MYCOPHAGOUS AMOEBAE IN A SUPPRESSIVE PASTURE SOIL IN RELATION TO THE TAKE-ALL DISEASE OF WHEAT

by

Sukumar Chakraborty

B.Sc.(Ag.) Honours, M.Sc.

Department of Plant Pathology
Waite Agricultural Research Institute
The University of Adelaide
South Australia

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SUMMARY

Amoebae were isolated, characterised and identified from soil of the Waite Institute permanent pasture plot which is suppressive in pot bioassays to the take-all disease of wheat. Nine species of amoebae belonging to eight genera were tested for their mycophagy against three plant pathogenic fungi including *Gaeumannomyces graminis tritici*. Members of the genera, *Gephyramoeba*, *Mayorella*, *Saccamoeba*, *Thecamoeba* and an unidentified species of the order Leptomyxida were mycophagous. All mycophagous amoebae, except the unidentified leptomyxid were able to feed on pigmented (melanised) fungal cells.

Populations of the various genera of soil amoebae were assessed from samples of the naturally suppressive pasture soil, a suppressive wheat-pasture rotation and three non-suppressive wheat-field soils. The suppressive soils showed higher populations of both mycophagous and other amoebae and a higher frequency of occurrence of mycophagous genera. Soil texture and water holding capacity were not related with population levels of amoebae in these soils.

Saprophytic survival of the take-all fungus was studied by burying fungal hyphae in suppressive and non-suppressive soils. Hyphal density and survival of pigmented hyphae declined at a faster rate in the permanent pasture soil than in the non-suppressive soils. Hyphae recovered from the suppressive soil showed a higher association of mycophagous and other amoebae and scanning electron microscopy of these hyphae showed extensive erosion and discrete perforations in their walls.

The decline in survival of the fungus was related to the rate of decline in the density of pigmented hyphae in suppressive soil, irrespective of the soil type.

Studies on the population dynamics of amoebae showed a higher rhizosphere population and a higher rhizoplane association of mycophagous and other amoebae in suppressive soils. Populations in the suppressive pasture-wheat soil did not correlate with soil moisture during two wheat crops and one inter-crop fallow.

Three mycophagous amoebae, *Gephyramoeba*, *Saccamoeba* and *Thecamoeba granifera* sub-species *minor*, alone or mixed effectively reduced take-all severity in pot bioassays. The reduction in disease rating and the increase in height and dry weight of plants were comparable to that obtained with the suppressive pasture soil. Higher populations of these amoebae in combination were able to further reduce disease severity and improve plant growth.

Mycophagous amoebae are proposed as a component of the suppressive factors in the permanent pasture soil. The observations that mycophagous amoebae associate themselves with the fungus in soils, can lyse both pigmented and hyaline hyphae during the pre-colonisation and parasitic phase of the fungus, and reduce take-all severity in pot bioassays substantiate this proposal.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and to the best of my knowledge and belief contains no material previously published or written by another person, except when due reference is made in the text.

S. Chakraborty

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I. INTRODUCTION

Leeuwenhoek (1677) was probably the first to record the occurrence of protozoa in soil and presented accounts of several protozoa including *Amoeba* (Leeuwenhoek, 1702). It was almost two centuries later that Russell and Hutchinson (1909) advanced their theory of 'soil sickness' and proposed that the increase in soil fertility after partial sterilisation was due mainly to the removal of bacteriophagous soil protozoa. Although current interpretations of partial sterilisation suggest that this explanation is incorrect, their hypothesis aroused great interest in the study of soil protozoa. Systematic research on soil protozoa began in 1915 at Rothamsted with the demonstration of active protozoa by Martin and Lewin. Cutler (1919, 1920) developed methods of counting soil protozoa and protozoa were found to be ubiquitous; the same species occurring in arctic, temperate and tropical soils. None of the soils examined were completely devoid of protozoa. Sandon (1927) reviewed the geographical distribution of soil protozoa and suggested that temperature, moisture, soil reaction and texture are of little importance in species distribution of majority of soil protozoa.

Soil protozoa have two major ecological roles: influencing the structure of the microbial community and enhancing nutrient cycling; both activities are related to their feeding on bacteria. Protozoa enhance mineralisation of nutrients immobilised in the microbial biomass by consuming bacteria and excreting excess nutrients thereby accelerating metabolic turnover in soils (Stout and Heal, 1967; Stout, 1973). Protozoa selectively limit the size of bacterial populations in soil (Singh, 1941, 1942; Danso and Alexander, 1975; Habte and Alexander, 1977; Sardeshpande *et al.*, 1977) and they are responsible

for the decline of *Rhizobium* (Danso *et al.*, 1975) and plant pathogenic *Xanthomonas campestris* (Pammel) Dowson in soil (Habte and Alexander, 1975). However, protozoa do not eliminate their bacterial prey (Danso and Alexander, 1975; Habte and Alexander, 1978a, 1978b).

The role of protozoa in the mineralisation of nutrients is well known. *Azotobacter* spp. can fix more nitrogen in the presence of protozoa (Cutler and Bal, 1962) or amoebae (Nasir, 1923) and mixed cultures of bacteria and protozoa (Skinner, 1927; Meiklejohn, 1932) produce more ammonia than the bacteria alone. Recent studies in soil microcosms have shown greater mineralisation of carbon, nitrogen and phosphorus in soil containing bacteria and amoebae than in soil containing bacteria without amoebae (Coleman *et al.*, 1977; Anderson *et al.*, 1978; Cole *et al.*, 1978; Coleman *et al.*, 1978; Elliott *et al.*, 1979). Recent reviews on the occurrence and activities of soil protozoa have been presented by Singh (1963), Pussard (1967), Stout and Heal (1967), Viswanath and Pillai (1968), Nicoljuk and Geltzer (1972) and Darbyshire (1975).

Protozoa are represented in the soil mainly by rhizopods, which include the naked and testate amoebae, flagellates and ciliates. In terms of number per unit mass naked amoebae constitute the dominant group. Their numbers vary from 2×10^3 to 1690×10^3 g^{-1} dry wt, whereas testate amoebae vary from 2×10^3 to 73×10^3 g^{-1} dry wt, flagellates from 0.5×10^3 to 56×10^3 g^{-1} dry wt and ciliates approximately 0.75×10^3 g^{-1} dry wt of soil (Darbyshire and Greaves, 1967; Heal, 1971). As a component of the protozoan fauna, soil amoebae contribute to the decomposition of organic matter (Aristovskaya, 1963; Ten, 1967) and increase rhizosphere phosphate activity (Gould *et al.*, 1979).

Soil amoebae are known to secrete heteroauxin (IAA) in lucerne rhizospheres (Nikoljuk and Tapilskaja, 1969), decompose chitin in soil (Okafor, 1966), and to transport animal viruses (Toomey *et al.*, 1948; Baron *et al.*, 1980). Of direct concern to mankind are the pathogenic amoebae which cause a variety of diseases, some fatal, collectively known as amoebiasis (Ewers, 1981). The free-living soil amoebae which cause diseases including amoebic meningoencephalitis have recently been reviewed by Culbertson (1971).

Oehler (1916) demonstrated that the yeast *Saccharomyces exiguus* Hansen was eaten by amoebae. In feeding trials with 12 species of soil moulds and 4 species of yeasts, Severtzova (1928) found that amoebae multiplied with 4 species of moulds and 2 yeasts and suggested that vegetative cells were preferred to spores. Castellani (1930), Negroni and Fischer (1941), Nero *et al.* (1964) and Bunting *et al.* (1979) showed that amoebae feed on yeasts but not on filamentous fungi. In 1963, Heal conducted feeding trials with 4 different amoebae using 16 filamentous fungi and 19 yeasts. Many yeasts were consumed, but only 2 fungi, *Paecilomyces elegans* (Corda) Mason and Hughes and *Polyscyalatum fecundissimum* Reiss. acted as food sources. Ingestion of fungal spores by amoebae and ciliates was reported by Esser *et al.* (1975). They found that conidia of *Alternaria, Curvularia, Helminthosporium* and *Fusarium* were ingested by a *Thecamoeba* sp. but only *Fusarium* spores were digested. Others have suggested an antagonistic action of amoebae towards plant pathogenic fungi (Tapilskaja, 1967; Mavljanova and Nasyrova, 1969). *Amoeba albida* develop in the rhizosphere of cotton plants, have a pronounced antagonistic effect on *Verticillium dahliae* Kleb. and protect plants against wilt (Tapilskaja, 1965; Nikoljuk and

Tapilskaja, 1967). Treatment of cotton seeds with amoebae after artificial contamination with *V.dahliae* increased the germination rate and had a favourable effect on the overall development of the plant.

Studies on the survival of soil-borne fungi have shown that spores of pigmented isolates of fungi survive longer in soil than those of hyaline isolates of the same fungus (Old and Robertson, 1970; Clough, 1975). This resistance towards lysis is due to the presence of melanin compounds in the walls of the pigmented isolates (Kuo and Alexander, 1967; Bull, 1970). However, direct observation of fungal spores, including pigmented ones, after incubation in natural soils revealed that spores become perforated by holes varying from 0.2 to 5.0 µm in diameter (Old, 1969; Old and Robertson, 1969; Clough and Patrick, 1972). This has been referred to as 'perforation lysis' in the review of Old and Wong (1976) and autolysis, mechanical puncture by soil animals and enzymatic lysis by microorganisms were considered as possible causes of perforations.

In 1977 Old showed that giant soil amoebae caused perforation lysis of spores of *Cochliobolus sativus* (Ito et Kuribay) Dreschl. ex. Dast. buried in natural soils (Old, 1977a). The amoeba was later identified as *Arachnula impatiens* Cienk. (Old and Darbyshire, 1980). The perforating and lysing activities of this amoeba during attacks on fungal spores have been described by Old (1977a) and Old and Darbyshire (1978). Of 30 fungal species tested, 21 were used as food. Conidia, chlamydospores, sporangia, teleutospores, basidiospores and hyphae of many fungi of a wide array of taxonomical groupings were suitable food for the amoeba. Anderson and Patrick (1978) also independently described mycophagous amoeboid organisms from soil that perforate spores of

Chalara elegans Nag Raj and Kendrick *(= Thielaviopsis basicola* (Berk. and Br.) Ferr.) and *C.sativus* and believed one to be a member of the genus *Vampyrella*. These authors described two other mycophagous amoebae of the genera *Theratromyxa* and *Vampyrella* which produced smaller perforations of 1 µm or less in diameter in conidia of *C.sativus* (Anderson and Patrick, 1980). One of these genera, *Theratromyxa* is also known to be nematophagous (Weber *et al.*, 1952; Zwillenberg, 1953; Sayre, 1973).

Among the known mycophagous genera of soil amoebae, *Arachnula*, *Vampyrella* and *Theratromyxa* belong to the family Vampyrellidae in the classification of the protozoa proposed by Honigberg *et al.* (1964). At least two other genera, *Thecamoeba* (Esser *et al.*, 1975; Alabouvette *et al.*, 1979; Pussard *et al.*, 1979) and *Cashia* (Pussard *et al.*, 1980) have mycophagous species. Homma *et al.* (1979) isolated vampyrellid amoebae from wheat field soil by burying pigmented hyphae of *Gaeumannomyces graminis* (Sacc.) Von Arx and Olivier. The amoebae caused perforations and annular depressions in the hyphae similar to those described by Old (1977a). Apart from these reports information on the significance of amoebae in the ecology and control of soil fungi is generally lacking (Old and Patrick, 1979).

The take-all disease caused by *Gaeumannomyces graminis* (Sacc.) Von Arx and Olivier var. *tritici* Walker *(Ggt)* is considered as the most important root rot of wheat throughout the world (Butler, 1961) and is second in causing crop losses after stem rust (Garrett, 1942). Although take-all was first recognised in 1852 in South Australia (Anon., 1868), it was Prillieux and Delacroix (1890) who reported that a fungus, *Ophiobolus graminis* Sacc., was the causal agent. Since then, take-all has been the subject of much research and comprehensive reviews on its various aspects appear in Garrett (1942), Butler (1961), Nilsson (1969), Walker (1975), Asher and Shipton (1981).

Despite considerable research, take-all is still a major limiting factor of wheat production in South Australia (Stynes, 1975). The development of take-all in the field is influenced by cropping history (Louw, 1957; Glynne, 1965; Slope and Etheridge, 1971) and less take-all occurs in wheat following oats (McAlpine, 1904; Richardson, 1910; White, 1947), peas (Zogg, 1969) and trefoil (Garrett, 1943) which makes crop rotation one of the main control measures against the disease (Butler, 1961).

In continuous cereal cropping disease incidence and severity tends to decline (Broadfoot, 1934a; Glynne, 1935; Garrett, 1943). However, White (1947) found an increase in the severity of take-all after 4 years of continuous wheat. A decline in the severity of take-all after a peak disease in cereal monoculture was reported by Slope and Cox (1964). Take-all decline (TAD), as it is now known, occurs in many countries including Netherlands (Gerlagh, 1968), France (Lemaire and Coppenet, 1968), Yugoslavia (Vojinovic, 1973), Switzerland (Zogg, 1959), U.S.A. (Shipton *et al.*, 1973) and England (Slope, 1963; Cox, 1963; Shipton, 1969; Pope and Jackson, 1973; Pope and Hornby, 1975). TAD soils can suppress the saprophytic and parasitic growth of *Ggt* in pot bioassays (Lester and Shipton, 1967; Shipton *et al.*, 1973; Pope and Jackson, 1973; Wildermuth, 1977).

Suppression similar to TAD has been reported under various soil, crop and environmental conditions and Walker (1975) distinguished six conditions of disease suppression of which three require the presence of both host and pathogen. Wildermuth (1977) found that suppression can also be induced by infection of wheat by *Gaeumannomyces graminis*

(Sacc.) Von Arx and Olivier var. *graminis* Walker and *Gibberella zeae* (Schw.) Petch. His work also emphasized that some natural soils, without a history of wheat monoculture, can be suppressive to take-all. Palti (1981) cited more than 20 plant pathogenic fungi which are prevented by soil suppressiveness from attacking crops. However, as Brown *et al.* (1973) pointed out, the development of an antagonism to *Ggt* in the absence of the host is not necessarily the same as TAD.

Interactions between members of the soil microflora and the pathogen have been the frequent explanations of the mechanism of suppression (Gerlagh, 1968; Pope and Jackson, 1973; Vojinovic, 1973; Cook and Rovira, 1976; Rovira and Wildermuth, 1981). Others have associated virus-like particles (Lapierre *et al.*, 1970), reduced virulence (Cunningham, 1975), changes in the NO_3-N:NH_4-N ratio of rhizosphere soil (Brown *et al.*, 1973), and hyperparasitism (Zogg and Jaggi, 1974) with reduced infection by *Ggt*. Hornby (1979) reviewed the literature and concluded that no single hypothesis adequately explained TAD. The demonstration of mycophagous activities of amoebae in TAD soils (Homma *et al.*, 1979) suggests that the role of soil amoebae in suppression of *Ggt* warrants further study.

Wildermuth (1977) found reduced numbers of infective hyphae of *Ggt* in the rhizosphere of wheat in the presence of several suppressive soils including soil from a permanent pasture plot at the Waite Agricultural Research Institute, South Australia. The amoebae of the suppressive permanent pasture soil and their role in the suppression of take-all have been investigated. Mycophagous activities of isolated amoebae were studied in detail and species which fed on *Ggt* hyphae *in vitro* were

tested for their ability to reduce the severity of take-all in pot bioassays. Saprophytic survival of the fungus in relation to amoebal populations in suppressive and non-suppressive soils was studied. Population dynamics of mycophagous and other amoebae in wheat rhizospheresin suppressive and non-suppressive soils was also studied to determine the association of these amoebae with the pathogen in its parasitic phase.

II. MATERIALS AND METHODS

PHYSICAL AND CHEMICAL PROPERTIES OF EXPERIMENTAL SOILS

The location, classification, particle size distribution, pH and cropping history of the three soils used are listed in Table 1. Particle size distribution was determined from three samples of each soil using the hydrometer method (Piper, 1950) for mechanical analysis. Three samples of each soil, passed through a 2 mm sieve were moistened to -0.98 kPa, placed on moist filter paper inside sintered glass funnels and suctions of -0.98 to -19.6 kPa (10 to 200 cm of water) were applied for two weeks before estimation of soil moisture contents. Suctions of -100, -500 and -1500 kPa were obtained using pressure chambers. Plastic conduits (2.5 cm long, 3.5 cm internal diameter) with the moist soils were placed on top of a high pressure ceramic plate in the chamber and pressure applied from a compressed air cylinder for two weeks. The moisture characteristic curve of each soil is shown in Fig.1.

ORIGIN AND MAINTENANCE OF FUNGI AND BACTERIA

Table 2 shows the origin of the different fungi and bacteria used in this work. The maintenance media for these organisms are as follows (composition of the media in Appendix I).

Cochliobolus sativus	Czapek-Dox agar (*In*: Raper and Fennell, 1965)
Gaeumannomyces graminis tritici	NDY/6 (Warcup, 1955)
Phialophora sp.	NDY/6
Phytophthora cinnamomi Rands.	V8 juice agar (Miller, 1955)
Saccharomyces cerevisiae Meyen and Hansen	2% malt agar
Klebsiella sp.	Tryptone-Glucose-Yeast agar (TGY, Cavender and Raper, 1965)

TABLE 1: Physical and chemical properties and cropping history of the experimental soils.

Locality	Classification	Particle size distribution (%) clay (<2μm)	silt (2-20μm)	sand (>20μm)	pH (1:5 water)	Cropping History[a]
Avon	Gc 1.11* solonised-brown soil**	16.9	8.2	74.8	8.6	WP
Murray Bridge	Gc 1.12* solonised-brown soil**	9.5	0.6	89.8	7.1	WPPBPPW
Waite Institute permanent pasture	Dr 2.23* red-brown earth**	24.5	17.6	57.8	6.4	PPPPPPP

* Factual key (Northcote, 1979)

** Great soil group (Stace *et al.*, 1968)

[a] W = Wheat
P = Pasture
B = Barley

Fig.1: Moisture characteristic curves of experimental soils.

Waite permanent pasture.

Avon.

Murray Bridge.

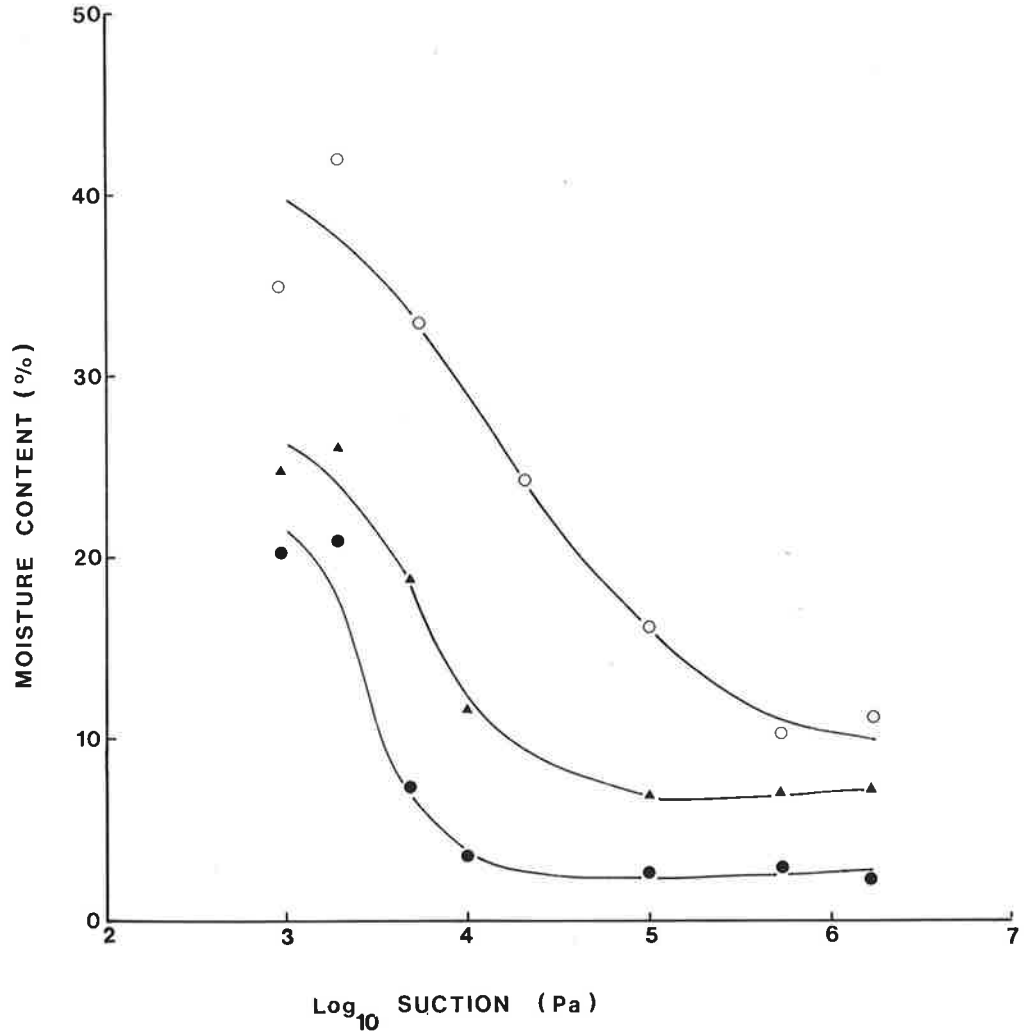

50
40
30
20
10
0
2
3
4
5
6
7
MOISTURE CONTENT (%)
Log10 SUCTION (Pa)

TABLE 2: Origin of the fungi and bacteria used.

Organism	Isolate No.	Origin	Supplier
Cochliobolus sativus	CS-2	Pine roots, A.C.T.	K.M.Old
Gaeumannomyces graminis tritici	G.1	Soil, Waite permanent pasture	S.Chakraborty
Phialophora sp.	PR-II (DAR 32098)	Maize, Cambridge, U.K.	P.T.W.Wong
Phytophthora cinnamomi	A-21	*Banksia* sp., W.A.	K.M.Old
Rhizoctonia solani Kühn	F91	Wheat, S.A.	J.H.Warcup
	WARH/6	Soil, W.A.	J.H.Warcup
Saccharomyces cerevisiae	729		J.H.Warcup
Klebsiella sp.			J.H.Warcup

TEST FOR SUPPRESSIVENESS OF SOILS

The pot bioassay (Shipton *et al.*, 1973) was used with modifications to test suppressiveness of experimental soils. A preliminary experiment was conducted using a sterile coarse sand (CS) instead of fumigated soil. Washed and dried sand was thinly spread in flat metal trays, covered with 'Alfoil' and autoclaved under dry cycle at 121°C for 1 h for 3 consecutive days.

Inoculum of *Ggt* was prepared by grinding oat grains colonised by the fungus. Oat grains soaked in distilled water overnight were autoclaved at 121°C for 1 h on each of three successive days. These were inoculated with agar cultures of the fungus and incubated in darkness at 25°C for 8-10 weeks until the fungus had extensively colonised the oats. The grain was shaken every two to three days to prevent it from matting together with mycelium. Colonised oat grains were dried in a laminar flow, ground and sieved through a 2 mm sieve.

Ground oat inoculum was added at a rate of 0.2% (w/w) to 1750 g CS and mixed thoroughly by shaking these in an inflated plastic bag. Soil of the permanent pasture plot (WPP) in the rotation experiment at the Waite Agricultural Research Institute, South Australia, was used as the suppressive soil (Wildermuth, 1977). The WPP soil was added at rates of 1% and 10% (w/w) and the mixing repeated. Three hundred and fifty g of the mixture was added to each 300 cm^3 plastic pot and 5 wheat seeds of cultivar Halberd were spread over the surface. In control pots, autoclaved ground oat inoculum (0.2% w/w) of *Ggt* was added. Seeds were covered with 50 g CS (without *Ggt* or WPP soil) in

each pot and 50 ml distilled water added. This resulted in 15% moisture in CS (oven dry wt basis). Five replicates were used for each treatment and pots were randomised. All plant growth experiments were conducted in growth rooms with a 12 h day-length, maintained at 15±1°C and provided a light intensity of 10760 lux at the height of the plants. Every second day, pots were watered to the original wt and height of plants measured.

Plants were harvested four weeks after seeding, roots washed free of adhering CS and rated for take-all (Cook and Rovira in Wildermuth, 1977). Details of the scale of disease rating is as follows:

Disease rating	Description of typical plants
0	No visible sign of disease
1	Lesions on < three seminal roots; no lesions on stem
2	Lesions on > three seminal roots; no lesions on stem
3	Lesions on all roots and discrete lesions on the base of stem
4	Lesions on all roots and lesions coalesced around base of stem
5	As 4 but necrosis around stem base more severe
6	Seedling so severely diseased that only the first leaf produced; leaf still green
7	Seedling completely necrotic

The growth rate of plants in the different treatments is shown in Fig.2. The results (Table 3) show that CS can be used as a growth medium to test suppressiveness of soils.

