminants.

2.42 Physical isolation

Alga-free culture of gametophyte shoots

The first stage of the technique used to obtain alga-free cultures of leafy moss gametophytes was as follows:

a) Culturing of leafy shoots of the gametophytes in Chu 10 G solid medium, the culture being incubated in the growth room for one week.

b) The culture stems (shoots) were then taken from the medium and shaken violently in several changes of distilled water for about one hour.

c) Stems were examined under the microscope and those which were still contaminated with algae were discarded.

d) The remaining stems were planted in fresh sterilized media and incubated for a week.

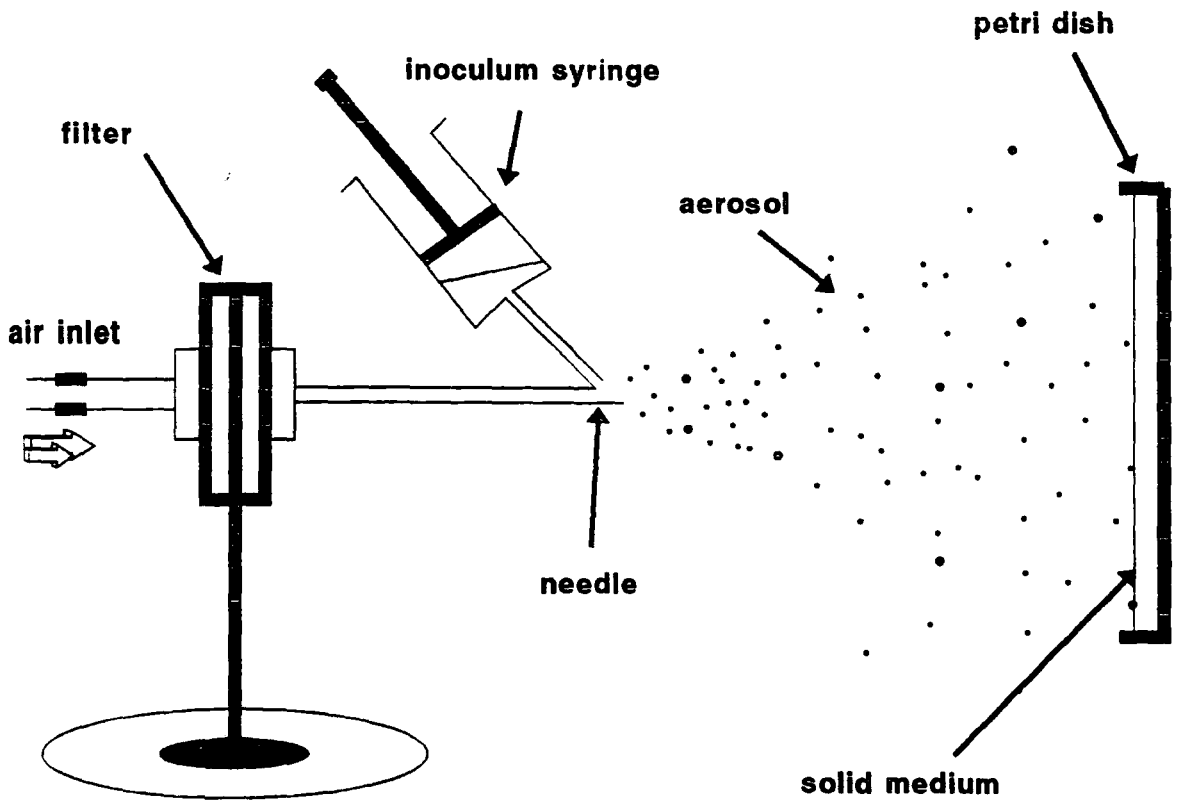
e) The apices of the stems were then cut off with flamed fine scissors and planted in fresh sterilized medium.

f) The above techniques were repeated several times on the same sample.

2.43 Spray planting

As long as the moss produced massive protonema and gemmae in liquid medium, it was found most convenient to use the spray planting methods (Fig. 2.1) in order to get axenic cultures. The protonema was centrifuged (Sorvall RC-5B refrigerated superspeed centrifuge) in sterilized medium several times and the supernatant poured out. Then the protonemal pellets were suspended in sterilized medium and sonicated (Soniprep 150 MSE) for 30 s to break them and to form an even suspension. The suspension was placed in pre-sterilized syringes and a jet of sterilized air was allowed to pass over the drop of sterilized medium which contained the protonemal suspension. The air forced the droplets to spread and they were then caught on sterile agar media. When the material was tested under the microscope on the bacteriological test medium, protonema were still contaminated with bacteria and fungus.

Fig. 2.1 Apparatus used in an attempt to obtain axenic culture of protonema



2.44 Antibiotic treatments of protonema and gemmae

The following antibiotic treatments in different combinations and concentrations were carried out in order to obtain axenic cultures.

Group 1	Group 2
1 benzyl penicillin - 504	1 chlorotetracycline
2 streptomycin - 504	2 polymixin B
3 neomycin - 504	
4 chloramphenicol	

Group 1 antibiotic mixtures were prepared (Droop, 1967) as a dry mix and were then dissolved in MilliQ Water just before use. Group 2 antibiotic mixtures were dissolved individually. Sterilization of antibiotics mixture solutions was carried out by passing them through an autoclaved 0.2 μM membrane filter in a plastic Swinnex filter holder. Antibiotics were then introduced to the moss protonema and exposure was given for 24 h in the dark.

The protonema were then centrifuged five times in sterile fresh medium in autoclaved centrifuge tubes and were subcultured into sterile fresh liquid medium under aseptic conditions in a laminar flow cabinet (Microflow Pathfinder). The cultures were incubated in the growth room for at least 10 days at 25°C prior to use.

Table 2.3

Antibiotic mixtures (group 1)

Antibiotics	Antibiotic conc. ($\mu g\ ml^{-1}$)		
	I	II	III
benzyl penicillin-504	400	200	80
streptomycin-504	80	40	16
neomycin-504	10	5	2
chloramphenicol	10	5	2
Total	500	250	100

Table 2.4 (Group 2)

Antibiotic	Antibiotic conc. ($\mu g\ ml^{-1}$)						
chlorotetracycline	1.0	5.0	10.0	20.0	50.0	100,	300
polymixin B	1.0,	5.0,	10.0,	20.0	50.0	100	300

2.45 Tests for purity

Tests were carried out after the isolation and antibiotic purification and also throughout the period of experiments; this was carried out on Bacteriological Agar testing petri dishes (Hoshaw & Rosowski, 1973). The media were:

1) nutrient broth
2) SST
3) peptone-glucose agar
4) yeast extract agar

Samples of cultures were taken and plated onto these various media in petri dishes, which were then incubated for two weeks at 32°C in the dark. After this period the petri dishes were examined visually, if there was no bacterial growth, further microscopic examinations were carried out. If on microscopic examination there were no microorganisms associated with moss protonema then the culture was considered as axenic.

2.5 Microscopy and photomicrography

Morphological examinations of moss field material were carried out on site using a Cooke-McArther field microscope fitted with micrometer eyepiece. Moss cultures were examined routinely using a Nikon Fluophot microscope fitted with a 35-mm camera. Small samples of moss protonemal and gemmae mat were mounted on microscope slides in a drop of medium in which they had been growing. When the filaments were densely interwoven they were gently teased apart with fine dissecting needles.

2.6 Water stress techniques

2.61 General methods for obtaining water stress

Polyethylene glycol (PEG) has been used successfully as an osmoticum to induce water stress in grown plants (e.g Michel & Kaufmann, 1973; Heath *et al.*, 1985; Kaufmann & Eckard, 1971; Resnik, 1970). Accordingly moss material grown in water solutions (growth medium) were subjected to water stress by introducing the moss material into the growth medium which were supplemented with PEG. Solutions of PEG with varying water potential were prepared according to Michel and Kaufmann (1973).

Table (2.5) Relationship between concentration (g l^{-1} water) and water potential of PEG solutions. (The effect of growth medium on potential is neglected)

PEG Concentration	Water Potential
g l^{-1} growth medium	10^6 Pa
196	-0.5
289	-1.0

2.62 Proline determination

Proline determination was carried out according to the methods described by Bates *et al.* (1973) and Troll and Lindsley (1955).

2.621 Reagents

Acid ninhydrin was prepared by dissolving 1.25 g of ninhydrin in 30-ml glacial acetic acid and 30-ml 6M phosphoric acid. The mixture was warmed to 70 °C in a water bath to insure that the ninhydrin was completely dissolved. The reagent was kept cool at 4°C. According to Troll and Lndsley (1955), it remains stable for 24 h.

2.622 Procedure

Samples of moss materials were ground in a mortar and pestle with acid-washed sand in 25 ml of 3% aqueous sulphosalicylic acid. This was then filtered through Whatman No 1 filter paper. Two ml of filtrate were added to 0.15 g Permutit in a test tube and shaken vigorously to remove the interfering basic amino acids. Two ml of glacial acetic acid and 2 ml of acid ninhydrin were added to the 2 ml of the filtrate, then heated in a water bath at 80°C for one hour, and the tubes were then removed from the heat and cooled in an ice-bath to terminate the reactions. A pink colour should result when proline reacts with acid ninhydrin. Four ml of the reaction mixture was placed in a clean tube containing 4 ml of the toluene and were gently shaken for 30 s. The upper toluene layer was separated into another clean tube and left to stand. The absorbance was then recorded in a spectrophotometer (LKB Biochem Ultrospec 4050) at 520 nm against a toluene blank. The concentration of proline was determined from a standard curve prepared using L-proline. Calculation of the proline concentration was obtained on a fresh weight basis using the following equation:

[(μg proline/ml x ml toluene)/ 115.5 μg/ mol]/ [(g sample)/2] = μmol proline/g fresh weight material.

2.63 Protein determination

Samples of moss were ground using a pestle and mortar with 4 ml of Tris buffer [0.04 M Tris (hydroxymethyl) methylomine, pH 7.5 plus 0.1 M magnesium sulphate and 0.025 M EDTA]. The homogenate was transferred into a centrifuge tube and centrifuged for 10 min in a bench top centrifuge (Griffin Christ) at c. 2000 x g for 10 min.

2.631 Extraction of soluble protein

The supernatant was decanted and the water-soluble protein in this fraction was precipitated by adding an equal volume of 10% Trichloroacetic acid (TCA) followed by standing for 30 min in an ice bath. The protein was pelleted by centrifugation at c. 2000 x g for 10 min, and was washed by re-suspending in 2 ml of 5% TCA followed by re-centrifugation. The washed protein precipitate was eventually dissolved in 1.0 ml of 1 N NaOH by warming to 80°C in a water bath.

2.632 Extraction of insoluble protein

The insoluble protein formed part of the pellet from the original centrifugation. In order to remove contaminating chlorophyll the pellet was washed in the following procedure:
The protein pellets containing chlorophyll were decolourised by washing three times in 2 ml of 1:1 (v/v) mixture of chloroform and methanol followed by one wash in 2 ml of absolute methanol. At each washing stage the pellet was obtained by centrifugation as above. The protein was then dissolved as for the soluble protein.

2.633 Protein measurement

Protein was measured by the method of Lowry *et al.* (1951). Five ml of copper sulphate reagent made up as follows: (0.5 ml of 1% copper sulphate plus 0.5 ml of 2% sodium potassium tartrate and 50 ml of 2% sodium carbonate) was added to each dissolved protein mixture and allowed to stand for 10 min. After this, 0.5 ml of 1 N Folin and Ciocalteu's phenol reagent was added and the mixture allowed to stand for a further 30 min. The absorbance of the blue coloured solution was measured at 520 nm, using a spectrophotometer with distilled water as blank. The spectrophotometer readings were quantified using a plot of the readings obtained from

standard protein solution of known concentrations. Standard curve was prepared using bovine serum albumin (BSA).

2.7 Assay for phosphatase activity

2.71 Preparation of moss material for analysis of phosphatase assays

Moss protonema were separated from the growth medium by centrifugation in 50 ml non-sealable polyethlene centrifuge tubes, in a SS-34 8 x 100 ml angle head rotor, using a sorvall RC-5B refrigerated superspeed centrifuge at 8000 x g for 10 min. The supernatant was decanted and made up to the required volume with MilliQ Water. The centrifuged medium was filtered through GF/C Whatman filter and regarded as the extracellular phosphatase fraction.

The moss protonema pellet was washed twice and resuspended in assay medium. The volume required to resuspend the moss protonema pellet would vary depending upon the concentration of protonema required in the assay. Moss protonema were then homogenized by sonication (Soniprep 150 MSE) at an amplitude of 26 μm for 30 s. During sonication the protonema homogenate was cooled with an ice jacket. The suspension was examined under the light microscope for cellular damage.

2.72 Assay procedure for acid phosphatase activity

2.721 Using MCC Plate Reader

For cell-bound phosphatase activity 50 μl moss sample was pipetted via a Titertek 8-channel pipettman (EFLAR, Finland) into a 96-microwell plate (no. 96F, Inter Med, NUNC, Denmark). All the microwell plates had lids (no. 96L, Inter Med, NUNC, Denmark)., which reduced contamination, prevented evaporation and acted as an insulator against heat loss. 100 μl of standard buffer was pipetted into the microwells (50 mM final

concentration). The plate was then incubated at 25°C for 10 min. Then 150 µl of 0.5 mM pNPP or bis-pNPP was pipetted out into the microwells. A t=0 min was taken and subsequent readings at 15 min intervals for 60 min. There was no termination of phosphatase activity. Subsequently, a time course was plotted from the readings. A value for phosphatase activity was taken from the linear part of the constructed graph. A calibration curve using p-nitrophenol (pNP) at pH 10.3 was constructed between 0.005-0.05 µmol. Activity was expressed as µmol pNP mg dry wt^{-1} h^{-1}.

2.722 Using snap cap vials and universal bottles

Snap-cap vials and universal bottles were used for phosphatase experiments which can not be carried out in the plate reader i.e temperature, desiccation and measurement of shoot phosphatase activity experiments. In those experiments the same substrate and buffer concentrations used were the same as when using MCC plate reader. Into each of the universal bottles and snap cap vials, 1.6 ml of buffer was pipetted. To this, 3-4 shoots 1.0 cm in 1.5 ml assay medium (when using shoots) or 1.5 ml of protonema homogenate in assay medium were added. This was left to equilibrate for at least 10 min. Assays were initiated by the addition of 1.5 ml pNPP substrate (pre-incubated at 25°C) and run for 60 min. Termination of the assay was achieved by addition of 0.35 ml of NaOH. Each assay was carried out with 4 replicates and a control unless stated otherwise. The control assay was set up by the addition of NaOH before substrate. This allowed for any optical variation in the sample.

2.73 Effect of pH on phosphatase activity

For determining the effect of pH, two different buffers were used for each pH unit to compensate for any inhibition of phosphatase activity by

the buffers used (Table 2.5). The buffers were 50 mM (final concentration), which was chosen by a suitable concentration for buffering physiological media (Dawson *et al.*, 1986).

PMEase and PDEase activity was monitored using pNPP and bis-pNPP. For assaying them 50 µl of moss and 100 µl of buffer were used. This was incubated at 25°C for 10 min. After the incubation period 150 µl of pNPP substrate (pre-incubated at 25°C) was added. The assay ran for 60 min and was terminated by the addition of 30 µl of 4.95 M NaOH, resulting in a final pH of *c.* 12.8 +/- 0.1. At each pH a t=0 line was set up by addition of NaOH before pNPP, which compensated for any optical variation. For the above procedure, calibration curves were set up at the requisite pH values using pNPP between 0.005-0.05 µmol

Table 2.6 Buffers used to investigate the effect of pH on phosphatase activity. Two buffers were used at each pH value (A+B). For abbreviations, see page 4.

pH	Buffer	Final Conc (mM)	Set	Buffering Capacity	pKa at 20°C
3.0	DMG-NaOH	50	A	3.2-7.6	3.66 & 6.20
3.0	Glycine-HCL	50	B	2.2-3.6	2.35 & 9.60
4.0	DMG-NaOH	50	A	3.3-7.6	3.66 & 6.20
4.0	Succinic acid	50	B	3.8-6.0	4.18 & 5.60
5.0	DMG-NaOH	50	A	3.2-7.6	3.66 & 6.20
5.0	Succinic acid-NaOH	50	B	3.8-6.0	4.18 & 5.60
6.0	DMG-NaOH	50	A	3.2-7.6	3.66 & 6.20
6.0	Succinic acid-NaOH	50	B	3.8-6.0	4.18 & 5.60
7.0	HEPES-NaOH	50	A	3.2-7.6	3.55 & 6.20
7.0	DMG-NaOH	50	B	6.8-8.2	7.50
8.0	TES-NaOH	50	A	6.8-8.2	7.5
8.0	HEPES-NaOH	50	B	6.8-8.2	7.5
9.0	AMeP-NaOH	50	A	9.0-10.5	9.69
9.0	Glycine-NaOH	50	B	8.6-10.6	2.35 & 9.60
10.0	AMeP-NaOH	50	A	9.0-10.5	9.69
10.0	Glycine-NaOH	50	B	8.6-10.6	2.35 & 9.60
10.3	AMep-NaOH	50	A	9.0-10.5	9.69
10.3	Glycine-NaOH	50	B	8.6-10.6	2.35 & 9.60
11.0	CAPS-NaOH	50	A	9.8-11.1	10.40
11.0	Na_2CO_3-$NaHCO_3$	50	B	9.2-10.8	10.33

2.74 Assay procedure for acid phosphatase activity using 4-MUP substrate

The assay was carried out in snap-cap vials. Reagents (buffer, assay medium and substrate) were allowed to equilibrate in a room at 25 °C for 15 min. The assay was carried out at this temperature using the shaker tank. 1.6 ml of buffer of the required pH was pipetted in the snap cap vial. To this 1.5 ml of protonema homogenate in assay medium was added. This was left to equilibrate for 10 min. Assay was initiated by the addition of 0.1 ml 4-MUP substrate and run for 30 min. The assay was immediately filtred, using 2.5 cm GF/D glassfibre filters, in order to separate protonema from assay medium. Termination of the assay was achieved by addition of 1 ml of K_2HPO_4 terminator (0.1 M K_2HPO_4 plus 0.1 M NaOH and 5 mM EDTA) to 1 ml of the filtrate. A value for phosphatase activity was taken from the linear part of the constructed graph. A calibration curve using 4-methyl umbelliferone (MU) was constructed between 0.25-25 μM. Activity was expressed as μmol 4-MU mg dry wt^{-1} h^{-1}.

2.8 Analysis of total filterable phosphorus

Analysis of the phosphorus fractions of the moss and medium was made according to Eisenreich *et al.* (1975). In a suitably acidified solution sodium molybdate and potassium antimony tartarate react with orthophosphate to form molybdophosphoric acid which is then reduced to the intensely coloured molybdenum blue complex by ascorbic acid and determined spectrophotometrically at 882 nm wavelength.

stock solutions:

1. H_2SO_4 antimony: 53.3 ml of concentrated H_2SO_4 was mixed with 500 ml MilliQ water and cooled; 0.748 g of K(SbO) $C_4H_4O_6.^1/2H_2O$ was dissolved in H_2SO_4 solution and diluted to 1 l.

2. Molybdate: 10.839 g $Na_2MoO_4.2H_2O$ was dissolved in about 500 ml MilliQ water and diluted to 1 l.

3. Digestion Acid: 100.0 ml concentrated H_2SO_4 was mixed with 500 ml MilliQ water and diluted to 1 l.

Reagents:

1. Digestion Reagent: 6.0 g $K_2S_2O_8$ was dissolved in about 80 ml MilliQ water containing 10.0 ml digestion acid and diluted to 100.0 ml.

2. Mixed reagent I. 25.0 ml each of the H_2SO_4-antimony and molybdate stock solutions were mixed with 0.2 g ascorbic acid and diluted to 100.0 ml.

3. Mixed reagent II. 25.0 ml each of the H_2SO_4-antimony and molybdate stock solutions was mixed with 10.0 ml of digestion reagent and 0.2 g ascorbic acid and diluted to 100.0 ml.

Procedure:

I. Filtrable reactive phosphate

A suitable aliquot of sample was diluted to 25 ml with MilliQ water in a 125-ml conical flasks. Then 5.0 ml of mixed reagent II was added and mixed thoroughly.

II. Filtrable total phosphate

A suitable aliquot of sample was diluted to 25 ml with MilliQ water in a 125-ml conical flasks. 5 ml digestion reagent was added to the sample. The flask top was covered with aluminium foil and autoclaved at 121 °C for 30 min. The sample was cooled to room temperature and 5.0 ml of mixed reagent I was added.

Calibration curves were prepared by treating two series of phosphate standards, as for filtrable reactive phosphate and filtrable total phosphate.

2.9 Estimation of yield

2.91 Dry weight

Moss material was separated from the growth liquid medium either by using a very fine forceps for shoots or by centrifugation in the case of protonema. The moss materials were then washed three times in distilled water prior to transfer to pre-dried acid washed, snap-top glass vials, which were previously weighed. The open vials were then placed in the oven at 105°C for 48 h to allow the moss material to dry. The vials were then removed from the oven and immediately placed in a desiccator to prevent absorption of water. The dry weight of the moss material determined by re-weighing the vials with moss material. The weight with the moss subtracted form the weight of the vial gives the dry weight of the moss.

2.92 Extraction and estimation of pigments

2.921 Chlorophyll *a*

Determination of the chlorophyll *a* as a parameter of growth was carried out by the following procedure:

a) Extraction

The moss was harvested by vacuum filtration through Whatman GF/C glass fibre filter papers. 30 ml universal bottles with moss and solvent (90% methanol) were incubated in a water bath at 70°C for 10 min. to allow extraction of pigments. The pigment extract was filtered through glass

fibre filter paper and made to a standard volume of 10 ml. Soon after the extraction of chlorophyll the optical density was recorded at 665 and 750 nm against a solvent blank using a spectrophotometer. The samples were then acidified with 100 ml of 1.0 N HCl and incubated in the dark for one hour, and the absorbance read again at 665 and 750 nm.

b) Quantity of chlorophyll *a*

The following formula given by Marker *et al.* (1980), was used for the calculation of chlorophyll *a*.

$$\text{Mg chl } a = (A_b - A_a) \times \frac{R}{R-1} \times k \times \frac{v}{L}$$

Where Ab = absorbance of pigments at 665 nm before acidification minus absorbance at 750 nm

Aa = absorbance of pigments at 665 nm after acidification minus absorbance at 750 nm

R = maximum acid ratio (A_b/A_a)

K = 1000 x the reciprocal of the specific absorption coefficient (SAC) of chl *a* at 665 nm in 90% methanol.

V = volume of solvent used to extract the sample in ml

L = Path length of the cuvette in cm

Marker *et al.* (1980) recommended a specific absorption coefficient for chl a in 90% methanol of 77, and maximum acid ratio of 1.59 for 90% methanol in 1.0 N HCl, therefore:

$$\text{mg chl } a = 2.69\ (A_b - A_a)\ 12.99 \frac{V}{L}$$

2.922 Total chlorophyll extraction and estimation

Method of extraction of total chlorophyll pigments was the same technique as for chlorophyll *a* extraction. The extracts were made to a volume of 10 ml and the chlorophyll absorbance was determined by reading at

645 and 633 nm in the spectrophotometer. Chlorophyll concentration was read at 652 nm, or determined using the absorbtion coefficients used by Arnon (1949) and checked by Bruinsma (1961) as follows:

(A) is the absorbance.

Chl *a* = 12.7 A663 - 2.69 A645

Chl *b* = 22.9 A645 - 4.68 A663

Chl *a* + *b* = 8.02 A663 + 20.20 A645

Growth rate have been expressed in terms of relative growth constant or specific growth constant (K') (Fogg, 1975).

$$\frac{\log_{10}N_t - \log_{10}N_0}{t}$$

Where t = time in days, from the time of inoculation

N_t = biomass after t days

N_0 = biomass at zero time

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

$$G = \frac{0.301}{K'} \text{ day}$$

3 STUDY AREA

3.1 Introduction

The Asir Mountains, located in south-western Saudi Arabia, are a territory with a relatively high number of bryophytes (more than 25 species) as well as other vegetation and as such form a convenient unit for botanical study. To date, no study has been carried out in the Tanumah area (Asir mountains) except that of Whitton *et al.* (1986) on water chemistry and algal vegetation. The authors mentioned abundant moss and tufa-deposition at the Tanumah site. It was therefore thought desirable to carry out an in-depth study of this region on the biology of specific mosses with a view to understanding their ecophysiology, especially their response to drought stress and phosphate limitation, both factors which seemed likely to be of importance in the field.

3.2 Background of Tanumah region

3.21 Location

Tanumah (Al-Dahna) (18°58′ N, 42°06′ E) is (*c.* 1920 m above sea level) located on the highlands of Asir Mountains, south-west Saudi Arabia. It is about 110 km north of Abha near (600 m) the Taif-Abha main road (Fig. 3.1). Although it is located in the highland region, the Tanumah site is lower than its surroundings.

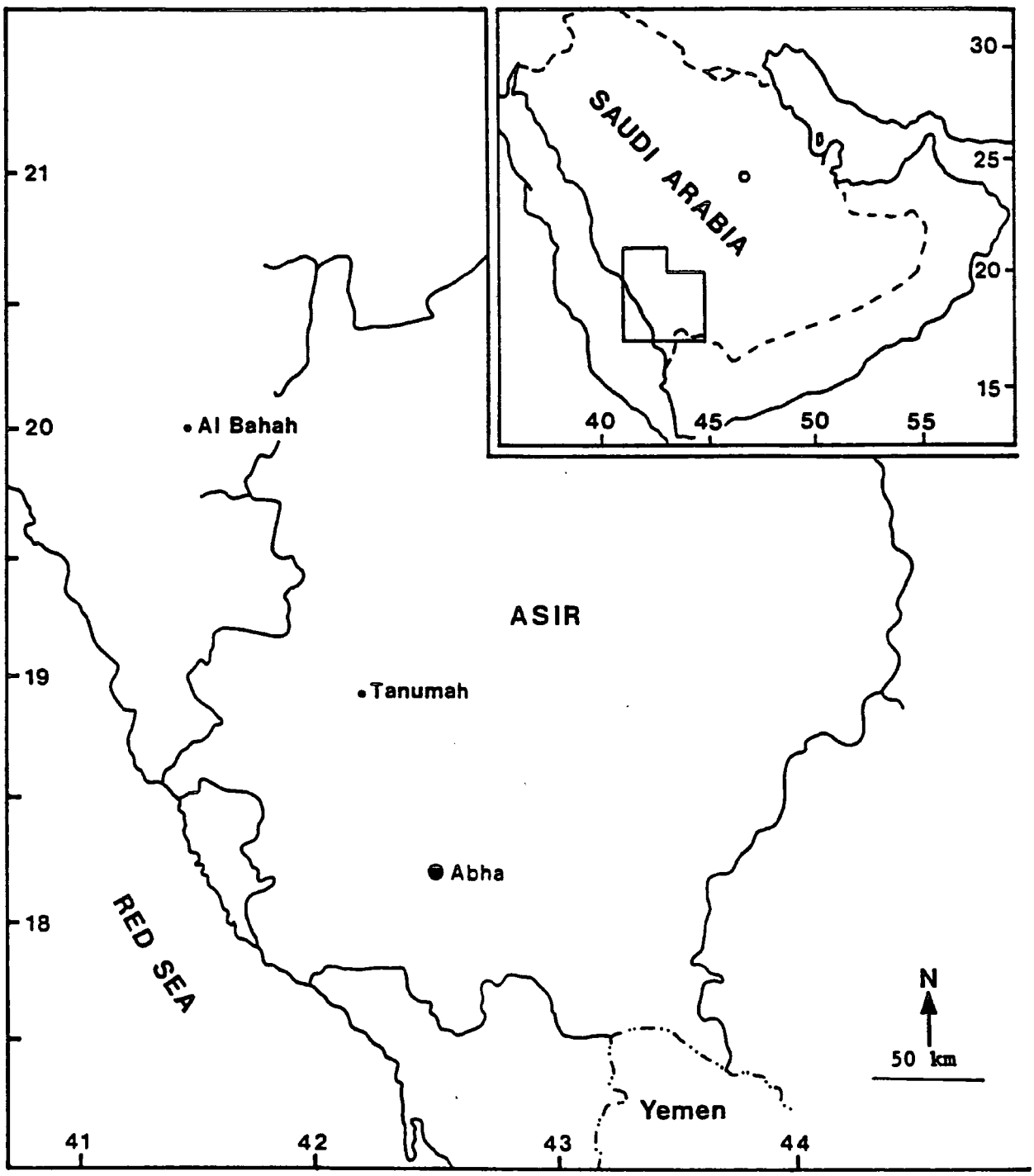


Fig. 3.1 Location of Tanumah (Site of Study)

3.22 Climate

3.221 Temperature

The mean maximum and minimum temperatures are 27.6 °C and 5.6 °C respectively, while the mean temperature is about 17.3 °C (Tanumah Agricultural Branch, Ministry of Agriculture, 1986).

3.222 Rainfall

The mean annual rainfall recorded in 1986 was 221 mm (Tanumah Agricultural Branch, Ministry of Agriculture, 1986). The rain falls throughout the year, but especially in spring and summer. However, according to Vesey-Fitzgerald (1955), there are three notable rainy seasons:

1. Monsoon (from August to mid-October). This rain is particularly useful to plants since it is accompanied by heavy clouds which come in contact with the mountain sides and crests and thus lessening moisture loss from flora throughout evapo/transpiration (Vesey-Fitzgerald, 1955).

2. Cyclonic Winter (around December). This is during the relatively cold season when southerly winds blow across the Red Sea against the Asir Mountains.

3. Early Summer (around May). This rain is accompanied by heavy thunderstorms which fall within a short time. This rain affects the soil and environment by causing violent floods.

These three types of rain can be light, erratic or may fall altogether over a period of years, but the monsoon rain seems most reliable for the Tanumah area.

3.23 Regional geology

The Asir Mountains are part of the Arabian Shield. This is a crystalline basement consisting of plutonic and gneissic complexes that intruded a series of steeply dipping metamorphosed volcanic and sedimentary rocks during the late Precambrian (Büttiker & Fergusson, 1983; Kürschner, 1984). Sedimentary cover rocks deposited from the Cambrian until the Early Tertiary have largely been removed by erosion, with only a few relics of Wajid sandstones and Khums limestones remaining.

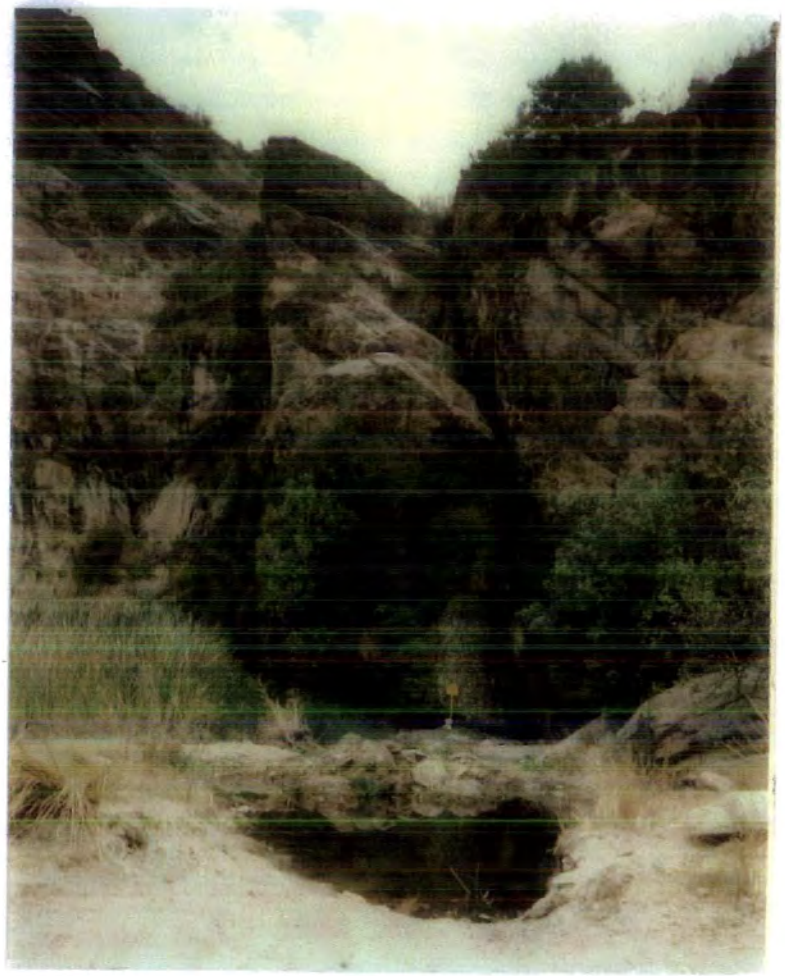
Most of the ridges in Tanumah area are often flat, consisting of the Wajid formation sandstone which lies non-conformally on the Precambrian Basement Complex. In some places, outcrops such as diorities and gabbros show up (Italconsult, 1969). Wadi Tanumah (Al-Dahna) flows on the flat surfaces upland plateaus and suddenly drops by some 60 m into a deep pool.

3.3 Site description

Al-Dahna twin waterfalls are sites (Fig. 3.2a) of tufa-deposits and are about 60-65 m tall. At the tufa-deposition, moss is the dominant species (Fig. 3.2b) and is seen to up to 20 m from the ground level. The tufa-deposition is wider at the bottom (3-4 m) than at the top (1-2 m) (Fig. 3.3a). The waterfalls are surrounded by cliffs from east to west and they face north-west. The quantity of water over the falls at a given time is controlled by the dam, which lies about 370 m away from the summit of the waterfalls (Fig4a, b). Of the two waterfalls, the one on the eastern side drops directly into a lake and the other into small shallow pool which joins the lake (Fig. 3.2a). The lake is about 30 m long, 20 m wide and 10 m deep. The water from the lake flows down the valley and is sometimes used for irrigation.

The vegetation of the waterfalls is dominated by the moss *Hydrogonium fontanum* (C. Müll.) Jaeg. (Pottiaceae) (Fig. 3.3b). Higher plants such as *Primula verticillata* were found growing within the waterfall zone. About 7-10 m from the waterfall three other moss species, *Trichostomum brachydontium* Bruch, *Pseudoleskea leipipiae* (C. Müll.) Par. and *Trichostomum erispulum* Bruch, were also found in abundance.

a



b

Fig. 3.3

a) Close view of the zone of tufa-deposition, showing the abundance of *H. fontanum*.

b). *H. fontanum* growing at the lower zone of the tufa-deposition.

a

b

Fig. 3.4

a) Al-Dahna dam, showing the date of construction (1984) and the capacity (200,000 m^{-3}).

b) Al-Dahna dam, general view.

a

b

3.4 Collection and storage of material

All four species of moss were collected from Tanumah waterfall during August and September 1987. Fresh material was kept in damp conditions in plastic containers and transported to Durham, where it was stored at about 4°C in a refrigerator. On the following day the materials were cultured in unsterilized conditions on a medium containing calcareous soil and peat in tap water, which was prepared from powdered dolomite obtained from a local quarry. The mixture was approximately 50% peat, which aimed to simulate the soil conditions where the plants were found. They were left to grow for a month in a growth room at a temperature of 24°C, 60% humidity and under a 16-h light cycle of intensity. Later further subcultures were made in sterile conditions in both liquid and solid Chu 10 D medium in controlled temperature growth room at 25°C. *Hydrogonium fontanum*, which was the main moss growing in the waterfall site, was found to grow better during culturing under laboratory conditions than the other species. It was also obtained in axenic culture. Therefore it was selected for further study.

3.5 Collection and analysis of water

Water samples from Al-Dahna waterfalls, Al-Dahna Dam and from the lake was collected on 16 October 1988 at 0830. The samples for pH, total alkalinity and sulphate measurements were taken by filling bottles to the brim, replacing the screw-caps immediately, maintaining the bottles in a cool-box until shortly before measurements were made later that day; the bottles were allowed to return to their original temperature before measurement was made using methods given by American Public Health Association (APHA) (1985). This manual was updated during the course of the study APHA (1989); however no changes were made to the methods used here.

Another sample of water for analysis was also collected on 9.October 1991 from the same sites. Clean polythene containers were used for the collection of the water samples. Two sets of samples were collected: a) total water which was directly decanted into sample bottles (acid-washed, snap-cap vials) from the container in which it was allowed to settle for ten min; b) filtered water which was obtained by collecting the water from the container using a 20-ml polypropylene syringe and passed through 0.22 mm porosity membrane filters. The first 5 ml of the water which passed through the filter was shaken in the sample bottle and discarded. The bottles were then filled with filtered water samples. The remaining quantity of unfiltered water was also collected in 250-ml iodized polypropylene bottles. All the water samples were transported to the laboratory at Durham under cooled conditions using freezer bags. On arrival at Durham, two drops of atomic absorption grade HNO_3 were added to each bottle, which was then stored in a refrigerator until analysed.

The results of the water chemistry at the sites is shown in Tables 3.1 and 3.2. The pH values at all three sites were above 7.0 and below 8.0 and were almost the same on the two dates.

Table (3.1): Chemistry of water at 3 sites in Tanumah Waterfall sampled on 16.10.1988, element concentration recorded as mg l^{-1} TDS = total dissolved Salt and 0 = No-detectable (ND).

Site	Waterfall	Lake	Al-Dahna Dam
pH	7.72	7.48	7.72
SO_4	14.81	9.81	11.52
Cl	60	60	50
Mg	16.32	17.2	8.64
Ca	44	35	29
$CaCO_3$	180	160	108
TDS	264	244	214
Mn	0	0	0
Fe	0	0	0
NH_3	0	0	0
NO_3	0	0	0
NO_2	0	0	0

Table (3.2) Chemistry of water at 3 sites in Tanumah Waterfall, samples on 9.10.91, element concentration recorded as mg l^{-1}.