SUPPRESSIVENESS OF EXPERIMENTAL SOILS

Suppressiveness of the Avon, Murray Bridge (MB), and WPP, along with soils from three other permanent rotation plots at the Waite

Fig.2: Mean height of wheat plants during growth in sterilized coarse sand, inoculated with dead or live *G.graminis tritici*, or live *Ggt* plus two levels of the suppressive Waite permanent pasture soil.

●—● Control (autoclaved *Ggt*).

■—■ Live *Ggt* plus Waite permanent pasture soil 10%.

□—□ Live *Ggt* plus Waite permanent pasture soil 1%.

○—○ Live *Ggt*.

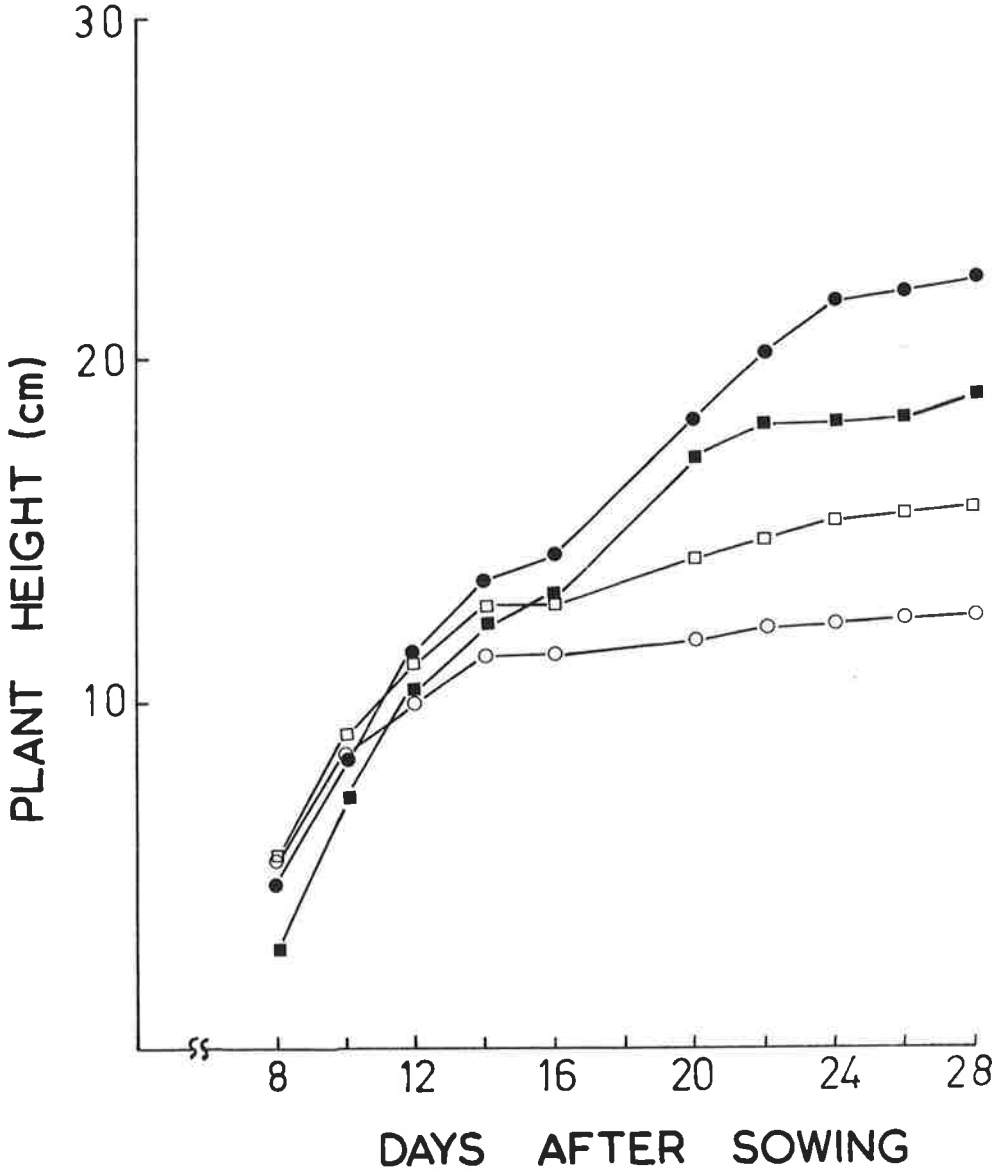

30
20
10
PLANT HEIGHT (cm)
8
12
16
20
24
28
DAYS AFTER SOWING

TABLE 3: Height and disease rating of wheat grown in sterilized coarse sand, inoculated with dead or live *Ggt* or live *Ggt* plus suppressive soil.

Treatment	Plant height (cm)	Disease rating
*LGgt + WPP soil (10%)	21.1	2.0
LGgt + WPP soil (1%)	16.8	3.2
LGgt	14.5	4.7
DGgt	24.0	0.0
Least significant difference P = 0.01	2.33	0.62

* L = Live
D = Dead

Institute was determined using the modified pot bioassay described earlier. The length of rotation in the Waite Institute plots was 27 years in WPP, 53 years in the continuous-wheat (CW), 53 years in the pea-wheat (PeW) and 27 years in the pasture-pasture-wheat (PPW). The test soils were used at a rate of 1% (w/w) in all cases. Results (Table 4) show that the WPP, PeW and PPW are suppressive, and Avon, MB and CW are non-suppressive to take-all.

TABLE 4: Disease rating and plant heights obtained with *Ggt* and different soils added at a rate of 1% to sterilized coarse sand.

Treatment	Disease rating	Plant height (cm)
*L*Ggt* + Avon soil	4.6	13.0
+ MB soil	4.4	13.4
+ WPP soil	2.9	16.6
+ PPW soil	2.0	16.5
+ PeW soil	3.3	15.1
+ CW soil	4.1	14.0
L*Ggt*	5.4	12.9
D*Ggt*	0.0	18.8
Least significant difference P = 0.01	1.42	2.0

*L = Live
D = Dead

III. STUDIES ON SOIL AMOEBAE

A. ISOLATION, MAINTENANCE AND CHARACTERIZATION OF AMOEBAE

The methods of examination and isolation of soil amoebae can be broadly placed into three groups - (i) direct examination; (ii) extraction from soil; and (iii) culture (Heal, 1971). Koffmann (1932) used direct microscopic examination for counting numbers of soil amoebae. Although this seems to be the logical method to overcome selective culture techniques (Bunt and Tchan, 1955), its application is limited mostly to freshwater rhizopods (Leidy, 1879; Page, 1976) as generally microscopical observation of soils does not reveal more than shells of testaceous rhizopods and occasional ciliates (Sandon, 1927). Martin and Lewin (1915) extracted trophic amoebae using picric acid and by bubbling a steam of air through soil suspensions. However, due to the ease of culturing and identifying amoebae on agar plates, soil plating (Sandon, 1927; Page, 1976) and soil dilution cultures (Cutler, 1920; Singh, 1946a) have been widely used.

For isolation of mycophagous amoebae, Old (1977b) deposited spores of *Cochliobolus sativus* on membrane filters, enclosed them in filter packets and buried these in soil. Amoebae from the recovered membranes were cultured with fresh spores of the fungus in water. Anderson and Patrick (1978) baited soil suspensions with conidia of the same fungus and isolated, by pipetting, digestive cysts of mycophagous amoebae that surrounded partially lysed spores. Pussard *et al.* (1979) used neomycin (10-100 $\mu g\ ml^{-1}$) and rifamycine (100 $\mu g\ ml^{-1}$) for selective isolation of the strictly mycophagous amoeba *Thecamoeba granifera* (Greeff, 1866) subspecies *minor* Pussard *et al.*, 1979.

Water agar (Cutler, 1920), nutrient agar and dilute hay infusion agar (Sandon, 1927; Bodenheimer and Reich, 1933), soil extract agar (Dixon, 1937), NaCl-agar (Singh, 1946a), silica gel (Singh, 1946b) and several other media (Page, 1976), enriched with a food bacterium, have been used successfully in culturing soil amoebae. Axenic cultures are difficult to obtain and although pure culture of amoebae has been known since 1898 (Tsujitani, 1898) few amoebae have been grown in axenic culture (Neff, 1957; Griffin, 1973; De Jonckeere, 1977; Surek and Melkonian, 1980). Most soil amoebae are maintained in mono-axenic cultures using bacteria, algae or other protozoa as food (Page, 1976). *Klebsiella pneumoniae* (Schroeter) Trevisan (=*Aerobacter aerogenes* (Kruse) Beijerinck) supports the growth of a wide number of amoebae (Singh, 1946a). Bacteria and small protozoa, which are associated with amoebae in soil and which usually grow in media used for culturing amoebae can also serve as food organisms (Page, 1976); although some accompanying bacteria can have antagonistic effects on amoebae (Groscop and Morgan, 1964).

Amoebae are most abundant in the top 0-10 cm of the soil profile (Crump, 1920; Stout, 1968) and both population and diversity rapidly decline with increasing depth (Sandon, 1927).

Isolation of amoebae

Soil was collected from the top 0-5 cm layer of the WPP plot and kept in sealed plastic bags. Four methods of isolation were used:

(i) The soil plate method

The method consists of incubating small amounts (approx. 0.5 g) of soil on a suitable agar medium enriched with a food bacterium and

examining for amoebae. Initially 1.0% Bacto agar in distilled water spread with a suspension of a *Klebsiella* sp. was used. Plates were incubated at 25°C for 7-14 days and examined using a phase contrast microscope. Only *Acanthamoeba* sp. was present on these plates. This was probably due to the osmotic shock in water agar (Page, 1976) and therefore the agar medium was amended with a salt solution. The modified medium was 1.0% Bacto agar in Prescott and James' solution (Prescott and James, 1955; PJA, Appendix I).

Petri plates poured with this medium were similarly enriched with bacterial suspension and inoculated with a soil crumb (about 0.2 g). On incubation several amoebae, including *Acanthamoeba* sp., *Platyamoeba* sp., *Thecamoeba* sp., and unidentified leptomyxid and vampyrellid amoebae, were observed on these plates. Although smaller species like *Acanthamoeba*, *Platyamoeba* etc. could be seen on the clear agar medium after about 1 week, the larger leptomyxid and vampyrellid amoebae took up to 2 weeks to appear. Similar results were reported by Page (1976), who recommends an incubation of at least 2 weeks and the same was followed throughout this work.

(ii) Isolation from soil suspension

Two grams of soil were suspended in 20 ml sterile modified Neff's amoeba saline (Page, 1967a; AS, Appendix I), shaken and allowed to settle for a few min (3-5 min). Small portions (about 50 µl) of the suspension were plated on PJA with a *Klebsiella* sp. Plates were incubated as previously and when

examined showed *Acanthamoeba* spp., *Echinamoeba* spp., *Gephyramoeba* sp., *Platyamoeba* spp., *Thecamoeba* spp., and the unidentified leptomyxid and vampyrellid amoebae.

(iii) Soil enrichment method

Soil moistened to -8 kPa was dispensed into 15 cm Petri plates to a depth of 7-8 mm. Mycelial mats of *Ggt*, obtained from one week old cultures grown in 0.4% malt extract broth at 25°C were washed twice in sterile distilled water (SDW), placed in autoclaved silk cloth bags (5x5 cm) and buried in the moist soil. After 3 weeks at 25°C, mycelial mats were recovered and homogenised to form a suspension, portions of which were streaked on PJA. *Acanthamoeba* spp., *Echinamoeba* spp., *Gephyramoeba* sp., *Platyamoeba* spp., *Mayorella* spp., *Thecamoeba* spp., and several unidentified amoebae besides the unidentified leptomyxid and vampyrellid amoebae which migrated on to PJA medium were isolated.

(iv) Membrane filter burial method

Five fungi and one bacterium, *Klebsiella* sp., were buried in the WPP and MB soils using the membrane filter burial technique of Old (1977b). Mycelial suspensions of *Ggt*, *Phialophora* sp. and *Rhizoctonia solani* (F91) were obtained by homogenising 0.4% malt extract broth grown cultures of the fungi after washing in SDW. Conidia of *Cochliobolus sativus* were harvested from Czapek-Dox cultures by flooding plates with SDW and lightly rubbing each colony with a sterile loop. Suspensions of conidia were filtered through sterile cheese cloth and washed twice in SDW. Suspensions of *Saccharomyces cerevisiae* and *Klebsiella* sp. were obtained by

washing 2% malt extract agar and TGY cultures respectively with SDW. One ml of suspension of each organism was drawn up with a sterile syringe and deposited on a 12 mm diameter millipore filter (pore size 0.45 µm) held in a 'swinex' membrane holder (Millipore Corpn., Bedford, Massachusetts). The millipore filter was sandwiched between two 25 mm diameter nuclepore filters, pore size 5.0 µm (Nuclepore Corpn., Pleasanton, California), the edges sealed with vacuum grease and buried in the test soils. WPP and MB soils were sieved through a 2 mm sieve, moistened to -8 kPa and -2 kPa respectively and dispensed into 9 cm diameter Petri plates before membrane burial. Petri plates were incubated at 25°C for two weeks. SDW was used as a control.

At recovery, membranes were trimmed to exclude the peripheral areas and each was cut into 4 approx. 1 mm squares using a sterile scalpel. These squares were plated on PJA, incubated at 25°C for two weeks and examined for amoebae. Several amoebae were isolated from these soils: genera like *Acanthamoeba*, *Platyamoeba* and the unidentified vampyrellid amoeba were isolated using almost all the test organisms, whereas, *Echinamoeba*, *Thecamoeba* and the leptomyxids were isolated only using fungi (Table 5).

The previously described mycophagous genera, *Arachnula*, *Theratromyxa* and *Vampyrella* (Old, 1977a; Old and Darbyshire, 1978; Anderson and Patrick, 1978, 1980) were not isolated from these soils using these techniques. Old (1977a) recovered *Arachnula* from a garden soil using the membrane filter burial technique and maintained cultures of the amoeba in SDW with suspensions of *C.sativus* conidia. Membranes recovered from WPP and MB soils were

TABLE 5: Percent recovery of different genera of soil amoebae from two soils using different organisms as baits

Organism	*Acanth-amoeba* spp.	*Echin-amoeba* spp.	Leptomyxid amoeba	*Mayorella* spp.	*Platy-amoeba* spp.	*Thecamoeba* spp.	*T.granifera* ssp. *minor*	Unidentified amoeba	Vampyrellid amoeba
Waite permanent pasture soil:									
Ggt	20	5	0	5	40	0	20	40	5
C.sativus	20	15	10	5	40	0	60	70	0
Phialophora sp,	75	30	15	0	20	5	45	90	30
Rhizoctonia solani (F91)	55	5	10	0	5	0	30	60	45
S.cerevisiae	30	10	0	0	30	5	10	60	30
Klebsiella sp.	35	0	0	0	15	0	0	35	15
Control	20	0	0	0	10	0	0	0	20
Murray Bridge soil:									
Ggt	55	0	0	5	55	0	65	100	90
C.sativus	75	0	0	0	20	5	25	70	70
Phialophora sp.	65	45	0	0	70	0	80	95	75
Rhizocotonia solani (F91)	70	20	0	0	70	5	75	85	50
S.cerevisiae	70	0	0	5	45	10	0	65	80
Klebsiella sp.	45	0	0	0	20	0	0	55	35
Control	10	0	0	0	10	0	0	15	0

therefore placed in SDW and examined with an inverted microscope using phase contrast. Both *Arachnula* and *Theratromyxa* were observed in the suspensions recovered from these soils. So far, attempts to grow these amoebae on agar media have been unsuccessful (K.M.Old, personal communication) and this may be the reason for not obtaining these genera on agar plates from the WPP and MB soil using these techniques.

Purification and Maintenance

Single trophozoites of amoebae from mixed cultures on isolation plates were marked and transferred to fresh PJA + *Klebsiella* plates by cutting out small blocks of agar. Single amoeba cultures were established by repeated transfers of single trophozoites and gradually freed from accompanying soil bacteria by using Neff's (1958) technique as modified by Culbertson (1971). Four to five pieces of filter paper, each soaked with approx. 20 µl of neomycin solution (concentration 3.5 units μl^{-1}) were placed on the periphery of a PJA plate inoculated in the centre with an amoebal culture. Amoebae which migrated to the bacteria-free zone around the filter paper were subcultured on PJA + *Klebsiella* plates. This method is suitable for removal of surface-borne bacteria but does not eliminate endosymbionts (Griffin, 1973), therefore, these cultures can not be referred to as monoaxenic cultures. The mycophagous *Thecamoeba granifera* sub-species *minor* was associated with a *Fusarium* sp. when isolated and was maintained with this fungus without *Klebsiella* sp.

Cultural characters of Amoebae

(i) Growth responses of amoebae to different temperatures

In many protozoa increasing temperature up to about 30°C favours metabolism and reproduction (Reich, 1933). Higher temperatures are adverse with a death point in the range of 35-40°C in some protozoa (Maguire, 1960), but cysts can survive much higher temperatures (Stout, 1955). The effect of temperature on the multiplication rate is different for different amoebae or even strains of the same amoeba (Hawkins and Danielli, 1963), however, most of the work on temperature sensitivity of amoebae has been done with pathogenic species. Temperature tolerance is often used to distinguish between pathogenic and non-pathogenic amoebae (Singh, 1975). Most soil amoebae grow within a range of 18 to 25°C and pathogenic strains need 37°C (Page, 1976), although not all amoebae which grow at 37°C are pathogenic (Griffin, 1972) and some potential pathogens do not grow at this temperature. Non-cystic pathogenic amoebae in tissue specimens are destroyed by ordinary freezing at -20°C (Ensminger and Culbertson, 1966) whereas cysts can survive storage in liquid nitrogen for up to seven years (Neal *et al.*, 1974). Simple relationships between temperature and amoebal activity in soil have not been shown.

Nine different amoebae were tested for their growth responses to temperatures from 5°C to 35°C. Five mm diameter discs from 2-5 weeks old cultures were inoculated to PJA + *Klebsiella* plates. Three replicates of each of the test amoebae were incubated at each test temperature for 10 days. Results on the colony diamters (Table 6) show that most of the amoebae grew

TABLE 6: Growth response of amoebae to different temperatures.

Amoeba	Mean colony diameters (cm) at various temperatures (°C)						
	5	10	15	20	25	30	35
Acanthamoeba polyphaga	0.6	8.2	8.5	8.5	8.5	8.5	0.7
Gephyramoeba sp.	0	0	3.5	5.1	4.2	5.4	0
Unidentified leptomyxid	0	1.8	8.5	8.5	8.5	0	0
Platyamoeba stenopodia	0	2.7	3.7	8.5	8.5	8.5	0
Saccamoeba sp.	0	5.6	7.5	8.5	8.5	0	0
Thecamoeba quadrilineata	0	8.1	8.5	6.0	5.6	0	0
T.granifera ssp. *minor*	0	1.4	8.5	8.5	8.5	8.5	0
Unidentified amoeba (Un.1)	0	8.5	8.5	8.5	8.5	0	0
Unidentified vampyrellid	0	0	0.7	2.9	2.0	1.0	0

well at temperatures between 15°C and 25°C, and only *Acanthamoeba polyphaga* (Puschkarew, 1913) was able to grow at temperatures of 5°C and 35°C. The results are in agreement with those obtained with other soil amoebae (Page, 1976). The majority of the amoebae grew well at 25°C and this has been used as the incubation temperature throughout this work.

(ii) Thermal death points of amoebae

Trophozoite and cyst suspensions of individual amoebae were prepared by washing the surface of 2 weeks old PJA cultures with 5 ml AS. Two ml of these suspensions were placed in small test tubes and partially immersed in a water bath equipped with a rotary shaker (Orbital shaking water bath, Model OW 1412, Paton Industries Ltd., S.A. 5069) which was pre-heated to a constant temperature. Control tubes had 2 ml of AS with a thermometer in them. Treatment time of 10 min was recorded from the time when the AS in the control tubes reached the water bath temperature. About 0.1 ml of cooled (room temp.) suspension was plated on PJA + *Klebsiella* plates. No bacteria were added for *T.granifera* subspecies *minor*. Plates were examined for amoebal growth after two weeks at 25°C.

Acanthamoeba polyphaga and *Echinamoeba* sp. survived temperatures of up to 84°C (Table 7). *Gephyramoeba* sp. was most sensitive and did not survive beyond 40°C. The majority of the amoebae were killed at or below 70°C. Of the cyst-forming amoebae, *Acanthamoeba* and *Echinamoeba* survived very high temperatures, whereas *Gephyramoeba* and the unidentified leptomyxid and the vampyrellid amoebae were killed at relatively lower temperatures.

TABLE 7: Thermal death points of amoebae.

Amoeba	Temperature (^{o}C)
Acanthamoeba polyphaga	84
Echinamoeba sp.	84
Gephyramoeba sp.	40
Platyamoeba stenopodia	70
Saccamoeba sp.	65
Thecamoeba quadrilineata	65
T. granifera ssp. *minor*	65
Unidentified leptomyxid	60
Unidentified vampyrellid	55

Although cysts are generally regarded as more tolerant to high temperatures than vegetative amoebae (Singh, 1946a; Stout, 1955), cysts of all amoebae do not have similar temperature tolerances (Bodenheimer and Reich, 1933; Sopina, 1968).

(iii) Effect of antibiotics on the growth of amoebae

A considerable amount of information is available on the effect of various antibiotics on free-living soil amoebae (Hawkins, 1973). These include both antibacterial and antifungal antibiotics (Loefer, 1951; Loefer and Matney, 1952; Blumberg and Loefer, 1952; Kalinina, 1969). Actinomycin D and other antibiotics produced by the Actinomycetales make these bacteria unsuitable as food for amoebae and also suppress amoebal growth in soil (Geltzer, 1963).

The effects of four concentrations of each of two antibacterial and one antifungal antibiotic on the growth of ten different amoebae were studied using the poison food technique (Bateman, 1933). Stock solutions of each of the three antibiotics, streptomycin, neomycin and actidione were mixed with 100 ml luke warm PJA to obtain final concentrations of 10, 50, 100 and 200 µg ml^{-1} and plates were poured with approx. 20 ml medium. All plates except those to be used for *T.granifera* sub-species *minor*, were enriched with *Klebsiella* sp. Plates were inoculated with a 5 mm diameter disc from 2 weeks old amoebal cultures. Colony diameters after two weeks of incubation at 25°C show that the responses vary with the antibiotic and the amoebal species (Table 8).

TABLE 8: Mean colony diameters (cm) of amoebae on PJA incorporated with different levels of antibiotics.

Amoeba	Control	Streptomycin (μgml^{-1})				Neomycin (μgml^{-1})				Actidione (μgml^{-1})			
		10	50	100	200	10	50	100	200	10	50	100	200
Acanthamoeba polyphaga	8.5	8.5	7.9	6.8	2.6	8.5	8.5	8.5	8.5	8.5	8.5	3.0	-
	+*	+	+	+	-	+	+	+	+	+	+	+	+
Gephyramoeba sp.	2.9	3.0	1.4	0.2	-	1.7	1.2	1.9	1.4	-	-	-	-
	+	+	+	-	-	+	+	+	+	+	+	+	+
Platyamoeba stenopodia	7.4	8.5	6.1	4.1	-	0.1	-	-	-	7.3	6.1	3.5	-
	+	+	+	+	-	+	+	+	+	+	+	+	+
Saccamoeba sp.	8.5	8.5	8.5	7.0	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5
	+	+	+	-	-	+	+	+	-	+	+	+	+
Thecamoeba sphaeronucleolus	8.5	1.5	0.4	3.7	2.6	8.5	0.3	-	-	4.2	0.6	-	-
	+	+	+	+	-	+	+	+	+	+	+	+	+
Thecamoeba quadrilineata	8.5	6.9	4.9	2.8	1.5	8.5	8.5	8.5	7.9	-	-	-	-
	+	+	+	+	-	+	+	+	+	+	+	+	+
T.granifera ssp. *minor*	8.5	8.5	8.5	8.5	1.1	8.5	8.5	8.5	8.5	-	-	-	-
	+	+	+	+	+	+	+	+	+	+	+	-	-
Unidentified amoeba (Un.1)	8.5	8.1	8.4	7.4	5.5	8.5	8.1	8.5	8.5	8.3	8.5	8.5	8.5
	+	+	+	+	-	+	+	+	+	+	+	+	+
Unidentified leptomyxid	7.5	8.1	2.0	1.5	0.7	5.8	7.4	7.8	7.7	-	-	-	-
	+	+	+	-	-	+	+	+	+	+	+	+	+
Unidentified vampyrellid	3.7	1.1	0.6	-	-	1.6	1.0	1.5	0.8	-	-	-	-
	+	+	+	-	-	+	+	+	+	+	+	+	+

*Growth of the food organisms; *Klebsiella* sp. for all amoeba, except for *T.granifera* ssp. *minor* in which case it was *Fusarium* sp.

The growth of the food bacterium was effectively inhibited at 200 μg ml^{-1} of streptomycin and that of the *Fusarium* sp.,used to maintain *T.granifera* sub-species *minor*, at 100 μg ml^{-1} of actidione. *Saccamoeba* and an unidentified amoeba (Un.1) were not affected by any of the antibiotics at the concentrations used, *Platyamoeba stenopodia* (Page, 1969), *Thecamoeba sphaeronucleolus* (Greeff, 1891) and *Acanthamoeba polyphaga* were affected at higher concentrations of some or all the antibiotics. One significant observation was the effect of actidione on the growth of *Thecamoeba quadrilineata* (Carter, 1856), *T.granifera* sub-species *minor*, *Gephyramoeba* and the unidentified leptomyxid and vampyrellid amoebae, which failed to grow even at the lowest concentration of 10 μg ml^{-1} of this antibiotic. With actidione treatments, although the food organisms grew at lower concentrations, 5 out of 10 amoebae failed to grow at any concentration of this antibiotic. Thus, it appears that the effect of actidione at all concentrations and streptomycin and neomycin at higher concentrations had direct effects on the growth of the amoebae. The effectivity of actidione treated soil in limiting protozoan activity is known (Habte and Alexander, 1975).

(iv) Suitability of various brands of agar for the cultivation of amoebae

Two concentrations of each of three different brands of agar, Difco bacto, Davis, Oxoid No.3, and agarose were tested for their suitability to support the growth of seven soil amoebae. Gel strength of 1.0% Difco bacto water agar was used as the standard and concentrations of other agars were varied to match their gel strength with that of the standard. Gel strength was subjectively

tested by cutting uniform thicknesses of each agar with a scalpel after they had solidified. Water agar plates were inoculated in the centre with a 5 mm diameter disc taken from a stock culture of one of the test amoebae after a uniform suspension of *Klebsiella* sp. had been spread on each plate. Colony diameters were measured after an incubation of 2 weeks at 25°C. Difco bacto and Oxoid no.3 proved to be better than Davis agar (Table 9). Agarose was the least effective and *Gephyramoeba* did not grow on either concentration.

The difficulties in obtaining axenic cultures of free-living amoebae is mainly due to the lack of knowledge on their nutritional requirements (Griffin, 1973). Reich (1948) found that the respiratory rate of fed *Mayorella palestinensis* Reich, 1933 is greater than that of starved cells and much greater than that of the cysts. The respiratory rate was also slightly higher in a nutrient solution than in a balanced salt solution and the amoeba could metabolise added peptone. Nutrient toxicity, however, is more common and amoebae generally do not grow well on high nutrient media which support the growth of many inedible and toxin producing bacteria (Singh, 1948). Ionic strength of a medium has a profound effect on the growth and reproduction of amoebae (Griffin, 1973). *Naegleria gruberi* (Schardinger, 1899) moves four times faster in 20 mM KCl than in deionised water and non-electrolytes do not affect locomotion (King *et al.*, 1979). Elevated osmotic pressure, Ca^{2+}, Mg^{2+} and a decrease in carbon source on the other hand, causes encystation in *Hartmannella rhysodes* Singh, 1952 (Band, 1963). A weak saline solution favours the growth of many soil amoebae (Singh, 1955; Page, 1976). Of the four brands of agar

TABLE 9: Growth of amoebae on various water agars.

Amoeba	Colony diameter (cm)							
	Agarose		Difco bacto		Davies		Oxoid no.3	
	0.5%	0.7%	0.8%	1.0%	0.8%	1.0%	1.0%	1.2%
Gephyramoeba sp.	0	0	4.0	3.0	2.5	3.1	5.3	2.9
Platyamoeba stenopodia	2.6	3.2	4.0	4.6	5.3	4.1	2.7	4.1
Thecamoeba quadrilineata	1.2	0.9	6.8	6.0	4.2	4.8	3.9	6.1
T.granifera ssp. *minor*	3.5	4.2	2.6	6.3	6.7	8.2	8.5	8.5
Unidentified amoeba (Un.1)	4.9	3.9	8.5	8.5	8.5	8.5	8.5	-*
Unidentified leptomyxid	4.1	4.4	7.1	8.5	6.0	6.0	7.2	7.1
Unidentified vampyrellid	1.7	0.8	2.0	1.3	0.6	0.6	2.4	0.7

*Not tested

tested, Difco bacto, which proved better in supporting the growth of the amoebae tested, had a higher concentration of Na and K than the other brands tested (J.H.Warcup, unpublished data).

B. DESCRIPTION AND IDENTIFICATION OF AMOEBAE ISOLATED FROM THE WAITE PERMANENT PASTURE

Dangeard (1900) considered that nothing is more difficult than to identify an amoeba. Similar opinions have been voiced before and since (Page, 1967a). The difficulty was attributed to the nature of the organisms (Gruber, 1885) and inadequate descriptions (Arndt, 1924; Jepps, 1956). Both Penard (1902) and Schaeffer (1926) held that amoebae do have characteristics making identification possible. Bovee and Jahn (1973) listed more than 40 characters, including biochemical and physiological ones, used by various workers to distinguish between amoebal groups. They considered that no genus based solely on morphology is adequate. However, most work on amoebal identification, including the more recent (Bovee, 1972; Page, 1972,1976,1977; Pussard and Pons, 1977; Page and Blakey, 1979), is based mainly on the morphological characters. Two distinct trends have occurred with respect to the criteria used: structure of pseudopods and method of locomotion, and patterns of nuclear division (Singh, 1952; Bovee, 1953; Page, 1967a; Pussard, 1973).

I have attempted identification of isolates using trophozoite and cyst morphology, locomotive behaviour and cytological structures. Nuclear division and amoebo-flagellate transformation were not studied.

Methods

The hanging drop technique, Cruickshank culture chambers (Sterilin Ltd., Teddington, Middlesex, England) and agar cultures were used to examine live trophozoites by phase contrast microscopy. Occasionally agar plates were also examined by bright field illumination by adjusting the condenser.

For preparing hanging drops, PJA cultures were flooded with AS and suspensions of trophozoites were drawn into sterile pasteur pipettes. Drops of suspensions were placed on cavity slides and vacuum grease applied to seal the coverslip placed on the cavity. Slides were left inverted for up to 6 h before examination. Measurements, except in most cases those of nuclei, were made on live trophozoites.

For studies of the nuclei, the orcein staining method for nematodes (Triantaphyllou, 1975) was used. Trophozoite suspensions deposited on glass slides were incubated in Petri dish moist chambers overnight at 25°C to allow better adhesion and production of well spread forms of trophozoites. Plates were flooded with 2.5% glutaraldehyde in AS and the amoebae fixed at 25°C for 6 h to overnight. Amoebae were stained with 1% aceto-orcein for 25-30 min, destained with 45% acetic acid for 3-5 sec, mounted in 45% acetic acid using a coverslip and sealed with nail polish.

The amoebae studied

Acanthamoeba polyphaga (Puschkarew, 1913)

Trophozoites are more or less elongated to triangular in shape with a broad hyaline ectoplasmic zone at the advancing margin (Fig.3a). Numerous slender and tapering pseudopodia, often with a pointed tip arise from the ectoplasmic zone (Fig.3a). Acanthopodia, as these are commonly called, are also present in the posterior part of the body. Often bright field observation provides a better outline of the amoeba with the acanthopodia. One to many contractile vacoules are prominent within the dense cytoplasm (Fig.3b). Trophozoites measure 10-25 µm in length and 6-15 µm in breadth. Rate of locomotion is of the order of

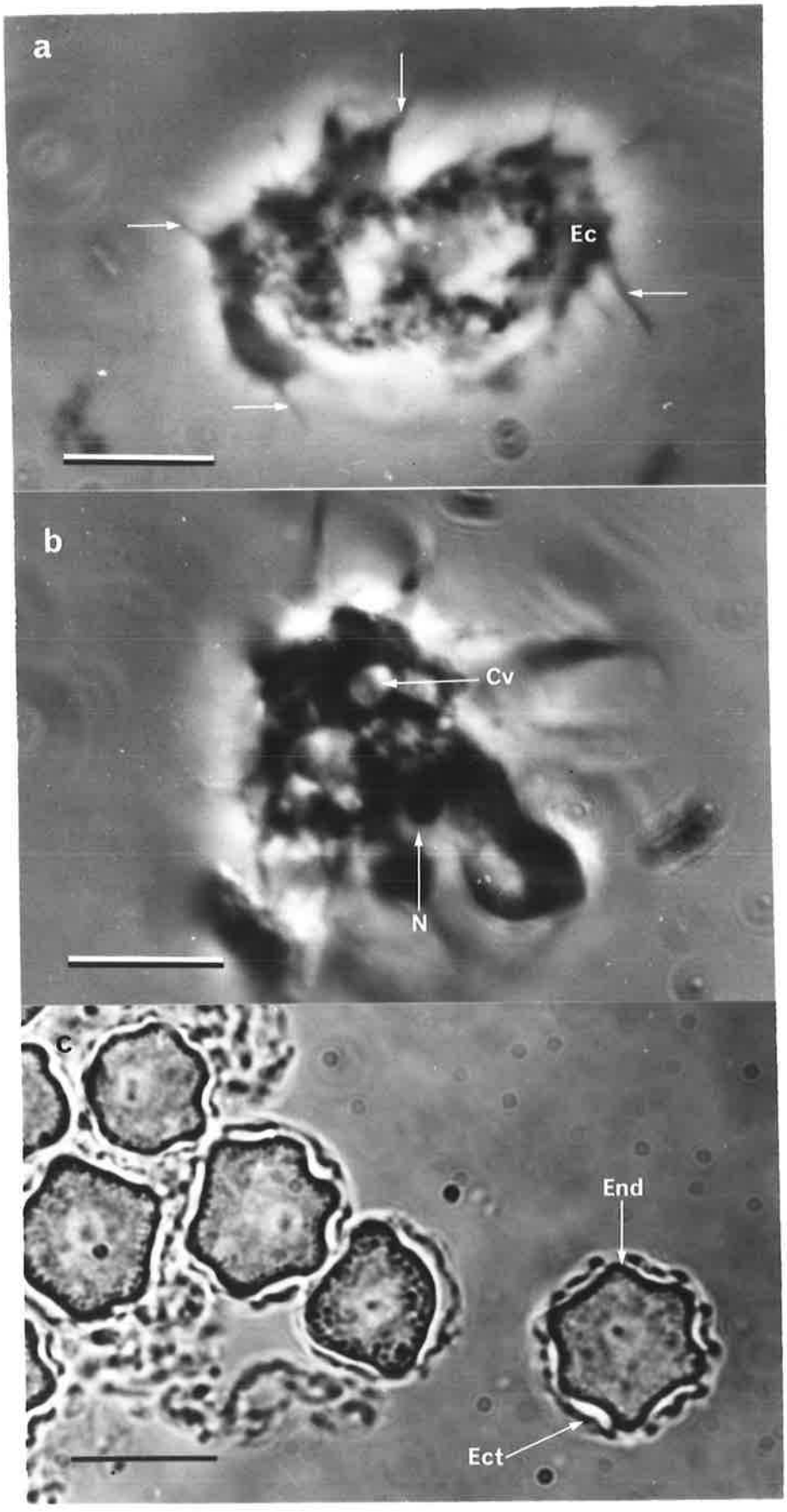
Fig.3: *Acanthamoeba polyphaga*.

a. A live trophozoite showing the hyaline ectoplasm (Ec) and acanthopodia (arrows).

b. Glutaraldehyde-fixed trophozoite after orcein staining showing a contractile vacuole (Cv) and the nucleus (N).

c. Cysts showing endocyst (End) and ectocyst (Ect).

(Scale bar = 10 µm for all Figs.)


a
Ec
b
Cv
N
c
End
Ect

0.33 - 0.9 µm sec^{-1} and is usually unidirectional. Trophozoites are uninucleate and nuclei can be observed in phase contrast. Stained nuclei (Fig.3b) are spherical to elongate, measure between 2-6 µm in diameter and contain one darkly stained nucleolus.

Cysts are rectangular to polygonal in shape with two distinctly visible walls (Fig.3c). The thick endocyst often appears as 4-6 radiating rays. The ectocyst is thin walled, often wrinkled and loosely applied and usually does not conform to the shape of the endocyst (Fig.3c). Cyst diameters range from 11 - 18 µm.

The genus *Acanthamoeba* Volkonsky, 1931 is well characterised, specially by the cyst which forms the basis for species differentiation (Page, 1975,1976; Pussard and Pons, 1977). According to the trophozite and cyst morphology, this amoeba resembles *Acanthamoeba polyphaga* (Page, 1976).

Echinamoeba sp.

Active trophozoites are somewhat triangular or elongate with a distinct anterior hyaline ectoplasmic zone. Several fine long and slender pseudopodia, often with a pointed tip, and commonly known as filopodia are produced from the ectoplasm (Fig.4a). The filopodia are generally longer and more slender than the acanthopodia typical of the genus *Acanthamoeba*. The flattened nature of the trophozoites is evident in bright field (Fig.4b). Their dimensions, including filopods range from 10 - 45 µm in length and 5-35 µm in breadth. Generally 1-5 contractile vacuoles are seen in the finely granular endoplasm. Trophozoites are uninucleate and the nucleus measures 2-4.5 µm in diameter with a darkly stained nucleolus 0.6 - 1.5 µm in diameter. Cysts are

Fig.4: *Echinamoeba* sp.

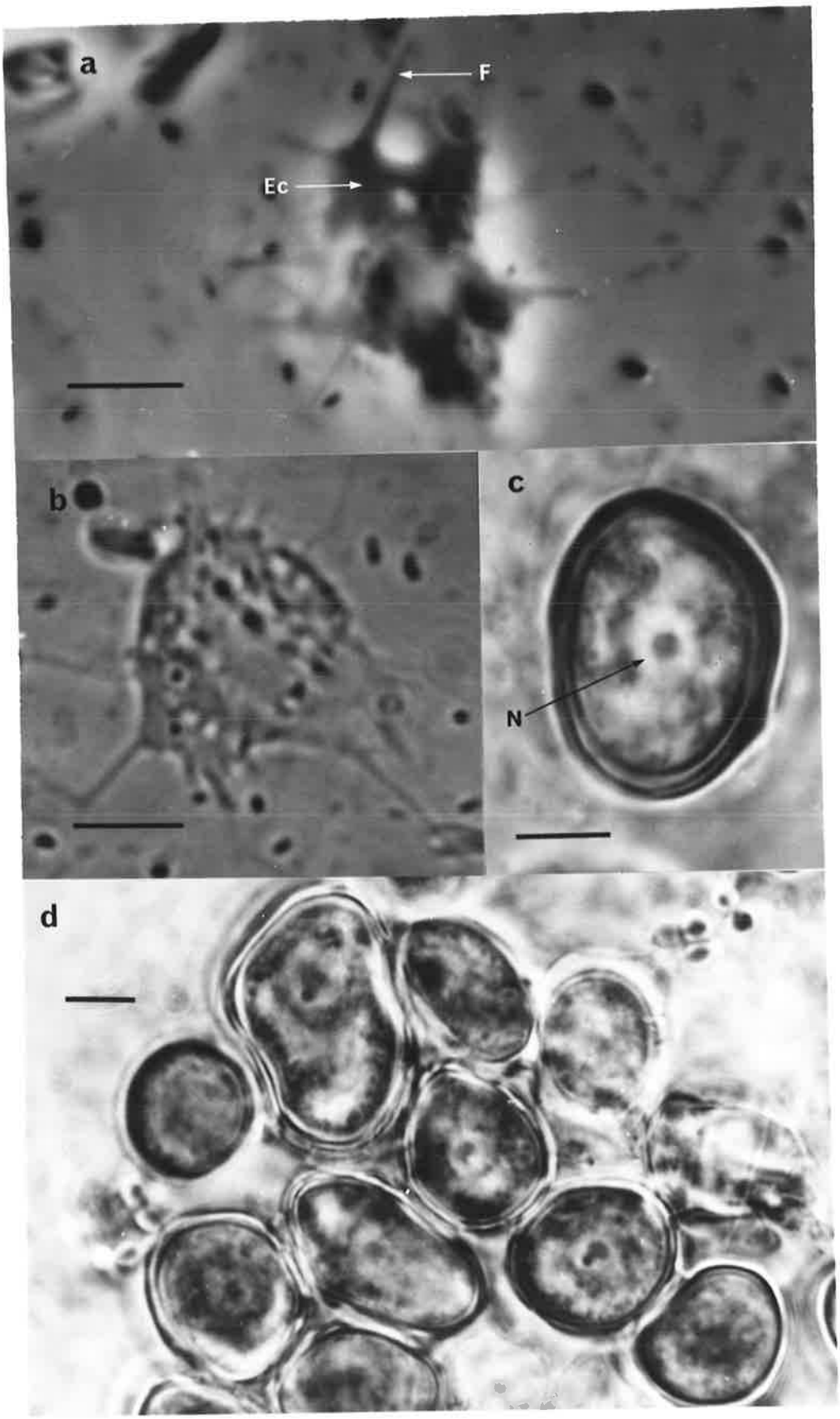
a. Live trophozoite showing the ectoplasm (Ec) and filopodia (F).

b. Live trophozoite in bright-field.

c. Cyst showing the single nucleus (N).

d. Cysts showing a range of commonly observed shapes and sizes.

(Scale bar = 5 µm in all Figs.)


a
F
Ec
b
c
N
d

generally oval, elongate or obpyriform (Fig.4d) and measure between 10-30 µm in diameter/length. They have a thick wall apparently consisting of three layers (Fig.4c). The prominent single cyst nucleus can be easily seen in wet mounts (Fig.4c).

In 1975, Page created a new family 'Echinamoebidae' to accommodate small solitary non-parasitic amoebae with fine, non-anastomosing pseudopodia. He included two of his newly described genera *Echinamoeba* and *Stachyamoeba* along with *Filamoeba* Page, 1967 in this family; differentiating them mainly on the basis of their filopod and trophozoite dimensions. While the cysts of *Stachyamoeba* are distinct from those of the other two genera, *Echinamoeba* and *Filamoeba* have some overlapping cystic and trophozoite characters. Trophozoites of my isolate match *Filamoeba nolandi* Page, 1967 (Page, 1967b) in their size, however filopodial and cystic characters are more like those of *Echinamoeba* and I have tentatively placed it in the latter genus. Two species of *Echinamoeba*, *E.exudans* Page, 1975 (=*Hartmannella exudans*, Page 1967) and *E.silvestris* Page, 1975, are known (Page, 1975) but my isolate does not entirely fit the description of either.

Gephyramoeba sp.

The trophozoites are more or less filamentous and branched and vary widely in their size due to anastomosis between individuals. In liquid medium trophozoites may extend over a wide area, up to 700 sq. µm, and branch profusely, with the branches generally of uniform width to give a ribbon-like appearance (Fig.5a). On agar, branching is limited and tends to be dichotomous (Fig.5b). A limax form has not been observed for this amoeba. The size of a trophozoite varies depending on the

Fig.5: *Gephyramoeba* sp.

a. Trophozoite in liquid medium showing its ribbon-like appearance.

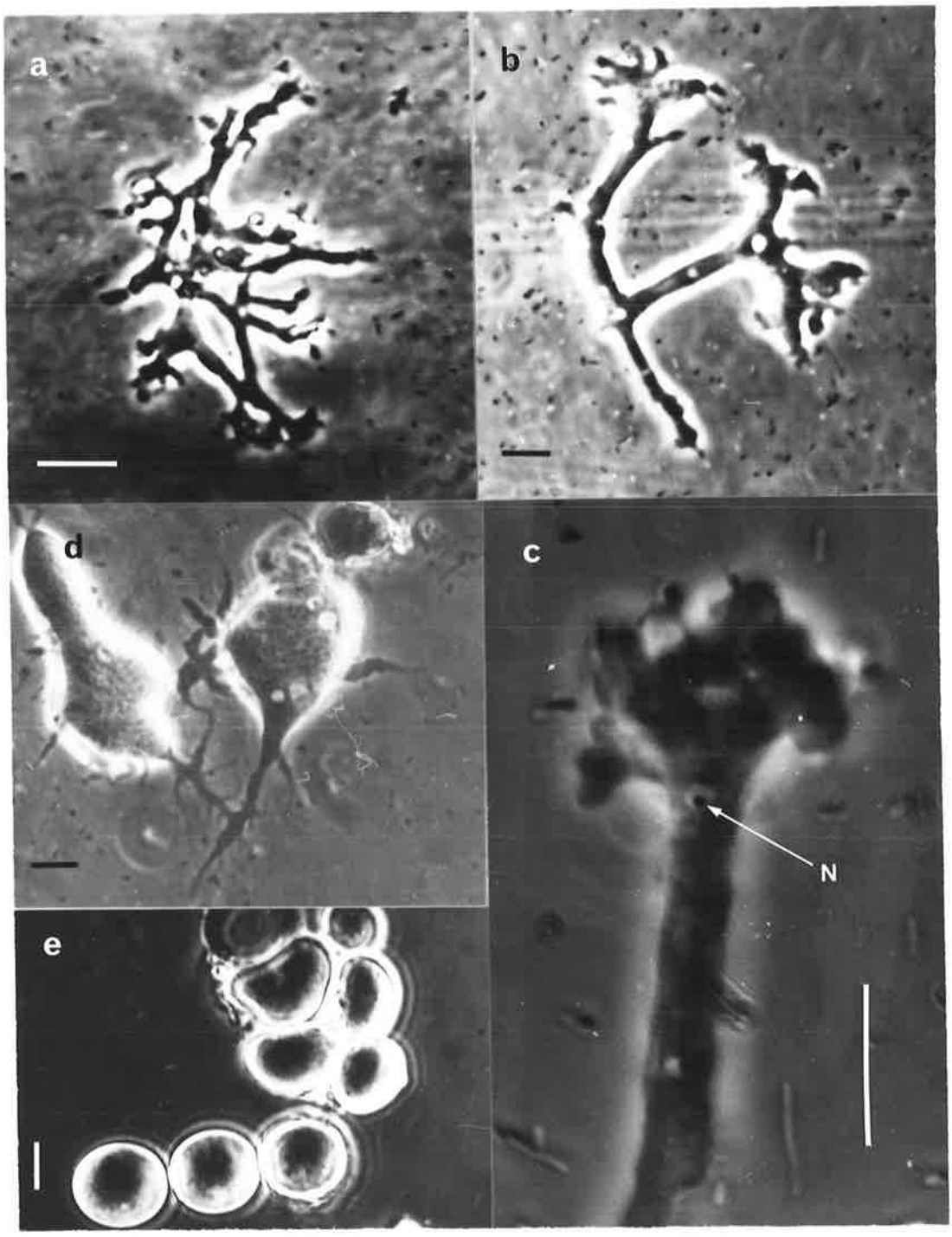
b. Dichotomously branched trophozoite on agar.

c. Part of a trophozoite showing an orcein-stained nucleus (N).

d. Trophozoites withdrawing pseudopodia to round-up as a result of limited oxygen supply.

e. Cysts.

(Scale bar = 10 µm in all Figs.)


a
b
d
c
N
e

number of anastomoses it has undergone. A range of 80 - 750 µm in length, and 50 - 130 µm in breadth has been observed in liquid medium.

In phase contrast, the cytoplasm of live amoebae appears to be devoid of any prominent organelles. The distinction between the hyaline ectoplasm and the finely granular endoplasm is only visible at high magnification. Pulsating vacuoles, 1 - 30 or more per trophozoite, with a diameter of approx. 1.5 - 17 µm, are the only cytoplasmic structures visible in live trophozoites. Nuclei can only be observed in stained specimens (Fig.5c). Young trophozoites formed by the excystment of uninucleate cysts are uninucleate but soon develop into multinucleated individuals by anastomosis. The number of nuclei in a trophozoite varied from 1 - 25 or more. The nucleus is spherical to oval in shape, with a diameter of 1.6 - 4.1 µm and contains one darkly stained nucleolus which varies from 0.8 - 2.5 µm in diameter (Fig.5c). The tips of pseudopodial branches often have a cluster of lobose pseudopodia (Fig.5c). The amoeba has a very slow rate of movement. Trophozoites in and around the centre of Cruickshank chambers often cease locomotion, withdraw pseudopodia, gradually round up (Fig.5d) and finally die, presumably due to the lack of oxygen. A similar pathological process has been observed in *Gephyramoeba delicatula* Goodey, 1915 (Pussard and Pons, 1976c).