Sites	Waterfall		Lake		Dam	
	Total	Filtered	Total	Filtered	Total	Filtered
pH	7.6		7.4		7.8	
Cond. ($\mu S\ cm^{-1}$)	449		474		483	
tot.alk, (meq l^{-1})	3.2		2.9		2.9	
Na	44	45	44	50	41	42
K	0.8	0.8	1.6	1.2	1.2	1.2
Mg	15	15	16	16	17	17
Ca	44	46	58	55	38	34

4. MORPHOLOGY AND GROWTH

4.1 Field material

4.11 Leafy shoots

Hydrogonium fontanum is the dominant plant in the Tanumah waterfall tufa-deposit. The semi-erect shoots reached 7 to 8 cm, but most were shorter (5-6 cm) (Fig. 4.1a). Shoots at the top of the waterfall were longer than those in the lower zone of the waterfall, where the tufa-deposition becomes firmer, these shoots reaching only 2.0-2.5 cm long. During the collection of the leafy shoots from the tufa-deposition, shoots were found to be embedded by the tufa layers, particularly, where the tufa becomes firmer. Young shoots were produced from the old branches (Fig. 4.1a). Each stem had 2 to 3 side-branches, which usually grew near the apex of the main shoot; these grew parallel to the main shoot, with their leaves in the lower part of the stem were yellowish-green to golden-brown. Leaves were lanceolate with lengths of 2 to 3 mm and widths of 0.4 to 0.7 mm (Fig. 4.1d, e). Under the microscope it could be seen that the nerve of the leaf continues to the apex occupying 1/6th of the basal leaf width. The leaf margin was plain with a subacute apex. The cells at the apex zone were thick-walled, short, and nearly square with dimensions of about 10 to 17 μm long and 8 to 12 μm wide. Those towards the base were rectangular and hyaline with dimensions of about 70 to 100 μm long and 12 to 16 μm wide.

4.12 Filamentous protonema

Filamentous protonema were few in the field samples. They were seen on the raised upper part of some shoots and in axils of the leaves. The basal zone of protonema was brown. However, the middle zone contained cells with green chloroplasts while the upper 3 to 4 cells of the filament were almost empty and form colourless cells.

Fig. 4.1

a) Shoots of *H. fontanum* collected from the field, showing young shoot at the apex of the main shoot.

Scale = 2 mm

b) Photomicrograph of the apex of a leafy shoot collected from the field.

Scale = 200 μm

c) Photomicrograph of gemmae of submerged leafy shoots collected from the field, showing numerous chloroplasts within the cells.

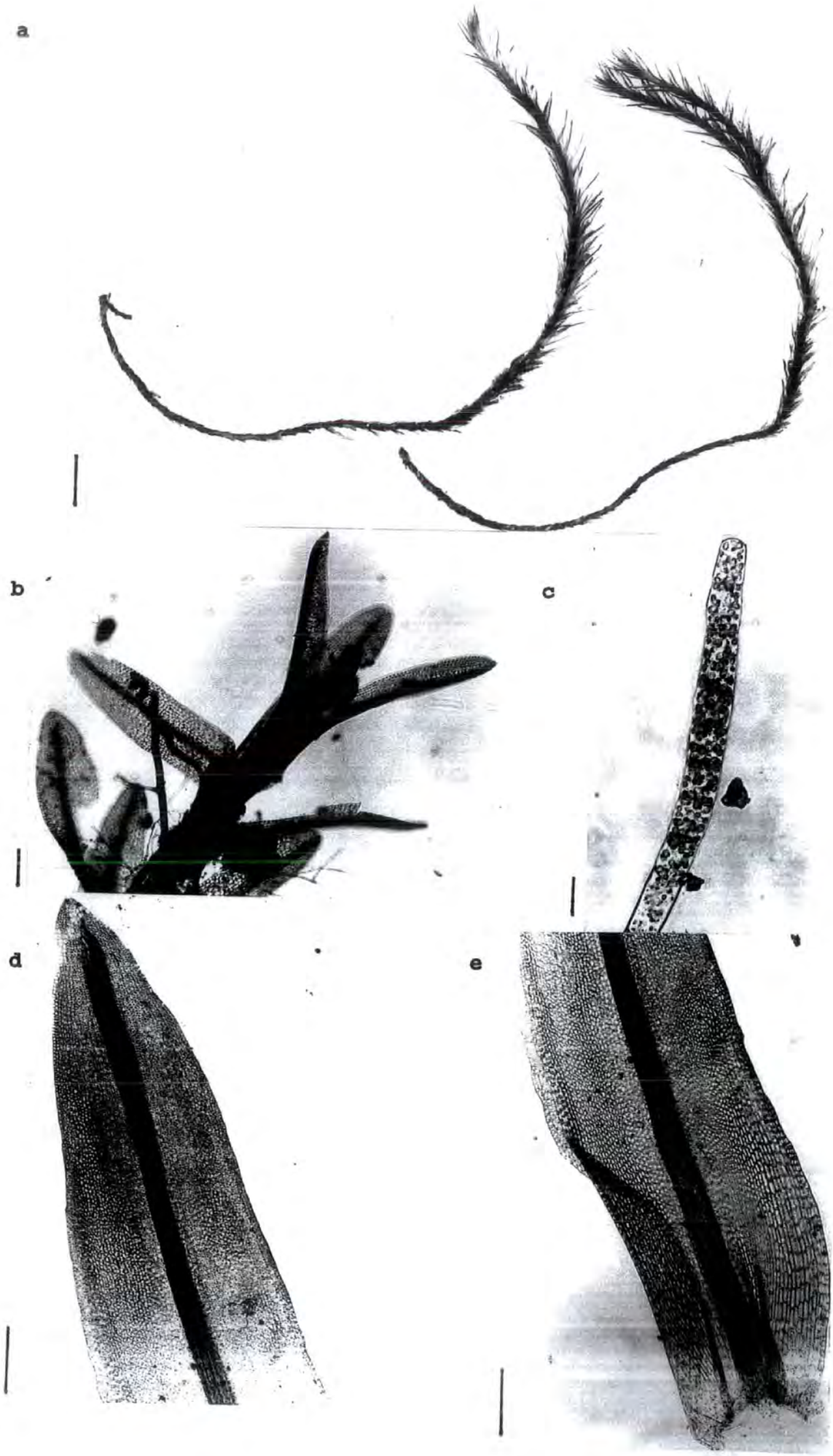
Scale = 20 μm

d) Photomicrograph of apex zone of the leaf of field material, showing the nerve continuing to the apex.

Scale = 200 μm

e) leaf base, showing the nerve occupying 1/6th of the basal leaf width.

Scale = 200 μm


a
b
c
d
e

4.13 Rhizoids

Rhizoids, which were brown with reddish cell walls, were formed all over the lower part of the stem. The main rhizoids bore short rhizoids with oblique cross-wall and were pale brown in colour. Rhizoidal cells ranged from 17-35 μm in width, and 70-160 μm in length. Some rhizoids of field material reached 2.5 cm in length and the rhizoidal cells ranged from 37.6-62.5 μm in width and 125-250 μm in length.

4.14 Gemmae

Examination for possible gemmae was carried out using a field microscope. Gemmae with numerous chloroplasts per cell were found on shoot, rhizoids, leaf axils and on the terminal cell of the protonemal filaments. Several were also seen detached from the plant. The gemmae ranged from 264 μm to 330 μm long (7 to 10 cells) and 23 to 29 μm wide (Fig. 4.1c).

4.2 Batch culture

4.21 Leafy shoots

4.211 Growing on stem

In the laboratory, secondary leafy shoots grew on the main stem. These usually occurred in the axil of the leaf. On solid cultures many new leafy shoots were observed growing all over the main stem, whereas in liquid culture the new leafy shoots were mostly on the upper part of the stem. Most of the leafy shoots were examined were found to have three or four new shoots at the apex of the main stem. In both liquid and solid cultures secondary leafy shoots were similar in morphology to the original shoot.

4.212 Development of leafy shoots on protonema

Leafy shoots were formed on the protonemal filaments (caulonemal type). These shoots were developed from the buds on the caulonemal filaments.(Fig. 4.2, b, c, d)

4.22 Protonemal filaments

Growth and development, under standard laboratory conditions, in Chu 10 G medium and the maintenance at 80-90 μmol photon m^{-2} s^{-1}, and 25 °C) led to the form of protonema known as choloronema (Fig. 4.3a), which later differentiated into caulonemal filaments (Fig. 4.3b). Some cells of these filaments formed buds, which then gave rise to the gametophyte's leafy shoots (Fig. 4.2d). The growth of protonema started within 2-3 days. The filaments were of the chloronemal type, with numerous round chloroplasts per cell. After a few days (5-6) of protonema formation, caulonemal filaments were seen with few chloroplasts.

Chloronemal cell types were slightly wider with cross cell walls (Fig. 4.3b). Size of cells range from 15.0-20.5 μm in width, and from 32.5-100 μm in length, whereas caulonemal cells were thinner (13.5-18.5 μm) with oblique cell walls (Fig.4. 3b). When the culture become old protonema turned brown with only a few chloroplasts in them.

Protonemal filaments were originated on the stem of the shoots in the axis of the leaf. The secondary protonema were also produced from the main filaments. This stage of growth was observed in both liquid and solid cultures. Due to the production of gemmae on the protonema many gemmae formed new protonemal filaments.

4.23 Rhizoids

In both field and laboratory material with solid and liquid media rhizoids developed on the lower part of the leafy shoot stem (Fig. 4.2b).

In liquid medium further clumps of rhizoids also grew at the points where the new shoots formed on the main leafy shoots.

Rhizoids usually grew towards the substratum. On some leafy shoots which were examined under the microscope rhizoids were, however, seen on both the upper and lower parts (Fig. 4.2c). Rhizoids were usually brown. The young rhizoids were pale brown, whereas the old ones were dark brown with thick reddish cell, oblique cross-walls (Fig. 4.2e). The last 3-4 cells of the young rhizoid were slightly green and contained several chloropasts with a distinct brown cell wall. Rhizoidal cells ranged from 20-37.5 μm in width, and 62.5-175 μm in length.

4.24 Occurrence and germination of gemmae

Protonemal gemmae were seen in both liquid and solid growth media before and after leafy shoots started to develop. They were also seen in field investigation when the moss was submerged.

The gemmae were spindle-shaped and green with numerous chloroplasts within each cell (Fig. 4.3c). They range from 8-10 single rows of cells, with well-defined thick walls. The cells in the middle were wider with more chloroplasts, those at the ends were narrower with comparatively few chloroplasts. The gemmae ranged from 264-330 μm long and 23-36 μm wide.

The gemmae usually germinated on protonemal filaments. Sometimes they separated from their point of attachment (Fig. 4.4a) to the protonemal filaments and germinated to form new protonemal filaments and in rare instances to form gemmae (Fig. 4.4b). This was observed both in liquid and solid cultures.

When the gemmae aged, they took on a brown colouration. Gemmae were also observed on some colourless or pale brown rhizoids.

Fig. 4.2 a) Standard liquid culture, showing leafy shoots of *H. fontanum* growing upwards from the floating mat of protonema and some growing inside the liquid medium.

b) Leafy shoot growing under laboratory conditions, showing the rhizoids growing only on the lower part of the stem.

c) Rhizoids formed all over the stem.

Scale = 2 mm

d) Photomicrograph of young leafy shoot developed from the protonemal filament.

Scale = 200 μm

e) Photomicrograph of rhizoid, showing the formation of young rhizoids.

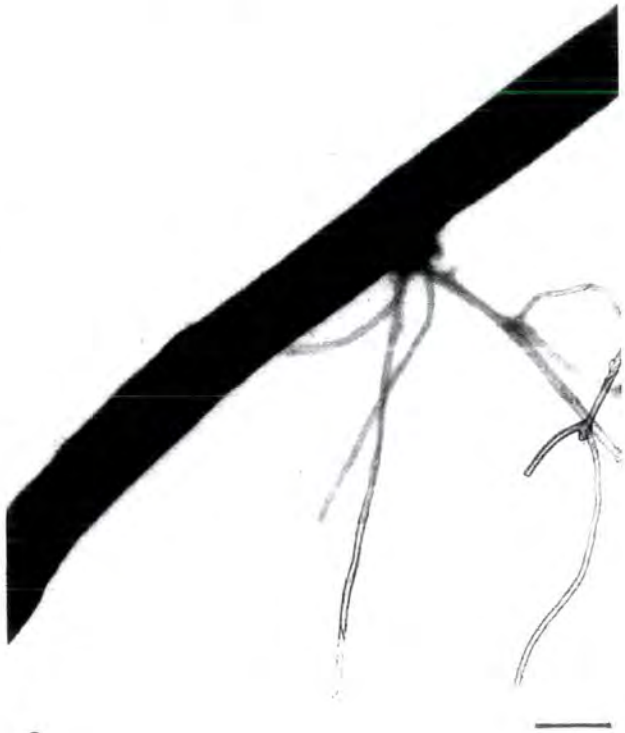
Scale = 100 μm

a

b

c

d



e

Fig. 4.3 Photomicrographs of *H. fontanum* protonemal growth stages, grown in standard medium taken at different stages from batch culture.

a) Young chloronemal filament (4-d old culture).

b) Caulonemal filament with oblique cell walls and elongate chloropasts; above the caulonemal filament is a chloronemal filament with perpendicular cell walls and rounded chloropasts (12-d old culture).

c) Protonemal gemmae with their barrel-shape, cells containing numerous chloroplasts developed on protonemal filaments (12-d old culture).

Scale = 20 μm

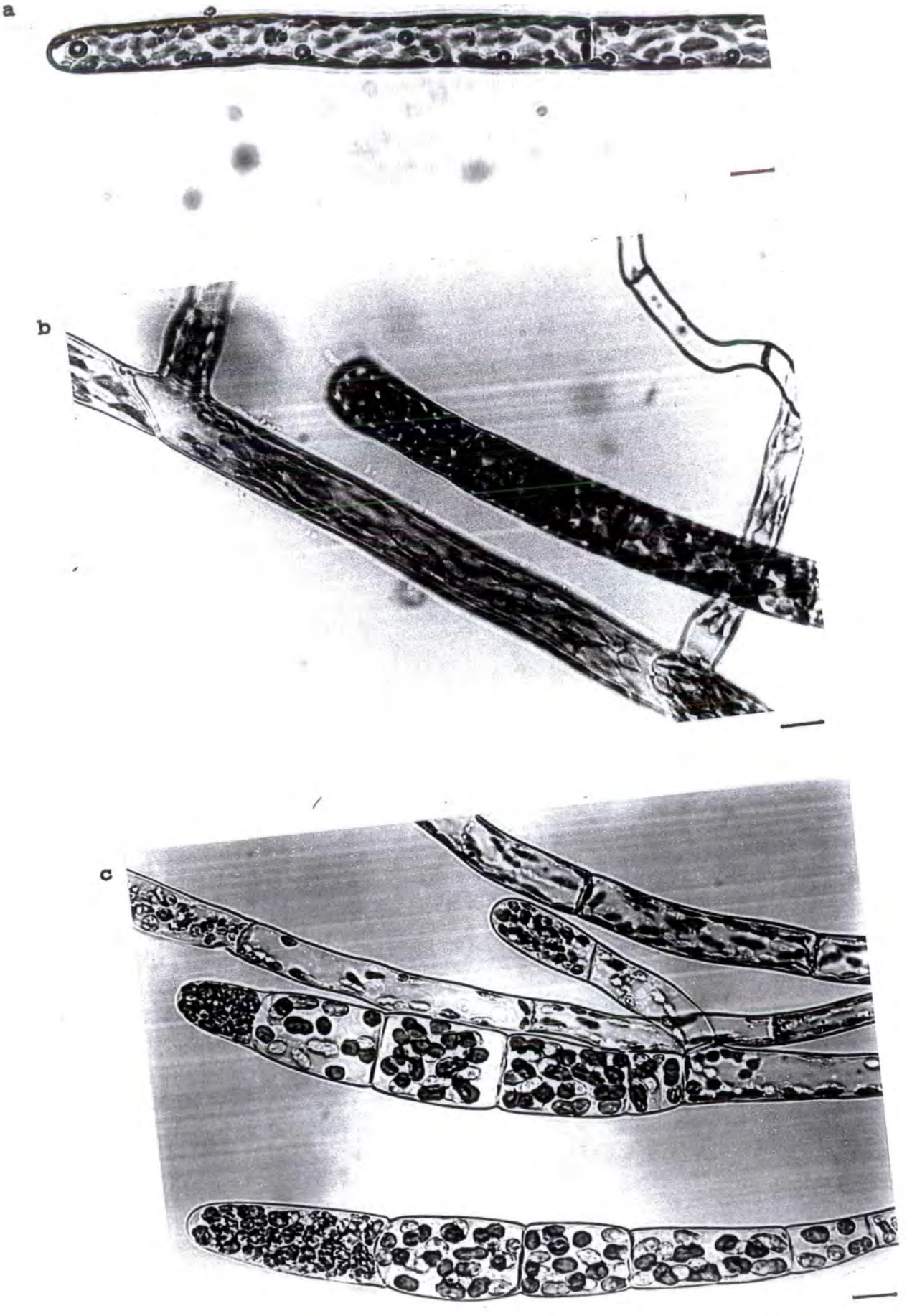

a
b
c

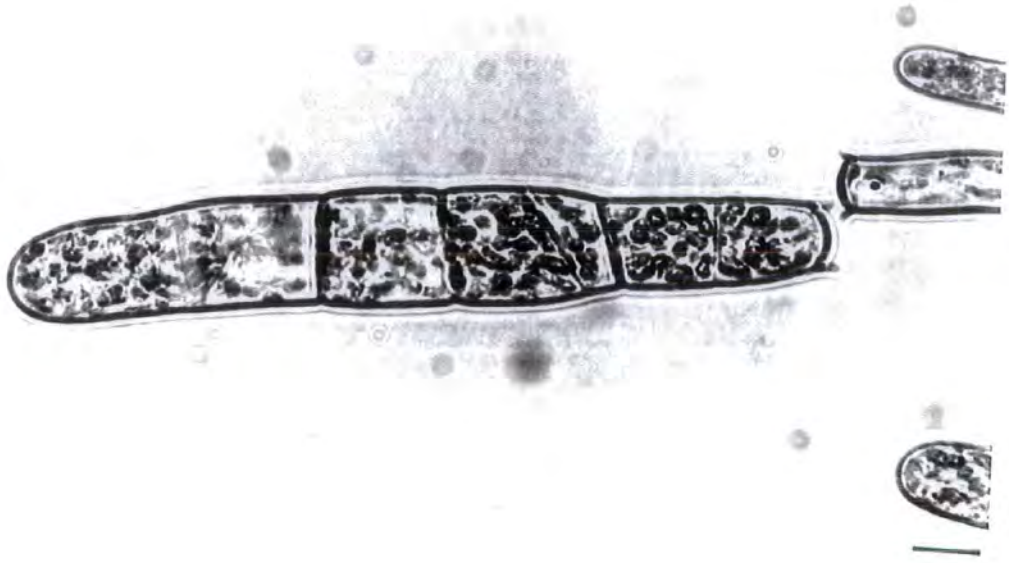
Fig. 4.4

a) Photomicrograph of protonemal gemma, showing its separation from the protonemal filament.

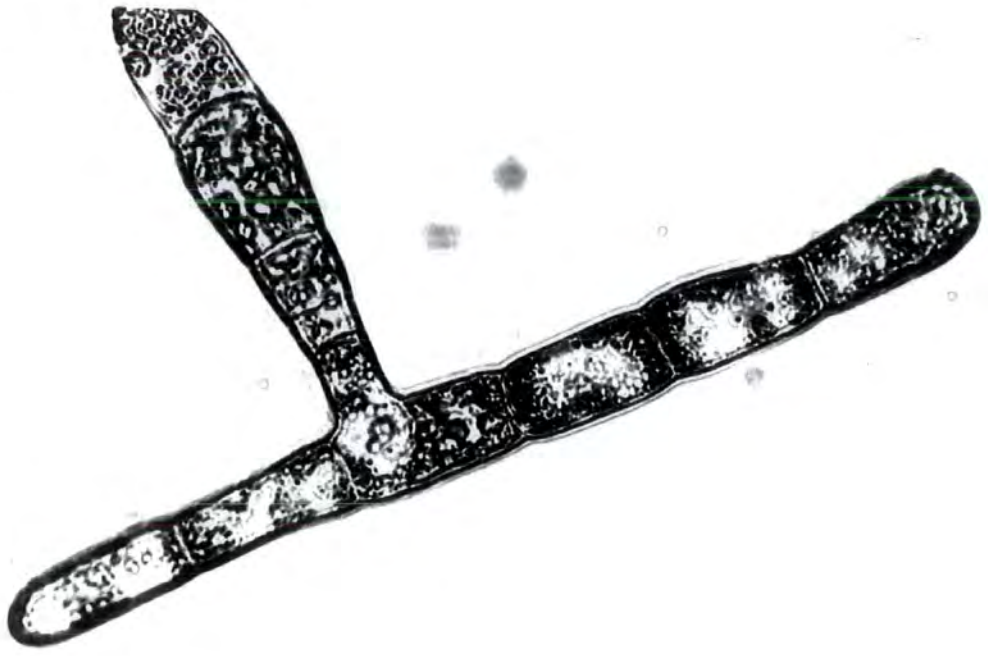
b) Gemma formation from another gemma.

Scale = 20 μm

a



b



4.3 Growth

4.31 Growth form

4.311 Liquid cultures

When grown in liquid culture leafy shoots produced protonema within 2-3 days; at this time gemmae were also seen. Protonemal filaments and gemmae were observed, sometime attached to the leafy shoots or growing at the surface of the liquid medium. During the period of growth, the protonemal filaments and protonemal gemmae were forming new protonema and gemmae. Rhizoids were observed after about 22-24 days from the time of inoculation

In liquid culture with a protonemal inoculum, leafy shoots take about 6-7 weeks to develop. When the cultures got old, protonemal filaments and gemmae formed a brown mat inside and at the surface of the liquid medium. From that brown mat of protonema, many leafy shoots were developed, some growing upward into the air as well as downward in the liquid medium.

The protonemal filaments near the surface area close to the glass walls of the culture flask grew attached to the glass and they produced leafy shoots which grew into the air and still attached to the glass wall.

When the moss was transferred from old to fresh medium, it formed very green healthy protonema and gemmae all over the culture within a few days (2-3 days).

4.312 Solid cultures

The inoculum used for solid culture was either shoots or protonema. Shoots were inoculated on the agar growth medium. About 3 days after the inoculation, shoots started to produce protonemal filaments and gemmae which grew either upward into the air or on the surface of the agar. After about 35 days, the protonemal filaments occupied about 80 % of the

surface of the agar; however many protonemal filaments did penetrate and grew inside the agar, even before this length of time.

As in the liquid culture, many protonemal filaments, which grew at the surface or upward into the air, had gemmae at the end of the filaments.

Shoot development from the protonemal culture on agar was seen after about 6-7 weeks. Most of the shoots grew upwards into the air, but a few shoots grew inside the agar. Rhizoids grew on the leafy shoot stems, on the surface of the agar from the protonema and inside the agar. Rhizoids were observed growing from protonemal filaments after about 11-13 day.

4.4 Growth rate

4.41 Introduction

Experiments were designed and carried out under standard conditions either in static or shaking conditions at 25 °C and continuous light (85 μmol photon m^{-2} s^{-1}: Section 2.36). The results are based on experiments with four replicates for each parameter under study.

4.42 Growth rate in solid and liquid cultures

The growth rate in solid and liquid cultures was studied under standard conditions. The pattern of growth was assessed by means of six harvests at regular intervals. The experiments were terminated after 28 days. Growth was expressed as changes in the chlorophyll and dry weight contents. The growth rate was calculated for the first 12 days from the growth constant (K') 1-4, 4-8 and 8-12 (Table 4.1);

Table 4.1 Growth rate constant (K') and doubling time (h) in liquid and solid media.

medium	time (d)	growth constant (K')		doubling time (h)	
		d. wt	chl	d. wt	chl
liquid	1-4	0.169	0.163	128.2	132.9
	4-8	0.135	0.115	160.5	188.4
	8-12	0.105	0.092	206.4	235.5
solid	1-4	0.124	0.136	174.8	159.3
	4-8	0.097	0.092	223.4	235.5
	8-12	0.084	0.080	258.0	270.9

The growth rate from this calculation showed a slight variation for the first 12 days. Increases of growth in relation to chl content and dry weight (Fig. 4.5) were observed up to day 16, after which chl content began to decrease. This was observed in both solid and liquid cultures. The period of fastest growth in the two cultures was, however, different; on solid culture this was between day 8-12, whereas in liquid culture it was between day 4-8.

4.43 Growth rate of shoots and protonema used as inoculum

Experiments were carried out using shoots or protonema as inocula. In the experiment with shoots, 10, 1 cm long shoots were used. Two growth parameters (dry weight and chlorophyll content) were determined at regular intervals.

The results are shown in Fig. 4.6. Chlorophyll content increased approximately in proportion to dry weight up to day 12, and after this period decreased. At the period of fastest growth (day 4-8) the moss almost doubled in dry weight in 4 days.

Fig. 4.5 The growth rate (± S.E) of *H. fontanum* in liquid and solid medium in relation to chl content and dry weight. (Inoculum 1 week old protonema; 85 μmol photon m^{-2} s^{-1}; 25°C)

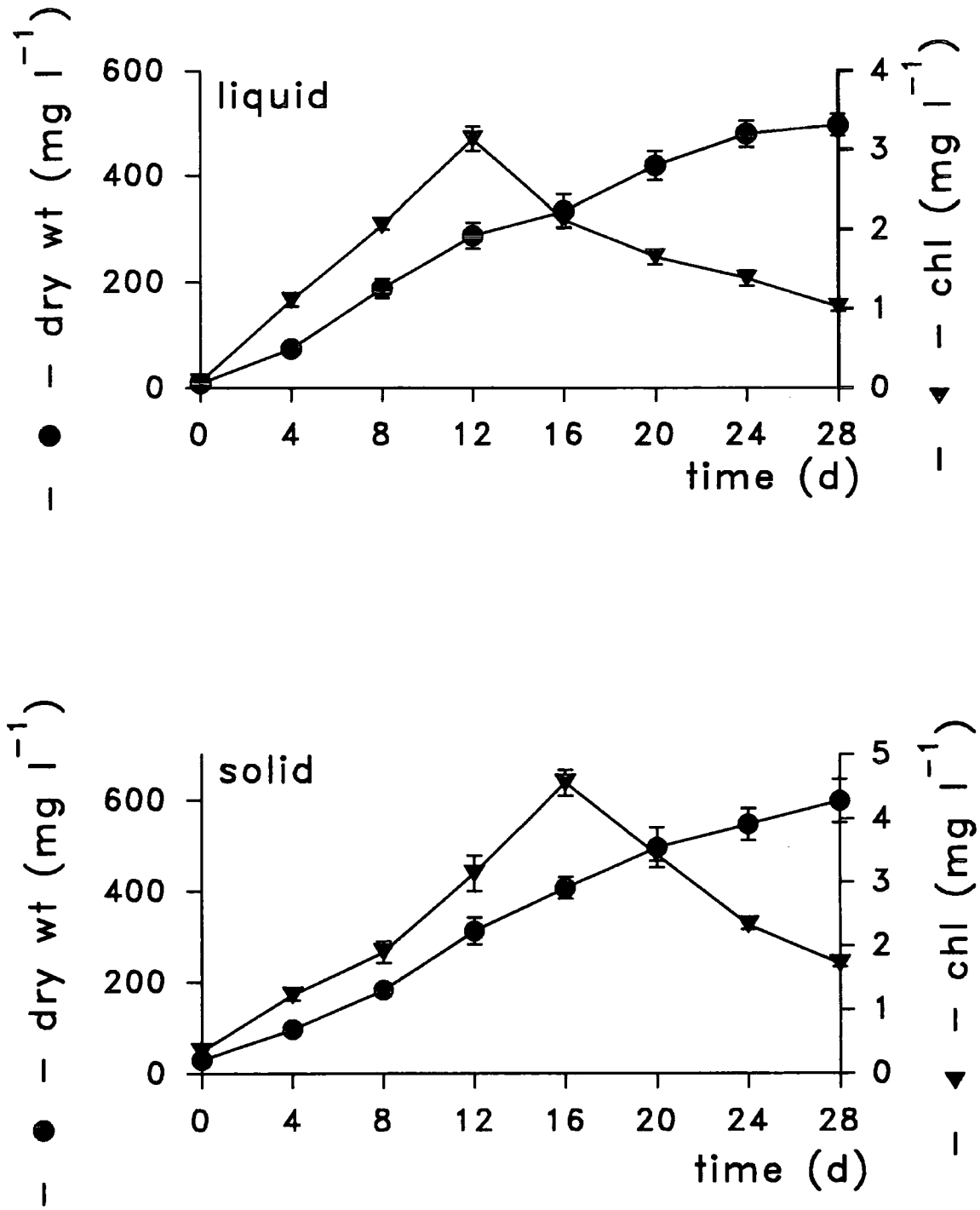
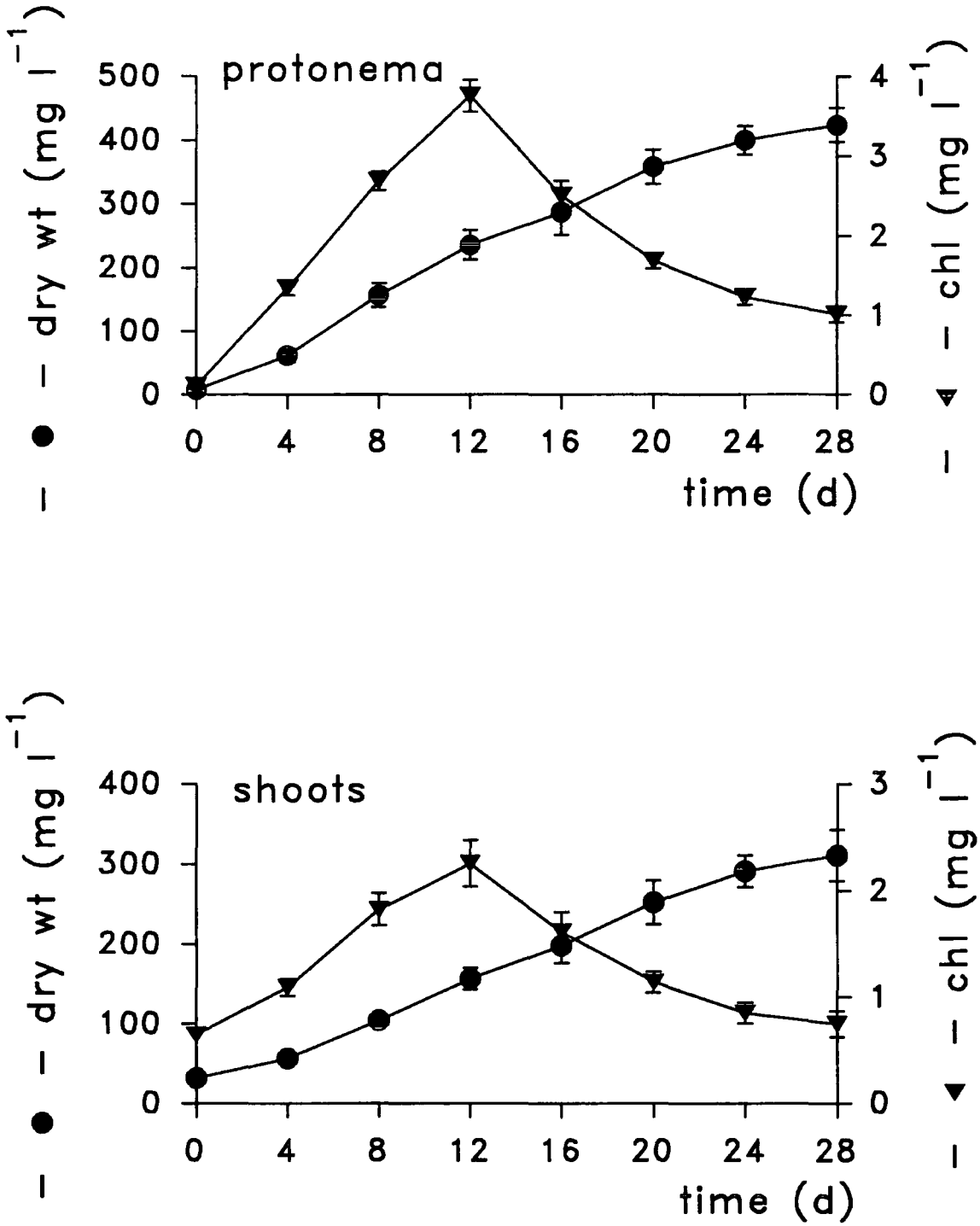


Fig. 4.6 The growth rate (± S.E) of *H. fontanum* in liquid medium in relation to chl content and dry weight. (Protonema and shoots were used; 85 μmol photon m^{-2} s^{-1}; 25°C)



In the case of inoculum using protonema, the growth rate as an increase in chlorophyll content and dry weight showed a close resemblance up to day 12; after that chlorophyll content began to decline sharply. The most rapid dry matter production was achieved between day 4-8. The maximum growth rate (K') attained was 0.202 d^{-1} with protonema, whereas in shoots experiments the maximum growth rate (K') was 0.07 d^{-1}.

4.5 Influence of macronutrients on growth

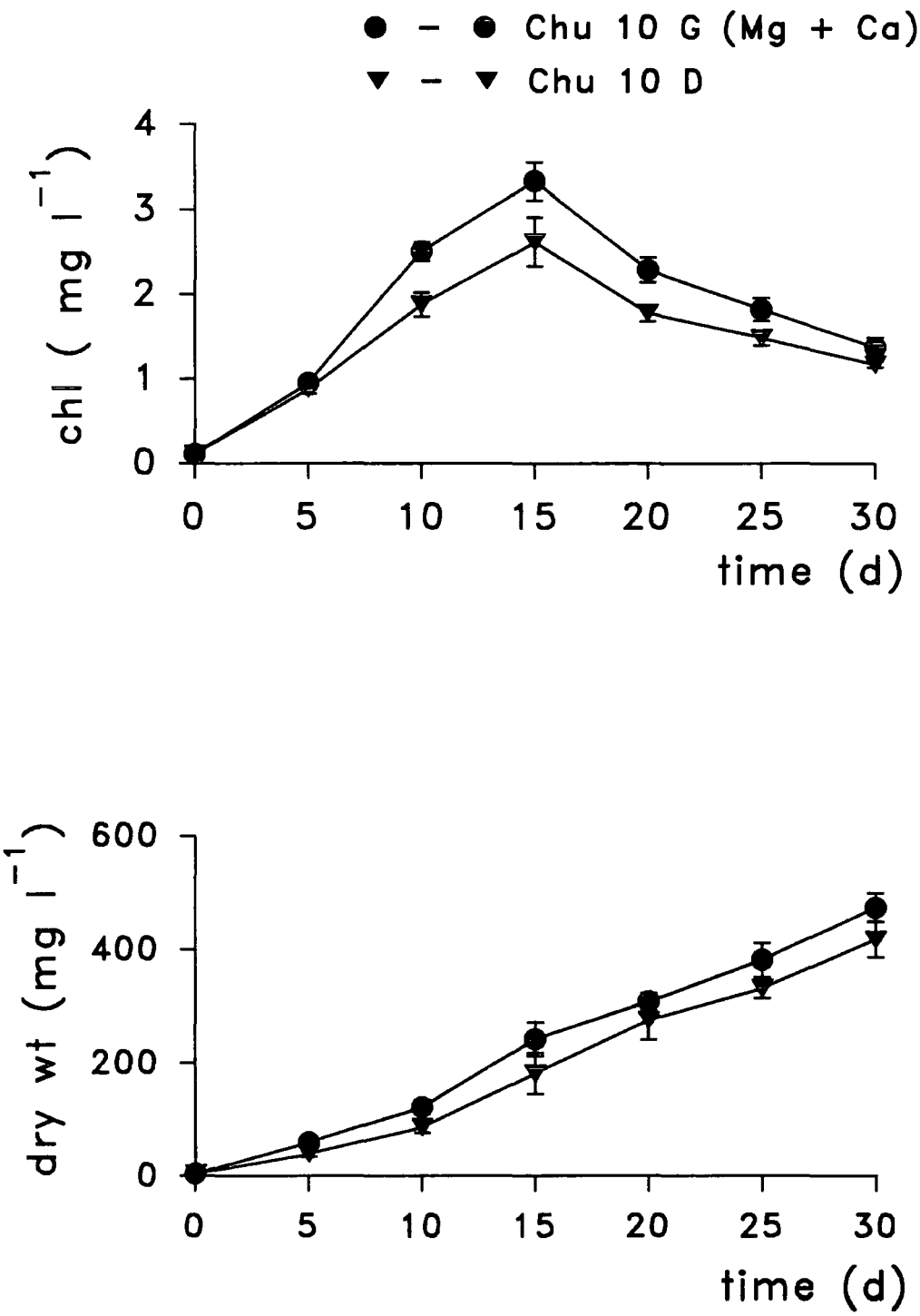
4.51 Influence of Mg and Ca on growth

This preliminary experiment was carried out to test the influence of Mg and Ca on growth of *Hydrogonium fontanum* and to establish the right concentration of these two elements for Chu 10 G growth medium. The increment of these elements concentrations were based on the result of the water analysis of the site, where the moss was originally collected (Mg 18.2 mg l^{-1} and Ca 33 mg l^{-1}). The results are shown in Fig. 4.7, it can be seen that increment of Mg (2.46-9.86 mg l^{-1}) and Ca (9.75-22.3 mg l^{-1}) showed an increase in both dry weight and chlorophyll content of the moss and this increase on the growth was seen after day 5 of the experiment. Growth rate, calculated for the first 15 days from growth constant (K') 1-5, 5-10 and 10-15 is given in Table 4.2.