The amoeba forms thin-walled spherical, oval or almost oblong cysts (Fig.5e), 12.8 - 37 µm in diameter, which resemble those formed by many other amoebae. Cysts are generally uninucleate. Of 100 cysts examined, only 6 had more than 1 nucleus (up to 4 in number). The cyst nucleus is specially prominent before germination.

Based on trophozoite and cyst morphology, this amoeba appears to belong to the family Gephyramoebidae (Pussard and Pons, 1976a), and it has been tentatively placed in the genus *Gephyramoeba* Goodey, 1915. The amoeba is similar to *G.delicatula* Goodey, 1915 in appearance as described by Pussard and Pons (1976c) but differs in being multinucleate and anastomosing.

Unidentified leptomyxid amoeba

The amoebal trophozoite exists in two distinct forms, a branched flattened form (Figs.6a,b,c) ranging from 80 µm to 150 µm in length and 28 µm to 95 µm in breadth; and a limax form (Fig.6b). The change between the two forms can be readily observed (Fig.6d). The limax form is common in aqueous media which are disturbed and can be found at the interface between medium and air. Amoebae in non-disturbed cultures tend to settle to the bottom of the dish and produce the branched form. The latter form is also common on agar. Limax forms are narrower at the posterior end than at the anterior one and measure from 50 µm to 180 µm in length and 20 µm to 40 µm in width. Both forms are uninucleate (Figs.6a,c,d), the nucleus measures 6 µm - 10 µm in diameter and contains an elongated nucleolus (3 µm - 5 µm). The protoplast is distinctly divided into a hyaline ectoplasm and a granular, compact endoplasm. The branched form contains one to many vacuoles up to 16 µm in diameter. The limax form normally has a single large vacuole at the posterior end of the cell.

From the advancing margin of the cell small eruptive pseudopodia of the type lobosa are formed by the ectoplasm into which the granular endoplasm flows. As the trophozoite advances the posterior margin forms

Fig.6: The unidentified leptomyxid amoeba.

a. Large flattened form of a trophozoite showing the anterior lobopodia (L), contractile vacuole (Cv) and single nucleus (N).

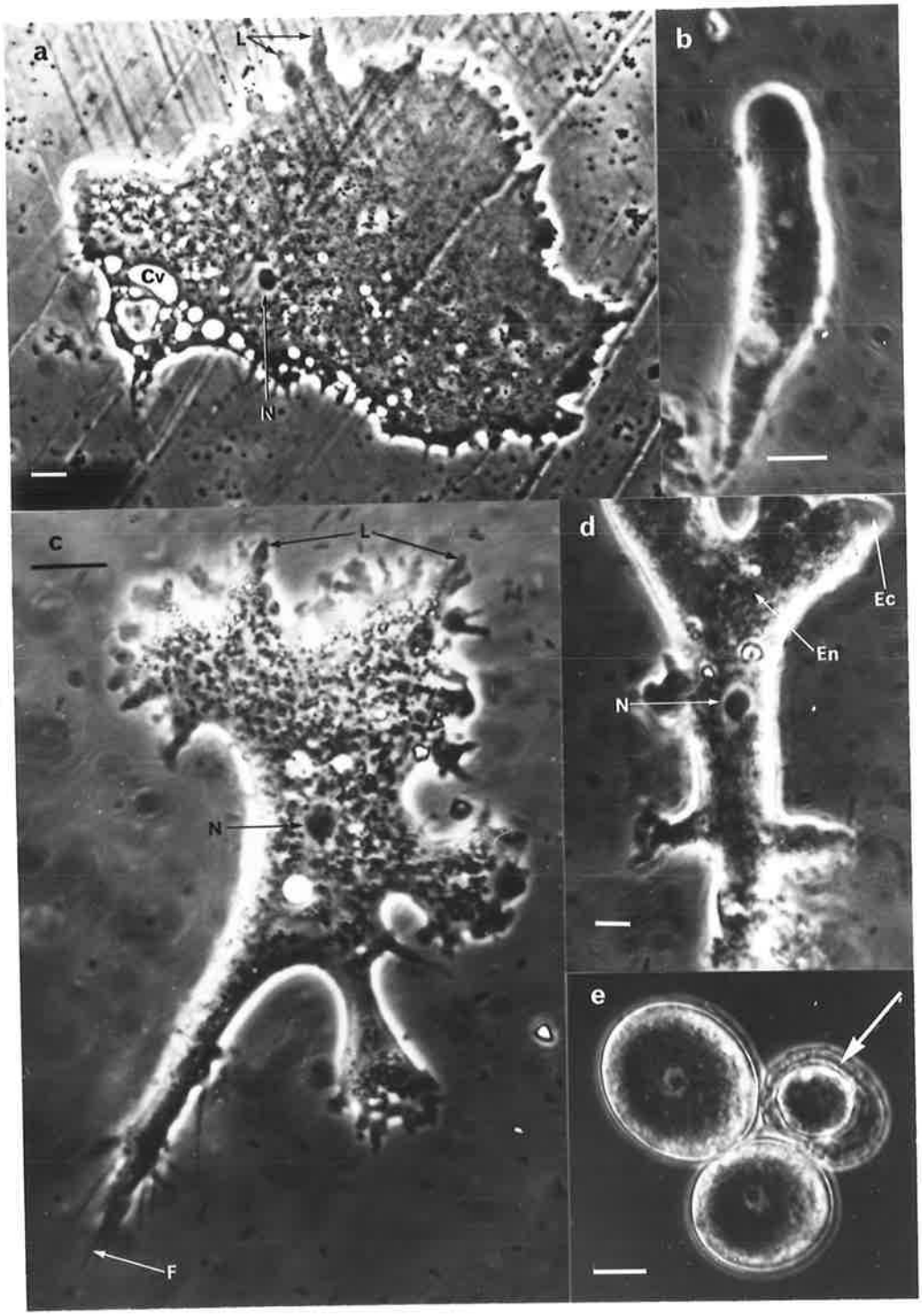
b. Tubular limax form.

c. Trophozoite showing anterier lobopodia (L), posterior filopodia (F), nucleus (N) and the typical branched morphology.

d. Form intermediate between branched and limax showing the single nucleus (N), hyaline ectoplasm (Ec) and granular endoplasm (En).

e. Cysts and endocyst (arrow).

(Scale bar = 10 µm in all Figs.)


a
L
Cv
N
b
c
L
N
F
d
Ec
En
G
N
e

a variable number of fine filopods (Fig.6c). The rate of locomotion can be as rapid as 5 µm sec^{-1} in branched forms. Pseudopods can be formed in a number of directions simultaneously, but ultimately flow in one direction. Similar filopods appear when the lobose pseudopod is retracted. True polyaxial locomotion has not been observed. Anastomosis between trophozoites has not been observed so far even though crowding is common on agar. During cell division the trophozoite extends linearly with pseudopods occurring at both ends. Two nuclei can be clearly seen at this stage. The central portion of the protoplast becomes constricted and finally two daughter cells separate. The generation time on PJA + *Klebsiella* sp. as food varies from 3-5 h.

The amoeba forms cysts which are usually spherical in shape (Fig.6e), but can vary if individuals are crowded or cysts form within the confines of lysed fungal cells. Cysts are smooth walled and measure 15-30 µm in diameter. The single nucleus can be seen very clearly just prior to germination. Rarely up to three nuclei have been seen at this stage. A single trophozoite invariably emerges from the cyst. Excystment is stimulated by placing the cysts into AS or on PJA + *Klebsiella* medium. In older cultures, a second type of cyst which is apparently smaller in size and formed inside an existing cyst wall, has been observed (Fig.6e). Similar 'endocysts' have been observed in other leptomyxid amoebae before (Pussard and Pons, 1976a, 1976b).

The order Leptomyxida includes amoebae which form thin spreading reticulate trophozoites, have polyaxial locomotion and produce lobose pseudopodia (Pussard and Pons, 1976a). The order contains two families, Leptomyxidae and Gephyramoebidae, distinguished on the basis of multinucleate trophozoites in the former and uninucleate trophozoites in the

latter. *Leptomyxa flabellata* Goodey, 1915 was retained within the same genus as *L.reticulata* Goodey, 1915, the type species (Pussard and Pons, 1976b), despite significant differences in trophozoite morphology. In *L.flabellata*, trophozoites are branched rather than reticulate and locomotion is not polyaxial. Another significant characteristic is the production of limax forms when growing in a water film. In all these characters this amoeba closely resembles *L.flabellata*. The principal difference appears to be in the number of nuclei. *L.flabellata* is reported to have 1-48 nuclei per trophozoite. Up to 3 nuclei were seen in cysts of this amoeba. As nuclear number is the basis for separating the families Leptomyxidae and Gephyramoebidae the amceba can not be attributed to *L.flabellata* and it has been placed under the order Leptomyxida as an unidentified amoeba of the order.

Mayorella sp.

Trophozoites are more or less elongated and flattened. In live trophozoites the single nucleus, 4-8 µm in diameter appears as the most prominent structure and is more or less spherical in shape with a rounded nucleolus measuring 2.5 - 5.5 µm in diameter (Fig.7a). The number of large contractile vacuoles, 2 - 11 µm in diameter may vary from 1 - 20 or more in one trophozoite and their abundance often gives a reticulate appearance to the trophozoite. Coarsely granular endoplasm covers the major part of a trophozoite and the hyaline ectoplasmic region is present in the pseudopodial projections and also appears as a fine film around the cell. From the hyaline ectoplasmic zone appear a number of pseudopodia in almost all directions. Pseudopodia are usually conical mamilliform but can be digitiform and sometimes quite extended with rounded end (Fig.7a). Sometimes trophozoites constantly change their

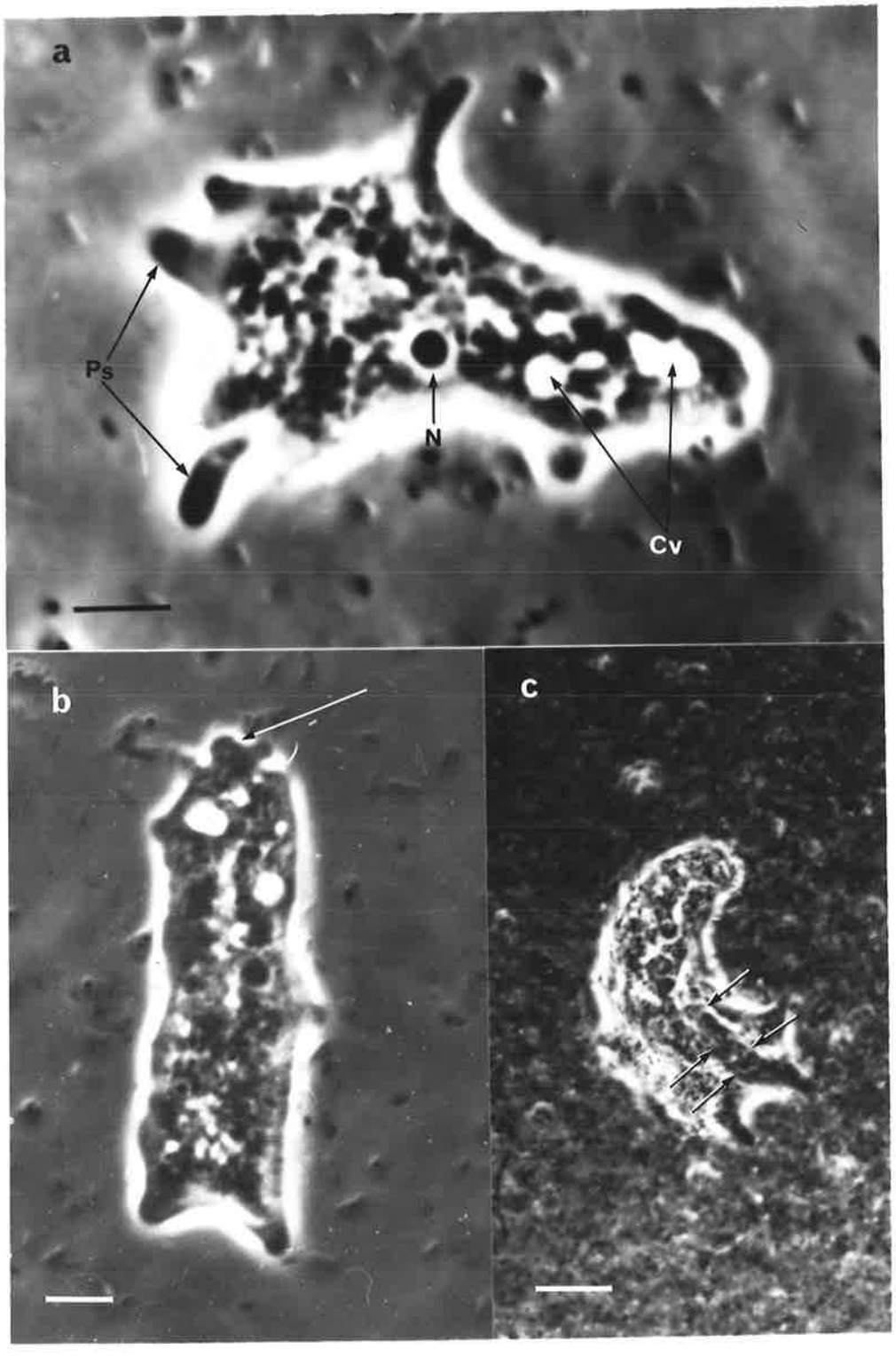
Fig.7: *Mayorella* sp.

a. Trophozoite with its prominent nucleus (N), digitiform pseudopodia (Ps) and contractile vacuoles (Cv).

b. Trophozoite showing the irregularly bulbous uroid (arrow).

c. Trophozoite showing parallel streaks (arrows) along its length.

(Scale bar = 10 µm in all Figs.)


a
Ps
N
Cv
b
c

direction of movement, while at other times a more or less unidirectional movement may continue for a longer period. Rapid unidirectional locomotion is common in many limax amoebae and although this amoeba does not have a limax form, the trophozoites move as fast as 0.5 - 1.8 µm sec^{-1}. Under continuous locomotion, the posterior end (uroid) appears irregularly bulbous (Fig.7b). Often the ridges formed on mamilliform pseduopodia extend beyond the pseudopodial length and can be seen as more or less parallel streaks along the entire length of the trophozoite (Fig.7c). Trophozoites measure from 50 - 130 µm in length and 20 - 100 µm in breadth. No cysts have been observed in culture.

Trophozoites of this amoeba resemble *Mayorella penardi* Page, 1972 (Page, 1976) except for the morphology of floating forms. Floating forms of *M.penardi* are irregularly rounded but those observed here of this amoeba have radiating slender pseudopodia.

Platyamoeba stenopodia Page, 1969

Trophozoites are generally elongated with a truncate anterior end (Figs.8a,b,d). The anterior portion covering half or more of the trophozoite's length is hyaline and appears flat whereas, the posterior half is granular and somewhat thicker (Fig.8d). Usually trophozoites are broader at the anterior half than at the posterior (Fig.8a). Floating forms commonly have blunt radiating pseudopodia which may be larger than the diameter of the central body and almost always have rounded ends (Fig.8c). The single nucleus and generally one contractile vacuole are the prominent features of the coarsely granular endoplasm. When stained, the nucleus appears oval, elongate or spherical in shape and measures between 1.4 - 2.5 µm in diameter with a nucleolus 0.9 to

Fig.8: *Platyamoeba stenopodia*.

a. Trophozoite showing the broad anterior ectoplasm (Ec) and a contractile vacuole (Cv).

b. Trophozoites. Note the granular endoplasm (En) and the truncate anterior (arrow).

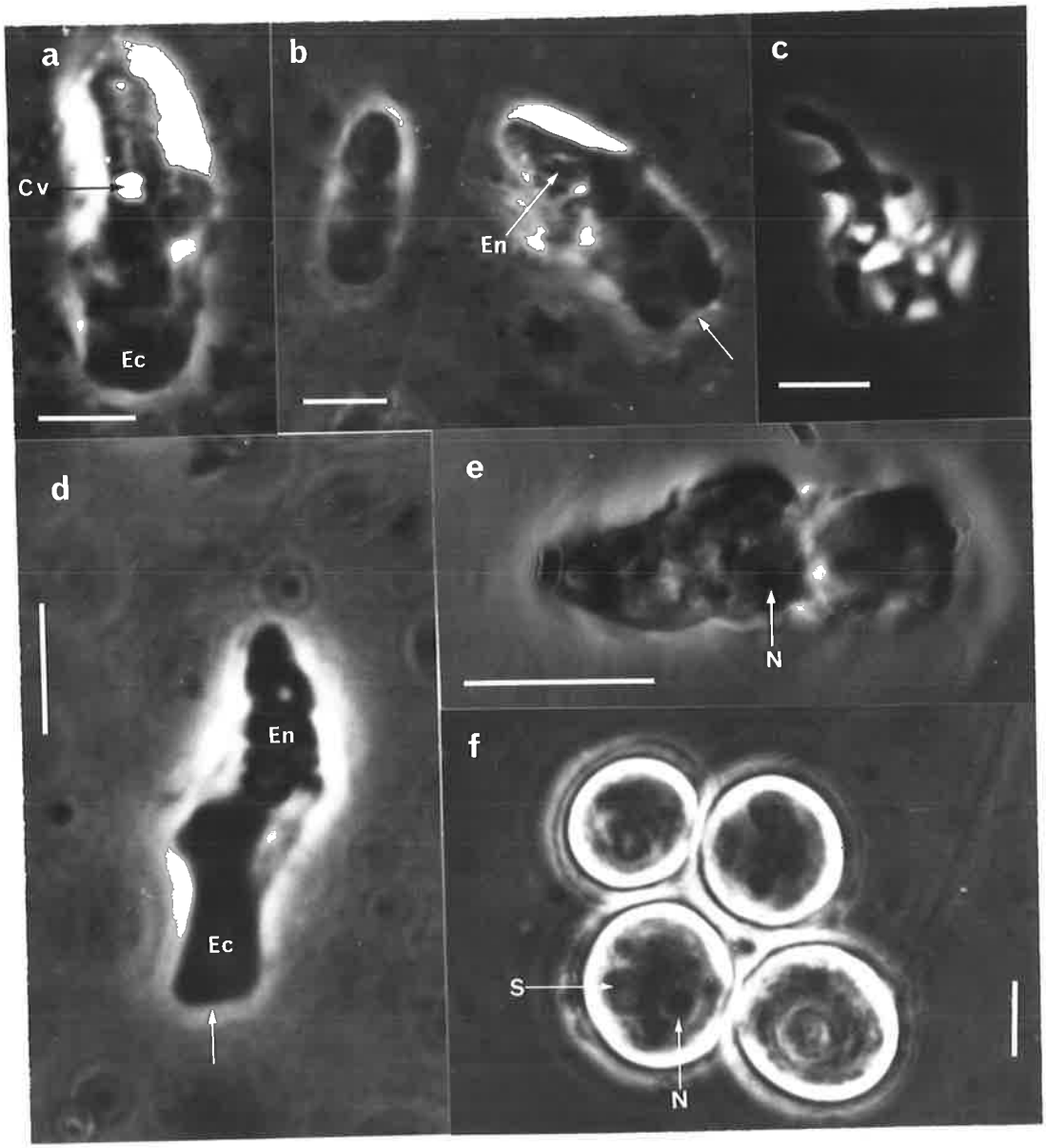
c. Floating form .

d. A typical elongated trophozoite in locomotion. Note the ratio of ecto- (Ec) and endoplasm (En) and the truncate anterior (arrow).

e. Orcein-stained nucleus (N).

f. Uninucleate cysts. Note the dense spherical body (S) besides the nucleus (N).

(Scale bar = 10 µm in all Figs.)


a
Cv
Ec
b
En
c
d
En
Ec
e
N
f
S
N

1.6 µm in diameter (Fig.8e). Trophozoites under continuous locomotion are elongated with the length breadth ratio often reaching 3:1 and have 2-3 folds approximately parallel to the longitudinal axis (Fig.8d). The rate of locomotion is between 0.2 - 0.4 µm sec^{-1}. Trophozoites range from 6 - 30 µm in length and 5 - 20 µm in breadth.

The amoeba forms spherical to oval cysts, 4-12 µm in diameter. Cysts are uninucleate and contain a dense spherical body apart from the nucleus (Fig.8f). Similar dense bodies have been reported in cysts of *Platyamoeba* spp. (Page, 1976).

Trophozoite and cyst morphology of this amoeba matches the description of *Platyamoeba stenopodia* Page, 1969, a freshwater and leaf litter species described by Page (1969).

Platyamoeba sp.

Locomotive forms are ovoid, ellipsoid, flabellate or triangular in shape with a rounded or flat anterior end (Figs.9a,b). Movement is more gliding than pseudopodial, although occasional short pseudopodia may be noticed. Rate of locomotion varies between 0.3 - 0.8 µm sec^{-1}. This is a small amoeba and cytological details are only visible at higher magnifications, although the distinction between the ecto- and endoplasm can be readily observed at lower magnifications. The hyaline ectoplasm extends as a broad fan shaped area at the anterior and often appears wrinkled or wavy (Fig.9b). The nucleus and usually one contractile vacuole (1.5 - 10 µm in diameter) are the prominent features of the finely granular endoplasm. Stained nuclei (Fig.9c) measure between 2.0 - 5.0 µm in diameter and nucleoli from 1.2 - 3.0 µm in diameter. Trophozoite dimensions: 15-90 µm in length and 8-55 µm in breadth.

Fig.9: *Platyamoeba* sp.

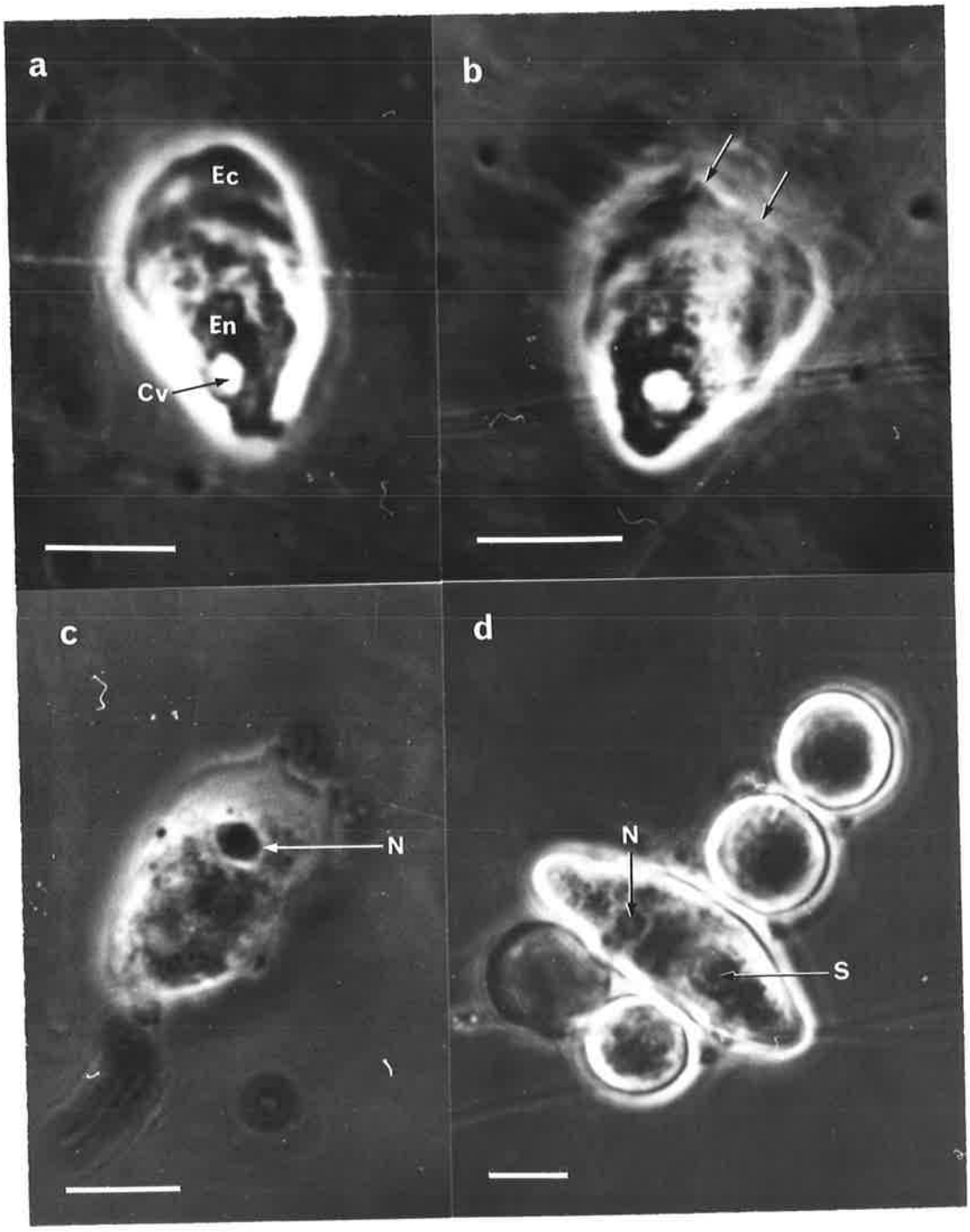
a. Trophozoite in locomotion showing ectoplasm (Ec), granular endoplasm (En) and contractile vacuole (Cv).

b. Trophozoite with wavy anterior end (arrows).

c. Nucleus (N) in a stained amoeba.

d. The spherical and elongated cysts. Note the dense spherical body (S) apart from the single nucleus (N).

(Scale bar = 10 µm in all Figs.)


a
Ec
En
Cv
b
c
N
d
N
S

Cysts of two distinctly different shapes have been consistently observed in single trophozoite cultures under similar conditions. More numerous are the spherical to oval cysts (Fig.9d) which measure between 7.5 and 20 µm in diameter. The other type is considerably larger and elongated in shape (Fig.9d) and has a maximum diameter around the middle and tapers towards the smooth and rounded ends. Their dimensions vary from 18-35 µm in length and 9-15 µm in breadth. Both types are uninucleate, have a smooth thin wall and contain a dense spherical structure apart from the nucleus (Fig.9d).

Trophozoites of this amoeba closely resemble those of the genus *Platyamoeba* Page, 1969 (Page, 1969). They also have resemblances with Schaeffer's *Rugipes bilzi* Schaeffer, 1926 as redefined by Sawyer (1975) as *Clydonella vivax* (Schaeffer, 1926) Sawyer, 1975. However, *C.vivax* is a much smaller amoeba, known only from marine habitats and does not form cysts. On the other hand, my isolate does not resemble any known *Platyamoeba* in cyst morphology and hence has been placed in this genus as an unidentified species.

Saccamoeba sp.

Trophozoites of this amoeba are long, slender and broader at the anterior than at the posterior end (Figs.10a,d). Occasionally it produces lobose pseudopodia both in liquid and agar cultures (Figs.10b,c). On agar however, trophozoites commonly appear as a rounded mass varying in shape and containing a number of refringent granules (Fig.10c); they either form small lobose pseudopodia or assume a limax form to continue locomotion. Trophozoites often penetrate into the agar medium. In a newly formed pseudopod (Fig.10c) and at the advancing margin of a

Fig.10: *Saccamoeba* sp.

a. Trophozoite with the ectoplasmic cap (C).

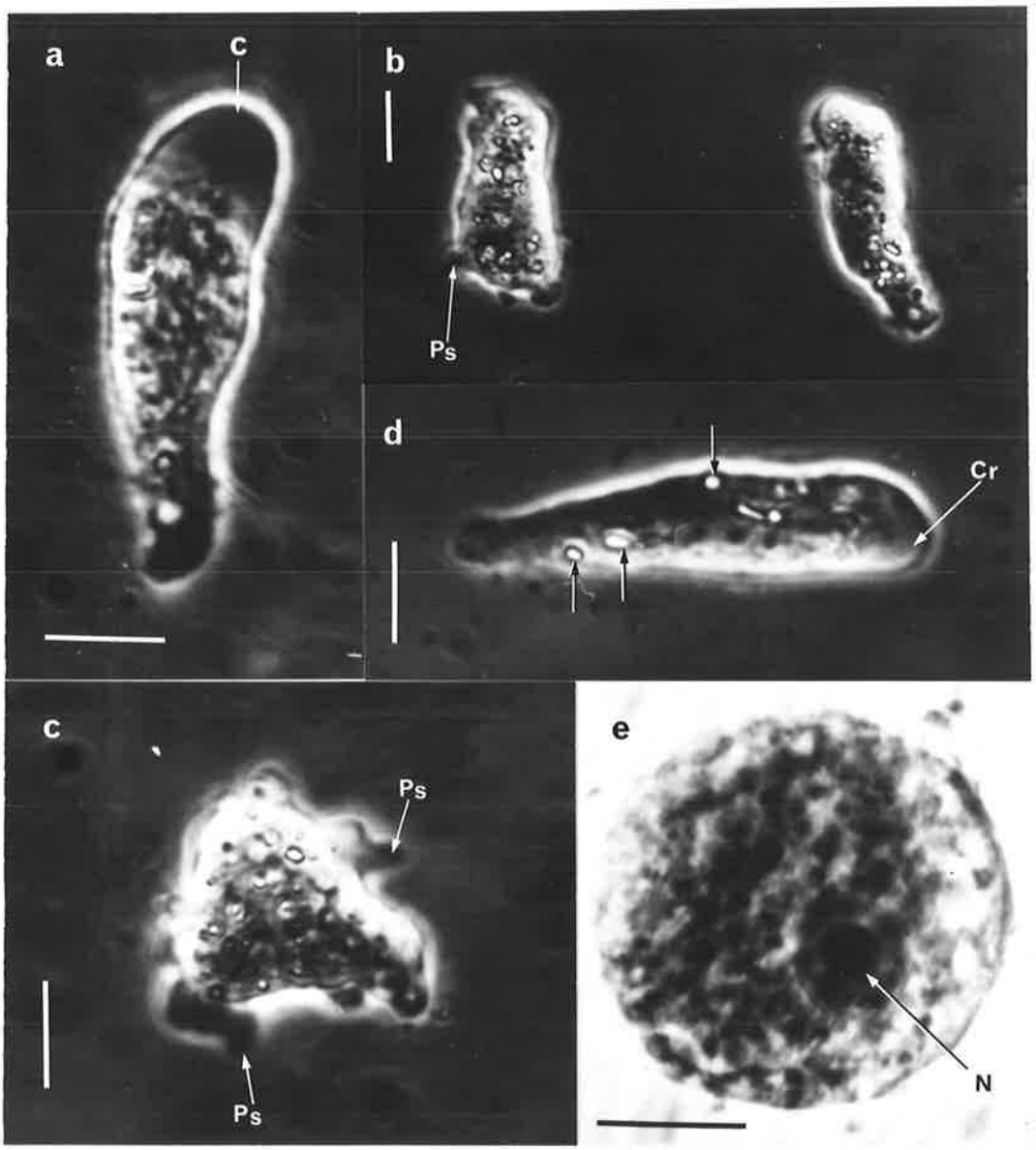
b. Trophozoites in liquid culture with short pseudopodia (Ps).

c. Trophozoite on agar appearing as a rounded mass containing refringent granules. Note the pseudopodia (Ps).

d. A typical limax amoeba in locomotion, showing the ectoplasmic crescent (Cr) and the crystals (arrows).

e. Orcein-stained nucleus (N) in a rounded-up trophozoite.

(Scale bar = 10 µm in all Figs.)


a
c
b
Ps
d
Cr
c
Ps
Ps
e
N

limax amoeba (Fig.10a), a hyaline ectoplasmic crescent is usually present. However, the ectoplasmic cap may be absent in limax amoebae under continuous locomotion. A characteristic feature of this amoeba is the occurrence of 10 - 28 rectangular to cuboidal crystals in its cytoplasm (Figs.10b,c,d). Crystals vary from 0.75 µm to 1.5 µm in length. Trophozoite dimensions vary from 24 - 45 µm in length and 8 - 33 µm in breadth. The single nucleus and its dense nucleolus can be seen in live trophozoites, but the abundance of cytoplasmic granules may obscure this. Stained nuclei (Fig.10e) measure between 3.2 - 8.8 µm in diameter and nucleoli from 1.6 - 7.2 µm. No cysts have been observed in cultures.

Trophozoites of this amoeba match the generic description of *Saccamoeba* Frenzel, 1892; emend. Bovee, 1972 (Bovee, 1972; Page, 1976). Number of crystals, trophozoite size and morphology of the villous-bulb uroid are used to distinguish between the non-cystic species of *Saccamoeba* (Page, 1976). The posterior end of the amoeba described here is smooth and round, and does not have villous bulb or rigid villi in the urodium. The size of the trophozoites is also smaller than the non-cystic species of *Saccamoeba* described by Bovee (1972) or Page (1976). The amoeba therefore has been placed in this genus as an unidentified species.

Thecamoeba quadrilineata (Carter, 1856)

Amoebae in locomotion are flattened, oblong or elongately elliptical in outline with broadest point usually at or near the middle. The advancing margin is usually rounded (Figs.11a,c), but can be wavy (Fig.11b) or truncate (Fig.11d). In locomotive forms 2-4 dorsal folds which form parallel along the length of the amoeba can be observed

Fig.11: *Thecamoeba quadrilineata.*

a. Trophozoite in locomotion showing the large contractile vacuole (Cv) and the anterior ectoplasmic zone (Ec).

b. Trophozoite with the wavy anterior margin (arrows).

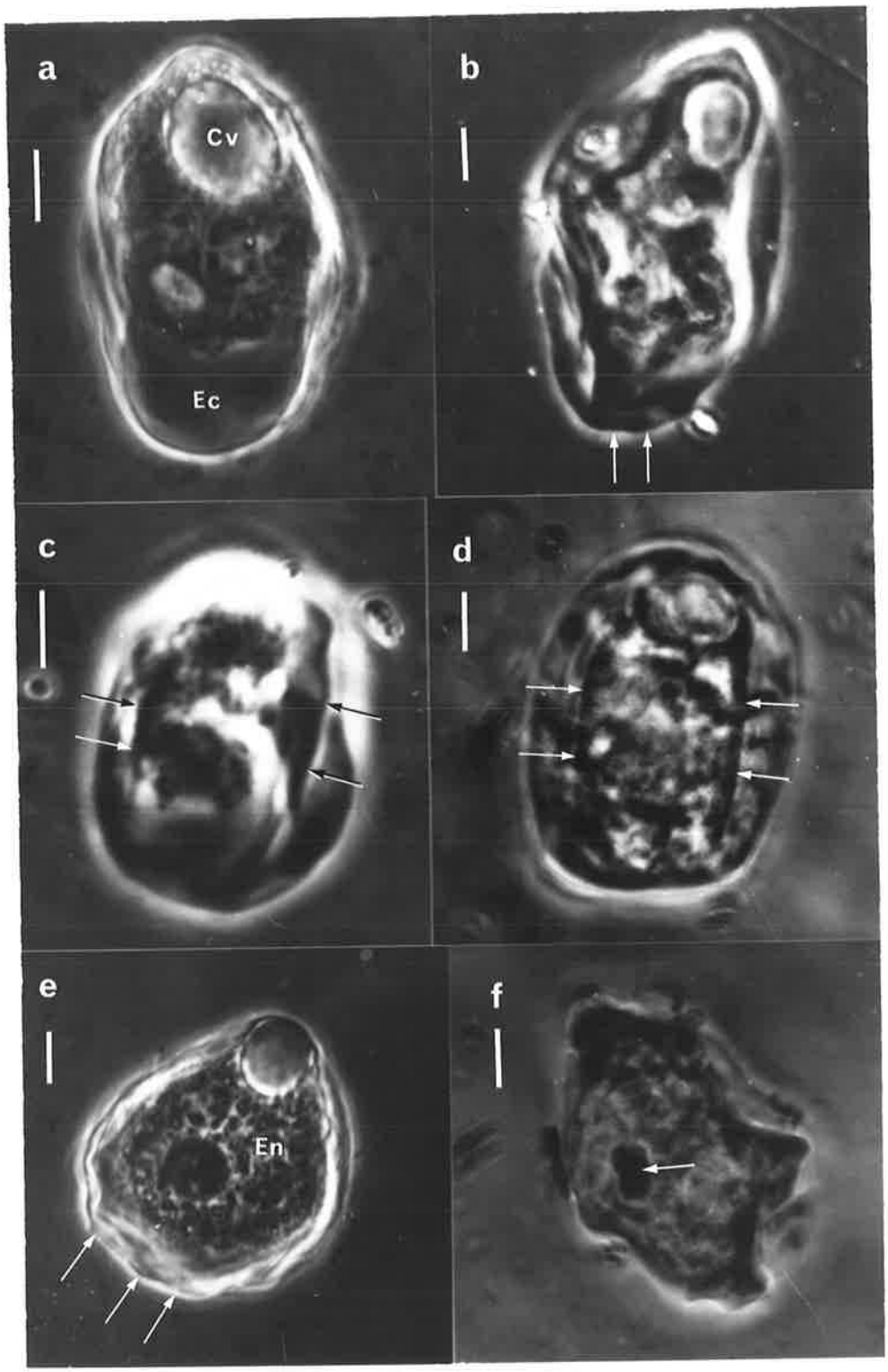
c. Live trophozoite showing two dorsal folds (arrows).

d. Glutaraldehyde-fixed trophozoite with two clear dorsal folds (arrows) and the truncate anterior.

e. Live trophozoite showing granular endoplasm (En) and wavy advancing margin (arrows).

f. Orcein-stained nucleus with a single elongated nucleolus (arrow).

(Scale bar = 10 µm in all Figs.)


a
Cv
Ec
b
c
d
e
En
f

(Figs.11c,d). Trophozoites usually move by gliding in one direction, however, occasional lateral shifts do occur on agar media whereby trophozoites aggregate together with their posterior ends oriented towards the centre. Lateral shifts are relatively faster and more common in young cultures. The rate of gliding varies from 0.2 to 0.9 μm sec^{-1}.

Cell cytoplasm is distinctly divisible into a granular endoplasm which contains refractile bodies, about 1.5 μm in diameter (Fig.11e), and the hyaline ectoplasm. The ectoplasm is broader at the anterior end. Usually one contractile vacuole, up to 20 μm in diameter is present at the posterior end of each trophozoite (Figs.11a,b,d,e). Amoebae are uninucleate and nuclei can be observed in live trophozoites; when stained, they appear elongated to oval in shape with an elongated nucleolus (Fig.11f). In live amoebae, reniform, triangular or spherical nuclei have also been seen.

The trophozoites match the generic description of *Thecamoeba* Fromentel, 1874 (Page, 1977). Two species of *Thecamoeba*, *T.striata* (Penard, 1890) and *T.quadrilineata* (Carter, 1856), have prominent dorsal folds in locomotive amoebae and nuclear structures have been used to distinguish them. In *T.striata* the nucleolus is made up of two or more distinct pieces, whereas, *T.quadrilineata* has a single nucleolus (Page, 1977). The isolate described here has been identified as *Thecamoeba quadrilineata*.

Thecamoeba sphaeronucleolus (Greeff, 1891)

The amoeba in hanging drop preparations often sink to the bottom during examination and in general poorly adhere to glass surfaces.

Floating forms appear highly wrinkled. Trophic forms, however, are smooth and flat (Figs.12a,b), and more or less oblong, oval or sometimes broad fan-shaped with an expanded anterior region. There appears to be a sharp line of demarcation between the ecto- and endoplasm in locomotive forms. As the trophozoite glides away, a series of wavy patterns which originate in the endoplasm and travel to the anterior extremity can be observed (Fig.12b). Rate of locomotion is 0.4 - 1.5 µm sec^{-1}. In general, there is only one contractile vacuole, 6-16 µm in diameter, located at the posterior of the cell (Fig.12a), but occasionally several smaller vacuoles can be seen as craters in the endoplasm (Fig. 12b). Trophozoite dimensions are 35 - 80 µm in length and 25 - 65 µm in breadth. Nuclei in live amoebae appear spherical, oval or elongate with centrally located lobed, spherical or elongated nucleoli. When stained the nucleolus, however, almost always appeared fragmented in to 2-3 lobes (Fig.12c). In live trophozoites nuclear diameter varied from 8-12 µm and that of the nucleolus from 5.5 - 9 µm.

Except for their size, trophozoites resemble *Thecamoeba sphaeronucleolus* (Greeff, 1891; Page, 1977). Trophozoites of *T.sphaeronucleolus* range from 65 - 140 µm (mean 98 µm) in length, whereas those of this isolate measure from 35 - 80 µm (mean 55 µm). The nucleus is also larger in size and contains a single or fragmented but centrally located nucleolus (Page, 1977). The main difference, therefore, appears to be in the overall dimensions of the amoebal cell and its nucleus. The isolate may be a sub-species of *T.sphaeronucleolus*.

Thecamoeba granifera (Greeff, 1866) sub-species *minor* Pussard *et al.*, 1979

The trophozoite of this amoeba is more or less elliptical, oval or elongated in shape (Figs.13a,b,c,d), measuring between 24.4 - 40.8 µm

Fig.12: *Thecamoeba sphaeronucleolus*.

a. A typical fan-shaped trophozoite on agar. Note the sharp line of demarcation (arrows) between the ecto- and endoplasm.

b. Trophozoite in locomotion showing wavy pattern in the anterior ectoplasm (Ec) and crater-like endoplasm due to the abundance of contractile vacuoles (Cv).

c. Orcein-stained nucleus with two nucleolar fragments.

(Scale bar = 10 µm in all Figs.)

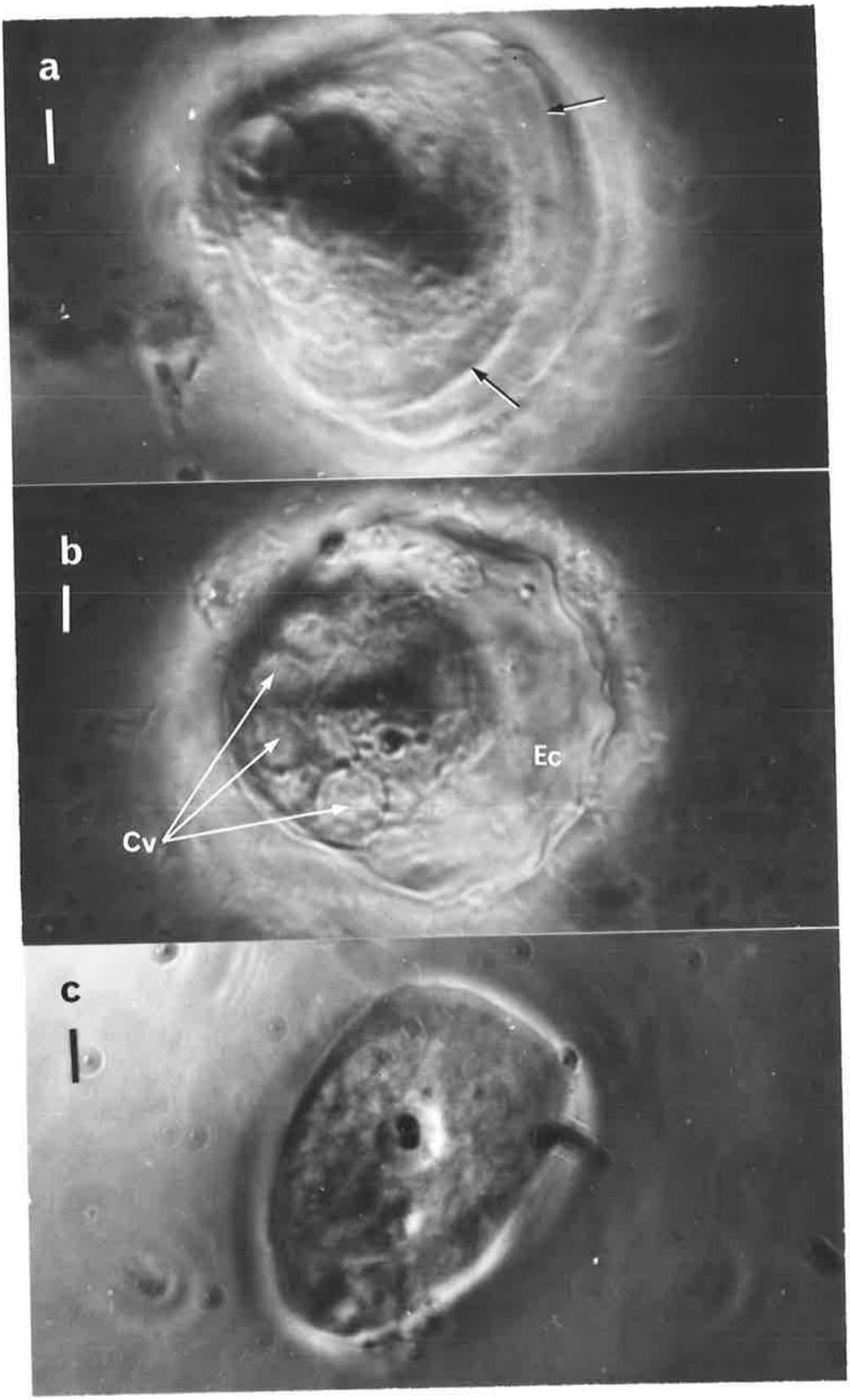

a
b
Ec
Cv
c

Fig.13: *Thecamoeba granifera* sub-species *minor*.

a. Trophozoite showing anterior ectoplasm (Ec) and the highly granular endoplasm (En).

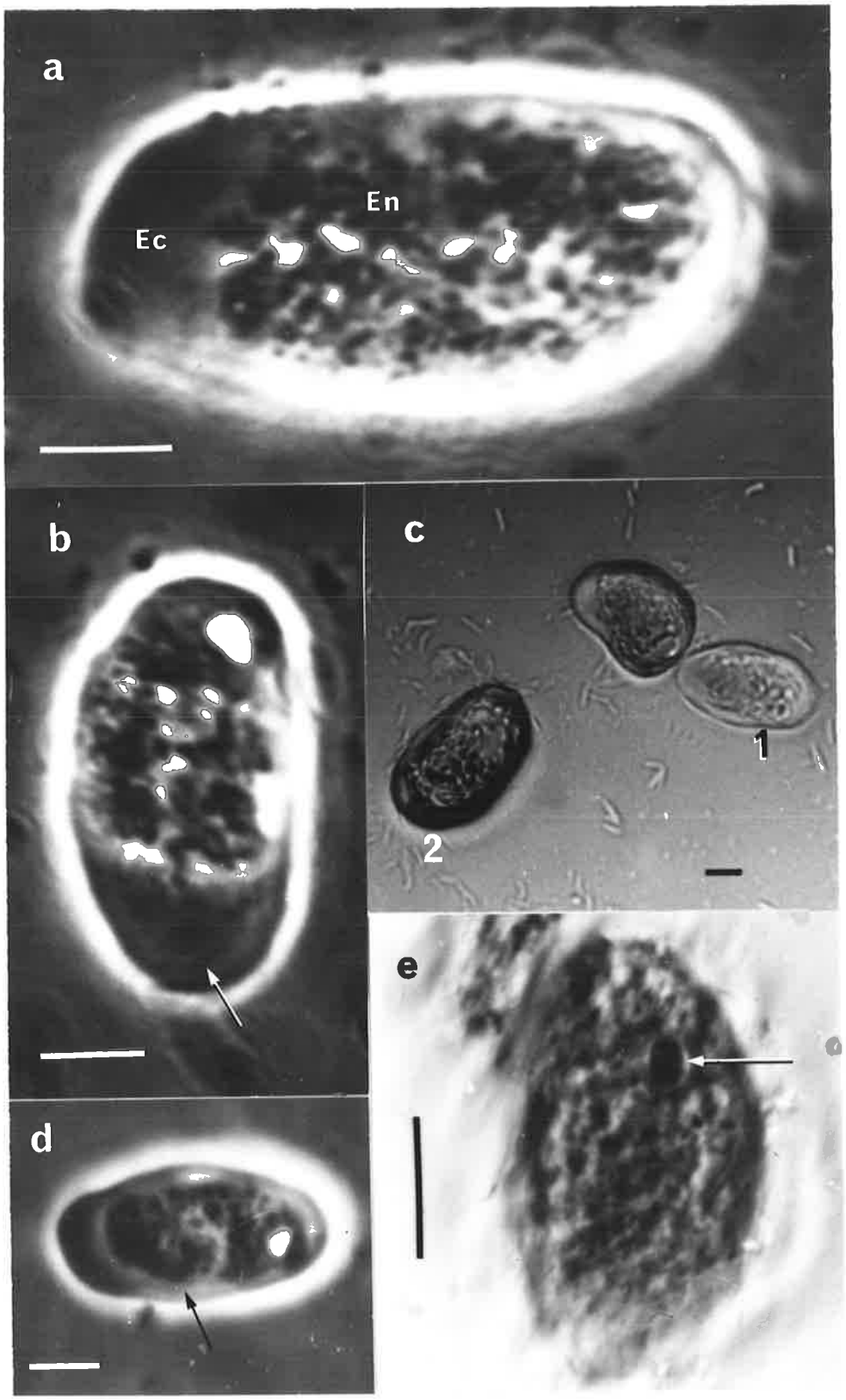
b. The ectoplasm appearing as a deep anterior crescent (arrow) in a trophic amoeba.

c. Trophozoites showing the variation in the number of granules in them. A young trophozoite (1) has less granules than a full-grown one (2).

d. Trophozoite showing the anterior ectoplasm crescent and its extension as a narrow border (arrow) towards the posterior region.

e. Orcein-stained nucleus (N).