Table 4.2 Growth constant (K') and doubling time (h) in Chu 10 D and Chu 10 G.

medium	time (d)	growth constant (K')		doubling time (h)	
		d. wt	chl	d.wt	chl
Chu 10 D	1-5	0.147	0.127	147.4	170.6
	5-10	0.093	0.11	233	197
	10-15	0.09	0.093	240.8	233
Chu 10 G	1-5	0.152	0.132	142.5	164.2
	5-10	0.107	0.122	202.5	177.6
	10-15	0.1	0.1	216.7	216.7

Fig. 4.7 Influence of low (Chu 10 D) and High (Chu 10 G) Mg and Ca on growth rate (± S.E) of *H. fontanum* in relation to chl content and dry weight. (Inoculum 1 week old protonema; 85 μmol photon m^{-2} s^{-1}; 25°C)



4.52 Influence of Na, Mg and Ca on yield

This experiment was conducted to investigate the effect of different concentrations of Na, Mg and Ca on yield. The normal Chu 10 G growth medium has the following concentrations:

Na 40.10

Mg 9.90

Ca 22.30

After 30 days (experiment period) growth was measured as yield (dry weight). The results (Table 4.3) showed that The lowest concentration of Na (10 mg l^{-1} gave the lowest yield, whereas the optimum growth was found over a wide range of Na concentrations (40-200 mg l^{-1}), increases above 200 mg l^{-1} gave a very slight reduction in yield.

An increase in the Mg concentration up to 40 mg l^{-1} gave the highest yield and a further increase did not affect the yield. The lowest concentrations (1 mg l^{-1}) resulted in a yield of 389.2 mg l^{-1} with growth rate (K'= 0.063 d^{-1}) whereas at a concentration of 40 mg l^{-1}, which was the optimum concentration, gave a yield of 418.8 mg l^{-1}, but with essentially the same growth rate (K'= 0.064 d^{-1}). The influence of Ca concentration on yield showed an increase over the range 10-80 mg l^{-1} Ca; the optimum yield was at 80 mg l^{-1} Ca; a further increase had no effect on yield.

Table (4.3) Influence of Na, Mg and Ca on yield of *H. fontanum*. (Inoculum 1 week old protonema; 85 μmol photon m^{-2} s^{-1}; 25°C)

Na concentration

(mg l^{-1})	$\bar{X}$	SD
10	376.0	22.41
40	448.0	51.34
100	449.2	30.76
150	442.6	21.28
200	442.0	46.17
250	421.6	36.12

Mg concentration

(mg l^{-1})	$\bar{X}$	SD
1	389.2	22.41
10	409.0	42.00
20	410.0	10.80
40	418.8	33.60
60	406.4	45.68
80	410.0	13.61

Ca concentration

(mg l^{-1})	$\bar{X}$	SD
10	393.6	26.81
22.3	416.0	10.04
40	427.2	25.61
60	452.0	47.54
80	462.0	16.85
100	431.6	39.65

4.53 Influence of NH_4 and NO_3 on growth rate

This experiment was set up to evaluate the availability of N as ammonia and as nitrate for growth. After 30 days (period of experiment), growth was measured as dry weight and chlorophyll content (Fig. 4.8). The growth of the first five days were more or less the same in both NH_4 and NO_3, with growth rates (k') of 0.287 and 0.2899 d^{-1}, respectively. After day 5 there was a difference in dry weight and chlorophyll content. The growth rate was higher with NO_3.

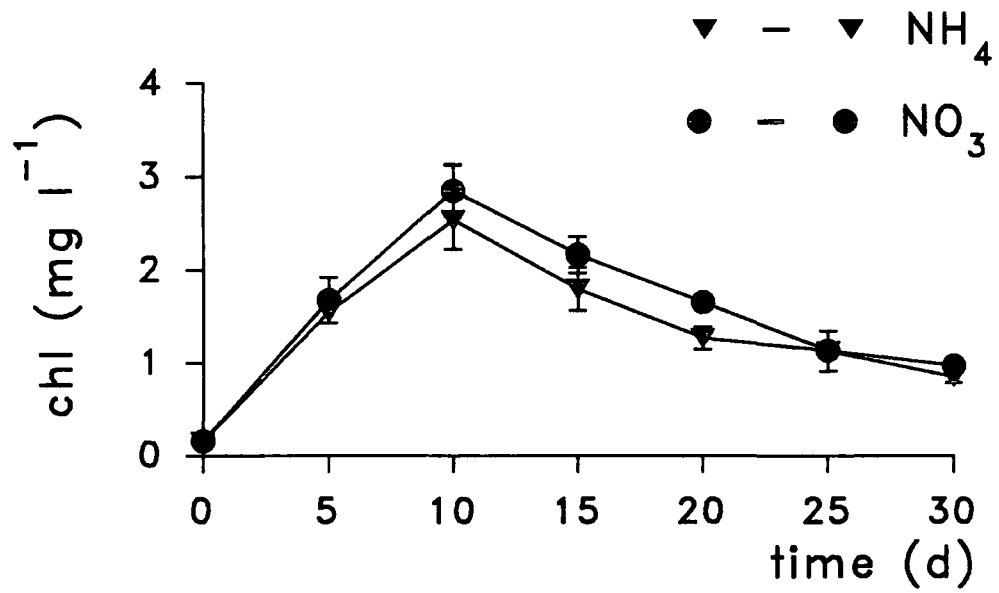
4.54 Influence of Si on growth rate

Initial experiments were carried out using Chu 10 G, which has 1.44 mg l^{-1} Si. In order to investigate the importance of this element for growth, experiment was set up both with Si (Chu 10 D) and without Si (Chu 10 G) in the growth medium. The growth was measured every five days as dry weight and chlorophyll content (Fig. 4.9). It can be seen that there was no difference in dry weight or chlorophyll content for the first 10 days. After this, particularly from day 10-25, the moss grew slightly better in Chu 10 G medium. However, at the end of the growth period (30-d) the dry weights of the moss grown in Chu 10 D and Chu 10 G were the same.

4.6 Effect of organic substrates on growth in dark

Carbohydrates (fructose, glucose, sucrose) and acetate were tested for their ability to support growth in the dark using Chu 10 G medium plus 10 mM substrate. An inoculum of 20 mg l^{-1} of the moss protonema was introduced to the medium with organic substrate. The cultures were then left for 6 weeks in the dark. Yield was recorded and the result showed that the moss protonema failed to grow heterotrophically: The yield was less than the initial inoculum (based on dry weight) in all cultures.

Fig. 4.8 Influence of NH_4 and NO_3 on growth rate (± S.E) of *H. fontanum* in relation to chl content and dry weight. (Inoculum 1 week old protonema; 85 µmol photon m^{-2} s^{-1}; 25°C)



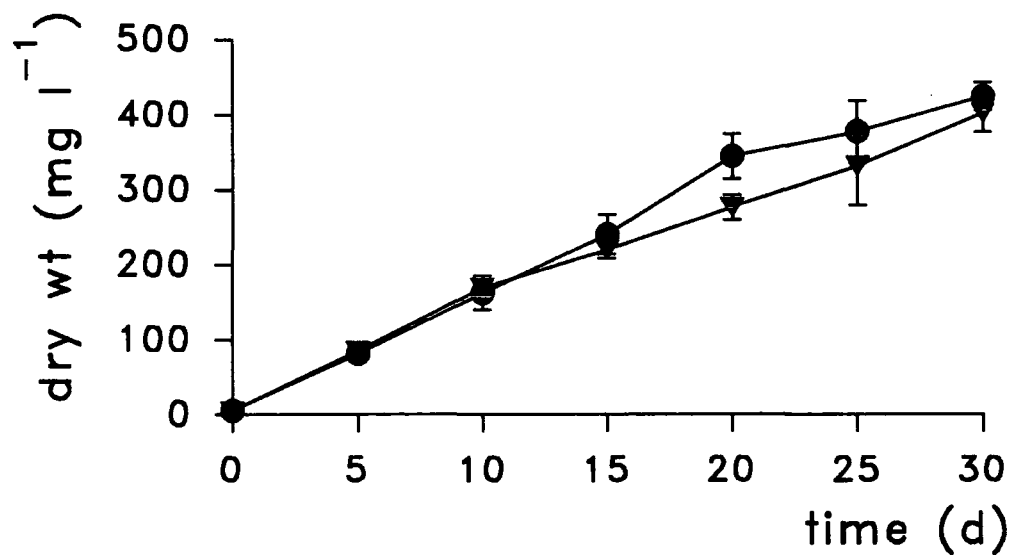
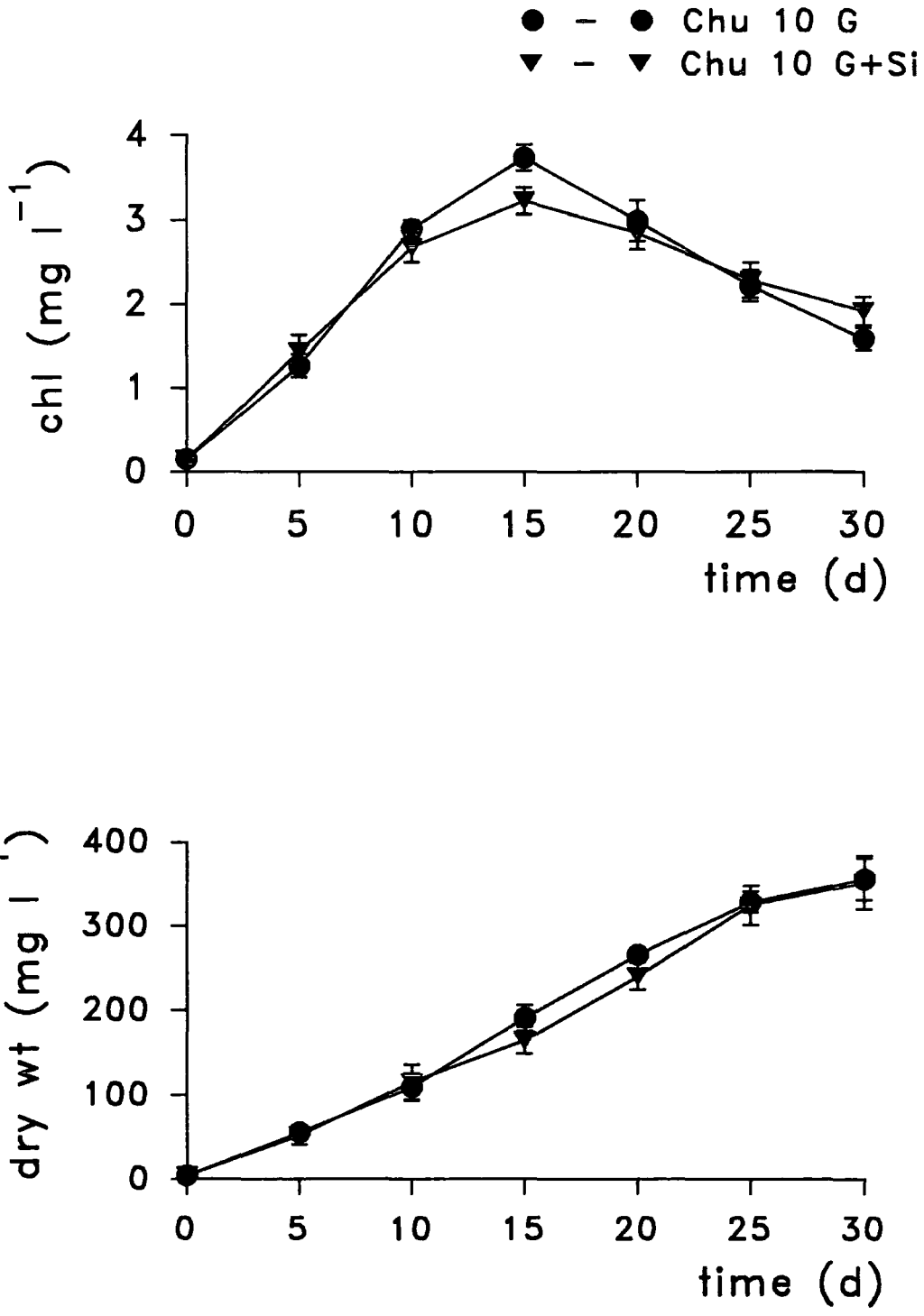


Fig. 4.9 Influence of Si on growth rate (± S.E) of *H. fontanum* in relation to chl content and dry weight. (Inoculum 1 week old protonema; 85 µmol photon m^{-2} s^{-1}; 25°C)



5 INFLUENCE OF ENVIRONMENTAL FACTORS

5.1 Introduction

Experiments were designed and carried out under standard conditions (Section 2.36), except for the environmental factors under investigation. Experiments were set up either in the shaker tank or in the growth room at 25°C and continuous light with intensity of 85 μmol photon m^{-2} s^{-1} except when the effect of light flux were being studied. The results are based on experiments with four replicates for each variable under study unless stated otherwise.

5.2 Influence of temperature on moss growth

Preliminary experiments were set up to establish the optimum temperature for growth under laboratory conditions. *Hydrogonium fontanum* shoots were cultured on solid growth medium on petri dishes in a temperature gradient chamber. Temperatures ranged from 18 - 41°C with six sets of temperature being used for the experiment viz 18, 21, 26, 32, 36 and 41°C. Growth was detected over the range of 18 - 32°C, shoots at 36°C and 41°C were obviously unhealthy by day 9 in that they appeared yellow. Although a few new shoots were observed at 32°C on day 9, the shoots turned yellow by day 12. Growth was most rapid at 26°C, but new shoots were also seen at both 18 and 21°C by day 6.

5.3 Influence of light on growth (yield)

Experiments were planned to test the influence of light flux (10, 20, 40, 60, 80, 90 μmol photon m^{-2} s^{-1}) on protonema for a period of 30 days. This was done to simulate the effect of potential submergence on the growth of the moss where light intensity might be reduced in its natural

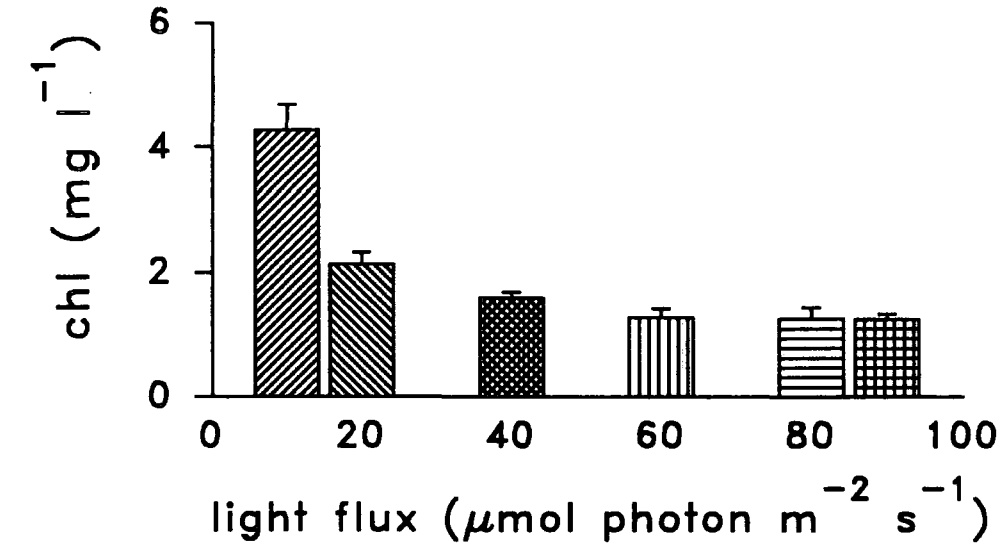
environment. Dry weight and chlorophyll content were recorded at the end of the experiment.

From the results shown in Fig. 5.1 it can be seen that the light intensity had a profound effect on the yield. At 10 μmol photon m^{-2} s^{-1} yield was very low. However, at this light intensity, total chlorophyll content at the end of the experiment was very high. Further, at light intensities of 60, 80 and 90 μmol photon m^{-2} s^{-1} there was no significant difference ($P < 0.05$) on yield. A similar effect was seen with regard to chlorophyll content as well. With respect to yield (dry weight) there was a significant increase with the increase in light intensity from 10-60 μmol photon m^{-2} s^{-1}.

The formation of gemmae under the different light intensities was also investigated. Gemmae formation was highest in the cultures growing under low light intensity (10 μmol photon m^{-2} s^{-1}) when compared with the cultures.

Another test experiment was carried out at a light intensity of 60 and 90 μmol photon m^{-2} s^{-1}. A comparison of the growth rate (as dry weight) and chlorophyll content was made using the shaker tank maintained at 25°C with the stated light intensities. The results (Fig. 5.2) indicated that the dry weight increased at both light intensities tested throughout the experimental period, whereas the chlorophyll content increased initially from day 1 to day 15 and then began to decline in both light intensities. At the end of the growth period (30 days) no significant effects of either light intensities as growth were detected.

Fig. 5.1 Influence of light flux on yield (± S.E) of *H. fontanum* in relation to chl content and dry weight. (Inoculum 1 week old protonema; 25°C)



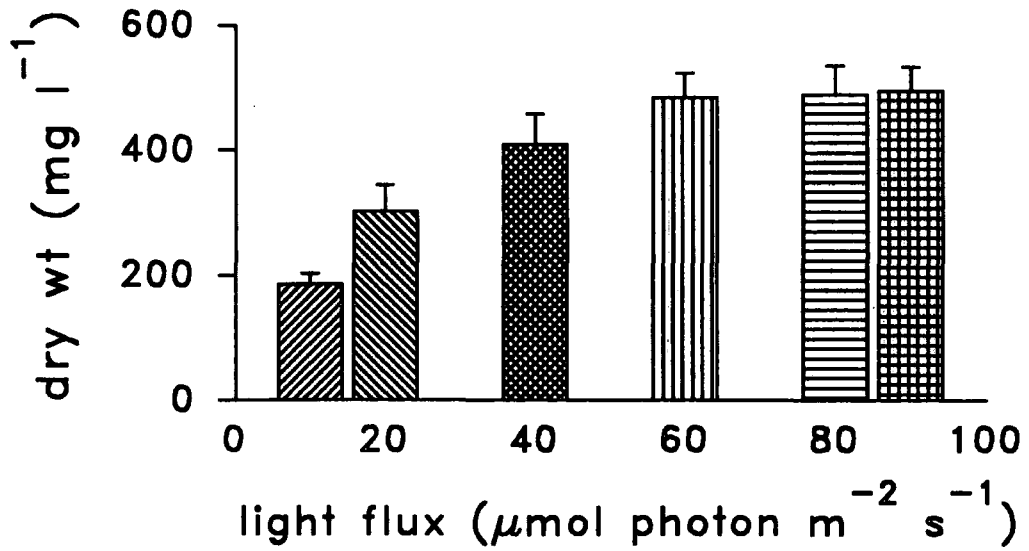
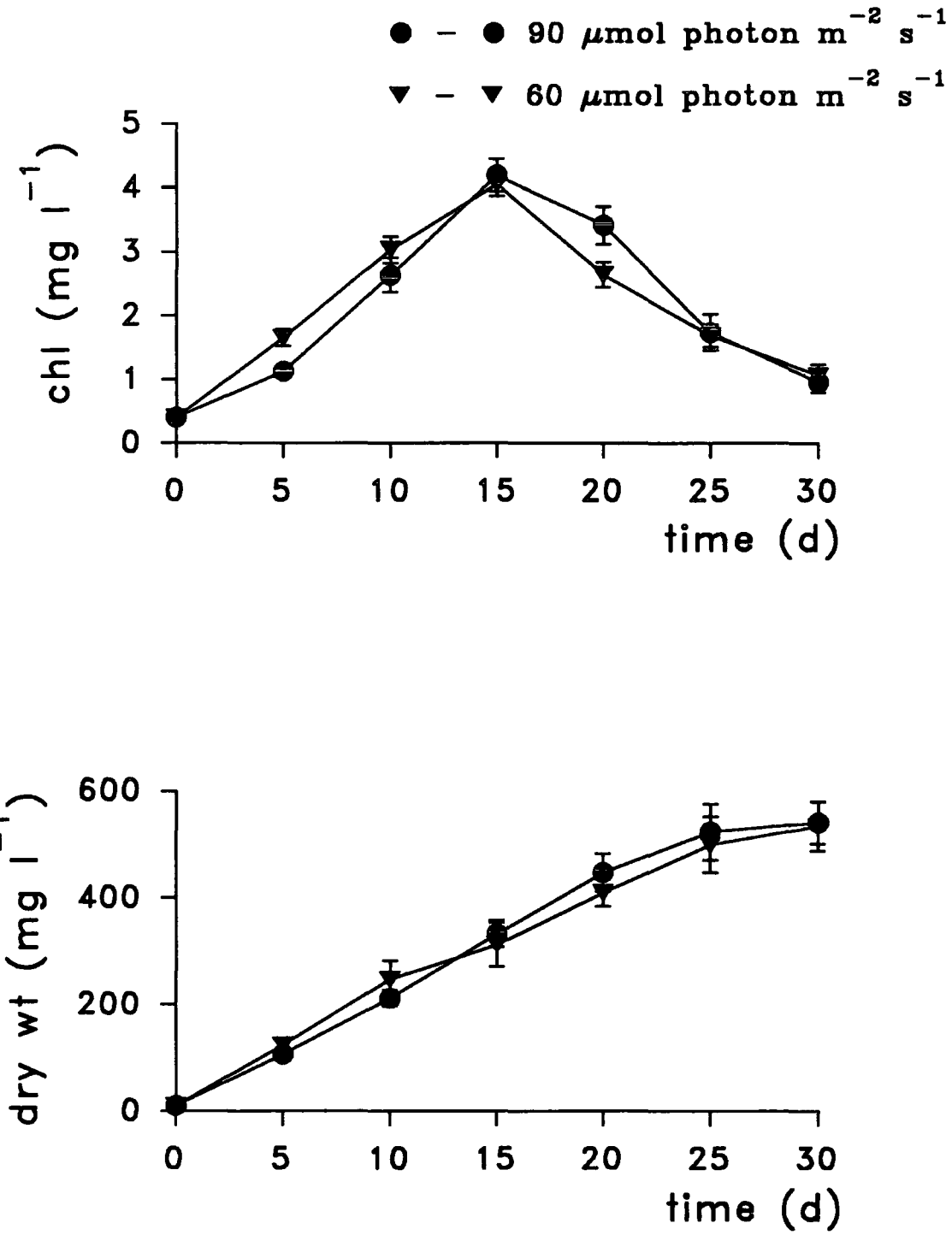


Fig. 5.2 Influence of light flux on growth rate (± S.E) of *H. fontanum* in relation to chl content and dry weight. (Inoculum 1 week old protonema; 2 light fluxes were used; 60 and 90 μmol photon m^{-2} s^{-1}; 25°C)



5.4 Influence of flooding on growth

5.41 Introduction

The waterfall where this species grows naturally shows seasonal changes with regard to the water levels since rainfall occurs especially in spring and summer (Vesey-Fitzgerald, 1955). In addition a dam has been constructed approximately 370 m above the waterfall with the result that water flow has become intermittent due to rainfall periods and extent of water retention by the dam. Therefore tufa-depositing mosses growing on the waterfall, are naturally subjected to fluctuating water levels. It was thought that these fluctuations might affect growth and therefore studies were carried out to see how flooding affected growth and morphology in the field and in the laboratory.

5.42 Method

Growth measurements of field material

The experiment took place at the waterfall site (lake) on 4.10.88 - 23.10.88. The flooding (submergence) was achieved by planting the shoots with their bases in pots of soil which were then placed at two different depths (20 and 50 cm) from the bottom of the lake. The technique of measuring the shoots' growth rate was similar to the method described by Russell (1984) and Kelly and Whitton (1987). This involved tying a piece of cotton at a known distance below the apex of the shoot and measuring elongation of the shoot from this point after a specific length of time. For the experiment, 100 shoots were tied individually and labelled. Fifty were left at a depth of 50 cm and another 50 at 20 cm from the bottom of the lake. The measurement of the growth rate was recorded at the end of the experimental period (19 d). Field measurement of temperature was taken twice during the period. Measurement of pH and analysis of water

for different elements were made in the Ministry of Municipal and Rural Affairs Laboratory in Abha (Section 3.5).

5.43 Results

The growth rate of *H. fontanum* subjected to two different depths (20 cm and 50 cm) was variable. At 20 cm depth most of the shoots were green and healthy and the increments in the length of shoots were obvious (Table 5.1). Side shoots were also formed in the apical zone reaching about 2 mm in length in both submerged samples. At both 20 and 50 cm depths a number of shoots showed rapid growth elongation up to 4.0 mm. However, several of the shoots maintained at 50 cm depth were either dead or gave no measurable growth increments. Visual examinations of the plants was made after measuring growth. Some shoots appeared (at 50 cm depth) less green and the leaves in the basal zone were yellow-brown.

Table 5.1. Growth rates (mm d^{-1}) of *H. fontanum* submerged to 20 and 50 cm depth (lake) and control (waterfall) during the period 4.10.88 - 23.10.88. * Significance (P) is in respect to control.

Position	No. of shoots	survival of shoot	mean growth	S.D	P.*
20 cm	50	44	0.18	0.0029	P>0.001
50 cm	50	35	0.17	0.0037	P>0.001
control	10	9	0.06	0.005	

When both treatments (50 and 20 cm in depth) were compared with the control, the latter showed that some shoots remained the same size while a few showed a slight increment.

Some specimens of both treatments were examined under the microscope to investigate if morphology was changed by this treatment. Gemmae were observed either to be detached from the plant or attached in the terminus of a few colourless cells on the shoot stem of the submerged plants but were absent in the controls. The survival of the non-submerged tissue was better than when submerged.

In the laboratory, experiments to study the effect of flooding (submerging the moss shoots) were carried out with 50 ml agar slopes in Chu 10 G medium in glass jars. Flooding was achieved by adding 100 ml sterile distilled water to one set of jars after planting the moss shoots in the agar medium. The experiment was carried out under the standard conditions described previously (Section 2.36). The growth was determined as chlorophyll *a*, *b* and *a+b* content and dry weight at regular interval (7 days) for 35 days.

The results (Fig. 5.3, 5.4) showed that the moss grew slightly better in unflooded conditions compared to the flooded. However, chlorophyll content started to decline at day 21 in unflooded cultures, whereas in flooded culture it began to decline at day 28. Although the difference in dry weight between the cultures was observed after day 7 in unflooded culture the increase was more marked after day 21. The maximum growth rate (using growth constant (K') for unflooded culture in terms of dry weight was 0.045 d^{-1}, whereas in flooded culture was 0.0374 d^{-1}.

Fig. 5.3 Influence of water depth on growth rate of *H. fontanum* (± S.E) in relation to chl content and dry weight. (Shoots were used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)

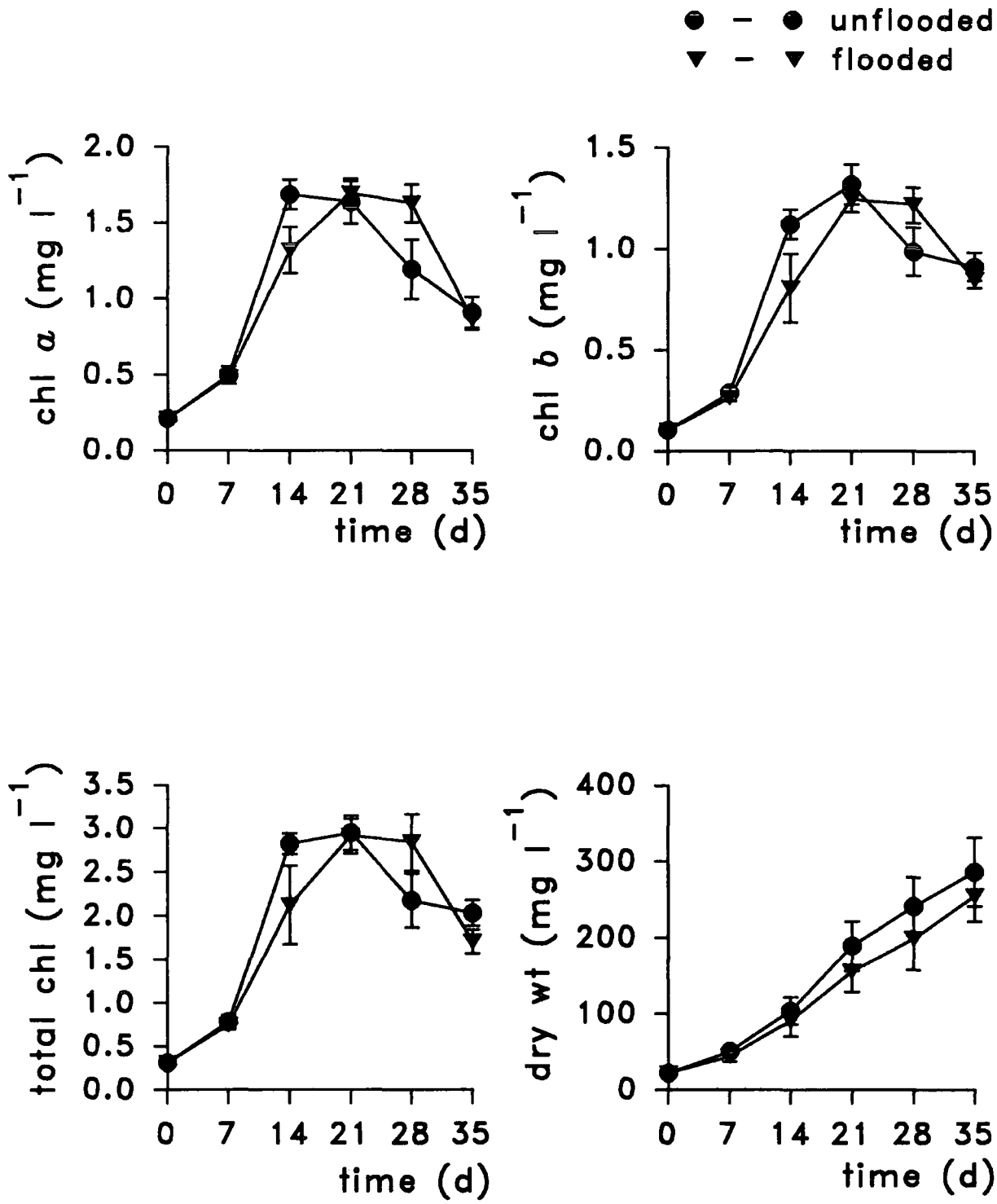
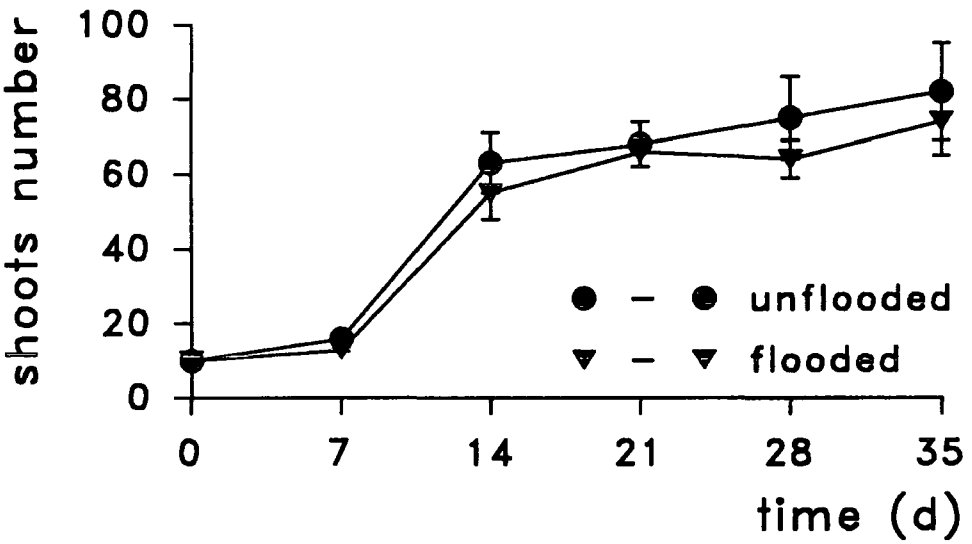
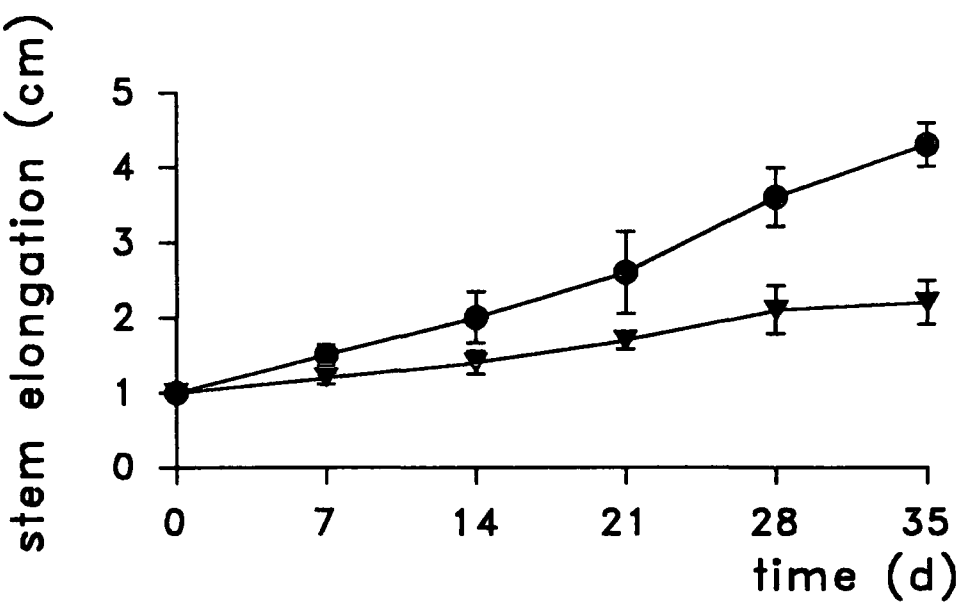


Fig. 5.4 Influence of water depth on growth rate (± S.E) of *H. fontanum* in relation to number of new shoots and stem elongation. (Shoots were used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)





Morphological observations, especially for the appearance of new shoots formation, stem elongation and production of protonemal gemmae were carried out during the experimental period. The new shoots developed on the original stem within the first week in both cultures. The mean number of new shoots was the same after day 14, but at the end of the experiment the number of new shoots formed in flooded culture was slightly higher (Fig. 5.4). Shoots grown in flooded conditions elongated 1.23 mm d^{-1}, reaching 4.3 cm at the end of the experimental period, whereas in the (control) unflooded culture shoots elongation was only 0.5 mm d^{-1}, reaching 1.7 cm at the end of the experimental period (Fig. 5.4).

Production of protonema and gemmae was observed in both cultures. In flooded samples, the production of gemmae and protonema was very low with only a few chloroplastes within the cells. In unflooded culture protonema and gemmae were abundant, and these appeared green and healthy.

5.5 Water stress

5.51 Influence on proline accumulation of shoots and protonema

Since the plants are subjected to fluctuating water levels there are periods when they will be exposed to water stress conditions. For this reason it was decided to examine the effects of water stress on the survival of the moss and on its ability to adopt metabolically by the production of stress metabolites, in particular proline. In order to test the effect of water stress on the moss a laboratory experimental system was set up where the plants were submerged in various concentrations of polyethylene glycol (PEG). Experiments were conducted to test the responses of moss to water stress (Section 2.6) in relation to proline accumulation and were carried out under the standard conditions described previously (section 2.36). The responses of the moss to water stress were

assessed as proline concentration and dry weight at regular intervals of 2 days for 6 days (Fig. 5.5). Even though the water stress conditions had a marked effect on the growth of the moss shoots, no accumulation of proline was detected in the tissues.

Another experiment was set up to test the effect of drought stress on proline accumulation of the moss protonema. Moss protonema samples of the equivalent of 4 g dry weight were placed in petri dishes. Half of the petri dishes (4 replicates used) were subjected to drought stress by withholding their water supply and the other half was kept under normal watering regimes. One week after treatment the proline content was determined every other day for 10 days. Again the results showed no detectable accumulation of proline.

5.52 Effect of water stress on protein content

Experiments were carried out to test the effect of water stress on protein accumulation. Water stress regimes were the same as mentioned in the previous experiments by subjecting the moss protonema to different concentrations of PEG (0, 196, 289 g l^{-1}) for different lengths of time (3, 24, 48 h). The results are shown in Figs 5.6-5.8. The protein content of protonema from all treatments after 3 h showed (Fig. 5.6) no significant differences. However, 24 h after treatment, the levels of protein became reduced significantly at both concentrations of PEG (Fig. 5.7). This reduction was greater after 48 h of treatment with both concentrations (Fig. 5.8), but the higher reduction in protein content was seen at 289 g l^{-1} PEG. The effects were paralelled by changes in the dry weight of the harvested protonema and probably reflect an effect on growth of the tissues.