(Scale bar = 10 µm in all Figs.)


a
En
Ec
b
c
1
2
d
e

in length and 15 - 24.5 µm in breadth. The endoplasm is characteristically granular with refringent granules, 1-3 µm in diameter, which appear yellow or brown in colour, the intensity of colour varying considerably between individuals. A small trophozoite may have few or no granules (Fig.13c) and as it grows the granules increase in number. The hyaline ectoplasm is broader at the anterior end and appears as a deep anterior crescent (Figs.13b,d) which extends posteriorly as a narrow border (Fig.13d) when in continuous locomotion. There is usually one pulsating vacuole, 1 - 6 µm in diameter, in the posterior part of the body. Locomotion is rapid and can be 1.5 µm sec^{-1}. In a live trophozoite the nucleus is rendered inconspicuous by the abundance of granules. When stained, nuclei appear elongate to oval in shape (Fig.13e) and measure between 8 - 12 µm in diameter. Cysts have not been observed.

The amoeba appears closely related to *Thecamoeba granifera* Greeff, 1866 as redescribed by Page (1976, 1977). The trophozoites of *T.granifera* however, are larger in size and almost always contain more than one nucleolus in their nucleus. Pussard *et al.* (1979) described a small cyst-forming mycophagous amoeba which closely resembled *T.granifera* except for its size and they named it *T.granifera* subspecies *minor*. They reported one to three nucleoli in their isolates. The amoeba described here also frequently associates itself with hyphae of *Fusarium* and other fungi on agar plates. The amoeba has been maintained in monoaxenic culture with an associated *Fusarium* sp. and the similarity in sizes of the amoebal cysts with that of the fungal chlamydospores probably explains the reason for the failure to distinguish cysts in cultures. The isolate has been identified as *T.granifera* subspecies *minor*.

Unidentified Vampyrellid amoeba

In this amoeba the concept of a trophozoite is almost non-existent due to the extensive anastomosis which occurs between individuals. Anastomosis between individuals leads to the formation of a giant reticulum, 10-12 mm in size, with an advancing front (Fig.14a) which is the most active part of the organism. Advancing fronts have fine filopodia which occur singly or in groups. Smaller individuals, several µm in length can be seen in liquid cultures (Fig.14b), but they soon anastomose and form the giant reticulum. The cytoplasm is finely granular and contains many small pulsating vacuoles 2.2 - 8 µm in diameter. No distinction can be made between ecto- and endoplasm. Locomotion is slow and in short term undetectable. If positions of filopods in an advancing margin are marked on the occular micrometer graticule, and observed after 2 - 4 h, they can be seen to have changed. More direct evidence of locomotion can be obtained by increasing the illumination. Then the reticulate form will round up and form cysts. Active movements however, occur in the terminal filopodia which are slowly and regularly formed and withdrawn.

On agar plates, this amoeba may be confused with the aphanoplasmodium of a myxomycete (Alexopoulos, 1969, Fig.2, page 222). Its proteomyxan nature is evident by the absence of a regular cytoplasmic streaming, formation of cysts and continuous formation and bursting of vacuoles in the cytoplasm. The amoeba is multinucleate (Fig.14d) and the number of nuclei varies with the size of an individual. Nuclei measure between 2.4 - 7 µm in diameter with a darkly stained nucleolus 1.6 - 4 µm in diameter.

Fig.14: The unidentified vampyrellid amoeba.

a. Advancing front of a giant reticulum formed on agar. Note the fine filopodia (arrows).

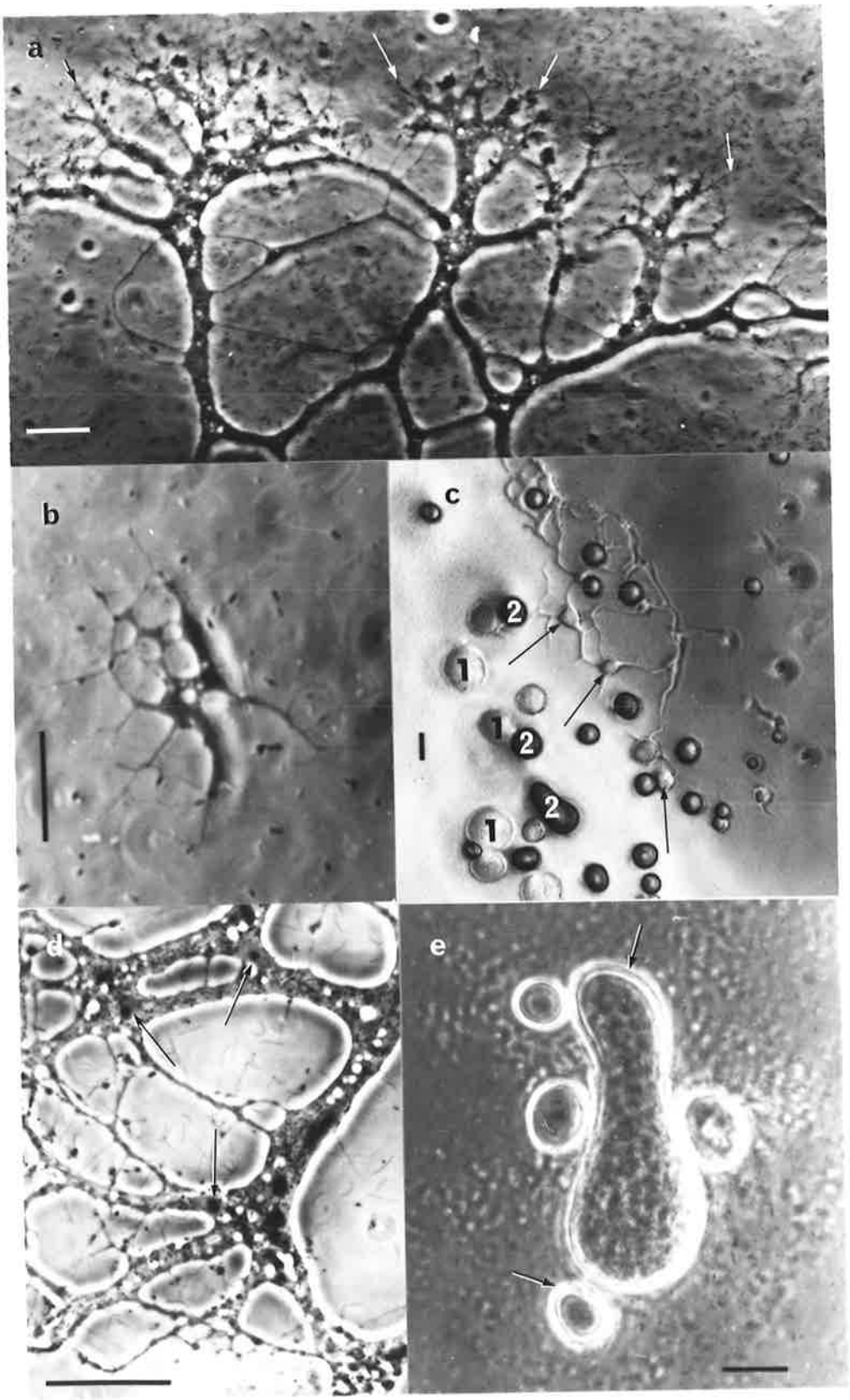
b. A smaller individual in liquid medium.

c. The giant reticulum of the amoeba has concentrated at several points (arrows) prior to cyst formation. Note the thin-walled (1) and thick-walled (2) cysts.

d. Stained nuclei (arrows) in a giant reticulum of the amoeba.

e. Thick-walled cysts showing the range of shapes and sizes and three distinctly visible layers of the cyst-wall (arrows).

(Scale bar = 25 µm in all Figs.)


a
b
c
2
1
1
2
2
1
d
e

The amoeba forms two types of cysts. During cyst formation, the whole mass of protoplasm concentrates at several points (Fig.14c) which gradually become detached from each other. These areas appear dense and finally round up to form single thin-walled cysts (Fig.14c). These cysts germinate easily. The other type of cyst has thick walls composed of three distinctly visible layers (Fig.14c) and is formed from the thin-walled cysts by further contraction of the protoplasm. Often the thin wall of the original cyst is visible as an outline outside the thick-walled cyst. Germination of these thick-walled cysts has not been observed. Cysts can be of any shape or size (Figs.14c,e). In the thick-walled cysts, a range of 10 - 369 µm in diameter or length, depending on their shape, has been observed. Cysts are commonly elongate to oval in shape.

Many earlier workers who observed freshwater and soil amoebae described them as new species and often erected new genera on the basis of differences in their specimens. Zwillenberg (1953) in describing a new species of *Theratromyxa* considered 19 genera as possible synonyms. Other genera with filopodia which received detailed modern treatment are *Arachnula* (Old and Darbyshire, 1980), *Vampyrella* (Anderson and Patrick, 1978, 1980), *Vampyrellidium* (Surek and Melkonian, 1980) and *Nuclearia* (Pernin, 1976; Mignot and Savoie, 1979). Two other genera, *Biomyxa* and *Gromia* also produce filose pseudopodia (Leidy, 1879; Hedley, 1962), of which *Gromia* is a testate amoeba and *Biomyxa* has a central body from which radiating filopodia are produced.

The family Vampyrellidae Zopf, 1885 includes organisms with hyaline filiform pseudopodia, often branching, sometimes anastomosing which do not produce spores and do not have a flagellate stage in their life cycle

(Loeblich and Tappan, 1961). The amoeba described here does not match any species description I have noted, however, its characters generally are similar to those of the members of the family Vampyrellidae and I have tentatively placed it in this family as an unidentified species.

C. *IN VITRO* INTERACTION OF SOIL AMOEBAE WITH *Ggt* AND OTHER SOIL FUNGI

There is a two way interaction between soil amoebae and fungi; firstly, several fungi, mostly Phycomycetes of the order Zoopagales, are predaceous and parasitic on soil amoebae (Drechsler, 1933, 1935, 1947, 1969; Dayal, 1975) and secondly, several of the terricolous amoebae feed on fungi. Among mycophagous amoebae particular interest has been shown in the genera *Arachnula*, *Vampyrella* and *Theratromyxa* (Old, 1977a; Old and Darbyshire, 1978; Old and Oros, 1980; Anderson and Patrick, 1978, 1980; Homma *et al.*, 1979) which belong to the family Vampyrellidae (Honigberg *et al.*, 1964). A number of other genera, *Thecamoeba* (Esser *et al.*, 1975; Alabouvette *et al.*, 1979; Pussard *et al.*, 1979), *Cashia* (Pussard *et al.*, 1980) and *Hartmannella* (C.Palzer, personal communication), have also been found to have mycophagous species. Although soil amoebae were known to feed on yeasts (Oehler, 1916; Severtzova, 1928; Castellani, 1930; Negroni and Fischer, 1941; Nero *et al.*, 1964; Bunting *et al.*, 1979), Heal (1963) was first to show that soil amoebae also feed on filamentous fungi. Drechsler (1936), however, had reported that the testate amoeba, *Geococcus vulgaris* France and *Arcella vulgaris* Ehrenberg, 1938 attacked oospores of *Pythium ultimum* Trow, and Miller (1963) found a ciliate which fed on the sporangia of *Pythium carolinianum* Matthews.

The mode of attack varies with the amoebal species. *Arachnula impatiens* was shown to penetrate the conidial walls of *Cochliobolus sativus* by erosion of an annular depression into the conidium wall (Old, 1977a, 1978). This disc of wall material is dislodged, allowing the spore contents to extrude into the amoebal trophozoite. The closely

related *Theratromyxa* encompasses whole spores within digestive cysts, the spore wall being penetrated by many small holes each about 0.5 µm in diameter (Anderson and Patrick, 1980; Old and Oros, 1980). *Mayorella* sp. has been shown to engulf up to five conidia of *C.sativus* and retain them within the trophozoite for many hours. Extruded spores are often lysed but little information is available on the feeding mechanisms of this genus (Anderson and Patrick, 1980). Pussard *et al.* (1979) have described in detail the penetration of hyphae and microconidia of *Fusarium oxysporum* Schlecht emend. Snyder and Hansen by *Thecamoeba granifera* sub-species *minor*. They regard this species as being strictly mycophagous, whereas Old and Darbyshire (1978) showed that *Arachnula impatiens* has an extremely broad range of potential prey including bacteria, algae, protozoa and nematodes.

Nine amoebae belonging to eight different genera which were commonly isolated from the WPP soil were tested for mycophagy in feeding trials using *Ggt*, *C.sativus* and *Phytophthora cinnamomi*.

The feeding trials

Feeding trials were conducted, using the three fungi in Petri plates containing the fungal substrate and amoebae suspended in AS. Mycelium of *Ggt* was grown in 0.4% malt extract broth for one week, washed twice with SDW and rinsed in AS. Mycelium was either left intact or homogenised. Conidial suspensions of *C.sativus* were obtained using the method described earlier. Mycelium and chlamydospores of *P.cinnamomi* were produced by inoculating V8 juice liquid medium containing 20 µg cholesterol ml^{-1} (D.D.Darling, unpublished information) with about 50 small pieces of agar culture of the fungus and incubating for up to three

weeks at 25°C in continuous light. Thalli were harvested, washed in AS and either homogenised or left intact. AS suspension of fungi were added to Petri plates to a depth of about 5 mm. At the outset of each trial, six or seven 12 mm diameter glass coverslips were placed into each Petri plate. PJA cultures of amoebae were flooded with AS and amoebal suspensions were drawn up into sterile pasteur pipettes. Drops of these suspensions were added to the fungal substrates and the cultures incubated for 4-5 days at 25°C.

Detailed observations of the interactions between trophozoites and fungi were made by transferring coverslips bearing these organisms to Cruickshank chambers and examining by phase contrast microscopy. After a feeding period of 3-14 days, coverslips were air dried and mounted on metal stubs for scanning electron microscopic (SEM) observation. Other specimens, including intact thalli and spore suspensions that had been incubated with the amoebae were fixed in 3% glutaraldehyde in 0.1M phosphate buffer overnight at 4°C, dehydrated in a graded ethanol series and critical point dried in a Polaron unit using liquid CO_2. Before SEM examination, all specimens were coated with approx. 900Å layer of gold-palladium using a Polaron E5100 sputter coating unit (Polaron Equipment Ltd., Herts. England). Specimens were examined with a Jeol JSMU3 scanning electron microscope.

Feeding activities of the amoebae

Of the amoebae tested, members of five genera, *Saccamoeba*, *Gephyramoeba*, *Mayorella*, *Thecamoeba* and the unidentified leptomyxid proved to be mycophagous (Table 10). The feeding behaviour of these amoebae are detailed as follows.

TABLE 10: Mycophagy of various soil amoebae on three plant pathogenic fungi.

Amoeba	Fungus	Mycophagy	Perforation (SEM)
Acanthamoeba polyphaga	*Ggt*	-	-
	Cs[a]	-	-
	Pc[b]	N.T.[c]	-
Gephyramoeba sp.	*Ggt*	N.T.	N.T.
	Cs	+	+
	Pc	+	+
Mayorella sp.	*Ggt*	+	-
	Cs	+	-
Platyamoeba stenopodia	*Ggt*	-	-
	Cs	-	-
Saccamoeba sp.	*Ggt*	+	-
	Cs	+	+
Thecamoeba granifera ssp. *minor*	*Ggt*	+	-
	Cs	+	+
Thecamoeba quadrilineata	*Ggt*	-	-
	Cs	-	-
Unidentified Leptomyxid	*Ggt*	+	+
	Cs	-	-
	Pc	+	+
Unidentified Vampyrellid	*Ggt*	-	-
	Cs	-	-
	Pc	-	-

[a] *Cochliobolus sativus*

[b] *Phytophthora cinnamomi*

[c] Not tested.

In cultures, the unidentified vampyrellid amoeba forms an extensive reticulum encircling many conidia of *C.sativus* (Fig.15) and *Ggt* hyphae and remains attached for a considerable period of time. However, no evidence of conidia or hyphae being perforated or affected otherwise has been observed. SEM observations of fungal propagules after they have been cultured with the amoeba have never shown perforations in their walls. The amoeba feeds on *Klebsiella* sp.

The unidentified leptomyxid amoeba

The amoeba employs two different mechanisms when feeding on *P.cinnamomi*. On hyphae, sporangia and hyphal swellings of the fungus feeding is rapid and completed in 35-45 min. Mature chlamydospores are digested within large digestive vacuoles over a prolonged period, between 21 and 36 h. These processes are quite distinct and are described separately.

When feeding on hyphae the trophozoite contacts the hypha, its movement ceases and then it adheres firmly to the surface. If hyphal fragments are encountered these may be dragged along the substratum attached firmly to the posterior filopods. When attacking an intact thallus the amoeba stretches itself along the hyphal length, attaching at any part (Fig.16a). The trophozoite becomes extremely thin and extended, eventually engulfing a portion of the hypha. This process takes 10-15 min, after which the amoeba contracts and masses around a small section of the hypha (Fig.16b). This process of extension and contraction may be repeated 2-6 times. The trophozoite then moves along the hypha and may concentrate on another part of the hypha (Fig.16c) or leave it altogether. At this stage, it can be seen that the portion of

Fig.15: Giant reticulum of the unidentified vampyrellid amoeba encircling spores of *Cochliobolus sativus*.

(Scale bar = 20 µm).

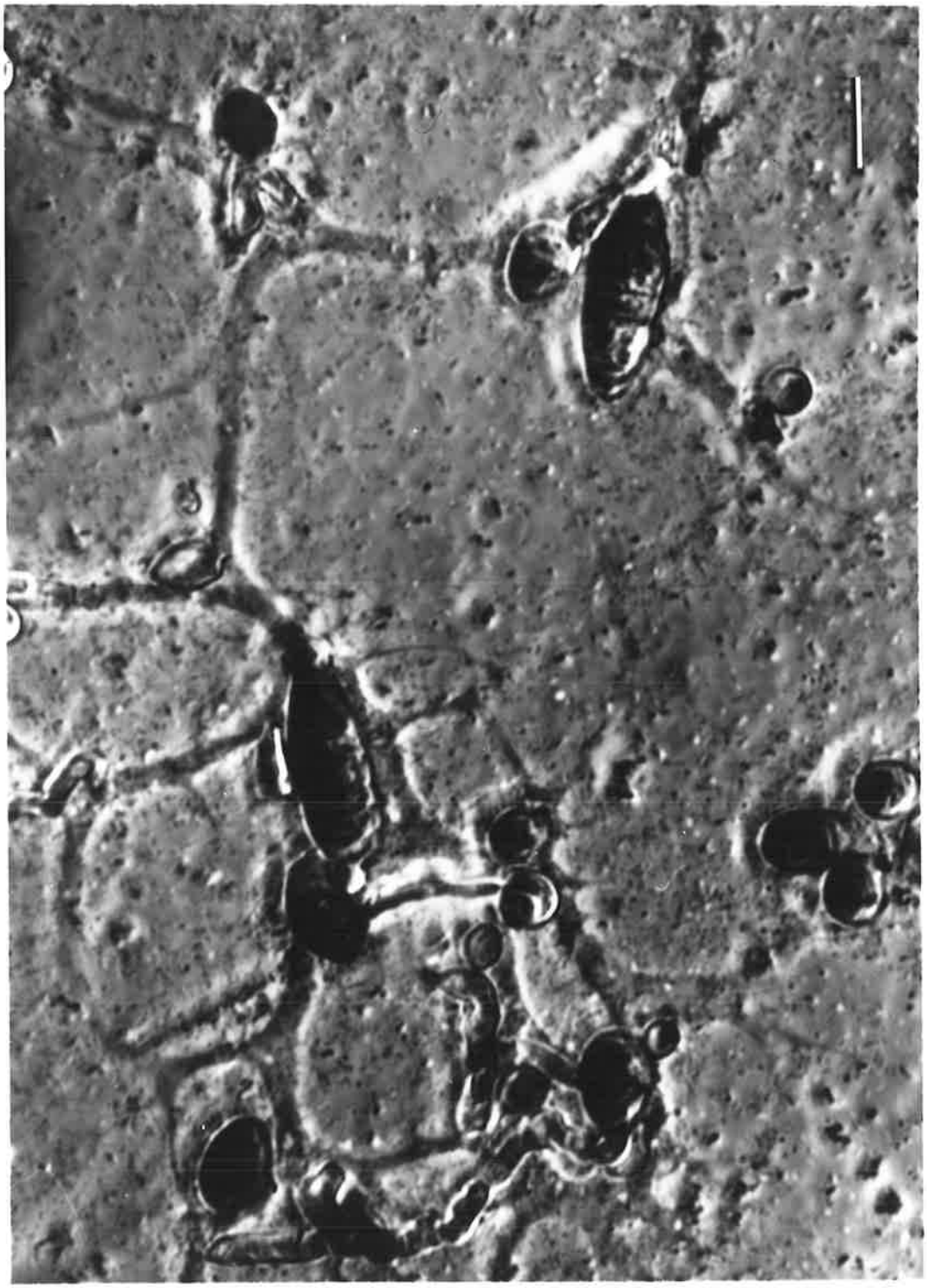


Fig.16: Feeding activities of the unidentified leptomyxid amoeba on *Phytophthora cinnamomi*.

a. A trophozoite has enveloped a length of hypha (arrows) of the fungus. Figs. 16a-d are a sequence of micrographs at the same magnification showing the lysis of the hypha.

b. The trophozoite has concentrated around the subterminal portion of the hypha (arrows).

c. The trophozoite has migrated back along the hypha and is concentrating at a new location (arrow).

d. The hyphal tip now lacks protoplasmic contents (arrow).

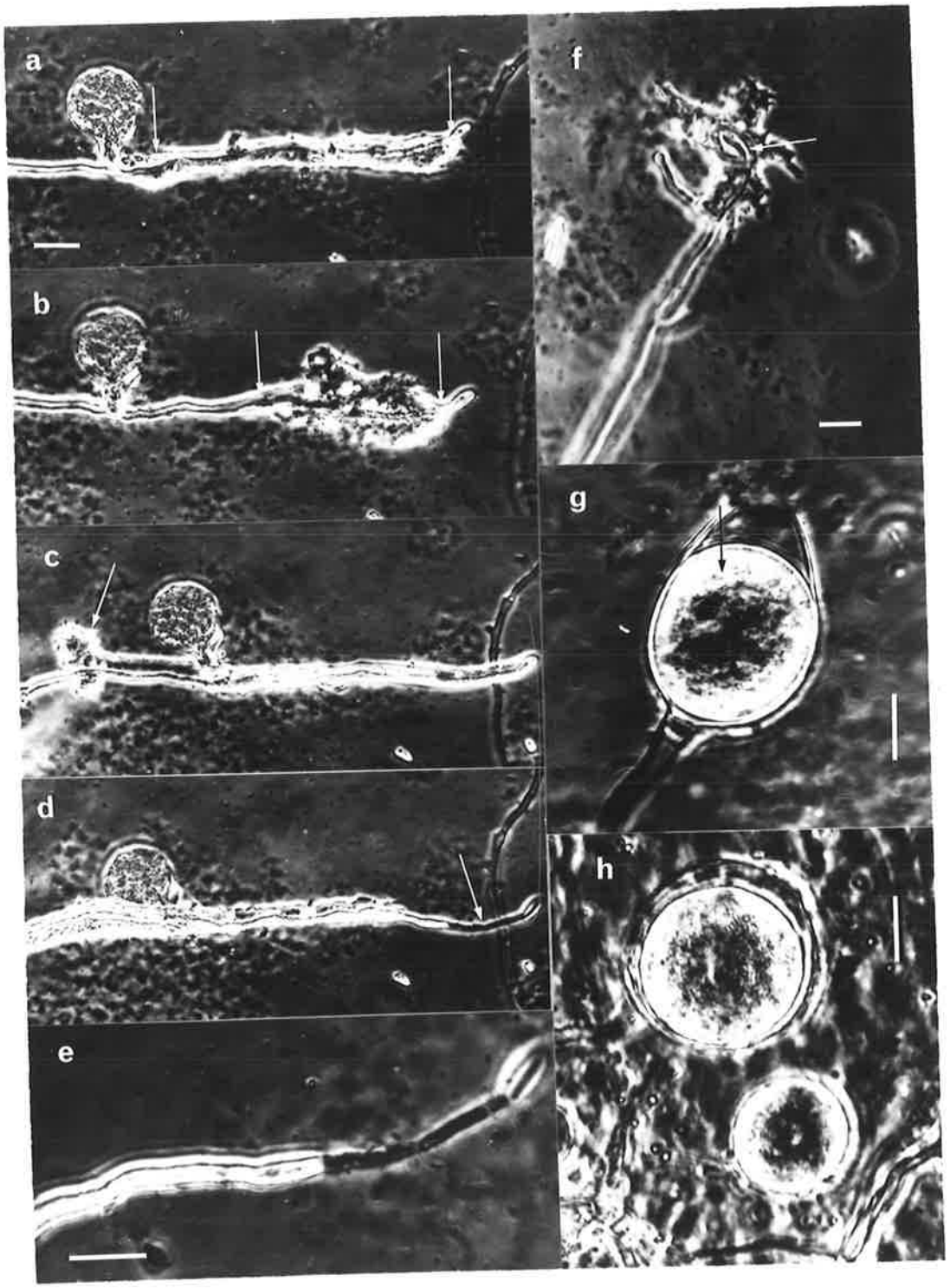
e. Detail of the hyphal tip.

f. Trophozoite exerting force to encompass the hyphal tip. Note the physically distorted hypha (arrow) inside the amoeba.

g. Amoebal cyst within empty sporangium (arrow).

h. Amoebal cyst within lysed swollen hyphae.

(Scale bar = 20 µm in all Figs.)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hypha where the trophozoite concentrated is completely devoid of cytoplasm (Figs.16d,e). The amoeba exhibits considerable mechanical force and free ends of hyphae may be physically distorted to encompass them within the body of the trophozoite (Fig.16f). The amoeba is very sensitive to bright illumination when feeding and photography and observation must be carried out with intermittent or low light intensity or feeding will be slowed or cease altogether.

Sporangia, hyphal swellings, or immature chlamydospores of *P.cinnamomi* are consumed by the amoeba in a similar way to hyphae, except that engulfment of the propagule may be incomplete. A similar series of extensions and contractions of the trophozoite occurs and the contents of the sporangium flow into the amoeba. The fungal propagule is rendered completely devoid of contents in 35-45 min after feeding begins and then the amoeba either moves away to feed on other cells or may migrate within the sporangium or hyphal swelling and form a cyst (Figs.16g,h). Scanning electron microscopy of fungal material after prolonged feeding showed that hyphae and other propagules were perforated by circular holes varying from 1.0 µm to 3.0 µm in diameter (Fig.17a).

The mode of feeding on *Ggt* hyphae varies according to the ability of the trophozoite to fully surround the hypha. When intact thalli are used, the amoeba envelopes sections of hypha and undergoes the extensions and contractions shown for *P.cinnamomi* hyphae (Figs.16a-d). Protoplasmic contents of these hyphal cells are lost and subsequent SEM examination shows that holes varying in size from 0.3 µm to 2.0 µm are present in the fungal cell walls (Fig.17b). Presumably these are the sites of attack by which the amoeba gains access to the fungal protoplast. When the fungal thallus is fragmented before feeding trials, the tropho-

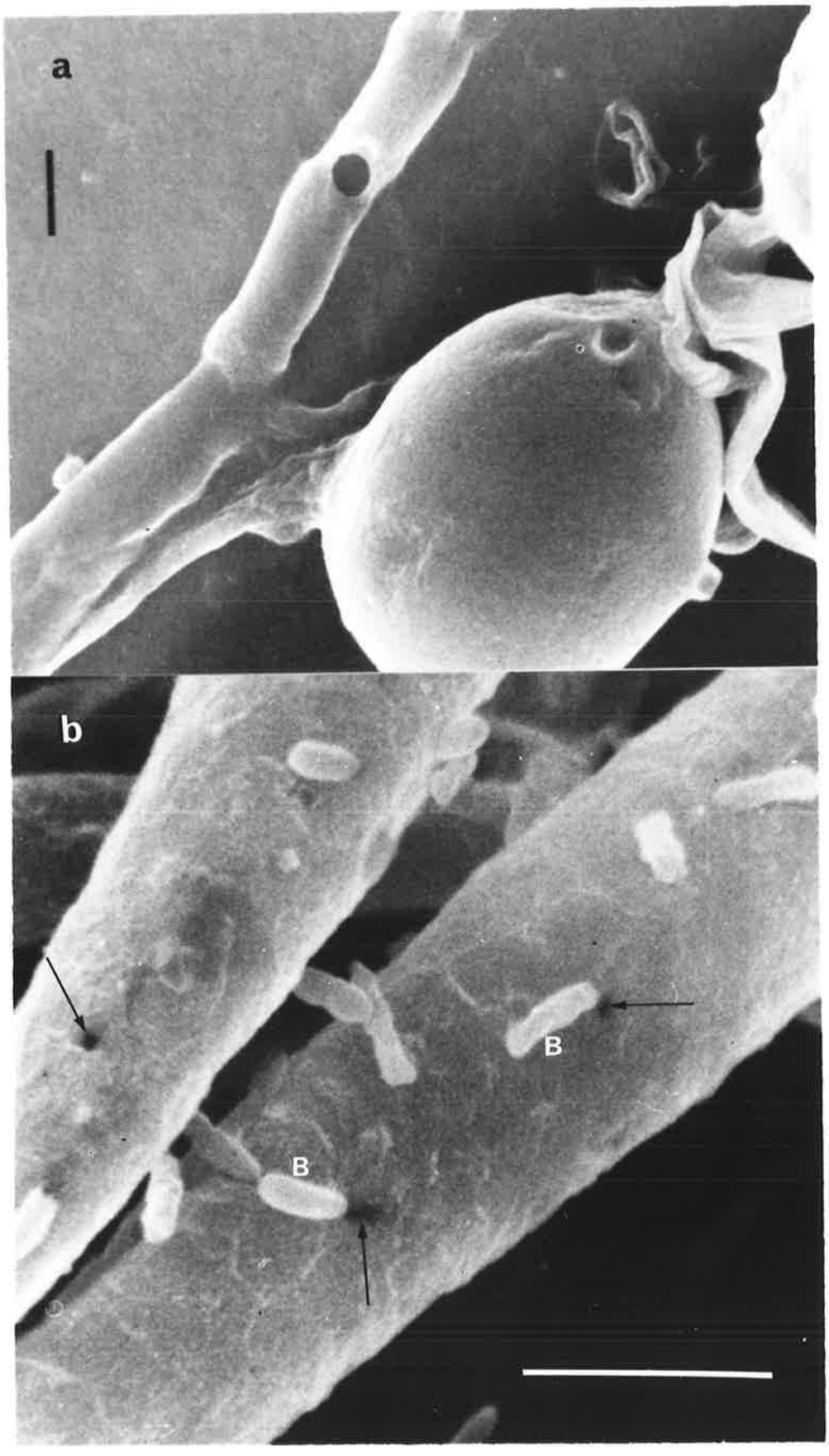
Fig.17: Scanning electron micrographs of hyphae from feeding trials with the unidentified leptomyxid.

a. Perforation in a hypha of *Phytophthora cinnamomi*.

b. Perforations in hyaline hyphae of *G.graminis tritici* (arrows).

The bacteria (B) are *Klebsiella* sp.

(Scale bar = 4 µm in both Figs.)


a
b
B
B

zoites ingest whole pieces of hypha. This is commonly achieved by twisting and bending the hypha until it is fully accommodated inside the trophozoite. The trophozoites continue to move across the substratum and in as little as 30-45 min the hyphal fragments are no longer recognisable and shortly afterwards undigested residues are extruded from the trophozoite.

Digestion of a mature chlamydospore of *P. cinnamomi* occurs by a trophozoite contacting and completely engulfing it. Formation and expulsion of the contents of the numerous small vacuoles within the amoebal protoplast become intense at this stage and result within approx. 20-35 min in the formation of a large fluid filled vacuole containing the chlamydospore. This vacuole is the subsequent site of digestion of the spore. During this initial phase of attack the amoeba is particularly light-sensitive and rounds up if illumination is too intense. Once the digestive vacuole is fully formed (Fig.18a) the amoeba is less sensitive. Microscopical observation of spores within digestive vacuoles show that delicate protoplasmic threads traverse the space between the main vacuolar membrane and the spore wall. These are not resolved adequately in Figs.18a-e. Contraction of these strands may be the cause of the rotation of the chlamydospore within the vacuole shown in Figs.18a-e.

The main activity seen within the amoebal protoplast at this time is the continuous formation of vesicles which develop bordering the digestive vacuole (Fig.18a) and discharge their contents into it (Fig. 18b). The other prominent feature, the nucleus, is also visible throughout the period of existence of the digestive vacuole (Fig.18d). At no stage did a cyst wall develop around the trophozoite in the manner

Fig.18: Digestion of a mature chlamydospore of *Phytophthora cinnamomi* by the unidentified leptomyxid amoeba.

Figs.18a-i are a sequence of micrgraphs at the same magnification.

a. A fully formed digestive vacuole around the chlamydospore. Note the formation of a vesicle (arrow) in the amoebal cytoplasm.

b. As in Fig.18a but with a mature vesicle about to discharge into the digestive vacuole (arrow).

c. Formation of a pseudopod.

d. The prominent nucleus (N) inside the amoebal protoplasm.

e. The digestive vacuole is reduced in volume after about 16-17 h from its formation.

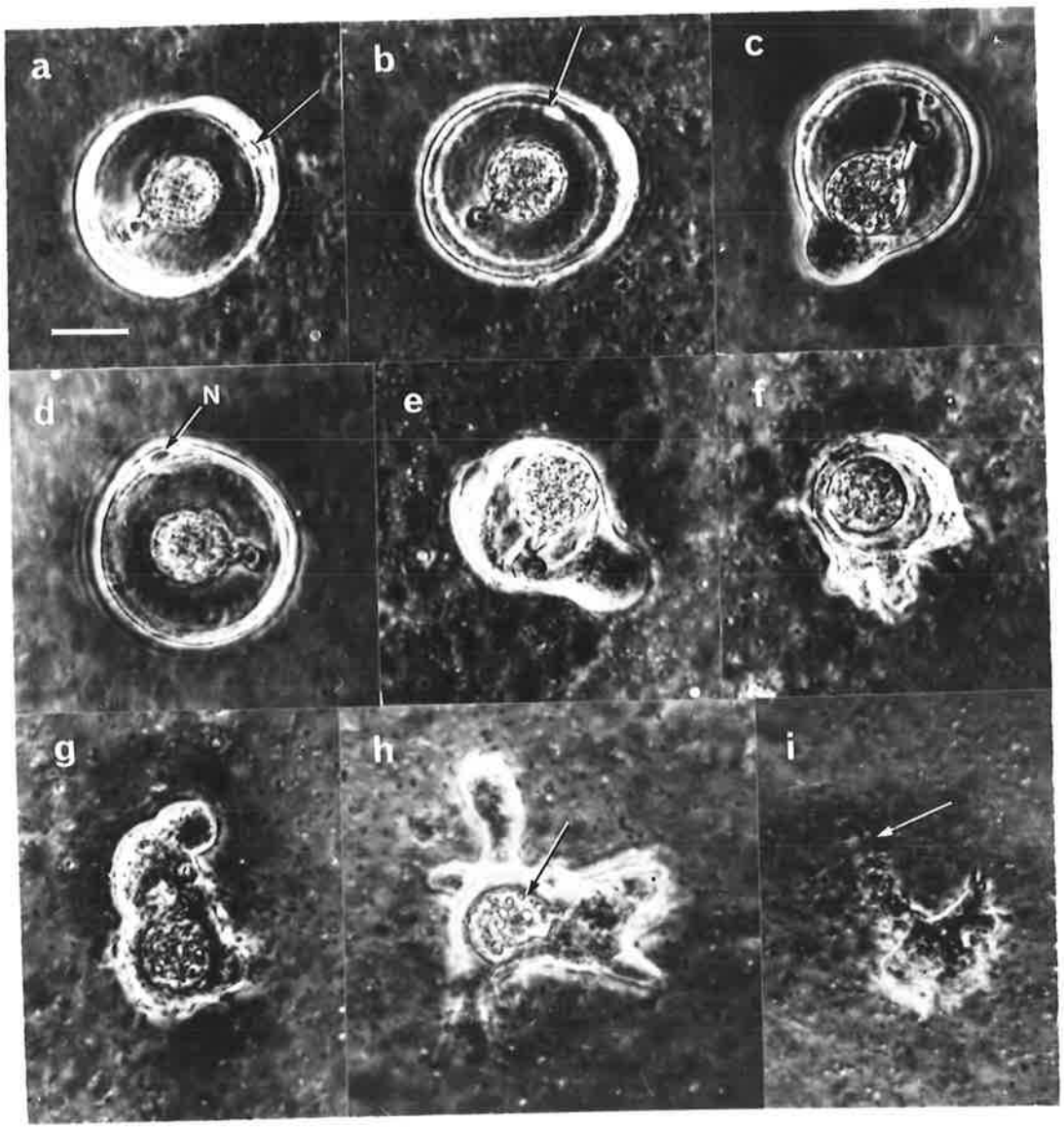
f. As the digestive vacuole is further reduced in volume, the amoeba becomes more mobile.

g. The fluid layer between the chlamydospore and the amoebal cytoplasm is lost.

h. The chlamydospore (arrow) has lost its structural integrity.

i. The trophozoite moves away leaving a trail of fungal cell debris (arrow).

(Scale bar = 20 µm).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described for *Theratromyxa* (Old and Oros, 1980). In fact, the outline of the amoeba was in a state of continuous though gradual change, and the trophozoite could be observed to migrate a small distance on the substratum, by normal pseudopodial movement (Figs.18c,e).

After about 16-17 h the diameter of the vacuole gradually reduces (Figs.18e-h). The amoeba resumes more active pseudopod formation (Fig.18f) and the generally spherical shape is lost. The fluid layer between the chlamydospore and the amoebal protoplast disappears completely and the contents of the chlamydospore appear disorganised (Figs.18g,h).

Typical trophozoite movement resumes (Fig.18h) and the remains of the digestive vacuole and its contents are conveyed along by the trophozoite. Finally, the digestive vacuole becomes completely disrupted and the remaining fungal protoplasm is taken into amoebal cytoplasm. As the trophozoite rapidly moves away, a trail of debris, presumably the undigested contents of the digestive vacuole, are extruded on to the substratum (Fig.18i).

With conidia of *Cochliobolus sativus* the amoeba often encircles them and spores also frequently adhere to the posterior of the trophozoite, apparently held by the group of filopods. Clumps of five or more conidia, short fragments of conidiophores and immature spores accumulate in this position and may be conveyed along by the trophozoite for many hours. Fungal cells are left behind from time to time and others accumulated. However, careful observation of many such trophozoites has never indicated that the amoeba is attacking these spores and digesting their contents. Examination by SEM of specimens from 14-day old

cultures containing conidia and very large numbers of active trophozoites has not revealed any perforated conidia.

A particularly interesting characteristic of this amoeba is its ability to employ two distinct feeding mechanisms in attacking different cell types of a single fungus. The criterion which directs the feeding option is the ability of the trophozoite to fully surround the propagule and the nature of the fungal wall. Once a propagule is fully surrounded, often by bending and coiling the hyphae by application of considerable force, then digestion of the cells is almost complete in 30-60 min. If however, the amoeba attacks an intact thallus and is unable to fully enclose the propagule, then discrete perforations in the wall are made and the protoplast only is ingested. Hyphal walls appear to be otherwise little damaged.

When preparations of mature chlamydospores (2-3 weeks old) are exposed to the amoeba the feeding mechanism is modified further. After complete encirclement of the chlamydospore a large digestive vacuole develops and the trophozoite remains almost immobile for a prolonged period (17-20 h) during which the entire chlamydospore is digested. Chlamydospores are widely regarded as survival structures, more resistant than hyphae to lysis, but the structural basis of this characteristic is not known. The stimulus to the formation of the large vacuole by this amoeba may be related to the susceptibility of the wall components to enzymatic digestion.

This amoeba, unlike *Arachnula impatiens*, *Theratromyxa* and *Vampyrella* sp. is unable to feed on *C.sativus* (Old, 1977a; Anderson and Patrick, 1978; Old and Oros, 1980). Opportunities for digestion of

these spores are commonly observed as the trophozoites adhere to conidia and convey them for considerable distances across the substratum. Hyphae of *Ggt* in old cultures become pigmented and an attempt was made to study their susceptibility to attack by this amoeba. Some perforated hyphae were found, but it was not possible to distinguish by SEM examination, pigmented hyphae from hyaline hyphae still present in the thallus.

Saccamoeba sp.

In this amoeba the type of damage to hyphae depends on the ability of trophozoites to accommodate these inside their bodies. In an intact thallus, attack usually starts at a hyphal tip. When a trophozoite comes in contact with a hypha of *Ggt*, it adheres with its posterior part, extends its body more or less at a right angle to the hypha and then bends back to form a loop. At this stage the trophozoite begins to contract and gradually forms a rounded mass containing many refringent granules. The portion of the hypha retained within the amoebal cell becomes twisted and bent at several points. Ingestion of the fungal cells takes about 10-15 min. After a period of approx. 25 - 40 min the amoeba moves on leaving the empty deformed section of the hypha on which it has been feeding (Fig.19a). When hyphal fragments are used in feeding trials entire segments are accommodated within trophozoites which surround the hyphae and bend and twist these into shapes which can be ingested. After about 15-20 min, debris, scarcely recognisable as hyphae, is extruded on to the substratum (Fig.19b). Frequently hyphal fragments are coiled around a trophozoite (Fig.19c) after the amoeba attaches itself to a portion of the fragment. This enables the trophozoite to feed on a larger area of the hyphae at once. Hyphae lose

Fig.19: Feeding activities of *Saccamoeba* sp. on *G.graminis tritici* and *Cochliobolus sativus*.

a. Trophozoite feeding on hyphae of *Ggt*. Note the lack of refringency (arrows) in parts of the hyphae.

b. Formless debris (arrow) extruded by the trophozoite after feeding on hyphal fragments of *Ggt*.

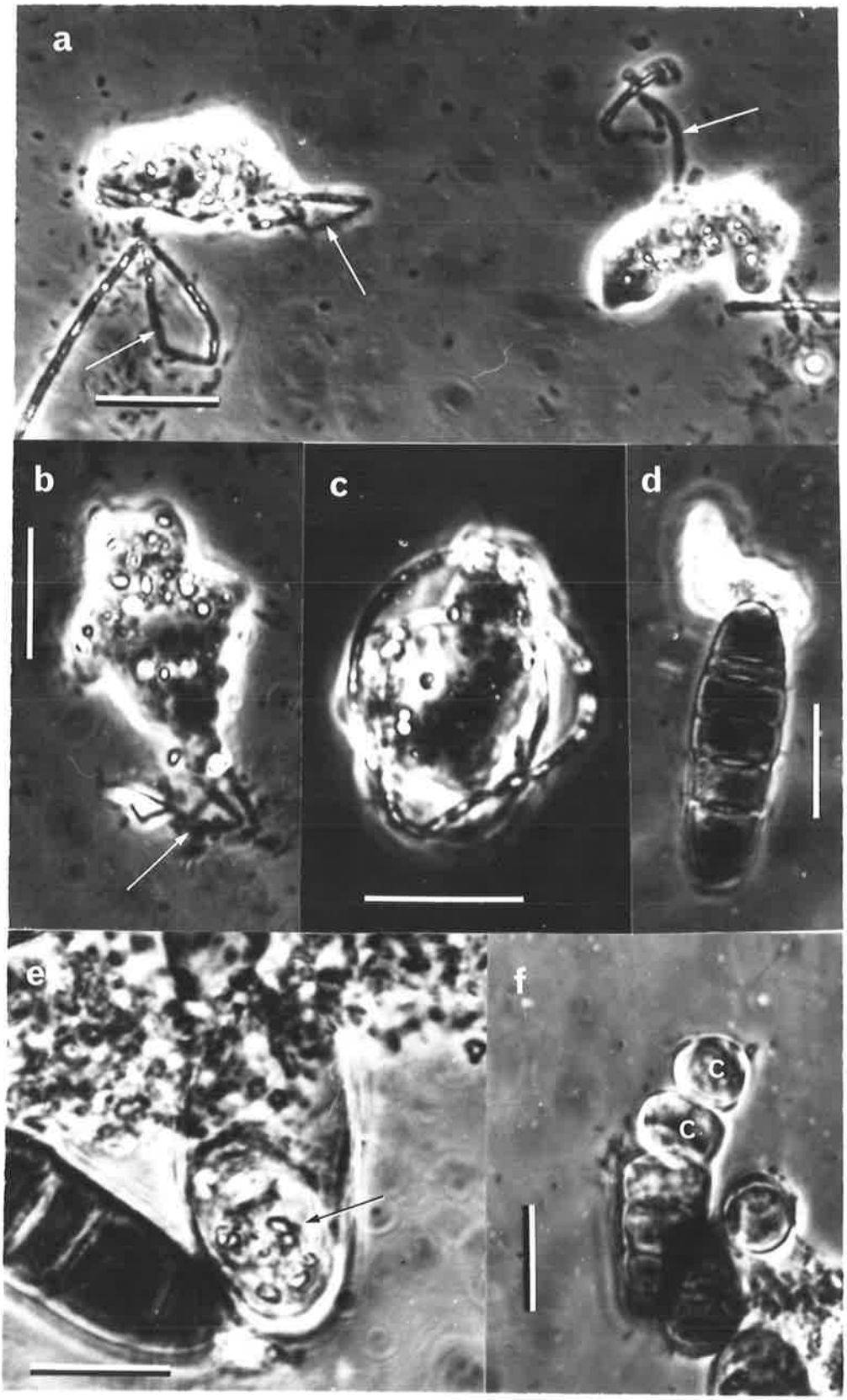
c. A hyphal fragment of *Ggt* coiled around a trophozoite as a result of attachments by the trophozoite.

d. A trophozoite forming a loop at one end of a *C.sativus* conidium.

e. Trophozoite (arrow) inside an empty *C.sativus* conidium.

f. *C.sativus* conidium ruptured by the amoeba; spheroplast-like bodies (C) arranged in a linear fashion.

(Scale bar = 20 µm in fall Figs.)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c

their refringency in about 20-30 min from the time the trophozoite established contact.

When feeding on *C.sativus* conidia, the amoeba establishes firm contact with the spore by forming a loop (Fig.19d) similar to that described above and stays attached to the spore for about 4 - 6 h as a round mass containing refringent granules. Sometimes the amoeba moves along the surface of the spore and very occasionally resumes the limax form of locomotion with the spore attached to its posterior end. After this prolonged period of contact, one or more cells in the conidium adjacent to the amoeba appear paler in colour. The septa of the spore are usually intact at this stage. The amoeba may leave the spore after emptying one or more cells. These conidia when examined with the SEM show perforations, 1.5 - 3.0 µm in diameter, in their walls.

On other occasions the amoeba enters the spore (Fig.19e), presumably through the perforation and stays inside the spore for periods ranging from 6 - 24 h. All compartments of the conidium may be entered and septa are completely disrupted. In other instances the spore is partially invaded and cells which survive attack may extrude from the conidium as spheroplast-like bodies (Fig.19f). The amoeba invariably ruptures the conidium wall as it emerges.

Gephyramoeba sp.

The branched trophozoite of this amoeba covers a wide area and this increases its chance of contact with food. Thus a trophozoite can contact one or more spores of *C.sativus* at once. The amoeba stays attached for up to 15 - 20 h (Fig.20a). The conidium wall is penetrated by a pseudopod which may be followed by the entire trophozoite

Fig.20: Feeding activities of *Gephyramoeba* sp.