Fig. 5.5 Influence of water stress on growth rate (± S.E) of *H. fontanum* in relation to dry weight. (Shoots were used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)

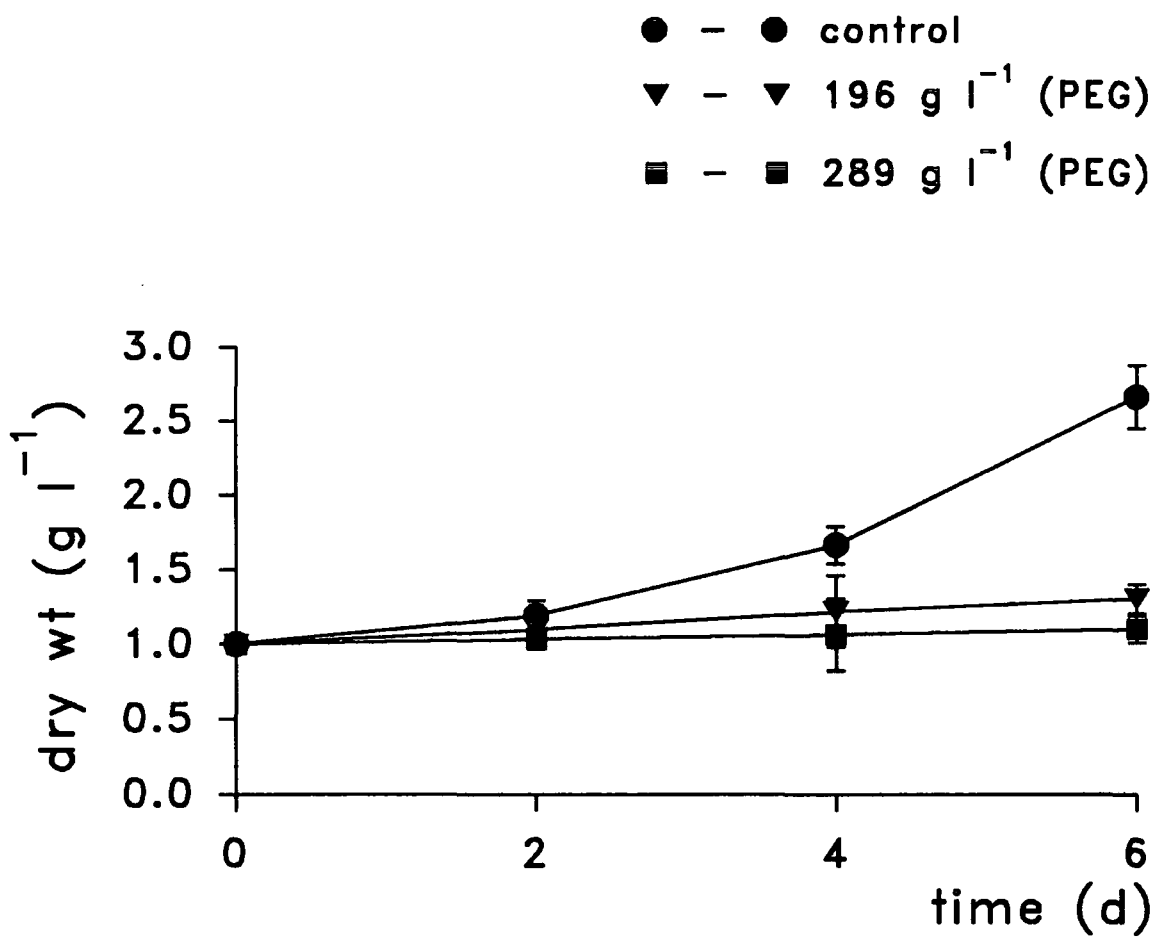


Fig. 5.6 Effect of water stress on protein content (± S.E) of *H. fontanum*. [(Inoculum 1 week old protonema; 85 μmol photon m^{-2} s^{-1}; 25°C) (protein determined 3 h after treatment)]

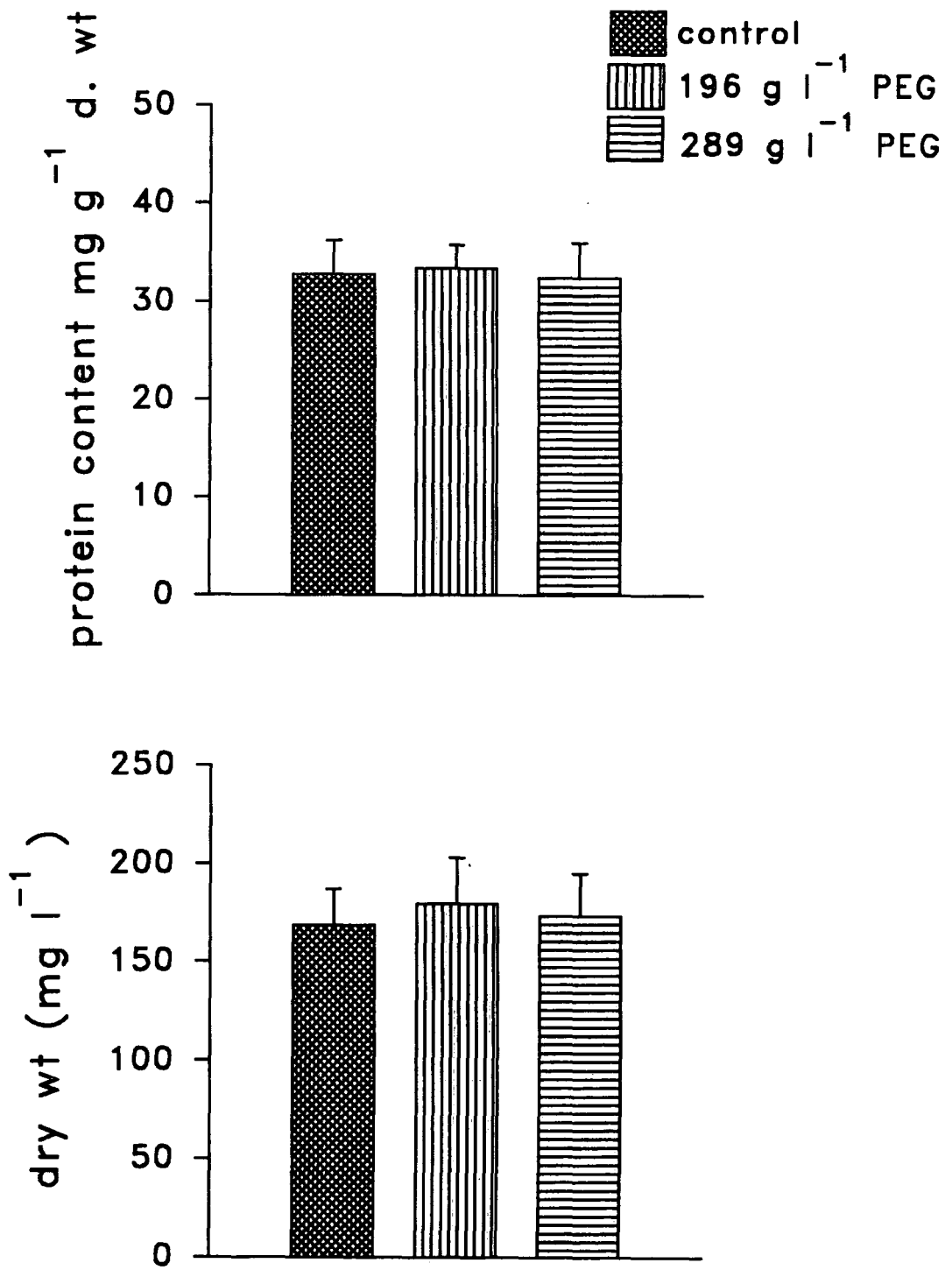


Fig. 5.7 Effect of water stress on protein content (± S.E) of *H. fontanum*. [(Inoculum 1 week old protonema; 85 μmol photon m^{-2} s^{-1}; 25°C) (protein determined 24 h after treatment)]

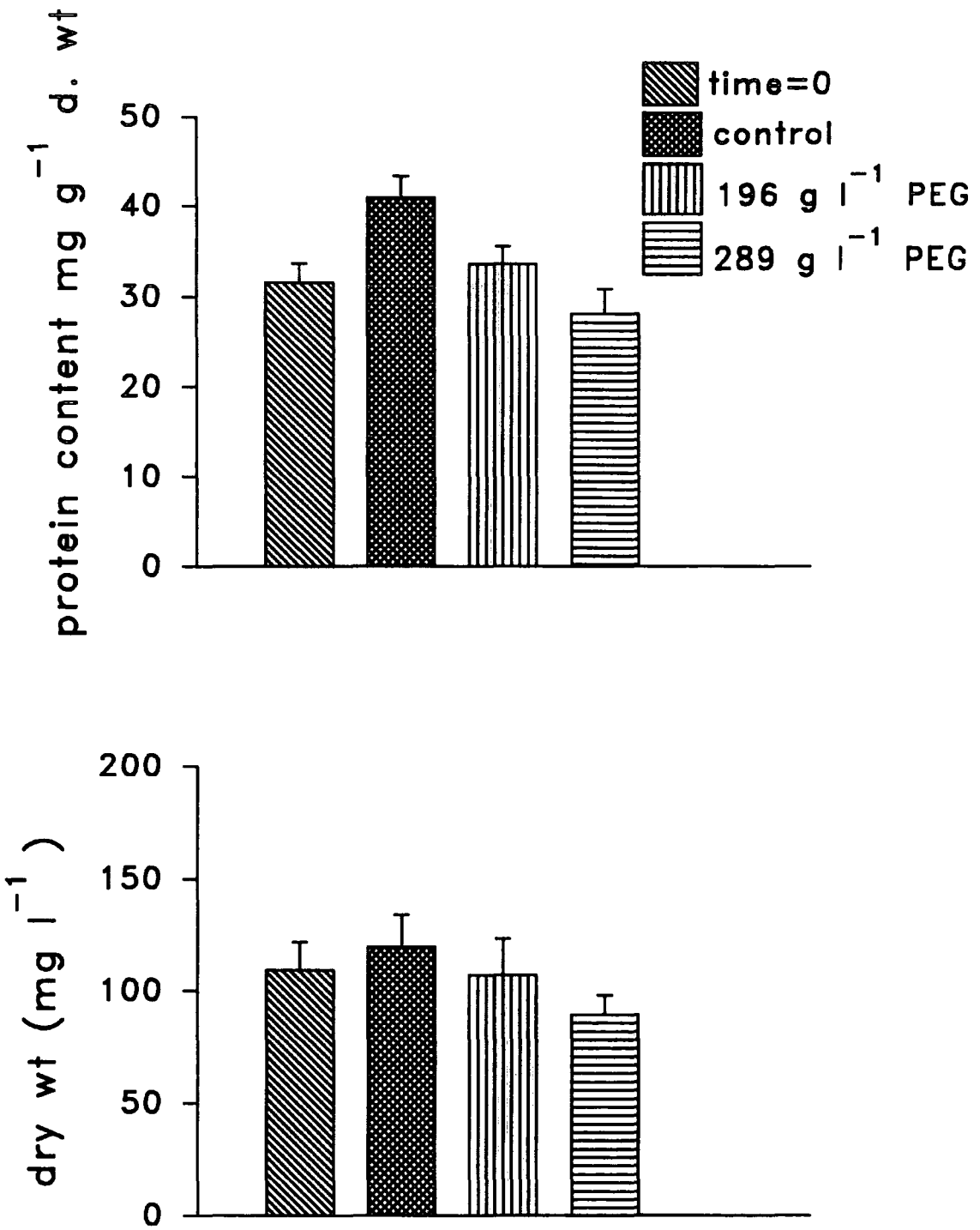
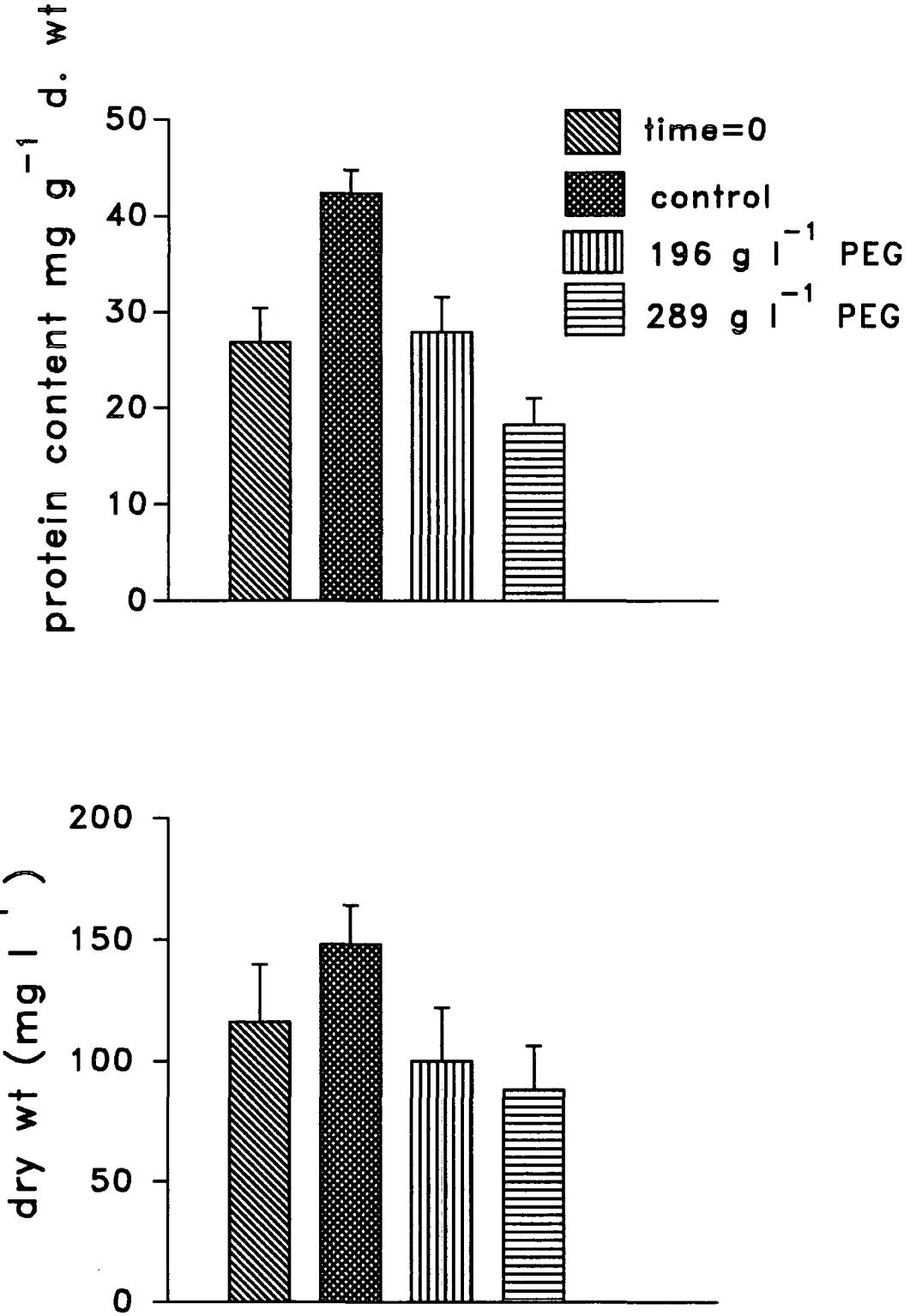


Fig. 5.8 Effect of water stress on protein content (± S.E) of *H. fontanum*. [(Inoculum 1 week old protonema; 85 μmol photon m^{-2} s^{-1}; 25°C) (protein determined 48 h after treatment)]



5.53 Effect of water stress on growth rate in relation to chlorophyll content and dry weight

The effect of water stress was studied through several experiments. The two different stages of the moss growth forms, shoots and protonema, were subjected to water stress (Section 2.6). Experiments were planned with the following objectives:

1 To determine the changes in growth parameters (chlorophyll content and dry weight) of protonema at different concentrations of PEG (water stress)

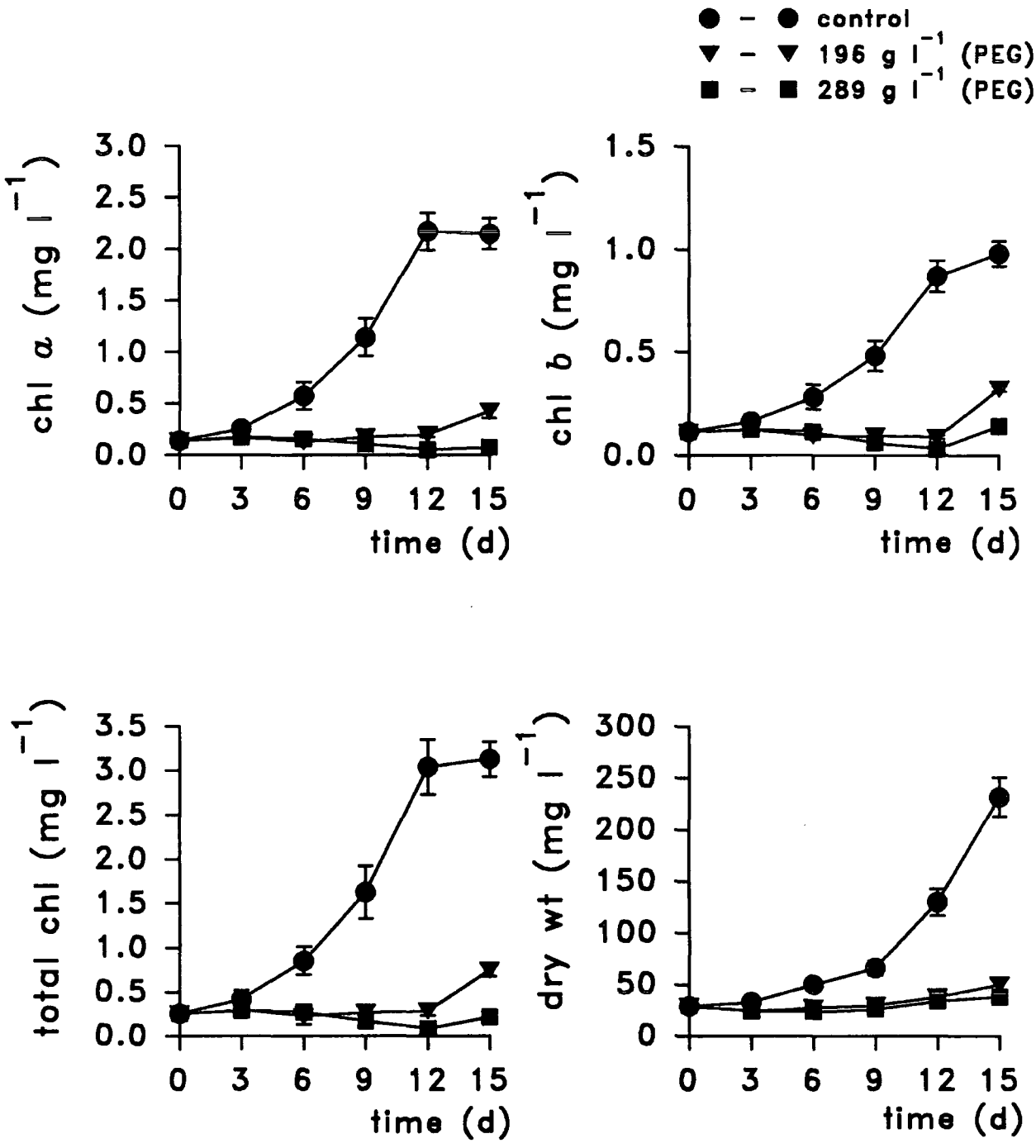
2 To determine the changes in growth parameters (chlorophyll and dry weight) of shoots at different concentrations of PEG

3 To determine the capacity for recovery by the moss after being subjected to water stress

5.531 Influence of water stress on growth parameters of moss shoots

Experiments were carried out to test the extent to which the moss shoots could grow and tolerate water stress. The experimental period was 15 days. Measurements of chlorophyll *a*, *b* and *a+b* and dry weight were made at regular intervals. The results are shown in Fig. 5.9. Chlorophyll contents of the control showed a marked increase from day 6 up to day 12 in respect to the other two sets of water stressed shoots cultures.

Fig. 5.9 Influence of water stress on growth rate (± S.E) of *H. fontanum* in relation to chl *a*, *b* and *a+b* and dry weight. (Shoots were used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)



Chlorophyll contents of the water-stressed cultures showed a slight decline from day 3 up to day 12, but then began to increase slightly. However, in the control chlorophyll content increased from day 3 up to day 12, then began to decline. The highest concentrations of chlorophyll content was seen in the control tissues, whereas the lowest was in the culture subjected to the highest concentration of PEG (289 g l^{-1}). The chlorophyll *a/b* ratio in treated shoots changed at different stages depending on time (Fig. 5.10a). The dry weight of the control, however, showed an increase from day 3 up to day 15 (experimental period) whereas in the water-stressed shoots cultures, increases in dry weight were observed from day 9 up to day 15.

5.532 Influence of water stress on growth parameters of protonema

Cultures, which had been subjected to the same concentrations of PEG (water stress) as the shoots were used in the case of protonema. The results showed (Fig. 5.11) that the growth pattern of protonema is more or less the same as the shoots growth pattern for the first 9 days except that the chlorophyll content of the water-stressed cultures showed a slight increase up to day 3 then began to decline after that time. The chlorophyll *a/b* ratio for treated protonema declined gradually (Fig. 5.10b). At day 12 the protonema of water-stressed cultures gave no detectable chlorophyll on extraction.

Fig. 5.10 Influence of water stress on growth rate (± S.E) of *H. fontanum* in relation to chl *a/b* ratio. (Shoots and protonema were used for experiments; 85 μmol photon m^{-2} s^{-1}; 25°C)

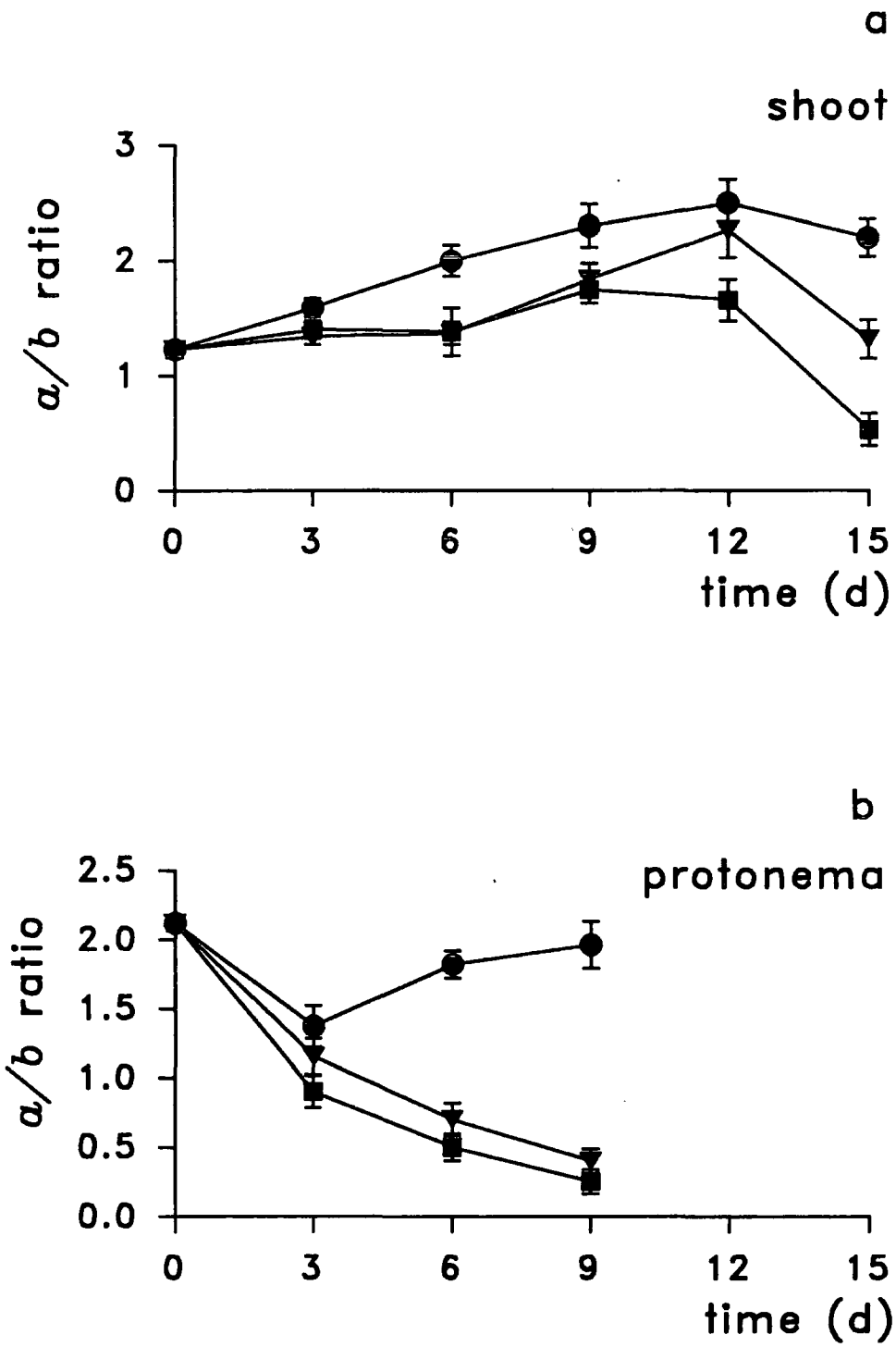
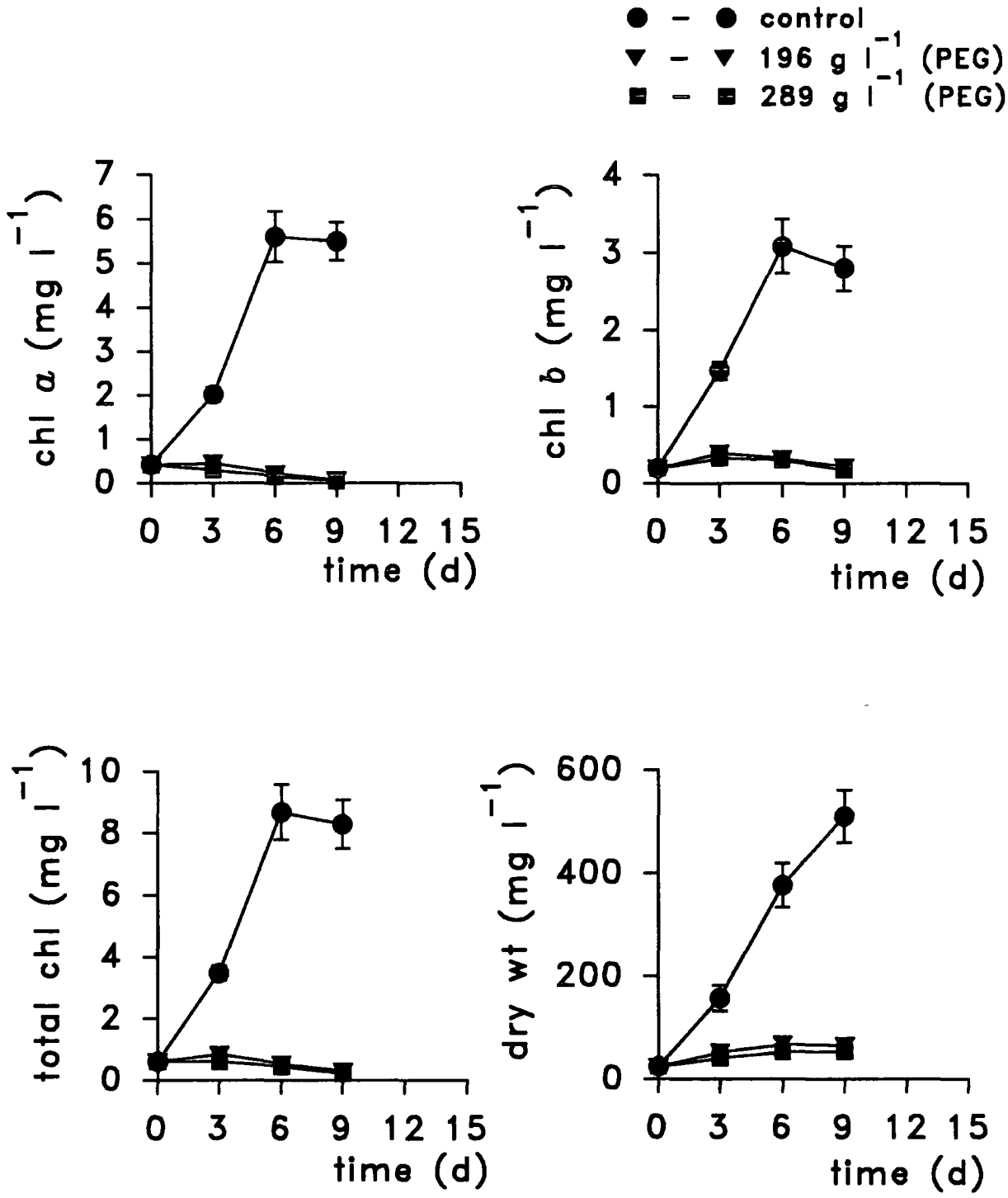


Fig. 5.11 Influence of water stress on growth rate (± S.E) of *H. fontanum* in relation to chl *a*, *b* and *a+b* and dry weight. (Inoculum 1 week old protonema; 85 µmol photon m^{-2} s^{-1}; 25°C)

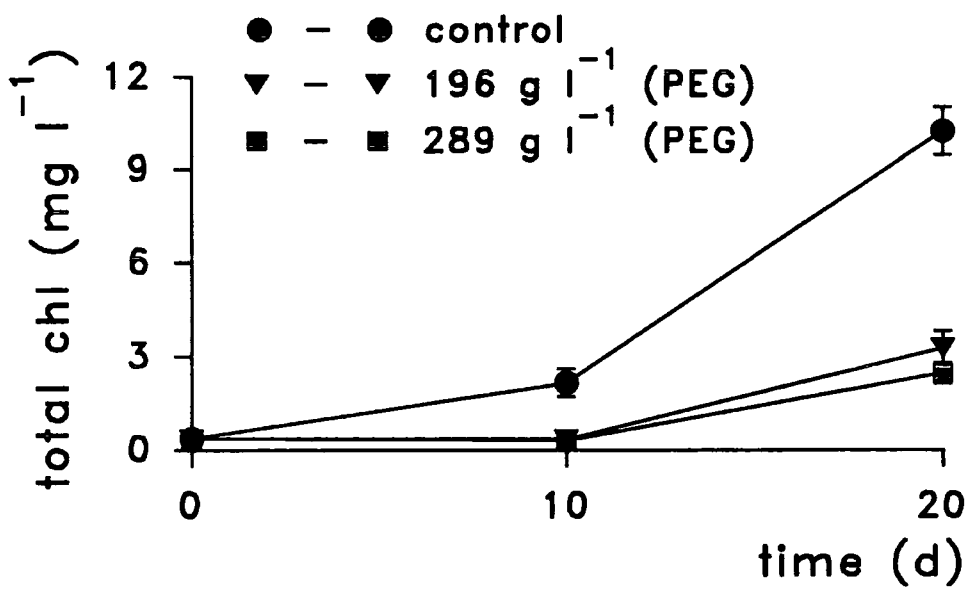


5.533 Recovery of the moss from water stress

Experiments were set up to determine the recovery of moss shoots and protonema cultures after being subjected to water stress. Cultures including the control were left to grow for 10 days, then 4 replicates for each culture were harvested and chlorophyll content and dry weight were determined. Similar replicates for each culture of shoots and protonema were subcultured into fresh Chu 10 G medium and left to grow for 10 days (end of the experimental period). Chlorophyll content and dry weight were again determined after this time. The results are shown in Figs 5.12 and 5.13. Chlorophyll content declined in both cultures (shoots and protonema) which were subjected to water stress for 10 days. Dry weight, however, had not changed. Ten days after being subcultured in a fresh medium, the moss shoots and protonema recovered, and the chlorophyll content and dry weight increased in all treatments. Although, increase of chlorophyll content and dry weight in the treated cultures was significantly lower in respect to the control in both cultures of shoots and protonema.

The shoots or protonema, which had been subjected to water stress, recovered after subculturing, but their rate of recovery was less than that of the control. This shows that there is a carry-over of the water stress treatment on the tissues.

Fig. 5.12 Recovery (± S.E) of *H. fontanum* after being subjected to water stress for 10 days. (Inoculum 1 week old protonema; 85 μmol photon m^{-2} s^{-1}; 25°C)



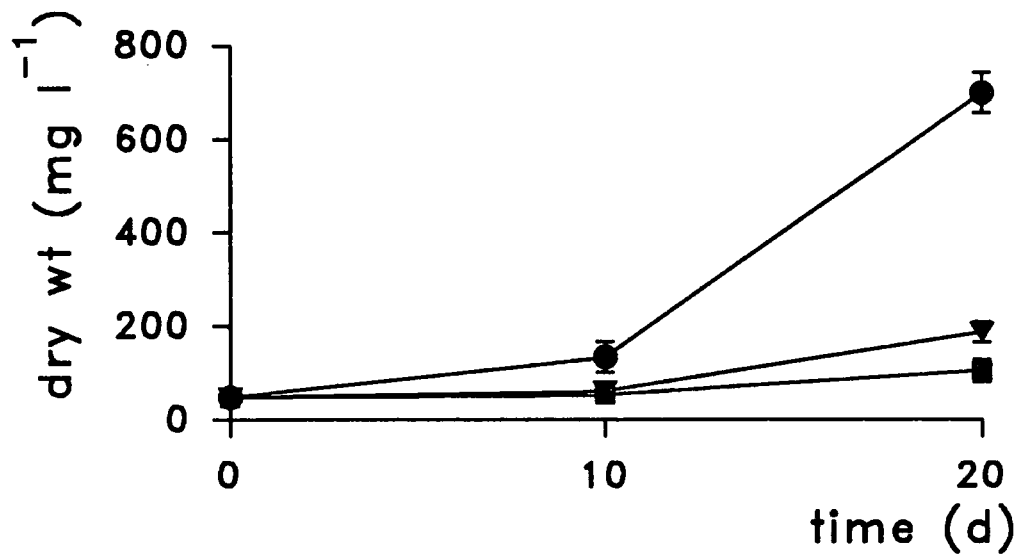
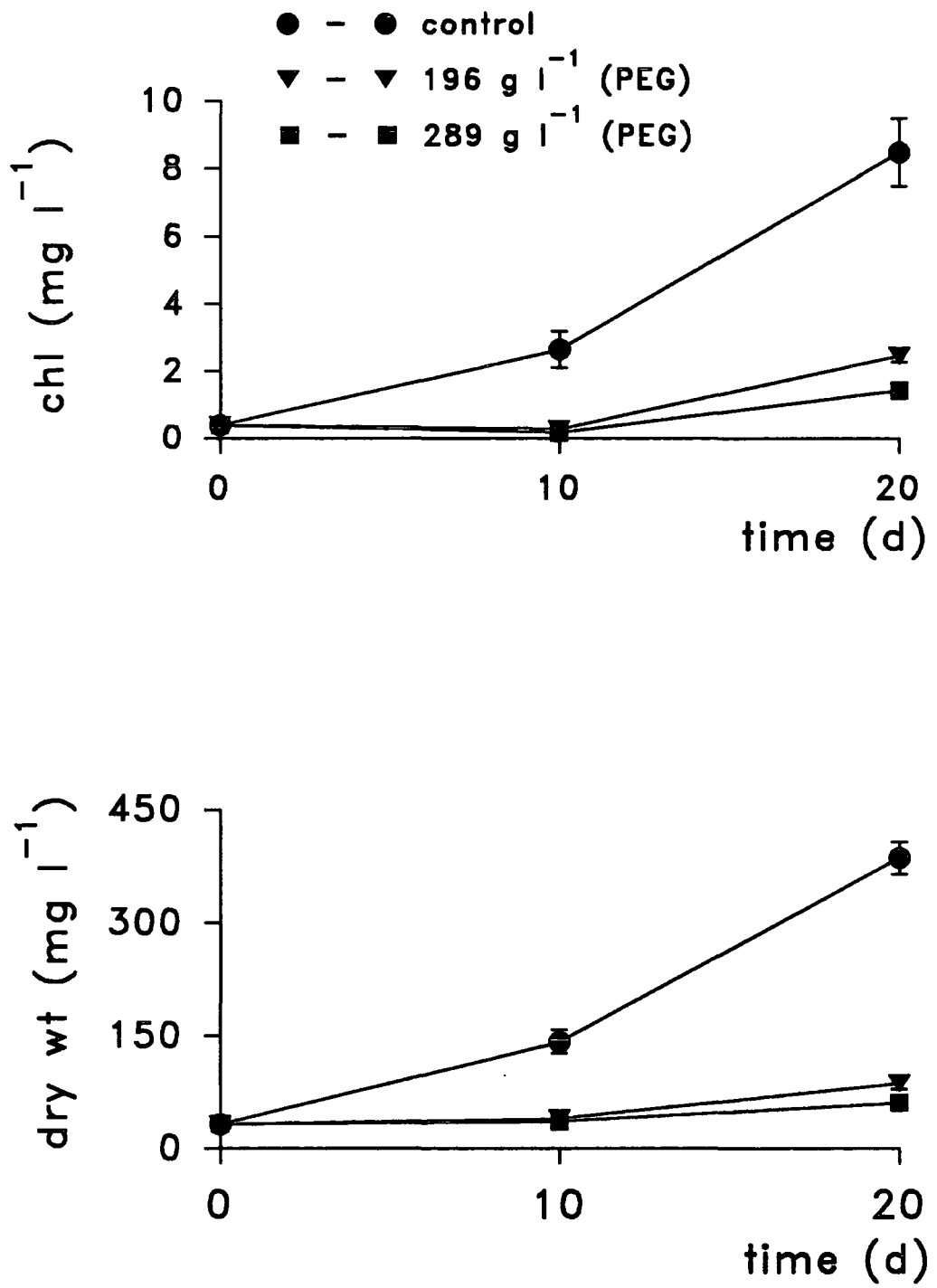


Fig. 5.13 Recovery of *H. fontanum* (± S.E) after being subjected to water stress for 10 days. (Shoots were used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)



6 PHOSPHATASE ACTIVITY

6.1 Introduction

The ability of blue-green algae and other organisms to produce phosphatase enzymes and to utilize organic phosphates for their growth has been demonstrated (Section 1.7). However, there have been only a very few study on bryophytes (Section 1.724). Studies were therefore undertaken on *Hydrogonium fontanum*. The specific objectives were:

(1) To determine which fraction (rhizoids, protonema, leafy shoots) of the moss has the highest phosphatase activities.

(2) To quantify the influence of environmental variables of phosphatase activities.

(3) To determine the ability of the moss protonema to utilize a range of organic phosphate substrates as P.

(4) To determine the influence of pNPP concentration on phosphomonoesterase activity of the moss protonema.

(5) To determine the influence of pH on PMEase using two different substrates (pNPP & 4-MUP) and to test different concentrations of 4-MUP on this activity.

6.2 Phosphatase activities of different moss fractions

Experiments were carried out with laboratory and field moss materials in order to see which fraction has the highest phosphatase activities. However, in the field material due to the low production of moss protonema only two fractions shoot and rhizoid were tested.

In the experiments of moss fractions, PMEase and PDEase activities were assayed at pH 6.0 (DMG-NaOH, 50 mM) and 25°C. Cellular material was harvested as in 2.71. The assays were carried out as in 2.721 for protonema and rhizoid and 2.722 for shoots. The tests were made on cell-

bound and extracellular PMEase and PDEase. The results are shown in Fig. 6.1. Rhizoids had the highest PMEase and PDEase, whereas shoots showed the lowest activities; this was seen in both field and laboratory materials. Although phosphatase activities were slightly lower in protonema compared to rhizoid, protonema was used for most of the phosphatase activities experiments and that due to its fast growth, high production and abundant in the culture. Extracellular PMEase and PDEase were not detectable in any fraction of the moss. Therefore further experiments were carried out on cell-bound phosphatase activities only (phosphatase activities).

6.3 Growth and phosphatase activities

In the previous experiments, the protonema formed both PMEase and PDEase activities. Since the protonema is the dominant stage of growth under laboratory conditions further experiments were carried out to determine the changes in phosphatase activities of this fraction in batch culture. With the determination of phosphatase activities cellular P content of protonema was also monitored, to relate the result with switch-on of phosphatase activities. The protonema was subcultured twice in Chu 10 G with 1 mg l^{-1} P at 3-d intervals to ensure that there was no detectable phosphatase activity. Experiments were carried out with 4 replicates under standard conditions (Section 2.36) in the shaker tank. Changes in dry weight, cellular P content and phosphatase activities of protonema were determined at 4 day-intervals for 36 days Figs 6.2a, 6.3a and 6.4. As a marked increase in phosphatase activities occurred between day 8 and 12, another experiment was carried out for 12 days to determine phosphatase activities on a daily basis (Figs 6.2b, 6.3b & 6.5).

Fig. 6.1 Phosphatase activities (± S.E) at pH 6.0 of 3 different fractions (rhizoids, protonema, leafy shoots) sampled at same time.

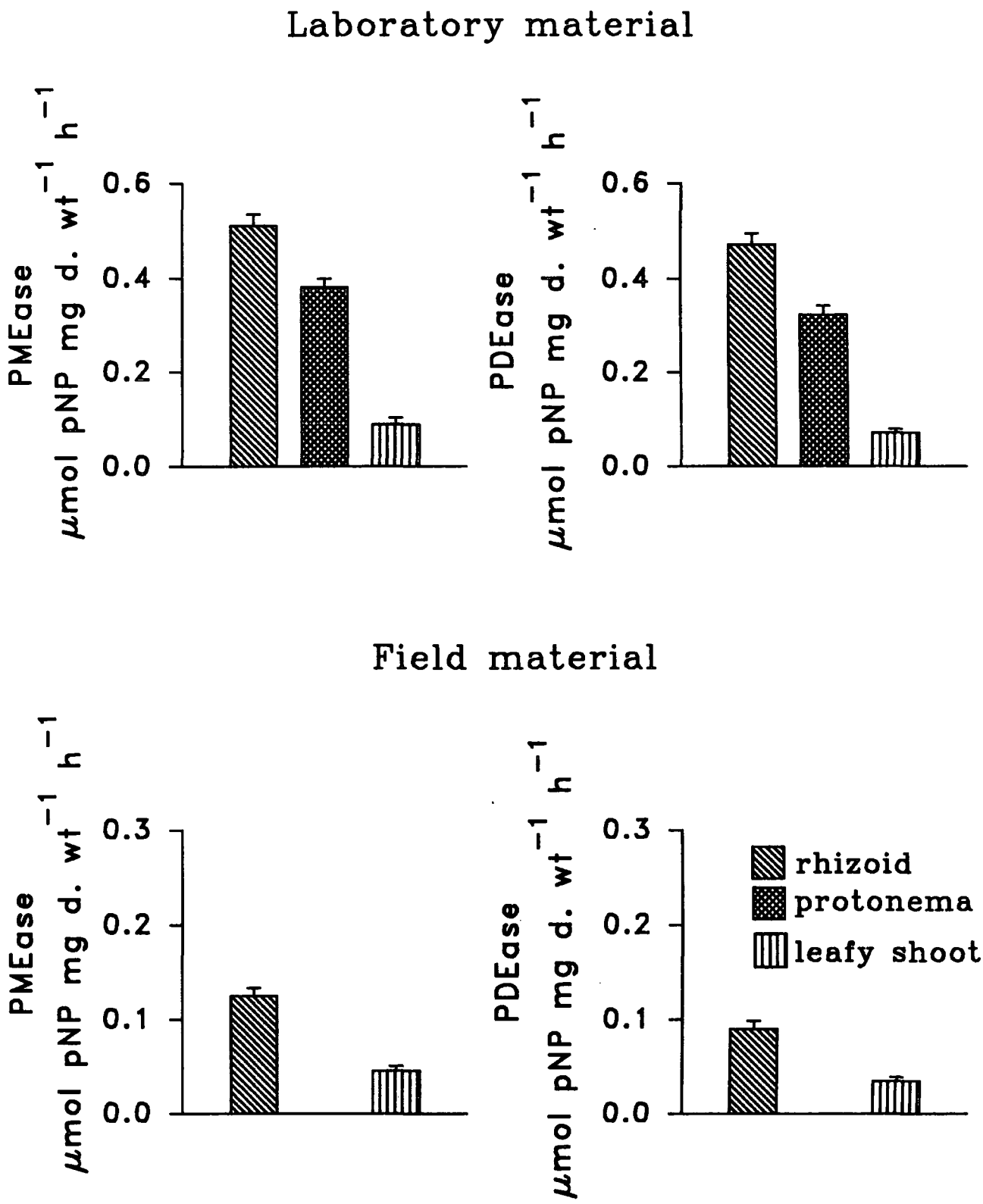


Fig. 6.2 Changes in dry weight (± S.E.) of *H. fontanum* in batch culture for (a) 36-d and (b) 12-d. (Inoculum 3-d old protonema; 85 μmol photon m^{-2} s^{-1}; 25°C)

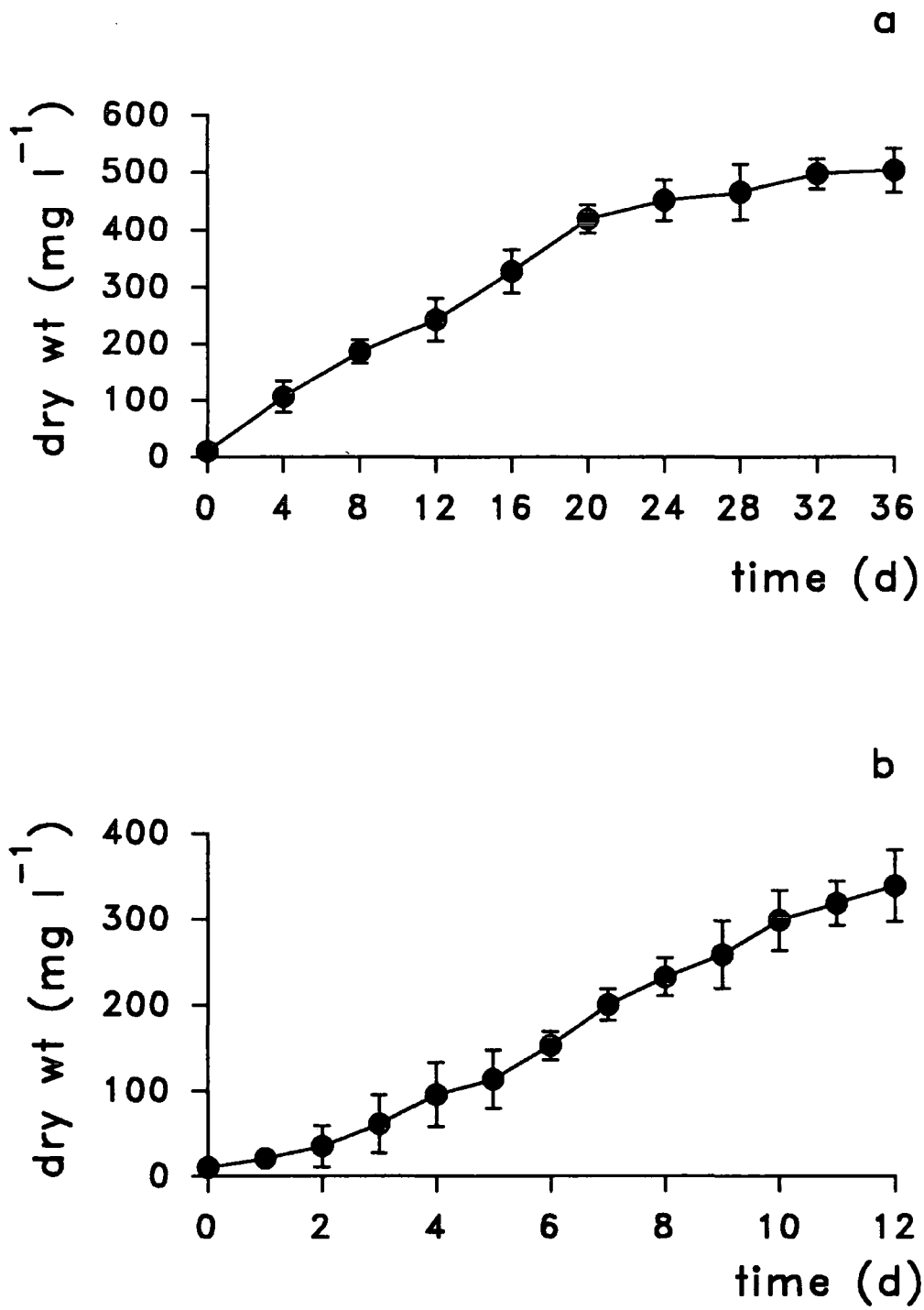


Fig. 6.3 Changes in cellular P content (± S.E) of *H. fontanum* in batch culture for (a) 36-d and (b) 12-d. (Inoculum 3-d old protonema; 85 μmol photon m^{-2} s^{-1}; 25°C)

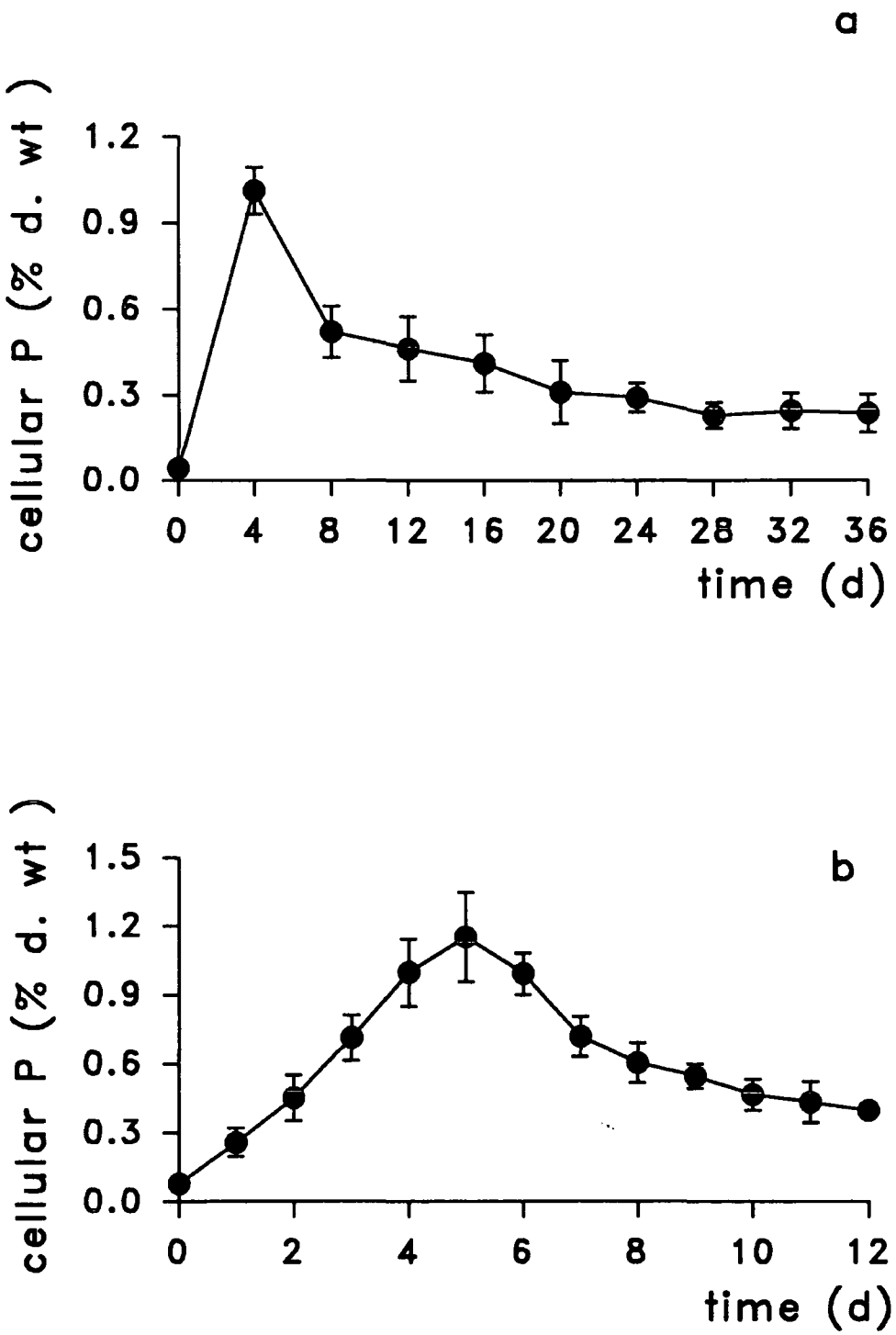


Fig. 6.4 Changes of phosphatase activities over 36-d (± S.E) of *H. fontanum* in batch culture. (Inoculum 3-d old protonema; 85 μmol photon m^{-2} s^{-1}; 25°C)

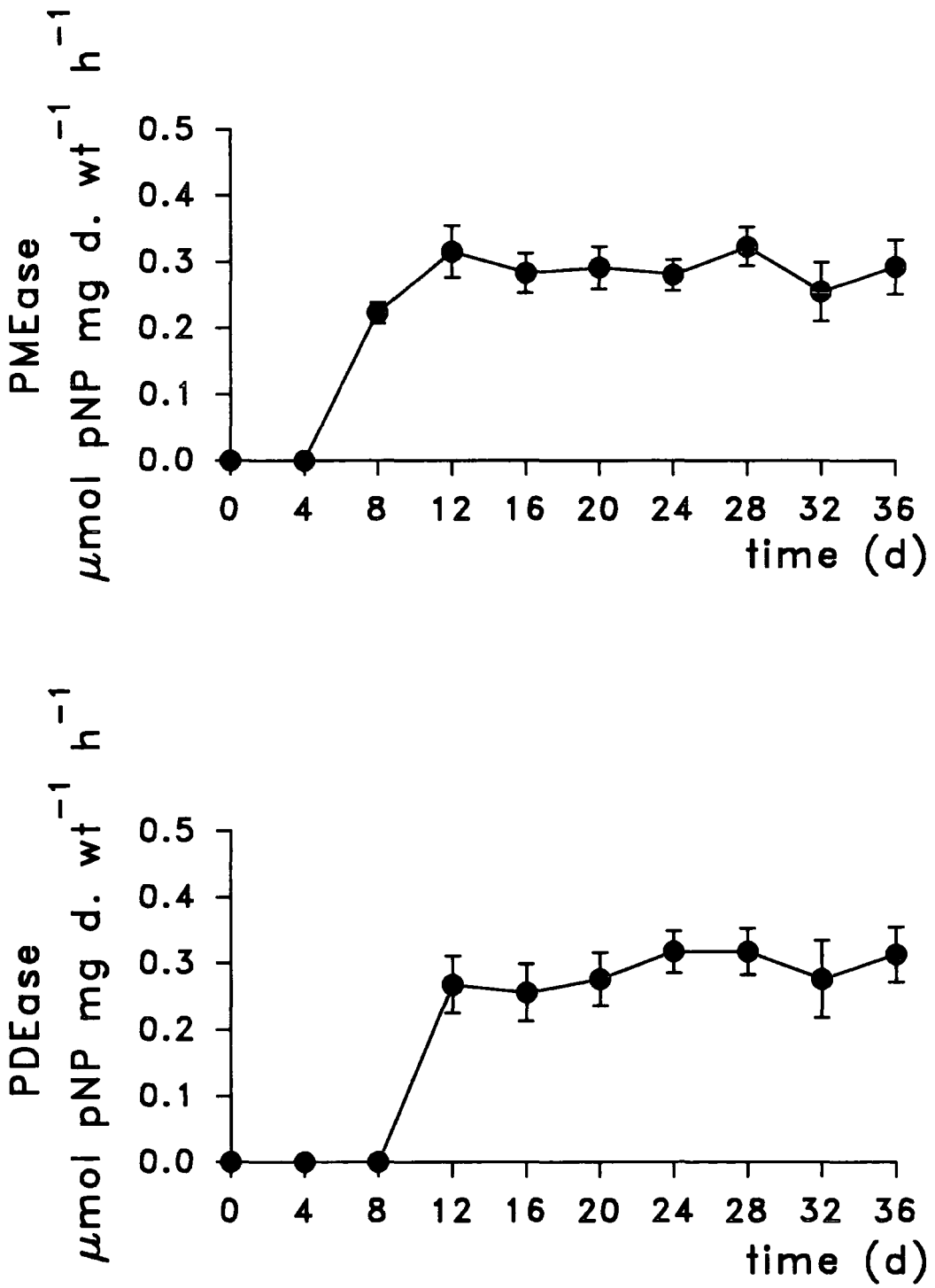
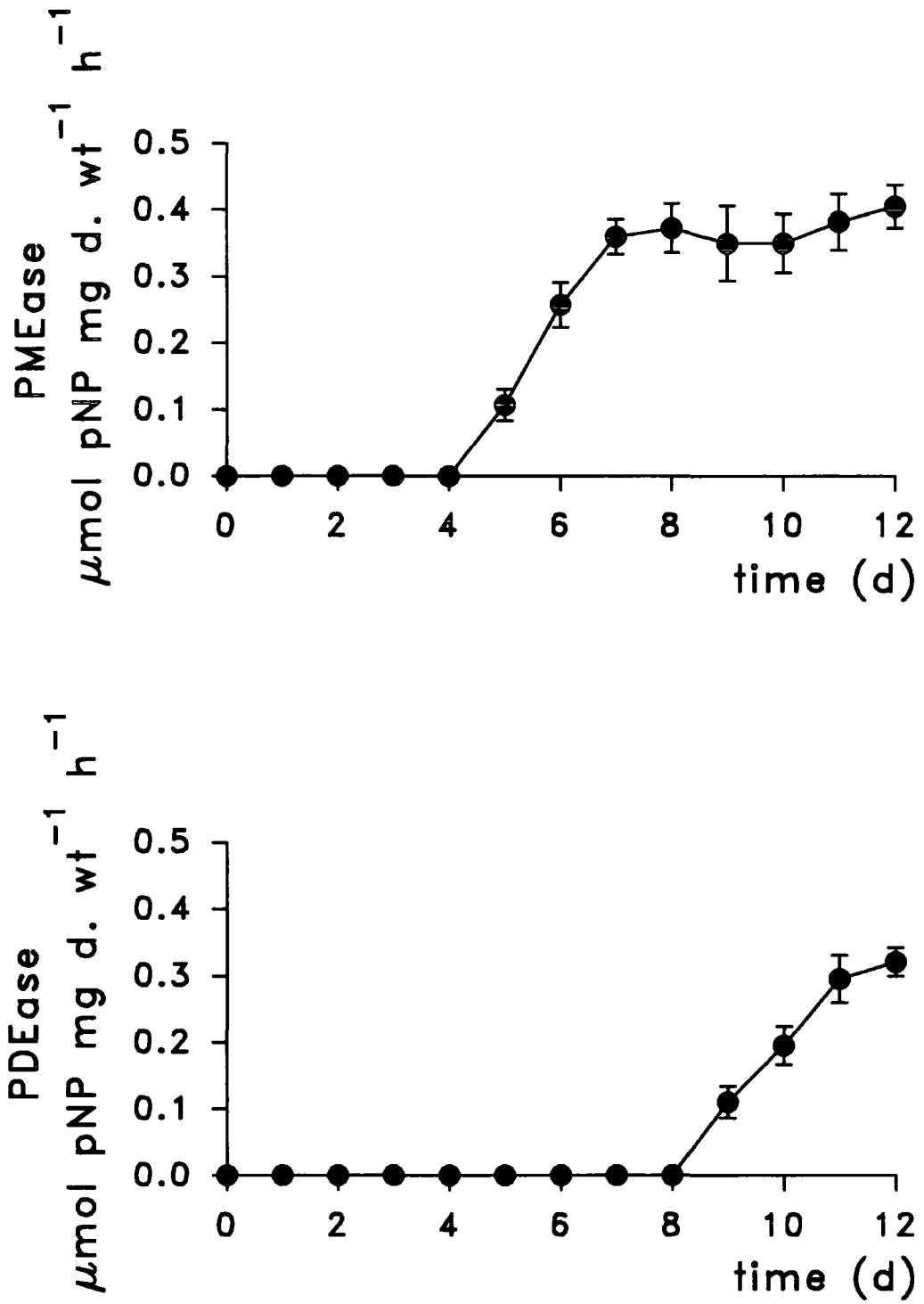


Fig. 6.5 Changes of phosphatase activities over 12-d (± S.E) of *H. fontanum* in batch culture. (Inoculum 3-d old protonema; 85 μmol photon m^{-2} s^{-1}; 25°C)



The protonema showed both PMEase and PDEase activities. Detectable PMEase activity was observed by day 5, whereas avoidance of PDEase activity was observed by day 9 (Fig. 6.5). There were no significant differences in PMEase activity between days 12 and 36 (Fig. 6.4). The rate of PDEase in protonema increased gradually up to day 12 (Fig. 6.5) and maintained almost the same rate to day 36 (Fig. 6.4).

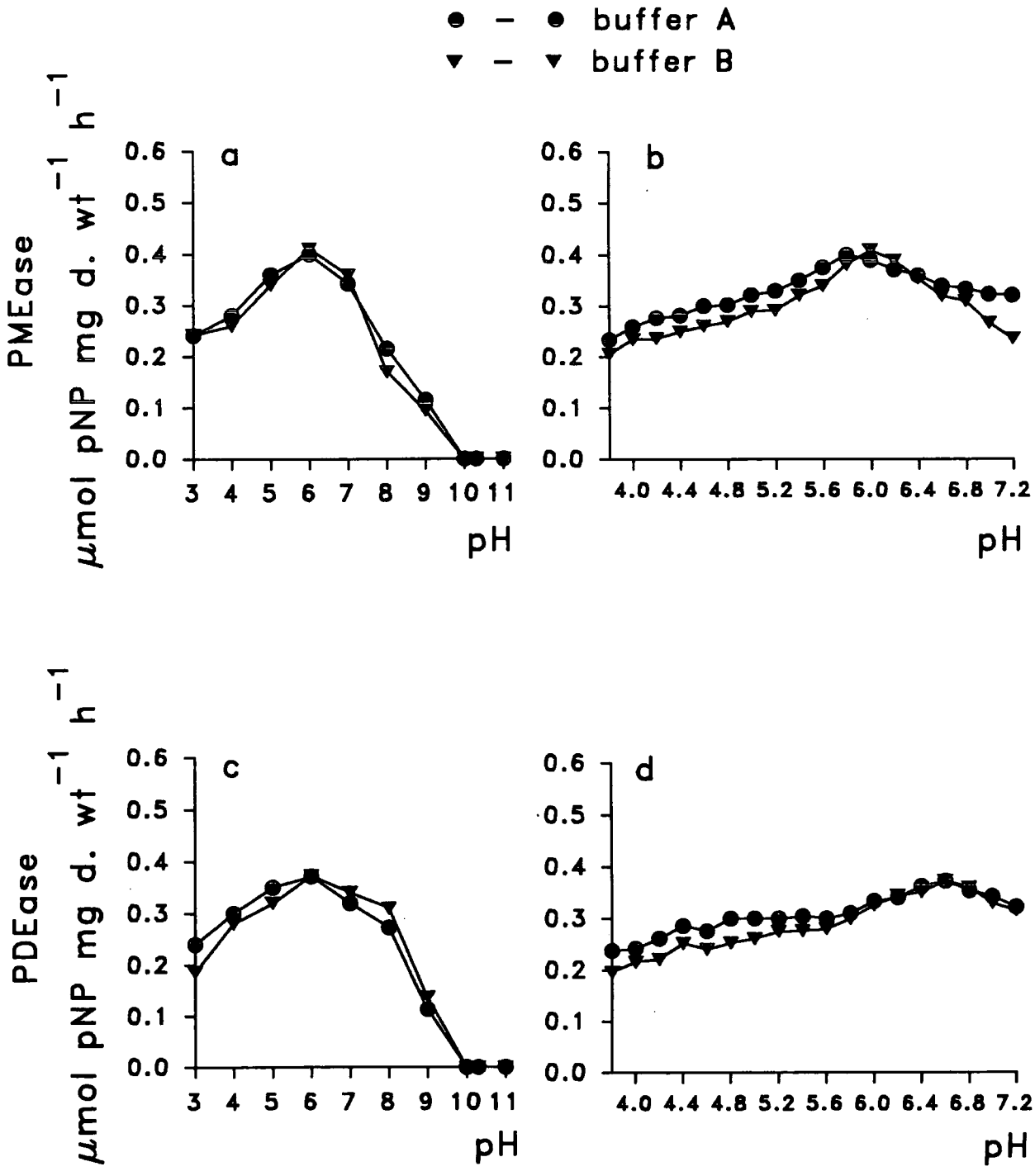
6.4 Influence of pH

The pH of the water of the field waterfall site where the moss grows showed changes with time (Tables 3.1 & 3.2). It was, therefore, felt necessary to test phosphatase activities of the protonema over a pH range from 3.0-11.0. The range from pH 3.8 to 7.2 was also tested at intervals of 0.2 pH units (Section 2.73). Two different buffer sets were tested at each pH value (Table 2.6). The influence of pH on phosphatase activity of the protonema was tested Fig.(6.6).

PMEase activity of the protonema was detectable between pH 3.0-9.0 (Fig. 6.6a) with the highest activity being at pH 5.8 (Fig. 6.6b) using buffer Set A (Materials & Methods, table 2.6). Whereas the optimum activity using buffer set B (Materials & Methods,table 2.6), was at pH 6.0 (Fig. 6.6b). PDEase activity was also detectable between pH 3.0-9.0 (Fig. 6.6c) with optimum activity at pH 6.6 (Fig. 6.6d). PDEase activity was slightly lower than PMEase activity at all pH values.

Initial experiments on changes in phosphatase activities of the protonema indicated that PMEase activity was detectable by day 5 at pH 6.0 (Fig. 6.5), which is the optimum pH for the PMEase activity. The experiment was set up to determine whether PMEase activity was detectable on other pH values at this early stage of growth.

Fig 6.6 Influence of pH on phosphatase activities of *H. fontanum*. (28-d old protonemal culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)



PMEase was detectable between pH 4.0-8.0 with optimum activity at pH 6.6 (Fig. 6.7).

6.5 Influence of pH on phosphatase activities of rhizoid, protonema and leafy shoot

The previous experiments (Fig. 6.6), used the protonema stage. However, other fractions (rhizoid, protonema, leafy shoot) make up the morphology of the moss and these similarly were tested for phosphatase activities. It was found that phosphatase activities were detectable in all these tissues. Therefore, it was decided to see if there were differences in the influence of pH for the moss fractions.

The influence of pH on activities was tested using a range of buffers of set A as this set of buffers showed better results and constant values than buffer set B (Table 2.6B) when they were tested through preliminary experiments. The moss fractions were taken from a 28-d old culture grown in Chu 10 G with P_i 1 mg l^{-1} at 25°C, 80 μmol photon m^{-2} s^{-1}. In the case of field material shoots with rhizoids were gently washed with MilliQ water, and rhizoids were separated from shoot stems with a fine forceps. Material was prepared for assays as in 2.721 for protonema and rhizoids and 2.722 for shoots.

PMEase and PDEase activities were detectable (Fig. 6.8) between pH 3.0-9.0 for rhizoids and shoots and pH 3.0-8.0 for protonema with highest activities at pH 6.0 for all three fractions (rhizoid, protonema, leafy shoots). In comparison with shoots, rhizoids showed higher activities at all pH values, protonema too compared well with rhizoids.

Fig. 6.7 Influence of pH on PMEase activity of *H. fontanum*. (5-d old protonemal culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)

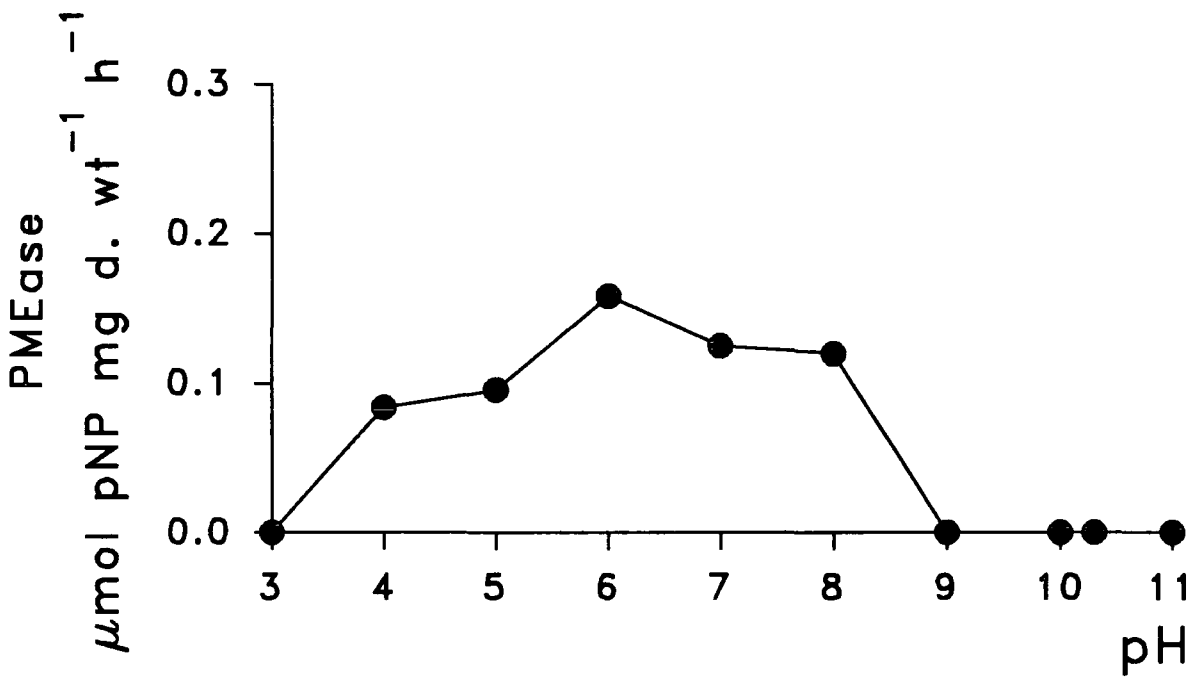
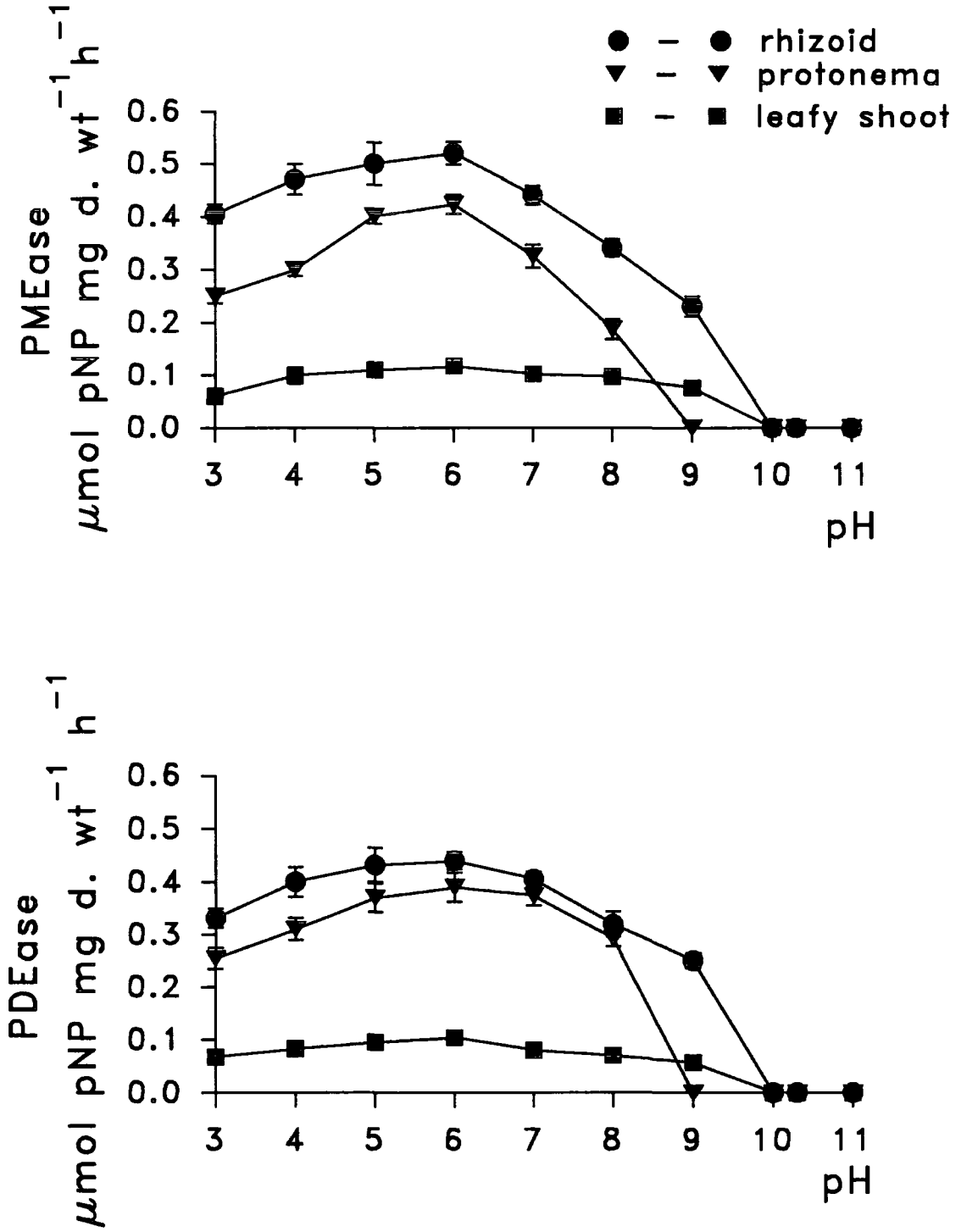


Fig. 6.8 Influence of pH on phosphatase activities of *H. fontanum*. (28-d old culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)



PMEase and PDEase of field material (Fig. 6.9: shoots and rhizoids) were detectable between pH 3.0-9.0 (shoots) and PMEase between pH 3.0-11.0 and PDEase between pH 3.0-8.0 (rhizoids) with optimum PMEase activity at pH 4.0 and 5.0 respectively. PDEase optimum for shoots was at pH 5.0 and at pH 6.0 for rhizoids.

In general, the PMEase and PDEase activities of the laboratory moss fractions (rhizoids, protonema, leafy shoots) showed higher activities than field moss fractions.

6.6 Influence of temperature

An experiment was carried out to test the influence of temperature on phosphatase activities of moss protonema. PMEase and PDEase were assayed using pNPP and bis-pNPP, respectively. To test the influence of temperature on phosphatase activities, aliquots of homogenate was pre-incubated for 30 min at 5°C intervals between 5-80°C and then incubated at the same temperature for a further 1 h (Fig. 6.10). The temperature optima for PMEase and PDEase were 60°C and 65°C, respectively.

6.7 Influence of ions

PMEase and PDEase activities were assayed using pNPP and bis-pNPP, respectively at pH 6.0 (DMG-NaOH, 50 mM). Phosphatase activities were assessed on day 28 and also at earlier stage in the growth of protonema in day 5, which is the earliest time when PMEase is detectable. As Mg, Ca, Zn, Na, K, and P are major elements in the growth medium, so the influence of these elements on phosphatase activities was tested. The concentrations tested in each case were 0.001, 0.01, 0.1, 1 and 10 mM. Cations were added as the relevant chloride or sulphate; phosphate was added as the sodium salt.

Fig. 6.9 Influence of pH on phosphatase activities of *H. fontanum*. (rhizoids & leafy shoots of field material)

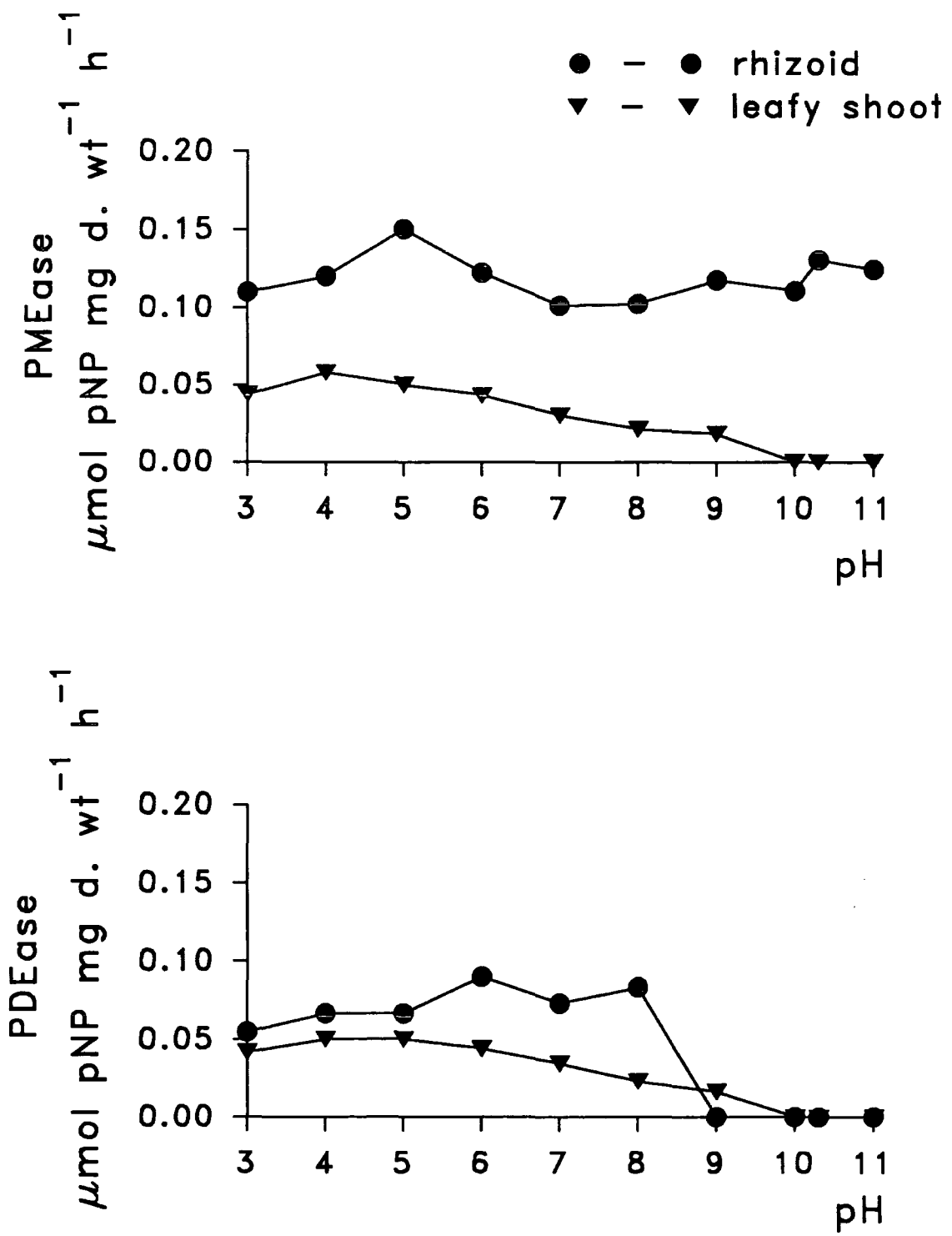
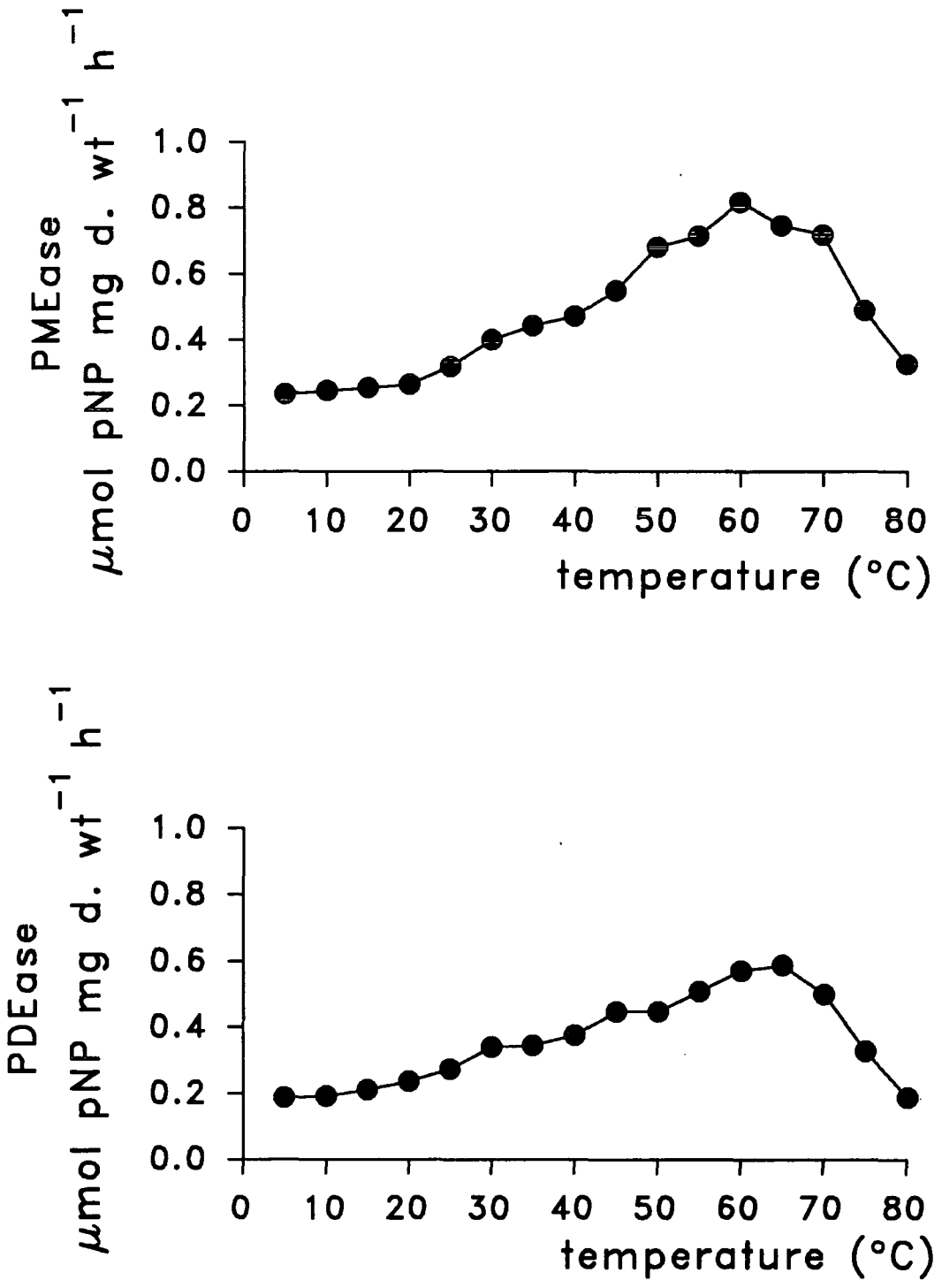


Fig. 6.10 Influence of temperature on phosphatase activities of *H. fontanum*. (28-d old protonemal culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)



The responses of all PMEase and PDEase activities are shown in Fig. 6.11 (5-d old culture) and Fig. 6.12 (28-d old culture).

5-day-old culture

In five-days-old culture PMEase activity was seen with all 6 elements tested (Fig. 6.11). An increase from 0.001 to 10 mM concentration of Na and K had no effect on PMEase activity. Zn and P began to gradually inhibit the activity from .01 to 1.0 mM concentration while P inhibited the activity completely at 10 mM. Ca and Mg in this activity still considerable at 10 mM.

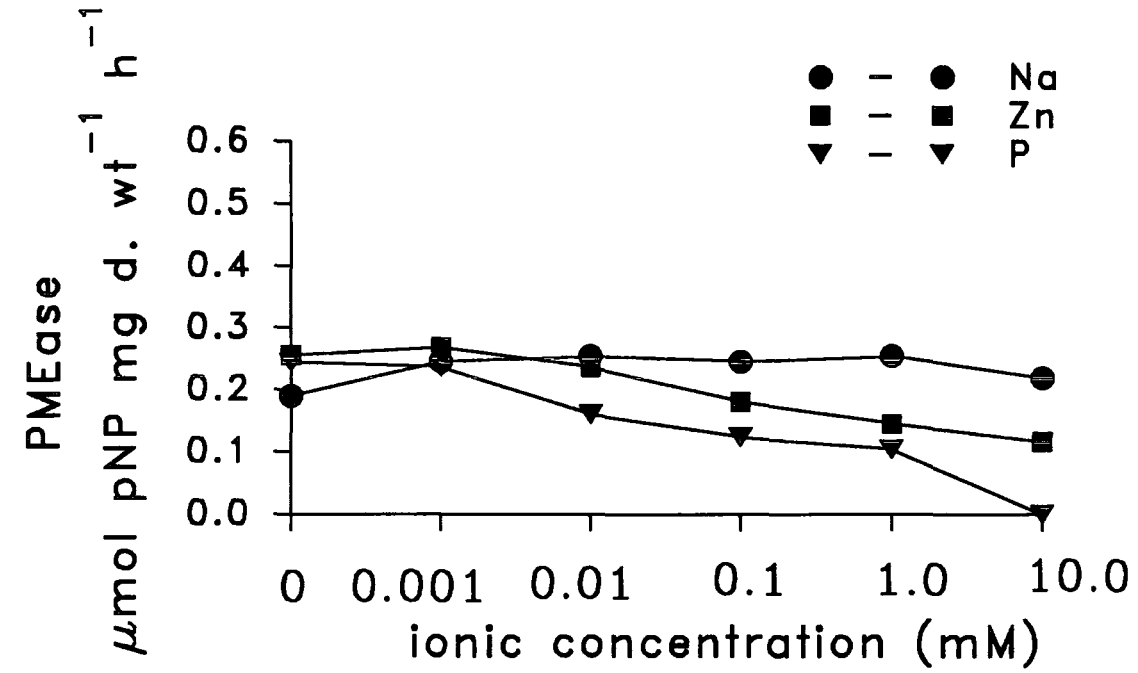
28 day old culture

(1) PMEase activity. Slight enhancement of activity occurred with 10 mM Mg. Zinc stimulates the activity up to 0.1 mM and inhibited it at 10 mM. Very slight enhancement of activity occurred between 0.01-0.1 mM K. Calcium showed complete inhibition of the activity at 10 mM. Phosphate showed gradual inhibition of PMEase activity up to 1 mM, and there was complete inhibition of this activity at 10 mM.

(2) PDEase activity

Ions in general had less effect on PDEase than on PMEase activity. With Na there was a slight increase in activity between 1.0-10 mM. K had a stimulatory effect on the activity between 0.1-10 mM. Phosphate between 0.001-0.1 had no apparent inhibitory effect, but in the range of 1.0-10 mM PDEase activity began to decline gradually and up to 10 mM there was a complete shut down of this activity. Ca had no effect. Mg had a slight inhibitory effect at high concentration 1-10 mM. Zinc inhibited completely PDEase activity at the high concentration of 10 mM, whereas a lower concentration had no inhibitory effect.

Fig. 6.11 Influence of ions on PMEase activity of *H. fontanum*. (5-d old protonemal culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25 °C)



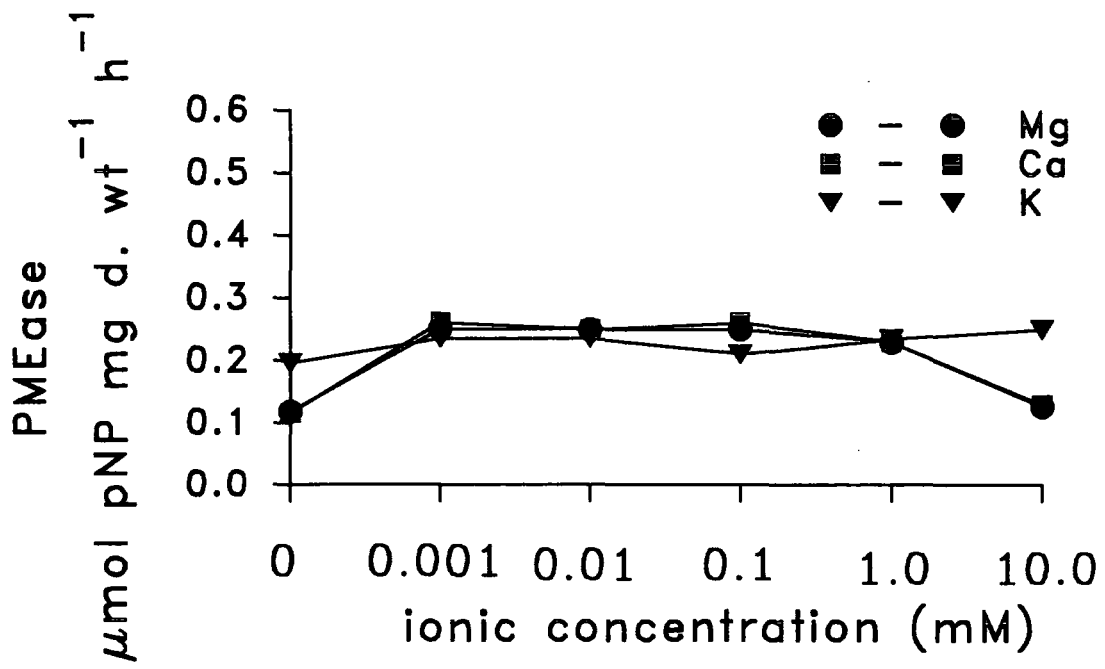
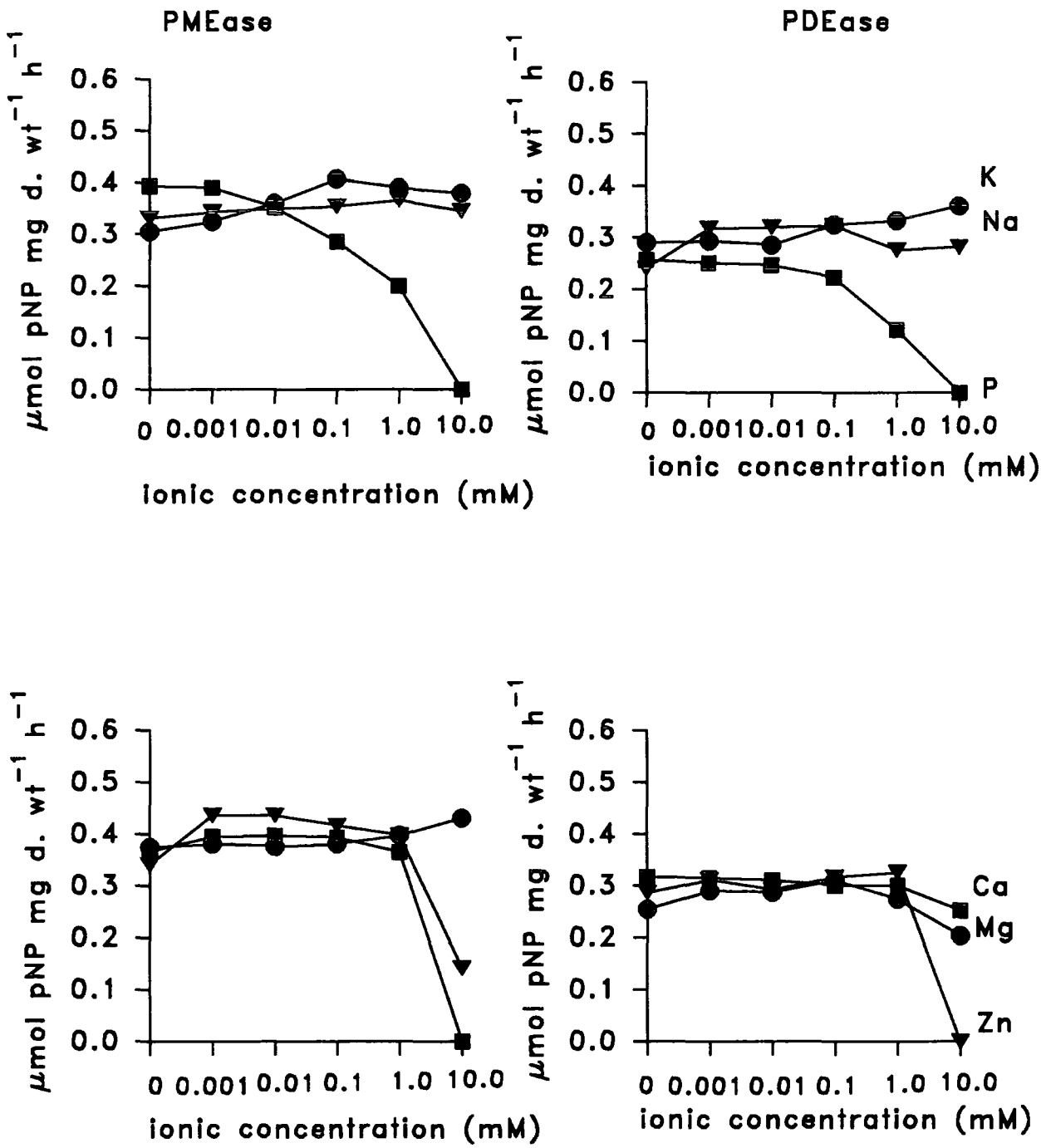


Fig. 6.12 Influence of ions on phosphatase activities of *H. fontanum*. (28-d old protonemal culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)



6.8 Influence of desiccation

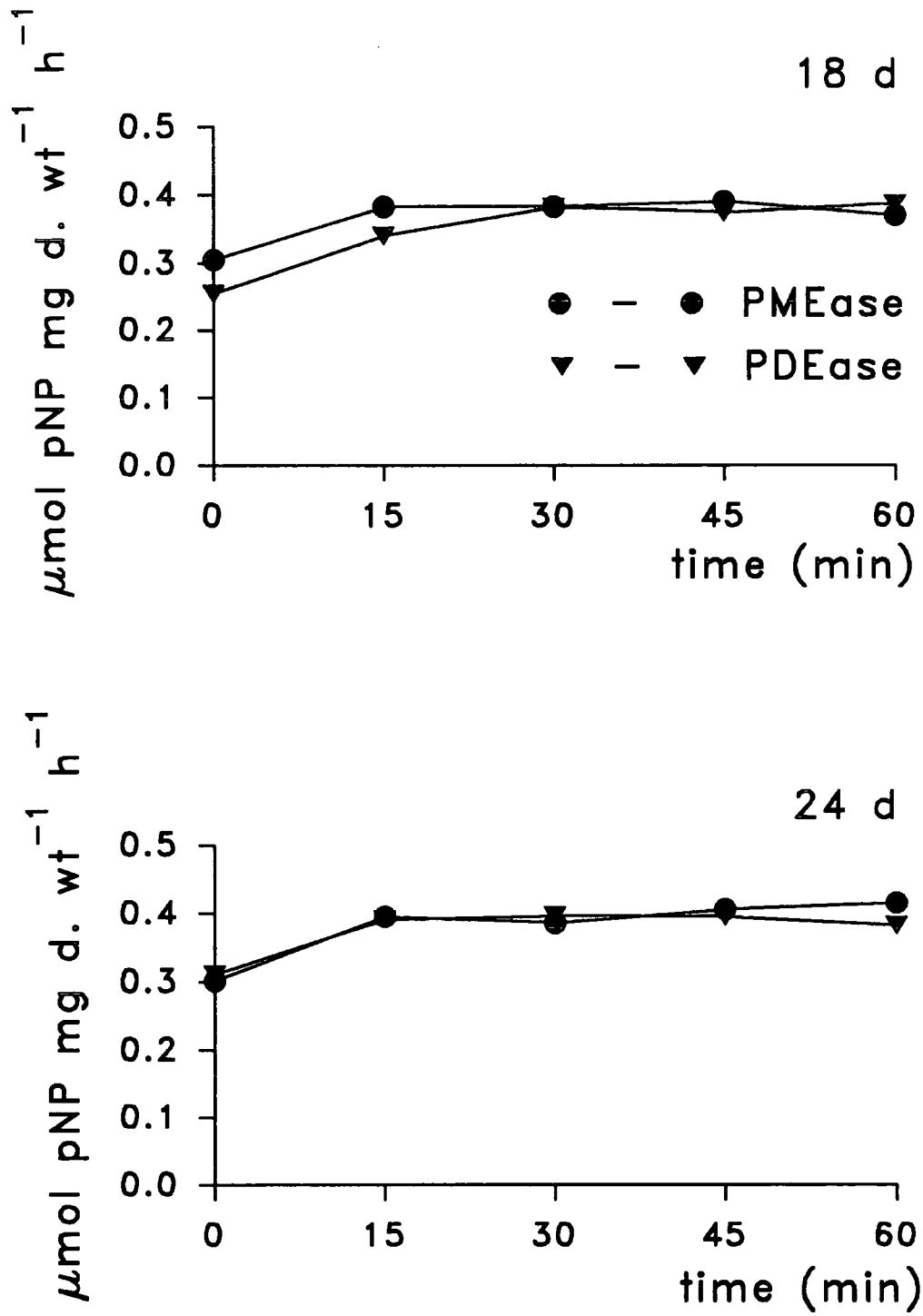
Of the different environmental factors that influence phosphatase activities of *H. fontanum*, are desiccation and drought which are ever present in the field where the moss grows. Therefore experiments were carried out to see the influence of desiccation on phosphatase activities in moss. Cellular material was harvested (section 2.71) on day 18 and 24. The samples were lightly homogenized, centrifuged and resuspended in assay medium. PMEase and PDEase were determined before the samples were dried at 25°C (under sterile conditions). The protonema then re-wetted 5 d later and assayed for 1 h, commencing at zero time, with subsequent assays at 15 min intervals for 1 h (Fig. 6.13).

Following the re-wetting of desiccated materials originally harvested on days 18 and 24, there was a marked decrease on PMEase and PDEase activities measured at zero time. The recovery of PMEase and PDEase to its pre-desiccation levels was observed 15 min after re-wetting. There was no significant difference from that values measured before the material was dried. The highest PDEase activity showed after 30 min after re-wetting. Although there was no significant difference in both PMEase and PDEase activities at time 15 min in 24-d old culture, there was slight increase in PMEase level in 18-d old culture.

6.9 Influence of drought stress

In the previous experiment, the influence of desiccation on phosphatase activities was tested and the recovery on re-wetting was found to be almost the same as the material that had not been desiccated. Further experiment on the influence of drought for a longer period (3 months) for the 3 moss fractions (rhizoids, protonema, leafy shoots) was carried out to see to what extent phosphatase activities respond to drought stress.

Fig. 6.13 Influence of dessication on phophatase activities of *H. fontanum* protonema harvested on day 18 & 24. [PMEase & PDEase were 0.417 & 0.389 (18-d) & 0.447 & 0.421 (24-d) μmol pNP mg d.wt^{-1} h^{-1} before dessication]



Moss material rhizoid, protonema and leafy shoots were harvested, at late growth stage (day 28) and then centrifuged to remove the growth medium (Section 2.71). Material was then dried at 25°C (under sterile conditions) for 3 months. Moss fractions were subsequently assayed separately at pH 6.0. The influence of pH on phosphatase activities was also tested. PMEase and PDEase activities in the treated (dried) moss fractions (rhizoids, protonema, leafy shoots) were significantly lower than the control (Fig. 6.14).The influence of drought on phosphatase activities over the full pH range is shown in Fig. 6.15. PMEase activity was detectable for all three fractions, rhizoids, protonema and leafy shoots between pH 3.0-10.0, 3.0-11.0 and 3.0-9.0, respectively. Optimum activity was at pH 6.0 for all fractions. PMEase activity of dried fractions was low compared with material before drying (Fig. 6.8) ($P > 0.001$). The activity decreased to about $1/3$ of the original values. PDEase activity was also detectable for all fractions, rhizoid, protonema and leafy shoot between pH 3.0-8.0, 3.0-10.0 and 3.0-7.0 respectively. The optimum activity was at pH 6.0 for protonema and shoot, whereas rhizoid showed the highest activity at pH 5.0. PDEase activity of dried fractions compared to control (undried fractions) also showed a significant decrease ($P > 0.001$).

6.10 Influence of water stress

Water stress is one of the factors which affects the growth and metabolism of *H. fontanum* (Section 5.5), so its influence was tested (0, 50, 100, 150, 200 g l^{-1} PEG) on material harvested on days 18 and 24.

Fig. 6.14 Influence of drought on phosphatase activities of *H. fontanum*. (28-d old culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)

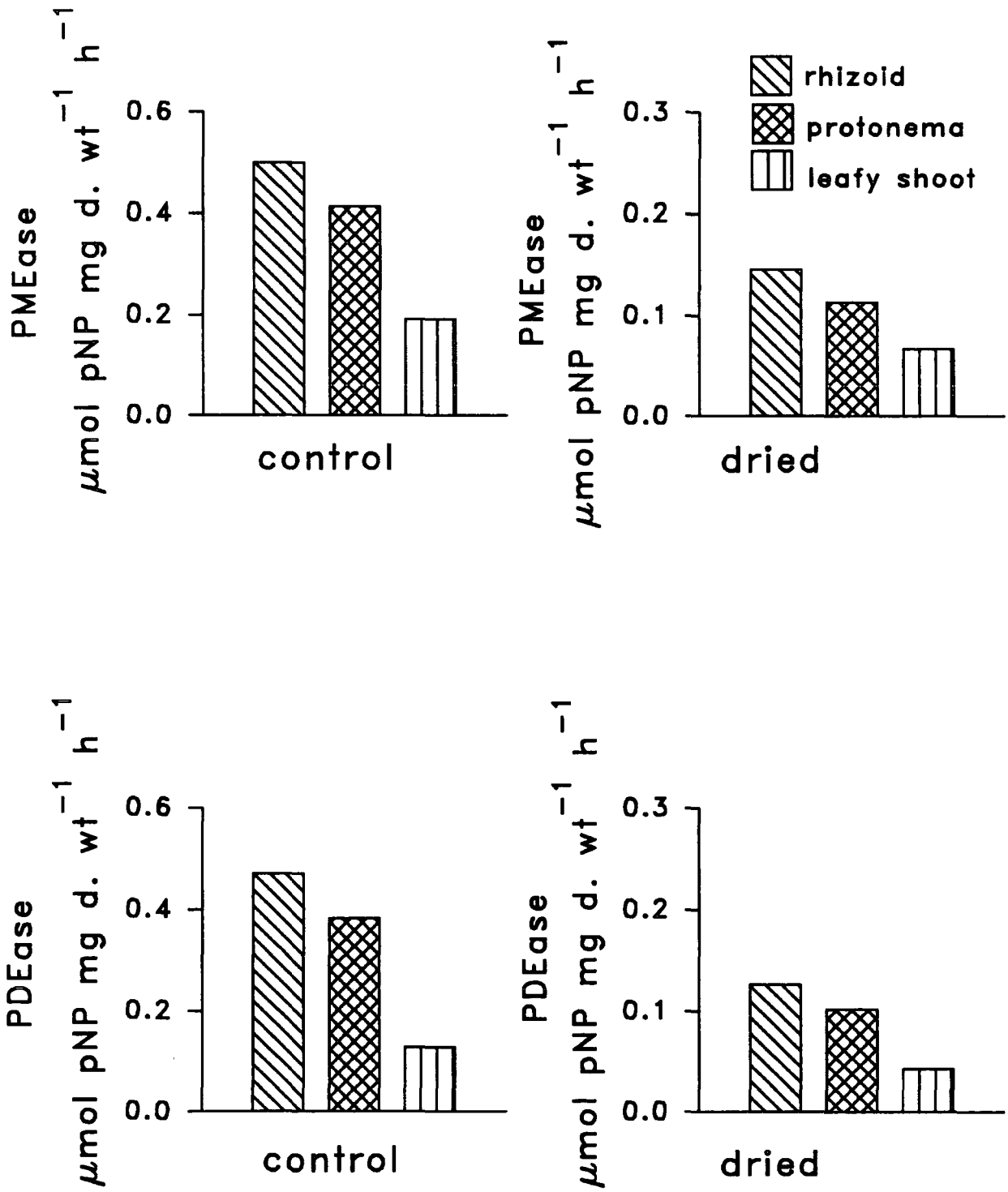
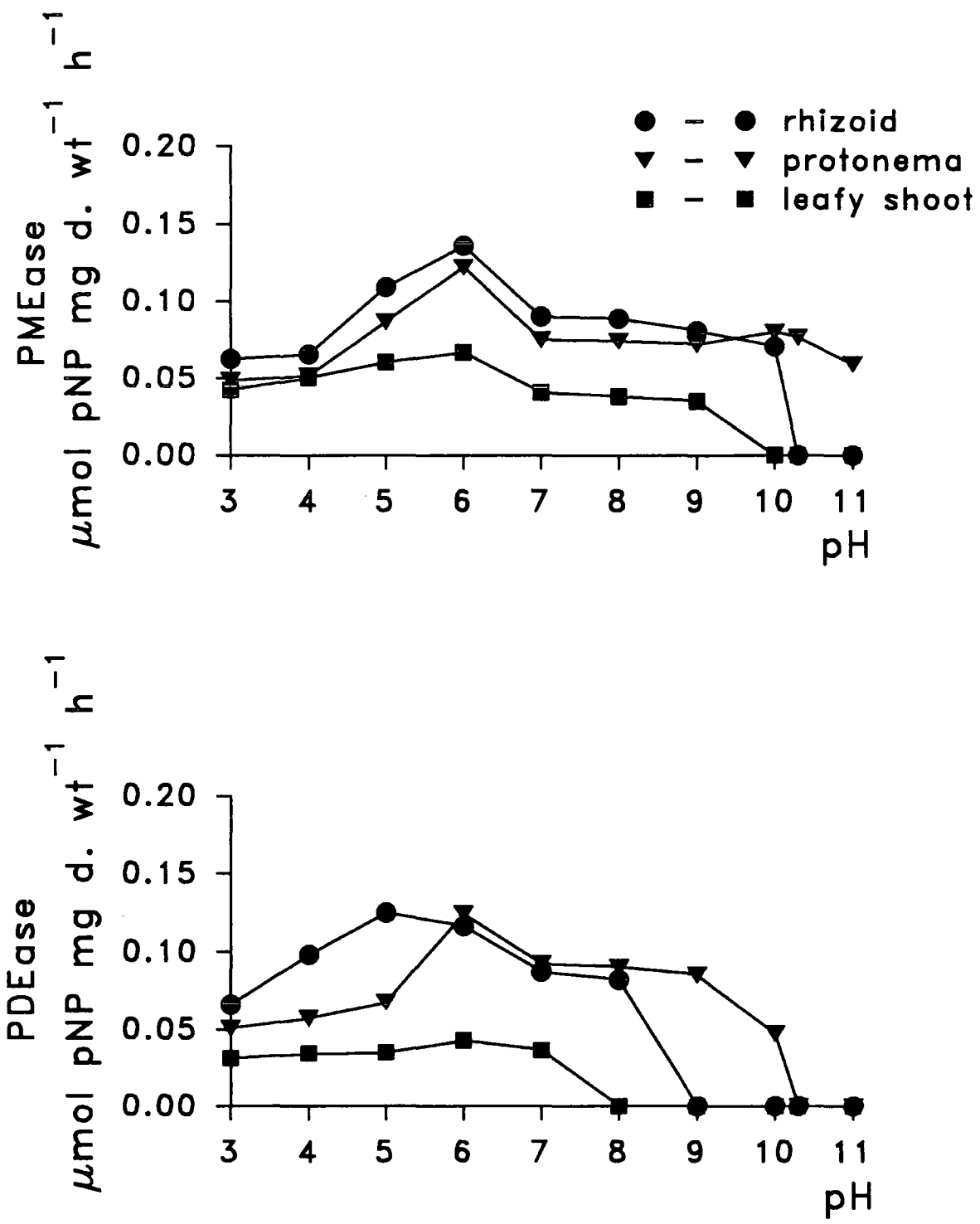


Fig. 6.15 Influence of drought on phosphatase activities of *H. fontanum*. (28-d old culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)



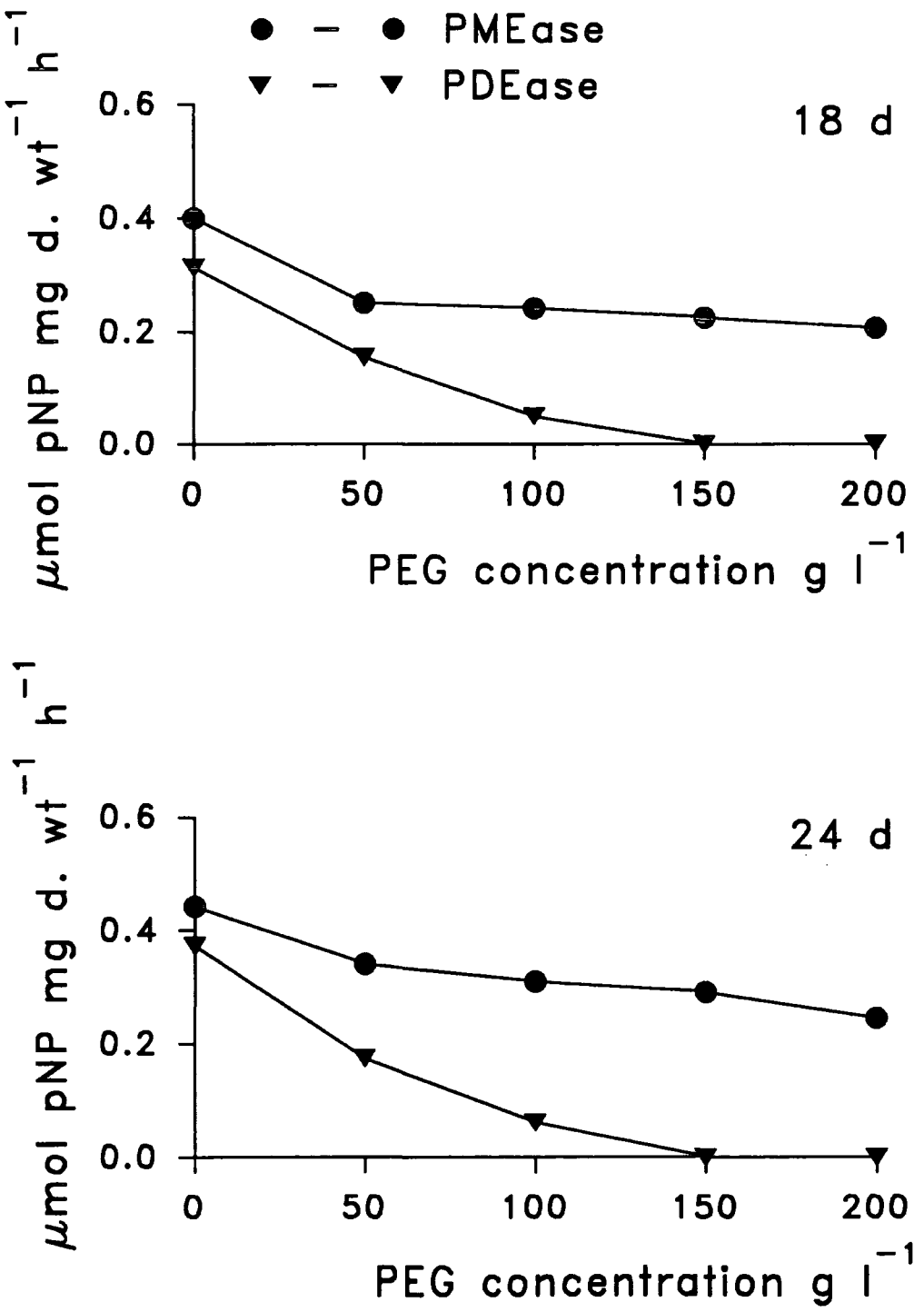
The results are shown in Fig. 6.16. PMEase and PDEase activities were higher in material harvested on day 24. The decrease of PMEase and PDEase activities of material harvested on day 18 at the lowest concentration of PEG (50 g l^{-1}) were 37% and 50%, respectively, and at the highest concentration (200 g l^{-1}) was 47% for PMEase whereas PDEase activity was not detectable. For materials harvested on day 24, the decrease at the lower concentration of PEG were 23% and 53% for PMEase and PDEase, respectively. At the higher concentration of PEG PMEase activity was decreased by 44% whilst PDEase was not detectable.

The effects of water stress, using different PEG concentrations, on PMEase and PDEase were significant ($P > 0.05$), even at the lowest concentration of PEG used.

6.11 Phosphatase activities of shoots sampled from three different sites on the waterfall

During collection of moss shoots from the field it was observed that there were differences in the shoot length of moss collected from different sites such as calcareous soil and clay soil in and around the waterfall. Therefore assays were carried out to investigate phosphatase activities of moss materials collected from calcareous and clay site at different locations.

Fig. 6.16 Influence of water stress on phosphatase activity of *H. fontanum*. (18 & 24-d old protonemal cultures were used for experiments; 85 µmol photon m^{-2} s^{-1}; 25°C)



Moss materials were sampled from three different locations on the waterfall, 1.5, 10, 58 m above the ground level. Ten samples with two replicates were collected from each. Samples were then dried at room temperature for 48 h then they were kept in sampling plastic bags until they were assayed for phosphatase activities. With the determination of phosphatase activities cellular P content of the moss shoots was also monitored.

The results are shown in Fig. 6.17. PMEase activity of the shoots showed significant differences from site to site in and around the waterfall. The highest PMEase activity was in the shoots which were collected from 1.5 m high of the tufa-deposition on the waterfall (calcareous soil). Whereas the lowest activity was in the shoots which were collected from 58 m high on the waterfall (clay soil). Conversely, the shoots from 1.5 m high (calcareous soil) had the highest tissue phosphorus concentration and the clay soil shoots had the lowest concentration.

PDEase activity had the same pattern as PMEase, although PDEase activity was slightly lower than PMEase activity in all shoots used.

6.12 Growth on different P substrates

Experiments were planned to determine the ability of protonema to utilize different organic phosphates for growth. Five different organic phosphate substrates were tested. One control (-P) and another with inorganic P were also included for comparison. Substrates were sterilized by filtration and added aseptically to Chu 10 G-P medium. Protonema was grown under standard conditions (section 2.36) and yield (dry weight) was determined after 16 days. The moss protonema showed moderate yield (compared with orthophosphate) with sodium-ß-glycerophosphate, p-nitrophenyl phosphate, bis (p-nitrophenyl phosphate and adenosine triphosphate), but showed very low yield with phytic acid (Table 6.1).

Fig. 6.17 Phosphatase activities and P content of *H. fontanum* samples from different locations on the waterfall.

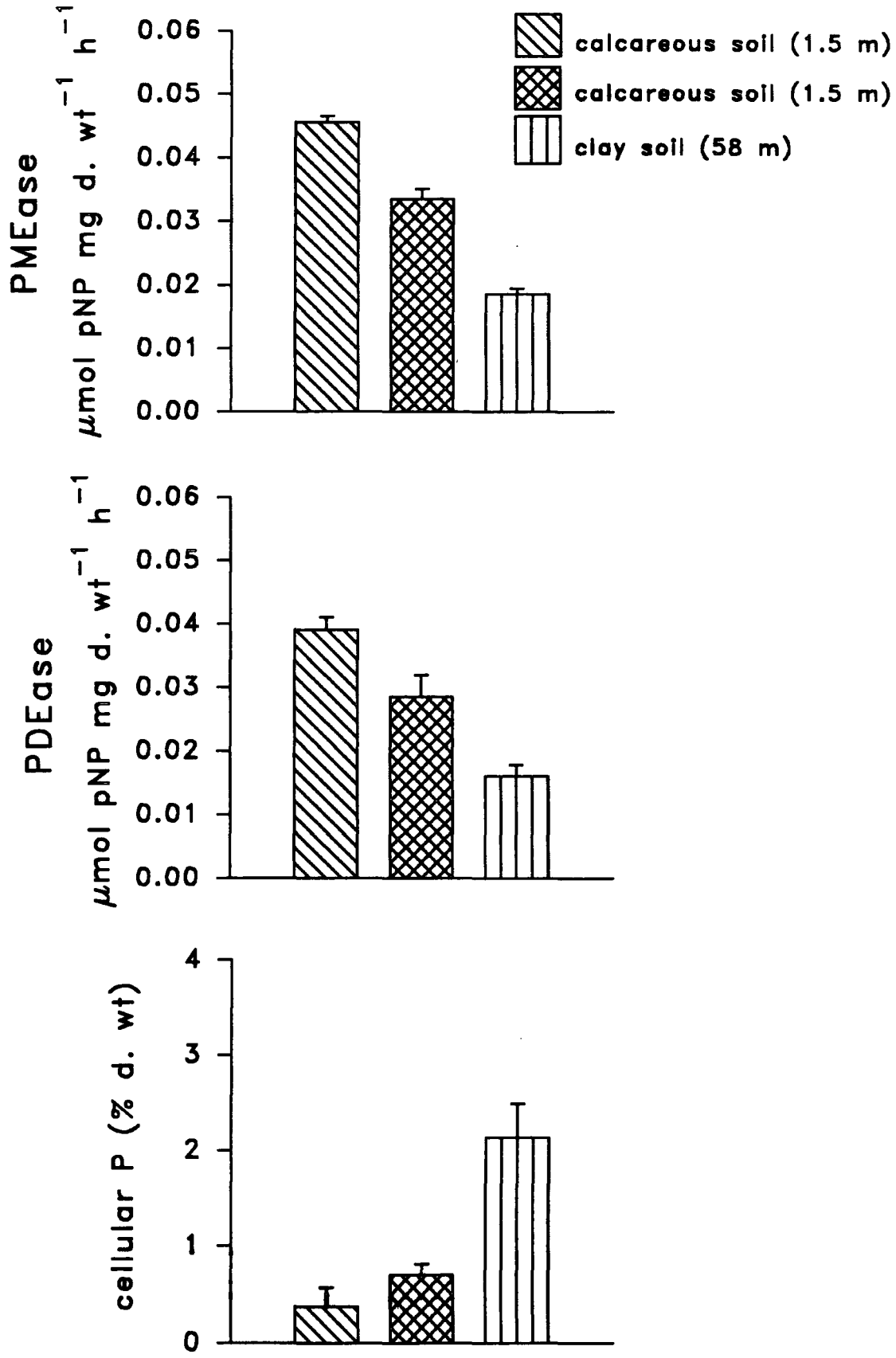


Table 6.1 Influence of P sources on yield of *H fontanum* 16 days of growth in batch culture inoculum 10 mg l^{-1} d. wt, 85 μmol photon m^{-2} s^{-1}, 25°C.

P Source	$\bar{x}$	S.D
-P	66.65	11.15
P_i	436.32	13.48
p-nitrophenyl phosphate	387.45	41.67
bis(p-nitrophenyl) phosphate	340.70	52.51
sodium-B-glycerophosphate	374.00	49.56
adenosine triphosphate	329.53	23.50
phytic Acid	78.50	6.78

6.13 Influence of pNPP concentration on phosphomonoesterase activity

In the case of variation in the rate of activity with substrate concentrations the reaction is represented by the Michaelis-Menten equation:

$$v = \frac{V_{max} . S}{K_m + S}$$

v is the rate at any substrate concentration. V_{max} is the maximum rate attained by the reaction at high substrate concentrations and K_m is known as the Michaelis Constant which is substrate concentration when the rate is half the maximum attainable rate.

V_{max} and K_m are experimentally determinable constants, provided the enzyme is studied under controlled conditions and are characteristic and constant for the particular enzyme. V_{max} and K_m is possible to be obtained directly from the graph of rate against substrate concentration, it is, however, more common to plot the reciprocal of the rate $1/v$ against

the reciprocal of the substrate concentration 1/s. This method of expressing the results is known as the Lineweaver-Burk equation.

The dependence of PMEase on the concentration of pNPP can be described on the basis of Michaelis-Menten kinetics. To determine the K_m and V_{max} a Lineweaver-Burk plot was constructed. The assays were carried out at pH 6.0 at 25°C. Cellular material was harvested as in 2.71

Use of the Lineweaver-Burk plot, $1/v$ versus $1/s$ allowed the calculation of half saturation values and concentration of pNPP (K_m) required to support half the maximum rates. K_m for PMEase was 2.101×10^{-4} M

6.14 Influence of incubation and protonema homogenate concentration on PMEase and PDEase activities

Influence of incubation

All phosphatase activities experiments were incubated for 1 h. In this experiment PMEase and PDEase activities were tested at different lengths of time in order to investigate the timecourse of activities. PMEase and PDEase activities were measured at 15 min intervals for 60 min. The activities were also determined 15 min before adding the substrates (Fig. 6.18). The rate of activities was calculated at half time of the intervals. There were no detectable activities either at 15 min before adding the substrates or at zero time. The maximum rates of both PMEase and PDEase activities were observed 1 h after adding the substrates.

Influence of homogenate concentrations

The standard volume of homogenate was 50 μl for all experiments using the plate reader. To standardize on this volume, determinations of PMEase and PDEase activities at different volumes (0, 10, 20, 30, 40, 50 60, μl) of the homogenate were carried out. The results (Fig. 6.19) showed that PMEase and PDEase activities increased gradually with the increase in

protonema homogenate concentrations. From the results it is apparent that 50 µl of moss protonemal homogenate gave considerable phosphatase activities. As PDEase activity showed no significant increase at 60 µl homogenate, 50 µl was selected as standard volume.

6.15 Influence of incubation on PMEase activity measured using pNPP and 4-MUP substrates

In these experiments PMEase activity was tested using pNPP and MUP substrates at different lengths of time before starting the assay to investigate the time-course of activity in order to determine the right amount of the protonema homogenate required. The time-course started after adding the substrates to the assays. PMEase activity was assayed at pH 5.5 with 250 µM of pNPP and 4-MUP, respectively. PMEase activity was measured at 15 min intervals for 60 min. The rate of activity was calculated at half time of the intervals. PMEase activity showed a linear increase over the incubation period. The maximum rate (Fig. 6.20) of PMEase activity was detected 1 h after adding the substrates.

The time-course assay at pH 5.5 and pH 6.0 using 1 µM 4-MUP was also carried out for 40 min with 10 min intervals. The maximum rate (Fig. 6.21) of PMEase activity was found 40 min after adding the substrate. PMEase measured using 1 µM showed the same pattern as when using 250 µM.

Fig. 6.18 Influence of incubation on phosphatase activities of *H. fontanum*. (28-d old protonemal culture was used for experiment; 85 µmol photon m^{-2} s^{-1}; 25°C)

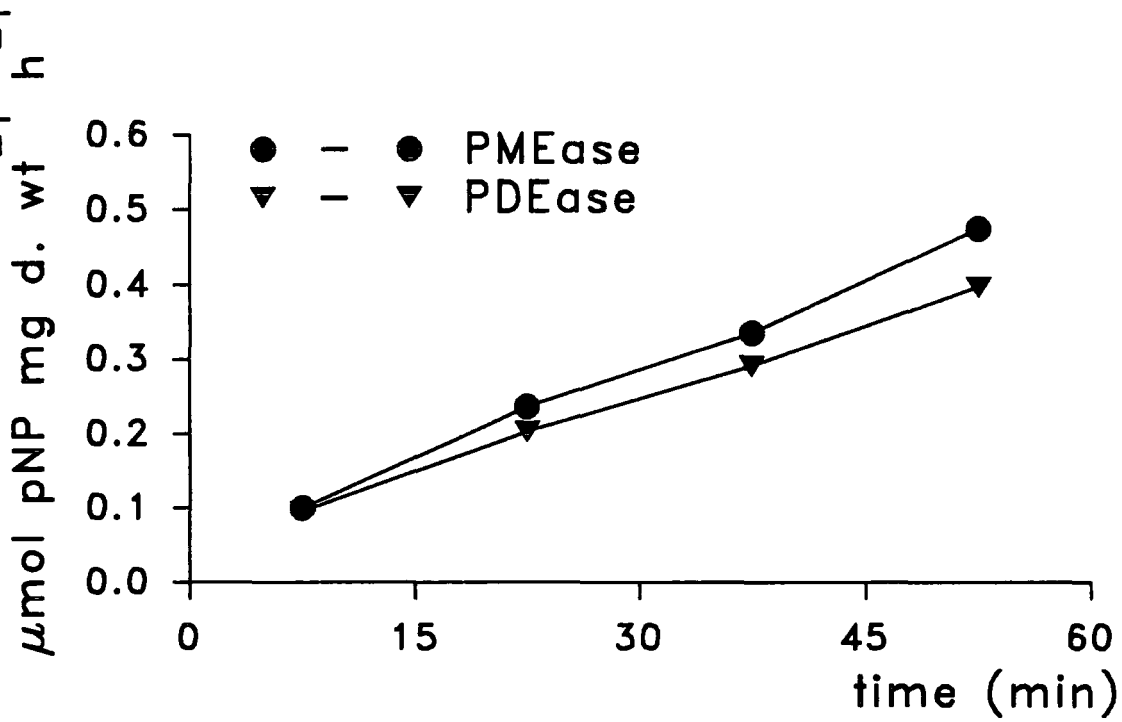


Fig. 6.19 Influence of protonema homogenate concentration on phosphatase activities of *H. fontanum*. (28-d old protonemal culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)

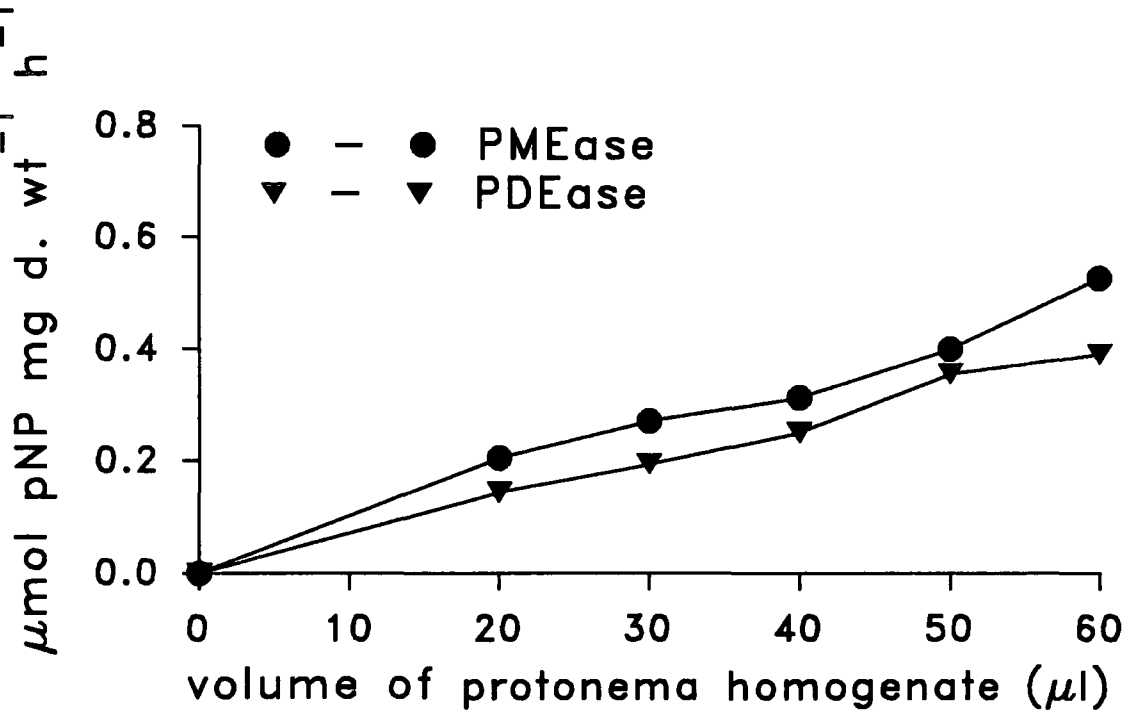
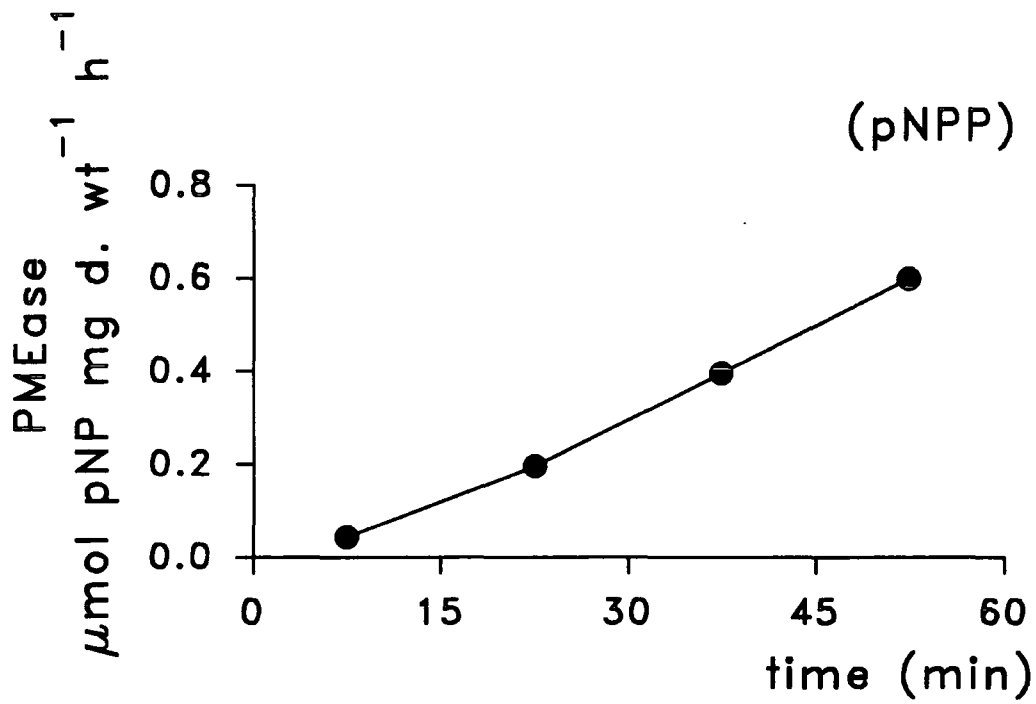


Fig. 6.20 Influence of incubation on PMEase actvity of *H. fontanum* using 250 μM pNPP and 4-MUP substrates at pH 5.5. (40-d old protonemal culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)



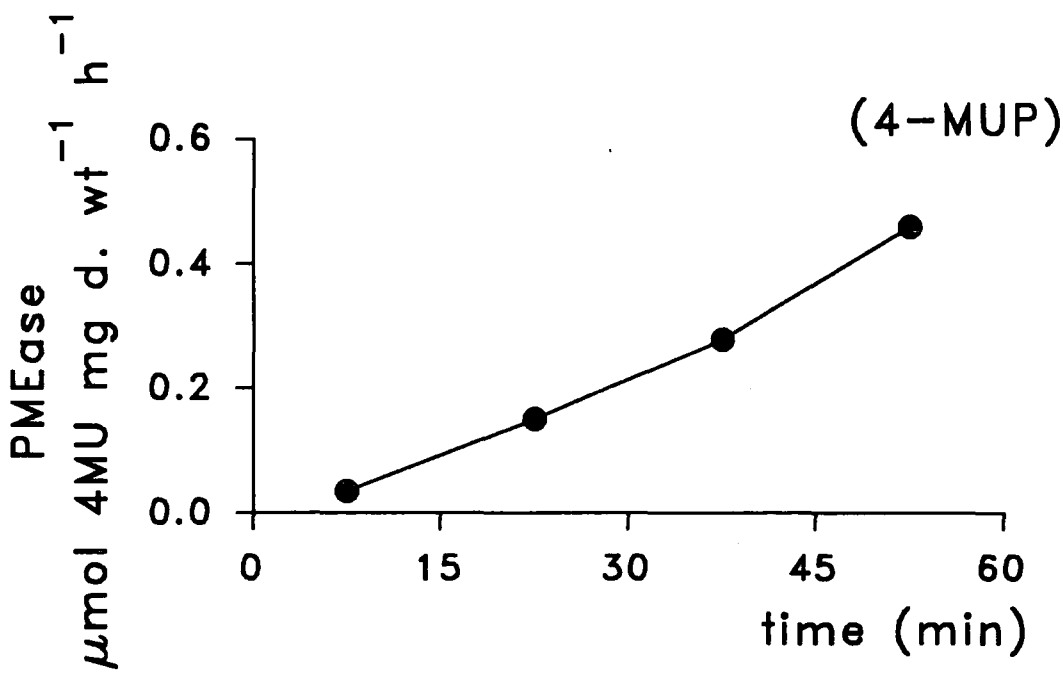
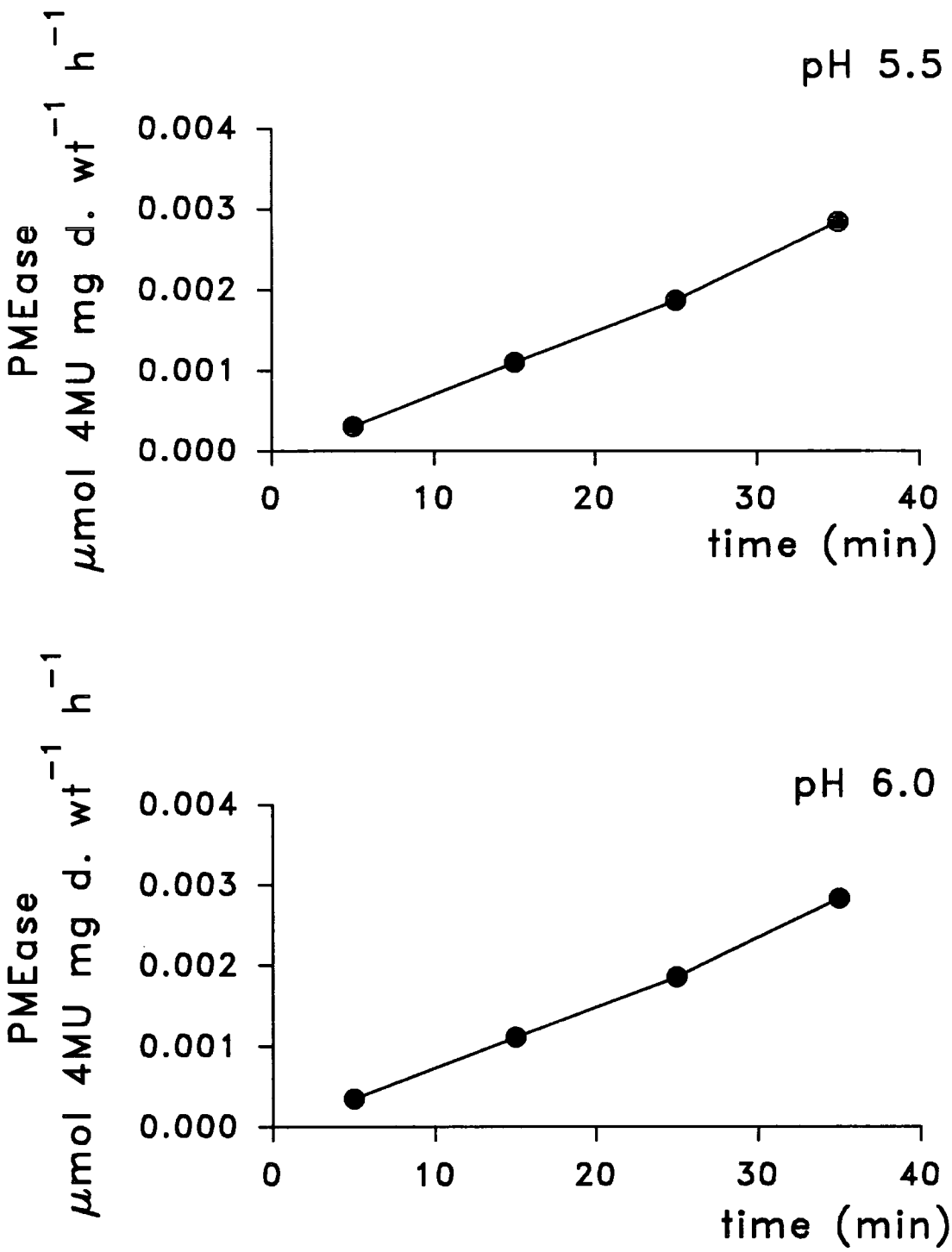


Fig. 6.21 Influence of incubation on PMEase activity of *H. fontanum* using 1 μM 4-MUP substrate at pH 5.5 & 6.0. (40-d old protonemal culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)



6.16 Influence of pH on PMEase activity using two different substrates (pNPP & 4-MUP)

Experiments were carried out with laboratory protonema. PMEase was assayed by using pNPP and 4-MUP substrates. pH values were tested over the range pH 3.0-11.0 with 0.5 intervals (Fig. 6.22). PMEase activity using pNPP was detectable between pH 3-8 with optimum activity at pH 5.5 after which the activity started to decline gradually. The activity using 4-MUP, however, was detectable between pH 3.0-11.0 with optimum activity at pH 5.5 and pH 6.0 after which the activity dropped sharply at pH 6.5 then declined gradually up to pH 8 and maintained the same rate up to pH 11.0. PMEase activity measured using 4-MUP substrate was about 60% of that measured using pNPP substrats.

6.17 Influence of different concentrations of 4-MUP substrate on PMEase activity

The previous experiment showed that the optimum pH for PMEase activity was between pH 5.5-6.0 at a concentration of 250 μM 4-MUP. As it has been reported that 4-MUP substrate has outstanding sensitivity, for detecting phosphatase activity, among several substrates tested including pNPP (Pettersson & Jansson, 1978). Therefore, different concentrations of 4-MUP were tested (1, 2, 5, 10, 20, 50, 100 and 250) at pH 5.5 and pH 6.0 (Fig. 6.23). PMEase activity was detectable at all concentrations and increased significantly with the increment of 4-MUP concentrations.

The influence of 4-MUP substrate concentrations, including 0.25 and 0.5 μM, on PMEase activity on all pH values was also tested. The results (Fig. 6.24) showed that the optimum activity for all concentrations was detected between pH 5.0-6.0 and the activity was detectable at all pH values. At lower concentrations (0.25 & 0.5) the optimum rate of activity was found over a wider range of pH values (5.5-7.0) with more or less the same rate of activity at these pH values.

Fig. 6.22 Influence of pH on PMEase activity of *H. fontanum* measured using 250 μM pNPP and 4-MUP substrates. (40-d old protonemal culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)

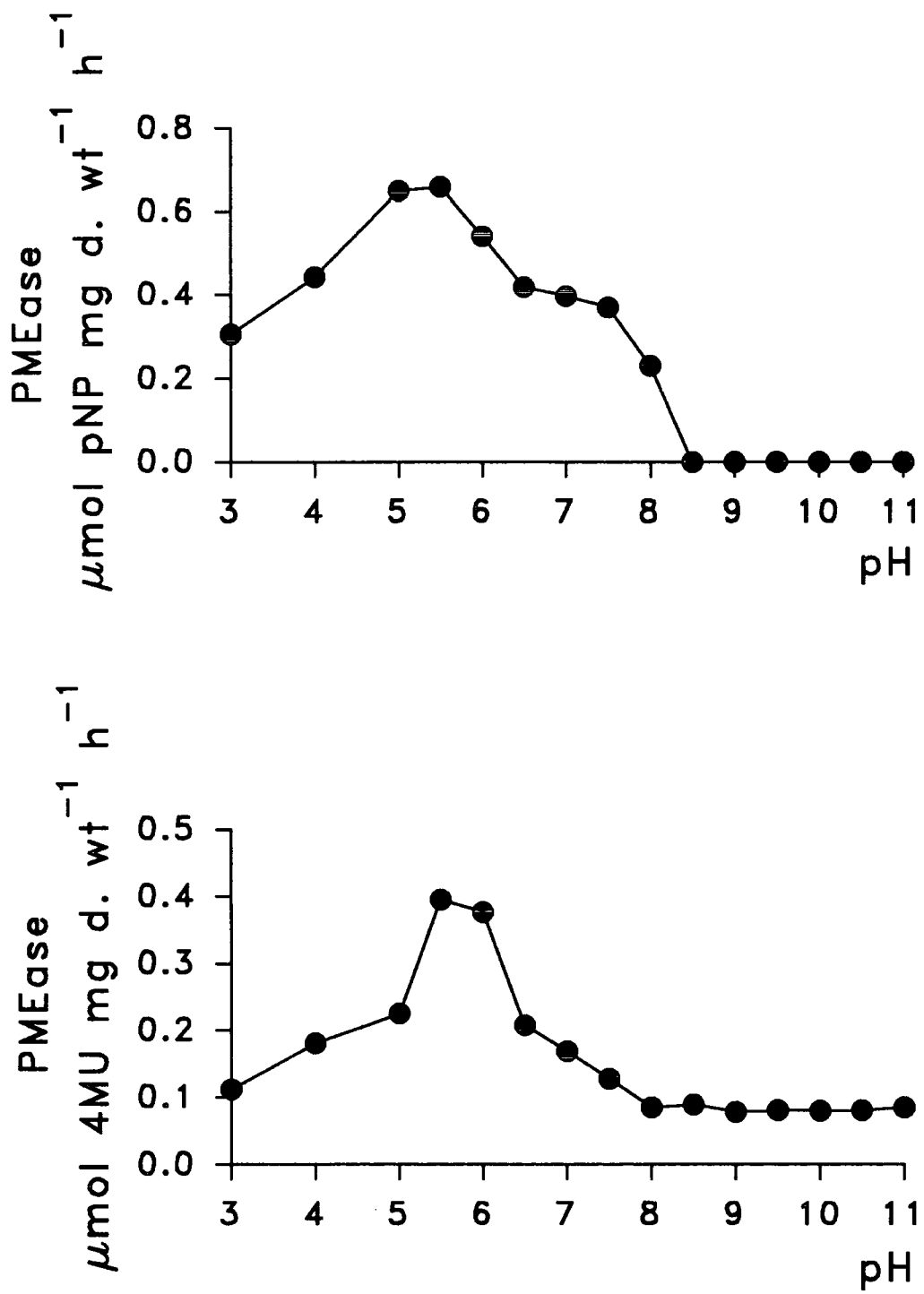


Fig. 6.23 Influence of different concentrations of 4-MUP substrate on PMEase activity of *H. fontanum* at pH 5.5 & 6.0. (40-d old protonemal culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)

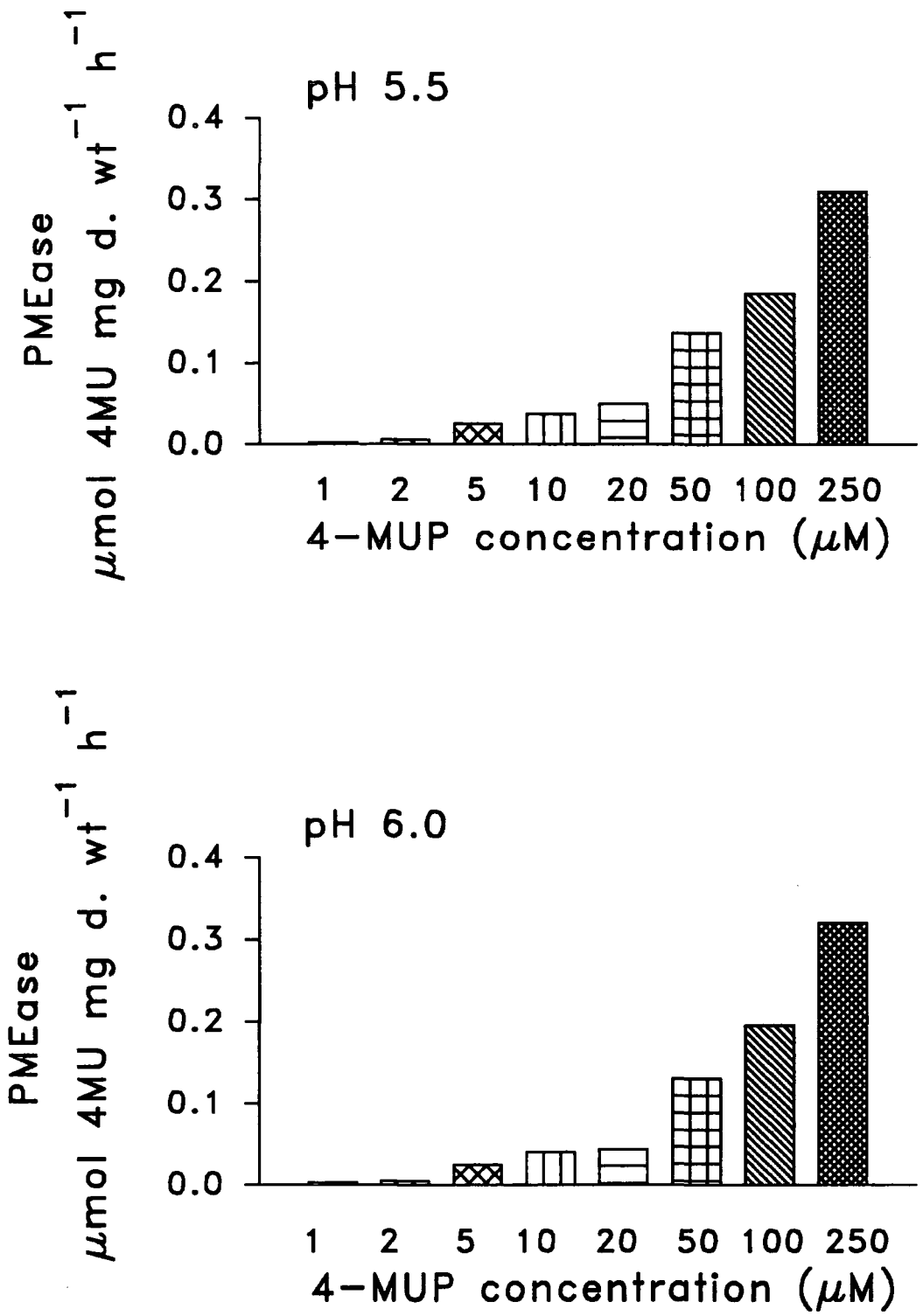
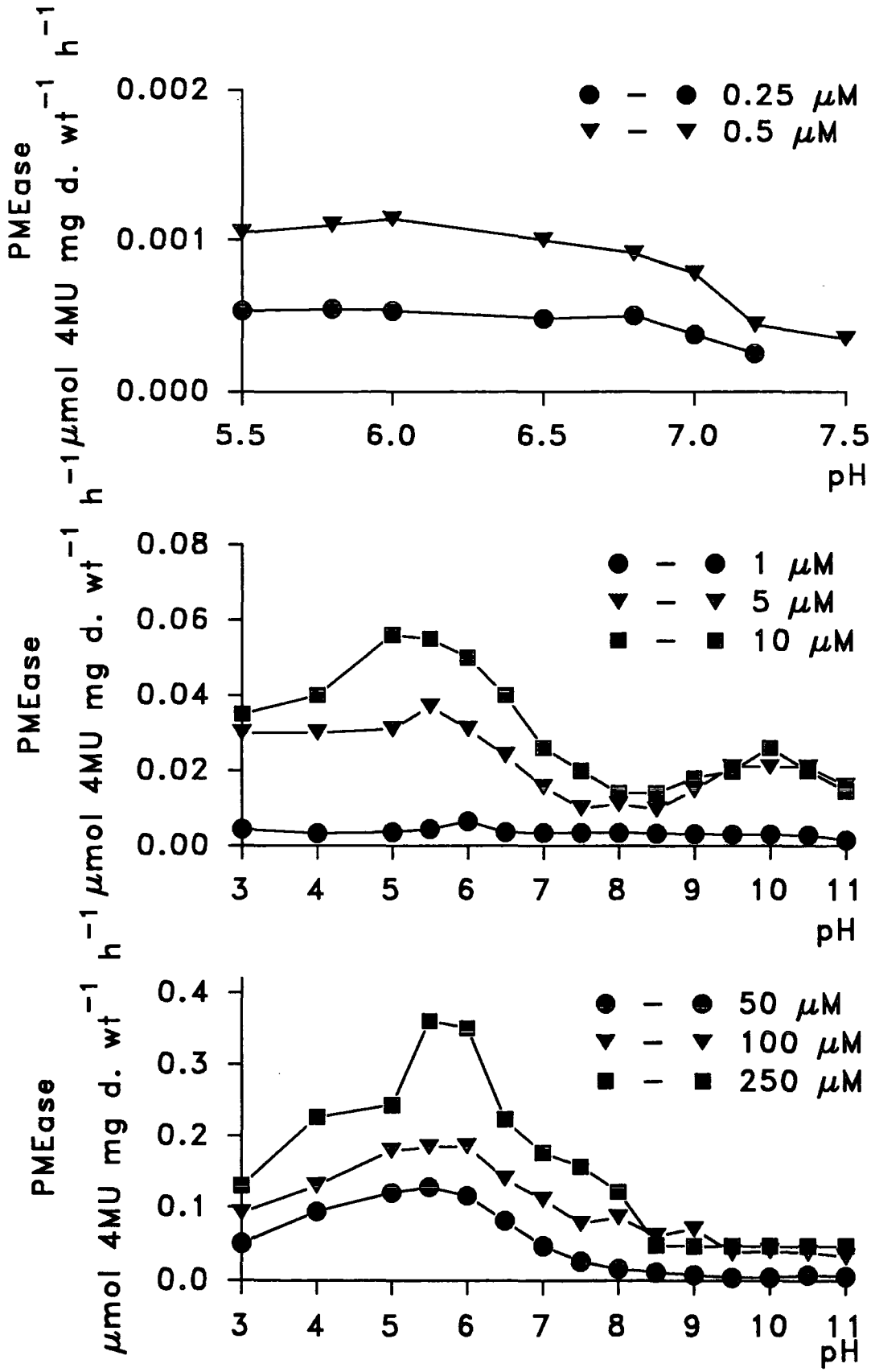


Fig. 6.24 Influence of different concentrations of 4-MUP substrate on PMEase activity of *H. fontanum* over pH range. (40-d old protonemal culture was used for experiment; 85 μmol photon m^{-2} s^{-1}; 25°C)



7 DISCUSSION

The laboratory studies on growth and phosphatase activities have quantified the influence of environmental variables likely to be important at the waterfall, where *Hydrogonium fontanum* is the dominant plant. Marked changes in the growth rate and phosphatase activity occurred with changes in environmental variables, including water stress.

Morphology and growth stages

The morphology of different members of the Pottiaceae has been reported to differ widely from one species to another (Smith, 1978; Watson, 1981). Growth stages of mosses also vary depending on environmental conditions such as light and flooding (Section 1.35). The results here indicate that *H. fontanum* collected from the waterfall habitat did respond to variation in environmental conditions.

H. fontanum from the field (waterfall) consisted very largely of stems and leaves. A third less pronounced component was the rhizoid system, which resembled closely the protonema in structure (Hébant, 1977; Schofield, 1981). These parts, stem, leaves and rhizoid, represented the gametophyte.

When *H. fontanum* was subcultured in the laboratory, protonema developed on the leafy shoots within two days; when protonema were subcultured in fresh medium two cell types could be identified in the protonemal filamentous stage. The first was the chloronema, which contained many chloroplasts with a cross wall perpendicular to the long axis of the cell filament (Fig. 4.3a, b). The second type was the caulonema, which contained fewer, spindle-shaped chloroplasts and had diagonal cross walls to the filament (Fig. 4.3b). The first cells

produced following tissue regeneration were chloronema; Ashton *et al.* (1979) reported a similar phenomenon in *Physcomitrella patens* and Cove (1984) in *P. sphaericum*. After a few days of growth under standard conditions (Section 2.36), the second type of cell, the caulonema, was formed. Many cells in the caulonemal filaments produced side branches of chloronemal filaments. At very late growth stages some caulonema filaments formed buds which subsequently become gametophores (Fig. 4.2d). Similar observations of these growth stages have been reported for other species of moss (Ashton & Coves, 1977; Cove & Ashton, 1983; Knoop, 1984).

In the field material of *H. fontanum* only a few protonemal filaments of caulonemal cell type were observed, at the time of moss collection, growing on the upper part of some leaf axis.

The gemmae of *H. fontanum* were spindle-shaped and green with numerous chloroplasts within each cell (Fig. 4.3c). They usually germinated on protonemal filaments. Sometimes they were observed to separate from their point of attachment to the protonemal filaments (Fig. 4.4a) and germinate to form new protonema, and in rare instances to form gemmae (Fig 4.4b). This form of development is not common to all mosses since Mueller and Rushing (1985) found that the gemmae of *Oedipodiella* produce apical cells directly from apical initials and there was no intermediate protonemal stage before the production of gametophores.

Gemmae of *H. fontanum* occurred in both liquid and solid medium. They were also seen in field investigations when the moss was submerged, and these observations match with the observation of Sharma and Chopra (1986) for *Hyophila crenulata* where they found that flooding of shoots resulted in production of abundant protonemal gemmae.

Rhizoids were seen in both field and laboratory material. In laboratory culture, both solid and liquid media, rhizoids were found growing on the lower parts of the leafy shoot stem (Fig. 4.2b). In contrast, in old (about 2-month) cultures rhizoids were formed all over the stem (Fig. 4.2c). Rhizoids were usually brown with thick reddish cell walls and oblique cross-walls. However, the young rhizoids were slightly green in colour and contained several chloroplasts; this feature made it difficult, at times, to differentiate between caulonema and young rhizoids. The rhizoidal filaments were, however, wider and usually grew towards the substratum. Profuse development of rhizoids from protonemal filaments was observed by about 15 days on solid culture, earlier than formed in liquid culture and this observation supports the observation of Odu (1978a, b) that mosses growing on hard surfaces produce more rhizoids than those growing among vascular plants. This may help to anchor the moss to the substratum.

Production of rhizoids varies in other mosses with habitat (Odu, 1978a). Floating, and submerged wetland plants often lack rhizoids (Watson, 1919; Odu, 1978b), but plants at the edges of lakes and in streams require them in order to remain fixed. Glime (1980) found that species of *Drepanocladus* ordinarily lack rhizoids when found in lakes, in fens and marshes or in carpets on lawns, but *D. fluitans* can be induced to produce them in culture on agar. She suggested that the character is under environmental rather than genetic control.

Growth

Changes in chlorophyll content and dry weight of *H. fontanum* have been studied in solid and liquid media (Fig. 4.5). The moss showed (Table 4.1) a faster growth rate in liquid than solid culture in relation to chlorophyll content and dry weight. The doubling time in liquid culture

for chlorophyll content and dry weight was 132 and 128 h, whereas in solid culture it was 159 and 174 h, respectively. A literature search has shown no similar work for comparison. The results reported here suggest that the faster growth rate in liquid medium could be due to easier uptake of nutrients. Further, in a liquid medium, the whole moss is submerged in the nutrient medium and may well be able to absorb the nutrients over its entire body, whereas in solid medium this uptake will be limited. In addition shaking of the medium helps to dissolve gases which will be easily available in the liquid compared to the solid medium.

Using shoots and protonema as inocula in liquid medium changes in dry weight and chlorophyll content were determined. Although the period of faster growth was the same in shoots and protonema at days 4-8, the growth rate in terms of dry weight was about 8 times that of the inoculum of protonemal cultures after day 4 (Fig. 4.6). However in shoot cultures, the moss only doubled in dry weight in the same period. The maximum growth rate (k') in protonema was 0.20 d^{-1} which is almost three times that of the growth rate (k') of shoots (0.07 d^{-1}). The higher growth rate may indicate suitable environmental conditions for protonema production.

Influence of macronutrients on growth rate and yield

Hydrogonium fontanum was subjected to different concentrations of major cations and anions under standard laboratory conditions. It was found that there was an improved growth rate in relation to chlorophyll content and dry weight (Fig. 4.7) with increased Mg (2.46-9.86 mg l^{-1}) and Ca concentration (9.75-22.3 mg l^{-1}), which was near the level of concentration in the water at the field site (Mg 18.2 mg l^{-1}, Ca 33 mg l^{-1}). Synnott (1987) stated that in fertilizer experiments, with some elements included, calcium is essential for subsequent bryophyte colonization. This suggests that a high Ca concentration is required for

this moss, whereas it appears that Mg is effective only at the lower concentrations and appears not to be limiting above 9.86 mg l^{-1}. Any increase in yield can thus be related to Ca concentration.

In *H. fontanum* increasing of Ca had a pronounced improvement on yield up to concentrations of 80 mg l^{-1}. In a comparison of *Scapania aspera* (calcicole) with *S. gracilis* (calcifuge), Harrington (1966a) considered growth of the former to be limited by low Ca. Nehira (1973) found that Ca accumulates at the rhizoid base and is required for differentiation. Glime (1981, 1982) found that in *Fontinalis* spp. rhizoids grow in summer when the moss is often stranded above water. Such a condition may provide a greater accumulation of Ca to develop in the moss and facilitate rhizoid production. *Cratoneuron commutatum* occurs only at alkaline pH where Ca is usually high (Bell & Lodge, 1963). The increase in Ca concentrations (Table 4.3) on *H. fontanum* indicated a significant increase in yield. This could be because the moss was originally from a calcareous site, and this factor may be responsible for its higher requirement of Ca. It is likely that at high natural Ca levels the moss has an effective exclusive mechanism for this ion. When growing in laboratory conditions sufficient Ca must be present to allow for the attainment of optimal tissue concentration of the ion.

Increased Mg concentration, from 1-40 mg l^{-1}, had a slight improvement on yield (growth rate (K') 0.063 d^{-1} at 1 mg l^{-1} and 0.064 d^{-1} at 40 mg l^{-1}). Na gave a marked increase in yield with optimum yield of 100 mg l^{-1} and showed almost the same yield at the highest concentration (250 mg l^{-1}) (Table 4.3). Although the mean concentration of Na in the waterfall is only 47 mg l^{-1}, which is about half the optimum concentration (100 mg l^{-1}) found in the laboratory experiments; therefore high concentrations of Na may be required for the growth of this moss.

H. fontanum was able to grow in both NO_3 and NH_4 as nitrogen sources. Press and Lee (1982) and Woodin *et al.* (1985) have demonstrated that nitrate is utilized by ombrogenous *Sphagnum* spp. and Brown (1982) concludes that nitrate is probably utilized by most other bryophytes. This agrees with the results for *H. fontanum* (Fig. 4.8). Schuler *et al.* (1955) reported that *Sphaerocarpos texanus*, which was grown in liquid medium containing (0.2 g) NH_4NO_3 (= 69 mg l^{-1} N) as a nitrogen source had taken and retained practically equal portions of ammonium nitrogen and nitrate nitrogen. Further Burkholder (1959) found that when inorganic sources of nitrogen were supplied either in the form of NH_4Cl, $NaNO_3$ or NH_4NO_3, growth of the moss *Atrichum* was about equally good at equivalent concentrations of nitrogen. Optimum growth occurred in the basal medium containing NH_4NO_3.

Peel (1974) stated that nitrate is the preferred form in which many plants absorb nitrogen. This applied *to H. fontanum*, which had a slightly higher growth rate in culture with NO_3 (Fig. 4.8).

It is interesting that Gigon and Rorison (1972) found that the calcifuge higher plant species *Deschampsia flexuosa* grew best on ammonium at low pH, whilst a calcareous race of *Scabiosa columbaria* grew optimally on nitrate at high pH.

Of the different organic carbon compounds tested for growth in the dark, the results indicated that the moss protonema failed to grow heterotrophicaly. Similar results have been reported for *Physcomitrella patens* Cove *et al.*, 1978; Jenkins & Cove, 1983a), and for *Amblystegium riparium* (Kato & Watanabe, 1982). On the other hand Szweykovska (1963), Valanne (1966) and Chopra and Gupta (1967) stated that in certain moss species, such as *Funaria hygrometrica* and *Ceratodon purpureus*, a significant level of germination is determined in darkness, and this is stimulated by the addition of carbon source.

Effects of Temperature

The effect of temperature on vegetative growth of *H. fontanum* was investigated at six temperatures (18, 21, 26, 32, 36, 41 °C). The shoots grew in a wide range of temperatures (18-32 °C) with rapid growth at 26 °C, but failed to grow above 32 °C after day 12. Furness and Grime (1982) found that the optima of the 40 species of bryophytes they tested was between 15-25 °C including a broad optimum of 15-28 °C for the aquatic *Eurhynchium (Rhynchostegium) riparioides*. High temperatures are detrimental to vegetative growth in *Fontinalis* (Glime & Acton, 1979; Fornwall & Glime, 1982).

The results reported here suggest that temperature is a factor of particular importance in controlling vegetative growth in *H. fontanum*. This probably relates to the fact that the moss was collected originally from a site where the mean maximum temperature is 27.6 °C, but where considerable seasonal variation in water temperature is seen.

Influence of flooding on the growth rate and morphology

Studies of *H. fontanum* in the field and laboratory were carried out to investigate the influence of flooding on growth and development since the collection site of this species is subjected to fluctuations in water levels (Section 5.4). The growth rate in respect to shoot elongation of the flooding shoots was significantly higher compared to the controls (Table 5.1) in both field and (Figs 5.3, 5.4) laboratory experiments. Similar results have been observed by other workers; Zastrow (1934) provided evidence that aquatic conditions cause plants to be taller. For example, *Drepanocladus exannulatus* has a shoot length four times as long in the submerged form (Zastrow, 1934) and that this was due to the longer internodes (Lodge, 1959). This increase in length might be due to lower light intensity (Lodge, 1959). Therefore, in *H. fontanum* growth responses

of the shoot during submergence might have been stimulated by environmental factors such as light intensity. The increase in shoot length was, however, slow in the field when the plants were submerged (Table 5.1), being approximately 1 mm per week. Much faster rates (at least 9 times) were found under the laboratory conditions when the moss shoots were submerged in distilled water (over agar) (Fig. 5.4). This was in part due to the fact that growth tended to be more etiolated under laboratory conditions, with the leaves more widely spaced. Seppelt (1983) found in field surveys in the Vestfold Hills that *Bryum algens* becomes easily etiolated when inundated by rising melt waters during the summer thaw period.

Nevertheless, it is almost certain that the moss could increase in dry weight much faster in the laboratory than it did during the period of field study. The most likely reason for this is because of relatively high, and stable, nutrient levels in the laboratory experiments (12 mg l^{-1} N, 1.76 mg l^{-1} P).

Production of Gemmae

Andrews and Bedfearn (1965) and Chopra and Rawat (1973) stated that the production of gemmae is expressed under conditions unfavourable for the initiation of gametophytic buds. Gemmae production is also influenced by environmental conditions such as when mosses are growing in shaded area (Edwards, 1978). Olarnmoye (1981) and Odu and Owotomo (1982) demonstrated that the production of protonemal gemmae in *Hyophila crenulata* appeared when the moss shoots were flooded.

Production of protonema and gemmae by moss shoots was observed in *H. fontanum* in both field and laboratory under flooding conditions. In laboratory samples flooded with distilled water, the production of

protonema and gemmae was very low with only a few chloroplasts within the cell. However, in the field the gemmae were green with numerous chloroplasts. The most likely reason for this, is probably the extremely low nutrient levels in distilled water used for flooding. In the laboratory experiments, when the moss shoots were grown in agar and flooded with Chu 10 G liquid growth medium, for comparison, shoots produced protonema and gemmae on the agar surface as well as in the growth liquid medium.

Light appears to play an important role in gemmae formation. Mosses such as *Schistostega pennata*, *Eucladium verticillatum*, *Barbula trifaria* (Whitehouse, 1980) produce gemmae in shade. Vashistha and Chopra (1984) found that low light intensity favours gemmae formation in *Didymodon recurvus*. In *H. fontanum*, too, low light intensity was seen to favour gemmae formation, since gemmae were produced effectively under low light intensity (Section 5.3). This could also have been the reason for the flooding response where there could be reduced light penetration in this habitat. This formation of gemmae under these conditions in *H. fontanum* supports the view of Chopra and Rawat (1973) that production of gemmae is an inherent characteristic of gemmiferous mosses which is expressed under conditions unfavourable to the initiation of gametophytic buds.

Changes in chlorophyll content and dry weight of *H. fontanum* have been determined at different values of light flux in batch culture (Figs 5.1, 5.2). The most pronounced effect of light flux on the moss was the increase in chlorophyll content at lower values of light flux at the end of the experiment period. At 10 μmol photon m^{-2} s^{-1}, the moss had almost three times the chl content than at 90 μmol photon m^{-2} s^{-1} (Fig. 5.1), whereas dry weight (yield) was significantly lower (one third lower) at low

light flux (10 μmol photon m^{-1} s^{-1}) than at high light flux (90 μmol photon m^{-2} s^{-1}) as shown in Fig 5.1.

The increase in chlorophyll content in mosses at low values of light flux compared to high light flux has been reported in previous research. Sand-Jensen and Madsen (1991) found that in submerged macrophytes, including *Fontinalis antipyretica*, the highest chlorophyll concentrations, were obtained at 3 and 11 μmol m^{-2} s^{-1} and concentrations declined gradually with higher photon flux density levels as well as in the dark.

Valanne (1977) observed that the chlorophyll content of protonema of *Ceratodon purpureus* was always higher at low light intensity. She also noted that cultures grown at low light intensity gave lower yields as measured by dry weight. This result is in agreement with the results reported here for *H. fontanum* when grown at low light intensity. The low chlorophyll content associated with high dry weight noted at high light intensity could be due to the degradation of chlorophyll *a* and *b* (Fig. 5.1). Further when growth rate was measured at 2 light intensities (60 and 90 μmol photon m^{-2} s^{-1}) there were no significant differences in relation to chlorophyll content and dry weight (Fig. 5.2). The greater chlorophyll content found at low light intensities has been interpreted as an increase in the size of the photosynthetic unit, and mainly as an increase in the bulk chlorophyll, the chlorophyll-protein complex II (Lewandowska *et al.*, 1976; Prézelin *et al.*, 1976).

Water stress

Proline accumulation

Proline accumulation as a result of drought stress appears to be a general phenomenon in higher plants (for example Singh, 1973). The ability for proline accumulation, however, differs between different

tissues of plants (Singh, *et al.*, 1973) and is strongly influenced by previous exposure to drought stress (Singh, *et al.*, 1973). Some workers e.g. Singh *et al.* (1972, 1973), Stewart and Lee (1974) reported positive correlations between proline accumulation and stress tolerance or adaptation to stress. The results of others such as Hanson *et al.* (1979), Hanson (1980) and Ferreira *et al.* (1979), however, indicate a negative correlation between the two characteristics. However, in *H. fontanum* the results of water stress experiments indicated that drought stress induced by withholding water or by PEG treatment showed that this species did not respond by producing proline.

Proctor (1990) stated that, when compared with vascular plants, mosses which subjected to water stress show little change in pools of solutes such as proline, carbohydrates or organic acids. Similar results were found in *H. fontanum* in terms of proline accumulation, since this compound was not detectable under water stress conditions.

It has also been pointed out by Aspinall and Paleg (1981) that proline accumulation is a primary response of living organisms to water deficit. However, the fact that no proline accumulation occurs in some drought-tolerant species of bryophytes, lichens, pteridophytes and flowering plants when subjected to severe water stress led Stewart and Larcher (1980) to conclude that proline accumulation can not be regarded as a universal response in water-stressed plants. The effects of various levels of water stress on protein content indicate that this treatment decreases protein content in *H. fontanum*, thus confirming the observations made by Dhindsa and Cleland (1975) on *Avena* coleoptile, Dhindsa and Bewley (1976) on moss *Tortula ruralis*, Dhindsa and Bewley (1977) on moss *T. ruralis* and *Hygrohypnum luridum* and Oliver and Bewley (1984) on *T. ruralis*.

Increases in water stress (PEG concentrations) over 48 h were accompanied by a significant decrease in protein contents when compared with the control (Figs 5.6-5.8). These results are in agreement with the results obtained by Dhindsa and Bewley (1976, 1977). The effects of water stress were also parallelled by changes in dry weight of the harvested protonema and probably reflect an effect on growth of the tissues.

Decreases in protein content during water stress of mosses due to decreased synthesis was observed by Dhinsa and Bewley (1976, 1977), Bewley (1979), Dhindsa (1987), Oliver and Bewley (1984) and Oliver (1991). Further Huffaker *et al.* (1970) are of the opinion that it is a biochemical adaptation to stress.

The effects of water stress on chlorophyll synthesis in *H. fontanum* shoots and protonema were considerable (Figs 5.9, 5.10). During the period of the experiments (15 days) unstressed shoots and protonema showed significantly higher levels of chlorophyll than did treated shoots and protonema. Water stress caused a very significant decrease in chlorophyll content of both cultures shoots and protonema, greater in the latter. As the period of water stress treatment was increased, so the level of chlorophyll declined. This was seen up to 9 days in the protonema, after which no chlorophyll could be detected. Shoot culture, however, showed a decrease of chlorophyll content over the period of 12 days (289 g l^{-1} PEG), while showing no change at low water stress (196 g l^{-1} PEG) up to this time but then showed a slight increase with further treatment time indicating that the tissue became acclimated to the stress conditions.

The continuous decrease of chlorophyll content in protonemal cultures could be due to the structure of protonema filaments being more fragile than the leafy shoots. Under water stress conditions there could be a loss of chlorophyll due to cell rupture and cytoplasmic damage. Shoot

cultures, however, were more robust physically and appeared to be more tolerant of such water stress.

The chlorophyll *a/b* ratio in treated shoots and protonema changed at different stages depending on the treatment (PEG concentrations) and time. Chlorophyll *b* appeared to be less affected by water stress than *a* in the case of protonemal culture (Fig. 5.11). These results support the observations reported by Gupta (1978), who stated that severe desiccation decreased the ratio of chlorophyll *a* to *b* and the damage to chlorophyll *a* being greater than that to chlorophyll *b*.

Dry weight, on the other hand, showed significant differences between the controls and treated tissues for both shoots and protonema. The significant increase in dry weight of the controls in protonemal cultures started at day 3 whereas in shoots culture started at day 6 (Figs 5.9, 5.10). Dry weight under water stress showed only a slight increase during the experimental period. The data reported here suggest that high water stress stopped the growth of the moss protonema, whereas in the shoots a slight increase was seen to start after day 12.

Recovery of the moss from water stress

When shoots and protonema were subjected to water stress for 10 days, growth in terms of dry weight did not change, but chlorophyll content declined. The shoots and protonema recovered after subculturing in fresh medium, the rate of recovery was significantly less in the stressed cultures compared to that of the control. This result suggested that there was a carry-over of water stress treatment on the tissues since they did not recover immediately.

Phosphatase Activities

Results of analyses for PMEase and PDEase in the three moss fractions indicate that rhizoids of both field and laboratory material produced the highest activities among the moss fractions (Fig. 6.1). Leafy shoots, on the other hand, showed the lowest PMEase and PDEase activities. Laboratory fractions showed significantly higher PMEase and PDEase activities than field material (Fig. 6.1). These results suggested that the low phosphatase activities detected from field fractions could be due to the high cellular P content (> 1.5% d. wt) of the tissues when compared with the tissues of the laboratory grown plants, which had very low P content (< 0.35 d. wt) when the assay was carried out. These results match those obtained by Press and Lee (1983), who found in *Sphagnum* species negative correlation of phosphatase activity with tissue phosphorus concentration. They also stated that phosphate enrichment reduced enzyme activity and phosphate starvation increased enzyme activity. Moreover, when the moss shoots were sampled from three locations on the waterfall (Fig. 6.17), two from calcareous soil and one from clay soil), the former showed the higher PMEase (0.05 μmol pNP mg d. wt^{-1} h^{-1}) and PDEase (0.042 μmol pNP mg d. wt^{-1} h^{-1}) activities and lower P content (0.4% d. wt). Similar results have been reported by Press and Lee (1983), who demonstrated that *Sphagnum papillosum* collected from Lupton, Cumbria, had comparatively low acid phosphatase activities and correspondingly high tissue phosphorus concentrations when compared with plants from other sites.

H. fontanum showed marked PMEase and PDEase activities with increasing phosphorus deficiency when growing in batch culture (Figs 6.4, 6.5), and as the data reported here in moss *H. fontanum*, phosphatase activities match those results achieved by Press and Lee (1983) it could be suggested that the activities detected represents inducible acid phosphatase activity,

since inducible phosphatases are enzymes whose synthesis is dependant on the P status of the organism, i.e. when the organism is P sufficient there is no new synthesis of phosphatase enzymes. Synthesis is switched on when the P status of the organism is reduced to a particular level (McComb *et al.*, 1979; Healey, 1982).

There were three phases with respect to phosphatase activities during growth in batch culture. However, the starting time of the phases was different for the PMEase and PDEase activities (Figs 6.4, 6.5). During the first phase (to day 4 in the case of PMEase and day 8 PDEase) there were no phosphatase activities. The second phase (day 5-8 PMEase and 9-12 PDEase) was one in which there was a rapid increase in phosphatase activities (Fig. 6.5). The third phase (day 9 PMEase and 12 PDEase onwards) was one where activities per unit dry weight maintained almost the same level (Fig. 6.4).

PMEase activity was first detected when the mean value for cellular P was 1.2% dry weight with only low activity of 0.117 μmol pNP mg d. wt^{-1} h^{-1}. PDEase appeared 4 days later when the mean value for cellular P was 0.68% dry weight with an activity of 0.12 μmol pNP mg d. wt^{-1} h^{-1}. These results suggested that the PDEase were influenced by cellular P concentration more than PMEase. There was a relatively similar rate for PMEase and PDEase from day 12 showing stable activities (Fig. 6.4) and that this could have been due to the relatively low but stable P concentration (Fig. 6.3). From these results it could be hypothesized that inducible cell-bound acid phosphatases were detected in moss *H. fontanum* suggesting that this moss would be very suitable as a P indicator organism in the environment.

Effects of environmental variables and ionic composition on phosphatase activities, were investigated in *H.fontanum*. Acid phosphatase

activities with a pH optimum of 5.8-6.0 for PMEase and higher pH optimum 6.4-6.6 for PDEase (Fig. 6.6) were detected. Siuda (1984) demonstrated that acid phosphatase activities were active at pH between 2.6-6.8. In the present studies PMEase and PDEase were detectable at higher pH values than those stated above, and this result supports the view of Jansson *et al.* (1988), who stated that different enzymes have different pH optima and does not mean that they are totally inactive at, or do not tolerate, other pH values.

The influence of pH on phosphatase activities in *H. fontanum* was also tested for the three fractions from laboratory and field materials (Figs 6.8, 6.9). The optimum pH for the laboratory material is pH 6.0 for both PMEase and PDEase activities with measurable up to pH 10.3. The activities in leafy shoot, however, showed little response to pH and had a significantly lower activity compared to protonema, with rhizoids having the highest activities. PMEase and PDEase activities in the field material (Fig. 6.9) showed a narrow range of activities between pH 3.0 and 9.0 with a slight increase (PMEase) at pH 10.3, this increase may have been associated with microorganisms on the leafy shoot and rhizoid, being undetectable (PDEase) between pH 9-11. Field material showed very low activities compared to the laboratory material. PMEase and PDEase maximum activities (rhizoids) at pH 5.0 and 6.0, respectively, were almost 3.5 times lower than those detected in the laboratory, and about 2 times lower than in field shoots. These results suggested that the low rate of phosphatase activities of the field material may be due to the high concentration of cellular P found within the tissue (>1.5% d. wt).

Temperature optima over a period of 1 h (Fig. 6.10) for PMEase (60 °C) and PDEase (65 °C) were very much above temperatures likely to be encountered in its original environment (the maximum 27.6 °C) (Ministry of

Agriculture, 1986). These results suggested that the temperature in the field is unlikely to limit phosphatase activity. PMEase and PDEase in *H. fontanum* are stable enzymes with cellular activities still detectable at 80°C. Similar results were reported in *Calothrix parietina* (Grainger *et al.*, 1989) and *Calothrix* strain D764 (Islam & Whitton, 1991). These results suggested that the maximum activity reported from laboratory experiments in relation to optimum pH and temperature (> 0.5, > 0.8 μmol pNP mg^{-1} d. wt^{-1} h^{-1}) would not be achieved in field conditions where the moss *H. fontanum* originates, where a mean water pH of 7.8 was recorded (Whitton *et al.*, 1986) at a mean maximum temperature of 27.6 °C. At these values of pH and temperature laboratory activities were about 45% and 50%, respectively, of the maximum. In the natural environment, for most of the year, activities may be expected to be significantly lower in respect to temperature.

Acid phosphatase is a non-metallic enzyme which is not activated by divalent cations (Hasegawa *et al.*, 1976). Absence of calcium (Fig. 6.12) from the assay medium had no significant effect on PMEase and PDEase activities, but high concentrations (10 mM) inhibited PMEase activity completely, although this ion has been reported to have great stimulatory effect in alkaline phosphatase activity in many cyanobacteria species (Healey, 1973; Doonan & Jensen, 1980; Grainger et al., 1989; Islam & Whitton, 1992). Ca concentration in the waterfall site reached only 1.09 mM. Inorganic phosphate (P_i) inhibited both PMEase and PDEase activities in *H. fontanum*, which is a common feature to all inducible phosphatase systems (Healey, 1973; Grainger *et al.*, 1989; Islam & Whitton, 1992). From these results it could be said that *H. fontanum* have inducible acid phosphatase activities.

Magnesium had a slight inhibitory effect on PDEase activity at 10 mM and had no effect on PMEase activity. These results agree with the results of Hasegawa *et al.* (1976) for young wheat roots. Zn had a significant inhibitory effect at high concentrations (10 mM) on PMEase and inhibited PDEase activity completely, at the same concentrations. These results matched the results for blue-green algae (Grainger *et al.*, 1989; Whitton *et al.*, 1990), where it was stated that Zn at 10 mM had marked inhibitory effect on the phosphatase activities. In wheat, it has been reported that Zn inhibited wall enzymes by 23-32% and showed a 36-39% inhibition toward cytoplasmic enzymes (Hasegawa *et al.*, 1976). The concentration of Zn reported for the waterfall was 0.045 μM (Whitton *et al.*, 1986) and 0.19 μM in the Chu 10 G medium, where at those concentrations the activities were not affected. Zinc is an important micro-nutrient for growth and metabolism in plants (Price *et al.*, 1972). If zinc is applied at high levels, however, it may be toxic (Whitton & Say, 1975; Dhruva *et al.*, 1977).

Desiccation, drought and water stress

It has been reported that water stress has a differential effect on enzyme levels for several tissues (Huffaker *et al.*, 1970; Takaoki, 1968; Todd & Yoo, 1964). Huffaker *et al.* (1970) found that water stress caused a great decrease in the level of nitrate reductase in barley leaves.

The PMEase and PDEase activities in moss *H. fontanum* were inhibited by about 23% and 21%, respectively, from the original activities before the desiccation (Fig. 6.13). The presence of this detectable lag in the activities subsequent to drying could be due to the depression in synthesis of the enzyme, as it has been reported by Dhindsa and Cleland (1975) that changes in enzyme levels in *Avenue* coleoptile were due to differential effects on synthesis. The results reported here suggested that the

decrease in the activities may be due to the decrease of the enzymes synthesis during the desiccation period. Nevertheless the enzymes were able to recover their activities as much as the control level within 15 min of rehydration indicating that either a very efficient reactivation of synthesis or re-establishment of activity takes place.

The inhibition of the PMEase and PDEase activities for the three fractions during longer period of drought (3 months) was significant (3.7-2.8 times) decrease in activities compared to the control (before drought) (Figs 6.14, 6.15). Since the inhibition in protonema PMEase and PDEase activities, subsequent to long period of drought (Fig. 6.14) (3 months) was 3.3 and 3.5 times higher, compared to the inhibition caused by desiccation (5 days) (Fig. 6.13) it could be suggested that the high reduction in PMEase and PDEase activities may be due to the longer period of drought stress. Although these results showed that phosphatase activities persisted through periods of drought, so enabling the plant to mobilize phosphate when water stress conditions were alleviated.

PMEase and PDEase activities in all 3 fractions were detectable over the full pH range tested (3-11). However, recovery of the activities after drought was only about 33% for rhizoid and protonema compared with the controls, with the shoots showing more tolerance to drought (Fig. 6.15). PMEase activity for protonema was seen even at pH 11 with a considerable activity and PDEase was higher between pH 6 and 10.3 than that detected in rhizoids and shoots. This may be attributed to the fine cells structure of the protonema, which possibly helps drought stress to cause some changes in wall or enzyme structure which renders the active site more accessible to the substrate.

Water stress using different concentrations of PEG also showed a significant reduction in protonema PMEase and PDEase activities. This is

accompanied by water stress increase (Fig. 6.16). Todd (1972) concluded from a list of some 25 enzymes affected by water deficits that severe stress or desiccation lowers enzyme levels generally.

PDEase activity (Fig. 6.16) was influenced by water stress more than PMEase activity, particularly at high concentrations of PEG (150 & 200 g l^{-1}), where the PDEase activity inhibited completely, whereas PMEase still showed considerable activity at those concentrations. From these results it could be suggested that PDEase is more susceptible to water stress than PMEase.

Comparison in phosphatase (PMEase) activity using two different substrates (pNPP & MUP)

The results (Fig. 6.22) of the influence of pH on PMEase activity measured using two different substrates (pNPP & MUP) indicated that the maximum rate of activity was obtained between pH 5.5-6.0 with the two substrates used. PMEase activity using MUP was detectable at all pH values (3-11) whereas PMEase measured using pNPP was only detectable between pH 3-8. The ratio between PMEase, using pNPP, to PMEase activity, using 4-MUP, was about 1.5. These results suggest that MUP substrate is more sensitive for detecting phosphatase activity, than pNPP, particularly at higher pH values where the activity was very low as in this organism. The high sensitivity of 4-MUP has been reported previously (Pettersson & Jansson, 1978). The effects of 4-MUP substrate concentrations (Fig. 6.23) indicated that the PMEase was detectable at all concentrations used which made this substrate useful particularly with very low phosphatase activity. When the influence of pH on phosphatase activity was measured using different concentrations of 4-MUP the results indicated (Fig. 6.24) that the pH-activity curves had more or less similar shape, and had optimum activity at the same pH for all concentrations of substrate. The optimum

rate of phosphatase activity was between pH 5-6 indicating that this substrate is suitable for measuring phosphatase activity, since there was no shift in pH optimum values. At low concentrations (0.25, 0.5 μM) of substrate, maximum activity was, however, obtained over a wider range of pH (5.5-7.0) than with high concentrations of substrate. There was no inhibition by excess of substrate in the concentration ranges, but at higher substrate concentrations inhibition was seen at high pH values.

Possibly due to the presence of both PMEase and PDEase activities, the moss protonema was able to grow on different organic P substrates (Table 6.1). Among the organic P substrates tested as phosphate sources for growth, none of them led to a similar yield on day 16 (Table 6.1) to that of protonema growth with P_i. The substrates used for enzyme assays, pNPP and bis-pNPP gave, however, 88.8% and 78% respectively of the yield obtained with P_i. It has been reported that some cyanobacteria species could not use ATP as a P source, but this was probably due to toxicity of ATP to those species at the concentration supplied (1 mg l^{-1} P) (Whitton *et al.*, 1990, 1991; Islam & Whitton, 1992). The moss *H.fontanum* appeared to be able to utilize ATP as a P source giving 75% of the yield obtained with P_i. Only phytic acid, among the organic phosphate substrates tested, did not support the growth. Many cyanobacteria species appear to be incapable of using phytic acid as a P source (Whitton *et al.*, 1990, 1991; Islam & Whitton, 1992). This may indicate the lack of availability of this substrate to planktonic organisms due to the low solubility of the molecule (Merck Index). This lack of solubility could be the same reason for the inability of the moss *H. fontanum* to utilize phytic acid for growth.

The K_m values usually reported for acid and alkaline PMEase are between 10^{-6} to 10^{-4} M (Gassing *et al.*, 1988). The K_m value for phosphomonoesterase in *H.fontanum* was 2.101×10^{-4} M, which is similar or

within the range to the values reported for *E. coli* (3×10^{-3} M) (Von Hofsten & Porath, 1962) and *Anabaena variabilis* (7×10^{-4} M) (Healey, 1973).

These results reported here show that *H. fontanum* is capable of mobilizing organic phosphate through the activity of phosphatases. However, further work should be carried out to characterize these enzymes fully in terms of cellular locations and induction at a molecular level.

SUMMARY

1) *Hydrogonium fontanum* was collected from a calcareous tufa-depositing waterfall in the Asir Mountains in south-west Saudi Arabia (18° 58′ N, 42° 60′ E). The compostion of water sampled in October 1988 and 1991 for Mg was 16.32 and 15 mg l^{-1}, respectively, and Ca concentration was the same in the two samples (44 mg l^{-1}).

2) The moss was the dominant plant in the waterfall tufa-deposition zone. Adult plants, consisting of stems, leaves and rhizoids, occurred in and adjacent to the waterfall, whereas protonema and gemmae occurred only when the moss was submerged.

3) The moss shoots were subcultured in the laboratory under standard conditions and the protonema were isolated and obtained in axenic culture. Leafy shoots were eventually formed from protonema.

4) In the laboratory, protonema grew faster than the shoots. The growth rate (measured as dry weight) was 0.22 d^{-1} for protonema after day 4. Shoot growth rate was only 0.06 d^{-1} after the same period.

5) Light intensity had a pronounced effect on chlorophyll content and yield. The moss had a chlorophyll content of 4.27 mg l^{-1} and yield of 186 mg l^{-1} when grown at low light intensity (10 μmol photon m^{-2} s^{-1}). In contrast when grown at high light intensity (90 μmol photon m^{-2} s^{-1}) the values were 1.24 mg l^{-1} and 496 mg l^{-1}, respectively.

6) In both field and laboratory, elongation of submerged shoots was about three times that of the non-submerged plants. In laboratory experiments the growth rate as dry weight was 0.045 d^{-1} for unflooded cultures and 0.037 d^{-1} for flooded cultures; this measurement was not made in the field.

7) Influence of water stress was greater in protonema than that of leafy shoots in respect to dry weight and chlorophyll content.

8) Drought or water stress had no influence on proline accumulation. However, increases in water stress over 48 h were accompanied by significant decreases in protein contents which were parallelled by changes in dry weight of the protonemal cultures.

9) Phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activities were detected in all moss fractions (rhizoids, protonema, leafy shoots) in the laboratory, and for rhizoids and leafy shoots in the field grown material; When different fractions were compared laboratory grown materials showed significantly higher activities than field grown materials. Rhizoids produced the highest PMEase and PDEase among the moss fractions.

10) The shoots with the lowest tissue phosphorus concentrations (0.37% d. wt), originating from the calcareous site and the highest phosphatase activities (0.05 μmol pNP mg d. wt^{-1} h^{-1}). Conversely the shoots from clay sites, having higher tissue phosphorus (2.13% per unit d. wt) concentrations, possessed lower phosphatase activities (0.02 μmol pNP mg d. wt^{-1} h^{-1}).

11) PMEase activity in batch culture was first detectable when the mean value for cellular P was 1.15% of dry weight (activity = 0.117 μmol pNP mg d. wt^{-1} h^{-1}). PDEase appeared 4 days later, when cellular P was 0.54% (activity = 0.12 μmol pNP mg d. wt^{-1} h^{-1}). PMEase and PDEase activities increased up to day 12, when the P content dropped to 0.43%, after which the activities maintained this level up to day 36 at which time the level of P was 0.26%.

12) The optimum temperatures, measured over a period of 1 h, for PMEase and PDEase activities were 60 °C and 65 °C, with pH optima of 5.8-6.0 and 6.4-6.8, respectively. The influence of six ions on the activities was also tested. Ca, Zn and P were found to inhibit the activities significantly at the highest concentrations applied (10 mM).

13) The protonema were capable of utilizing four different organic phosphate substrates (pNPP, bis-pNPP, ATP, and soudium-ß-glycerophosphate) for growth, but could not grow with phytic acid.

14) Desiccation for 5 days followed by re-watering inhibited PMEase and PDEase activities in protonema by about 23% and 21%, respectively, compared to the activities before desiccation. PMEase and PDEase activities detected in moss fractions subsequent to a long period of desiccation (3 months) were 3.7 and 2.8 times, respectively, lower than the original activities.

15) Water stress (PEG treatment) significantly ($P > 0.05$) reduced protonema PMEase and PDEase activities and PDEase was found to be influenced more than PMEase activity, particularly at high PEG concentrations (150, 200 mg l^{-1}), where the PDEase was not detectable.

16) The optimum pH for the hydrolysis of 4-MUP substrate was stable with decreases in the concentrations of substrate, though with low concentrations maximum activity was obtained over a wider range of pH (5.5-7.0) than (5.0-6.0) with high concentrations.

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