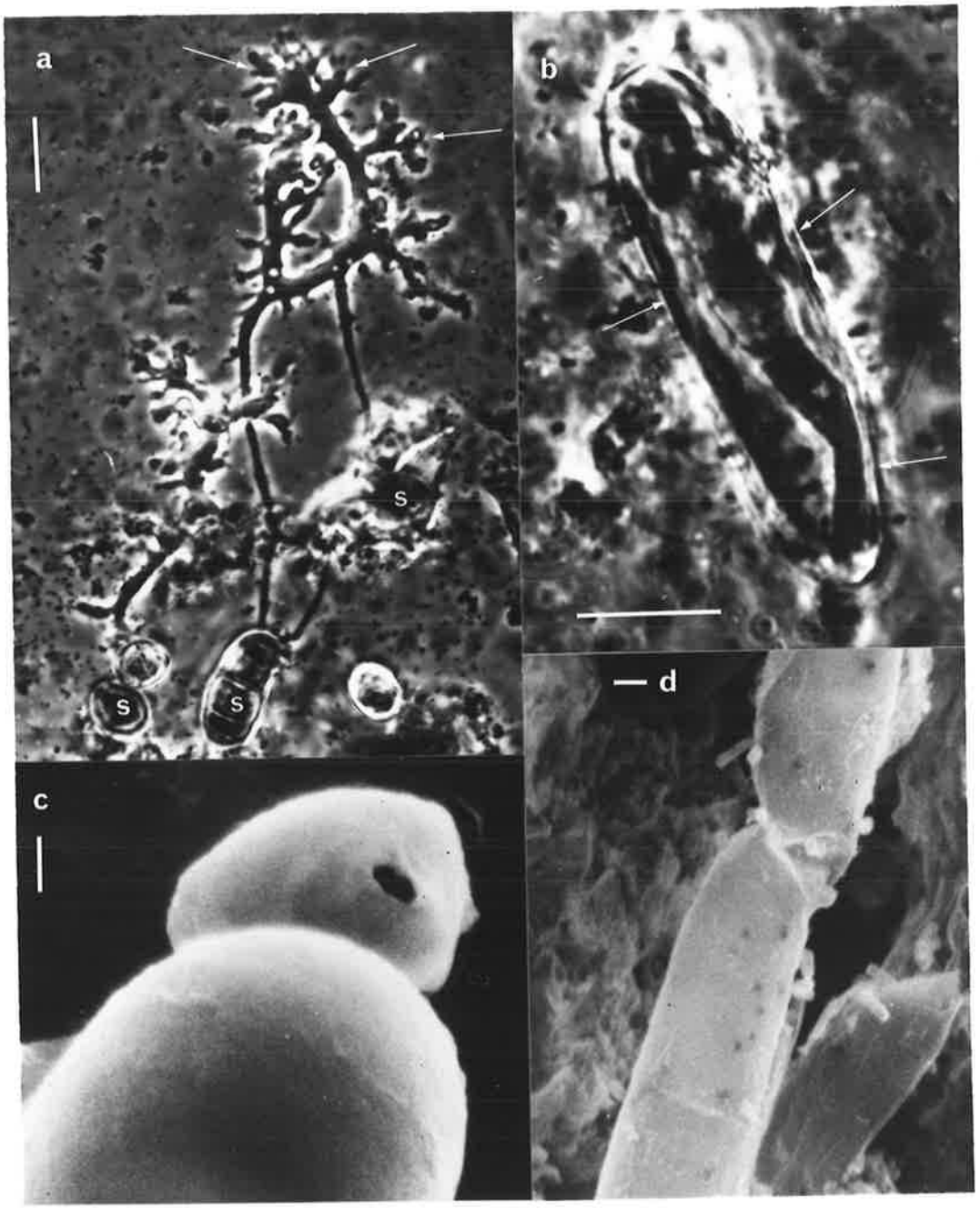
a. A branched trophozoite in contact with *Cochliobolus sativus* conidia (S). Note the pseudopodial clusters (arrows) at the tip of a pseudopodial branch.

b. A trophozoite inside an empty *C.sativus* conidium. Note the intact conidial wall (arrows).

c. Perforated conidium of *C.sativus*.

d. Perforations in hyphae of *Phytophthora cinnamomi*.

(Scale bar - 20 µm in Figs. a and b;
2 µm in Figs. c and d)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(Fig.20b). The trophozoite feeds on the conidial cells and if all the cells are attacked, the conidium loses its septa and appears darker in colour; otherwise some cells may survive attack and a few septa may still be visible. The sequence of invasion of a conidium is difficult to observe as the amoebae are extremely light sensitive. They respond to bright light by withdrawal of pseudopodia and by rounding up to form an inactive amoebal mass. Trophozoites leave spores through perforations in the wall and do not disrupt empty spores. Spores, when examined with SEM, show perforation in their walls varying from 1.0 to 3.2 µm in diameter (Fig.20c). Similar perforations are also observed in the walls of chlamydospores and hyphae of *P.cinnamomi* (Fig.20d) after they have been incubated with the amoeba for 7 - 10 days.

Thecamoeba granifera sub-species *minor*

Feeding on hyphae of *Ggt* is similar to the feeding of this amoeba on *Fusarium oxysporum* as described by Pussard *et al.* (1979). After encircling a length of hypha (Fig.21a), the amoeba becomes rounded and stays in contact for 10 - 20 min, after which it moves along the hypha and the fungal cell encircled appears empty. Small pieces of *C.sativus* hyphae which are usually present in conidial suspensions of this fungus are also emptied in the same way (Figs.21b,c).

The amoeba can perforate conidia of *C.sativus* and digest their contents. Depending on the size of a conidium, it may be partly or completely engulfed by the trophozoite which stays attached without movement for up to 45 min, at the end of which the conidium appears paler in colour. SEM observation shows perforations in conidial walls which range between 1.2 - 5.0 µm in diameter (Fig.21d). After prolonged

Fig.21: Feeding activities of *Thecamoeba granifera* sub-species *minor*.

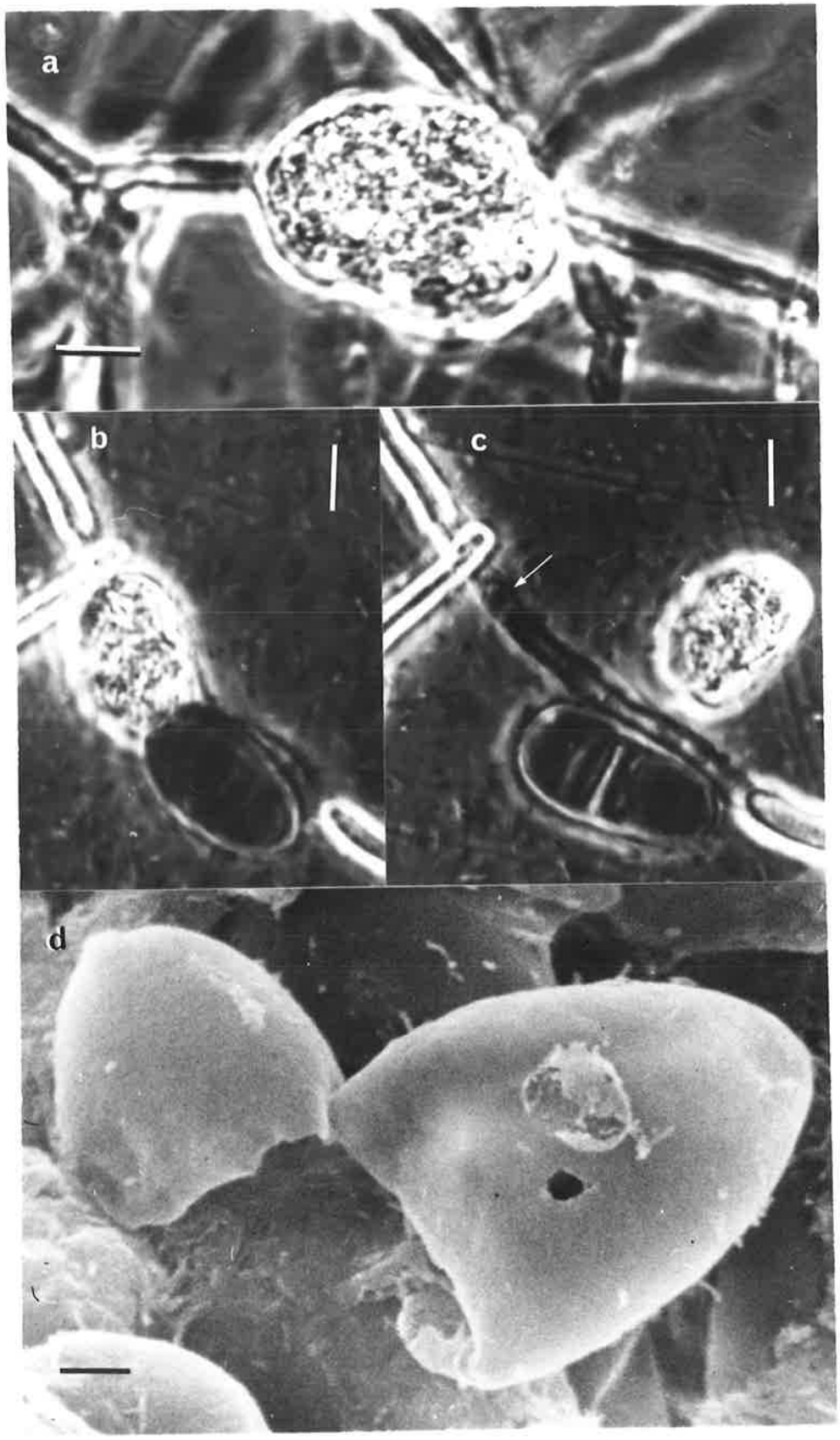
a. A trophozoite encircling a length of *G.graminis tritici* hypha.

b. A trophozoite encircling a portion of a conidiophore of *Cochliobolus sativus*.

c. As Fig.21b but the portion (arrow) of the conidiophore encirlced by the trophozoite has lost its protoplasmic contents and hence lacks refringency.

d. Electron micrograph showing a perforated and disrupted conidium of *C.sativus*.

(Scale bar = 10 µm in Figs. a,b and c;
4 µm in Fig.d)


a
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feeding by the amoeba, many *C.sativus* conidia become disrupted.

Mayorella sp.

As a trophozoite comes in contact with a *C.sativus* conidium it stops and encircles the spore. Ingested spores may be carried for long periods and ejected by the trophozoite without any apparent damage. On other occasions, the amoeba after ingesting one or more *C.sativus* conidia gradually assumes a more or less spherical shape with a number of prominent vacuoles in the cell cytoplasm (Fig.22a). The vacuoles discharge their contents and a fluid layer appears between the spore and the amoebal cytoplasm to form a large vacuole (Fig.22b). This vacuole resembles the digestive vacuole formed by the unidentified leptomyxid amoeba while feeding on chlamydospores of *P.cinnamomi*. After the vacuole is fully formed around the conidium the trophozoite may continue its usual locomotion (Figs.22c,d). The spore can be seen to rotate randomly inside the vacuole and often the vacuolar membrane invaginates and touches the spore (Fig.22c). The shape and the diameter of the vacuole, therefore, changes continuously. This is unlike the digestive vacuole formed by the leptomyxid amoeba which stays more or less constant in size and shape for a considerable period of time. After about 6 - 8 h, the septa in the spore are no longer visible (Fig.22e), and the conidium also appears paler in colour. The amoeba however, carries the spore inside for a much longer period of time even after the disappearance of the septa (up to 10 h). Finally the spore is ejected. SEM observations of spores taken from feeding cultures however, have not provided evidence of perforation. Bacterial populations in cultures have been very high and a film of bacterial cells and mucilage obscured surface detail of the conidia.

Fig.22: Feeding activities of *Mayorella* sp.

a. A trophozoite with a *Cochliobolus sativus* conidium inside one of the many prominent vacuoles in its cytoplasm.

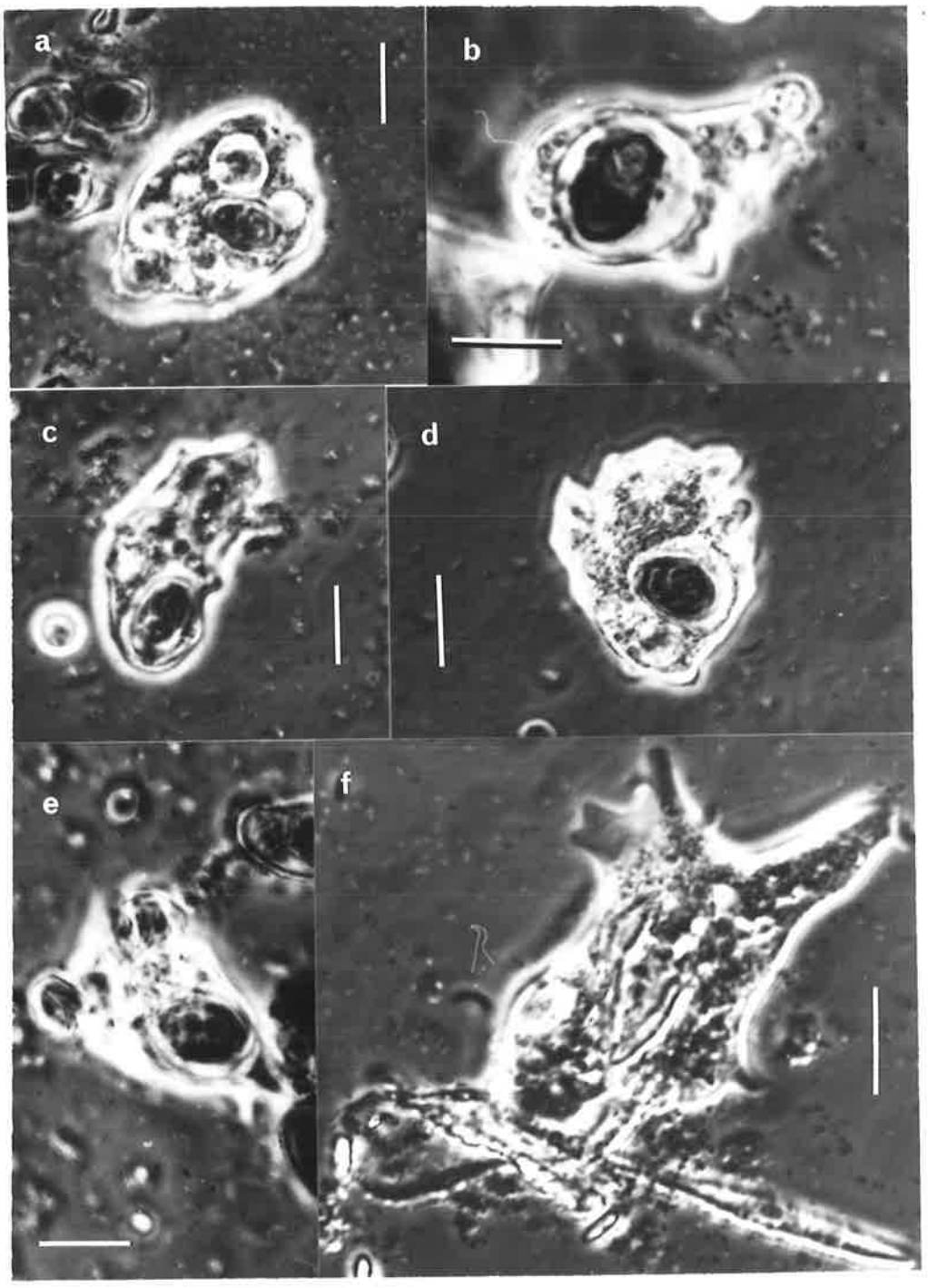
b. The same conidium inside a large vacuole formed by the dissolution of the small vacuoles.

c-d. The trophozoite continues its usual pseudopodial locomotion with the conidium inside.

e. The conidium after 8 h from the formation of the vacuole. Note the lack of septum in the conidium.

f. Trophozoite with engulfed *G.graminis tritici* hypha. Note the twisting and bending the hypha has undergone.

(Scale bar = 20 μm in all Figs.)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When macerated hyphae of *Ggt* are used in feeding trials, *Mayorella* engulfs portions of hyphae by twisting and bending these fragments (Fig.22f). Hyphal fragments are retained inside for up to 6 h and ejected fragments lack refringency in some areas. However, the mechanism of feeding is not clear.

Species of *Mayorella* have been reported to ingest and transport fungal spores (Heal, 1963; Anderson and Patrick, 1980). Heal regarded feeding as unlikely and Anderson and Patrick suggested mycophagy and parasitism of *Mayorella* on other amoebae. Observations here support the contention that *Mayorella* can lyse spores of *C.sativus* contained within large vacuoles in the trophozoite. Disorganisation of septa and protoplasts of conidia within vacuoles has been recorded. Further work is needed to determine whether cell wall perforation occurs during this period. The ability of *Mayorella* to feed on other amoebae is confirmed. In mixed cultures of soil amoebae containing *Mayorella* trophozoites, cysts of other amoebae, especially the readily identified *Acanthamoeba* cysts, have been seen within vacuoles of *Mayorella*.

Feeding of mycophagous amoebae on other soil fungi

Two different isolates of *Rhizoctonia solani* (WARH/6 and F91) and one isolate of a *Phialophora* sp. (PR-II) were tested as food for five mycophagous amoebae using feeding trials similar to those described above. After allowing a feeding period of 10 days, cultures were fixed, processed and examined with the SEM for perforations in hyphal walls.

Perforations were observed in hyphae of *Phialophora* sp. with *T.granifera* sub-species *minor* and *Gephyramoeba* sp. and in hyphae of *Rhizoctonia solani* (F91) with *Saccamoeba* sp. and the unidentified leptomyxid (Table 11).

TABLE 11: Perforation in hyphal walls of *Phialophora* sp. and *Rhizoctonia solani* caused by mycophagous amoebae.

Amoeba	Fungus		
	Rhizoctonia solani WARH/6	F91	*Phialophora* sp. PR-II
Thecamoeba granifera ssp. *minor*	-*	-	+
Saccamoeba sp.	N.T.**	+	N.T.
Unidentified leptomyxid	-	+	-
Gephyramoeba sp.	-	-	+
Mayorella sp.	-	-	-

*- No perforations observed;
+ perforations observed;
** N.T. Not tested.

F91 is an isolate of the strain that causes bare patch of wheat and WARH/6 has strongly pigmented hyphae even when they are young.

Discussion

Although the mechanism of feeding varies with the amoebal species and the fungi involved, mycophagous amoebae generally employ the following basic steps in fungal feeding:

1. Attachment of trophozoites to fungal propagules, which appears to be a matter of chance. There seems to be no strong chemotaxy or thigmotaxy. In all genera so far studied, amoebae may engulf spores or hyphae which are potentially food, only to move away leaving the propagules unharmed.

2. Engulfment of the propagule, partially or completely. The giant trophozoites of *Arachnula*, *Vampyrella* and *Theratromyxa* are able to completely engulf spores or sections of the fungal thallus (Old, 1977a; Anderson and Patrick, 1978, 1980; Old and Oros, 1980). The smaller *Saccamoeba* and *Thecamoeba* attach to restricted parts of hyphae or spore walls and penetrate through to the protoplast.

3. Digestion, which may be by motile trophozoites which penetrate cell walls as in *Arachnula*, *Thecamoeba*, *Saccamoeba*, *Gephyramoeba* and the unidentified leptomyxid, or by digestion of fungal cells within specialised food vacuoles. These may form within cysts, for example, *Arachnula impatiens* forms digestive cysts after a feeding period. A large central vacuole forms in the cyst within which fungal cell residues are digested as completely as possible.

In *Theratromyxa*, the whole process of wall penetration and digestion of spore contents proceeds within a cyst. The unidentified leptomyxid forms large vacuoles within which complete chlamydospores of *Phytophthora cinnamomi* are reduced to formless debris. *Mayorella* also forms food vacuoles within trophozoites but indications so far are that *C.sativus* spores remain largely intact and only the spore contents are digested.

The unidentified leptomyxid amoeba did not lyse pigmented conidia of *C.sativus* despite the opportunities that existed during its close association with the spores.

In many fungi the brownish to black pigment, melanin, confers resistance to microbial lysis (Lockwood, 1960; Lingappa *et al.*, 1963; Bartnicki-Garcia and Reyes, 1964; Kuo and Alexander, 1967). The ability of a number of genera of soil amoebae to lyse pigmented fungal propagules is particularly significant and has been discussed by Old and Patrick (1979). With the demonstration of lysis of *C.sativus* conidia by *Saccamoeba*, *Thecamoeba*, *Gephyramoeba* and *Mayorella* the number of genera able to attack pigmented fungal cells is increased to seven, although the taxonomy of some of these amoebae remains in doubt.

The time taken by different amoebae to lyse fungal propagules by different feeding mechanisms varies markedly. *T.granifera* sub-species *minor* emptied a conidium of *C.sativus* in less than 1 h, whereas *A.impatiens* took approx. 4.5 - 6 h (Old, 1978). The unidentified leptomyxid took about 30 min to lyse *P.cinnamomi* hyphae but needed 22 - 36 h to digest chlamydospores of the same fungus. Conidia of *C.sativus* were contained within *Mayorella* trophozoites for more than 16 h during which time rupture of internal septa and protoplast disorganisation occurred.

IV. ROLE OF SOIL AMOEBAE IN SUPPRESSION OF GGT

A. SOIL AMOEBAE AND SAPROPHYTIC SURVIVAL OF *Ggt*

That antogonistic microorganisms may have a role in the decline of virulence of field inoculum of foot rot fungi has been known for some time (Simmonds, 1928; Broadfoot, 1931; Winter, 1940). Soils may differ in their intrinsic 'biological antagonism' and thus contribute to the variation in take-all from soil to soil (Garrett, 1937); while others may develop higher populations of antagonistic microorganisms and suppress *Ggt* following the addition of organic materials (Garrett, 1934; Fellows and Ficke, 1934); ammonium nitrogen (Smiley and Cook, 1973); monoculture of wheat (TAD, Slope and Cox, 1964; Cox, 1965) or cultivation of non-hosts (Zogg, 1969, 1972). Whether some or all of these conditions of disease suppression are related and therefore have similar underlying causes is not known and it is undesirable to presume homology between suppression in pots and various kinds of suppression in the field (Hornby, 1979).

Walker (1975) distinguished six conditions of take-all suppression and concluded that the term TAD should be restricted to suppression in the field. Gerlagh (1968) recognised two types of take-all suppression, 'general', which is the interaction between the pathogen and increasing numbers of non-specific organisms, and 'specific', which followed wheat monoculture with severe incidence of take-all. Accepting these concepts, Cook and Rovira (1976) characterised general and specific suppression and Rovira and Wildermuth (1981) suggested that the main characteristic which distinguishes these suppressions is whether they can be transferred into natural or fumigated soil and pointed out that

TAD should be included in specific suppression. These authors outlined three causes for transferable suppression:

(a) continuous wheat with take-all (to include TAD soil);

(b) addition of *Ggt* mycelia to soil;

(c) addition of other fungi to soil, e.g., *Gibberella zeae*, *G.graminis* var. *graminis*.

However, since the development of an antagonism to the parasite in the absence of the host is not necessarily the same as natural TAD (Brown *et al.*, 1973); the term 'contrived suppressiveness' has been recommended for laboratory models until corroborated by field studies (Schroth and Hancock, 1981).

Suppression similar to specific suppression in its characteristics has been obtained by adding soils from fallow, wheat, pasture and continuous pasture rotations to fumigated soil and the induction of transferable suppression in a soil under pasture indicates that the wheat plant *per se* is not necessary in the induction of a transferable suppression (Wildermuth, 1980). This suggests that the suppressive factors are active in the saprophytic as well as the parasitic stage of the pathogen. Considering this, Palti (1981) rightly defined suppression as 'the resistance offered by certain soils to the survival, saprophytic and pathogenic activity of phytopathogenic fungi'. The definition of Rovira and Wildermuth (1981) for suppressive soil, i.e., 'soils with a micro-flora which reduces the level of the disease caused by the take-all fungus' does not consider the saprophytic survival of the fungus which is often reduced in a suppressive soil (Pope and Jackson, 1973; Wildermuth, 1977) and the possible role of micro-fauna

(Homma *et al.*, 1979) in suppression. In postulating the mechanism for specific suppression, Rovira and Wildermuth (1981) however, suggested that the 'major site of specific suppression is associated with plant residues which carry both the pathogen and the suppressive microorganisms....'.

According to Shipton (1981), since the parasitic and saprophytic modes of nutrition are so interdependent and inter-related in the field, with alternation from one to the other, it would be unrealistic, if not misleading, to consider either behaviour in isolation.

Several environmental and nutritional factors influence the survival of the fungus. Availability of nitrogen is probably the single most important factor, and the fungus dies early in N-deficient soil (Garrett, 1938; Butler, 1953, 1959; Chambers and Flentje, 1969; Scott, 1969; Weste and Thrower, 1971). Water potential, texture and temperature of soil, in general, indirectly influence the saprophytic survival of *Ggt* through their effects on microbial activity and antagonism. The fungus otherwise is not appreciably affected by extremes of temperatures (-29^{o}C to 71^{o}C, Fellows, 1941) or moisture (30% to 80% saturation, Garrett, 1938) alone, but is rapidly eliminated in wet hot soil (-10 to -20 kPa and 35^{o}C, MacNish, 1973). Among other factors, according to Lal (1939), mycelium of the fungus disappeared more rapidly from acid soils (pH 4.8 to 5.0) than in sand or alkaline soils (pH up to 8.0). However, Zogg (1959) found that neither pH (5.9 to 8.1) nor humus content or soil type altered the rate of elimination of inoculum; decline in survival was related to cropping history and greater numbers of microorganisms (Garrett, 1938).

Survival of *Ggt* decreases independently of fertility levels with increasing numbers of consecutive crops of wheat (MacNish, 1976) or barley (Smith, 1979). Cunningham (1975) found evidence of loss of competitive saprophytic ability in isolates from long sequences of wheat or wheat and barley as compared to those from the first, second and third crops after permanent pasture. Intensified development and activity of microflora (Vojinovic, 1973) causes reduced survival of inoculum (Gerlagh, 1968) and results in fewer infective particles in TAD soils (Pope and Jackson, 1973). Shipton (1981) considers that the phenomenon of TAD can at least in part be explained by the effects on saprophytic survival and associated soil microflora. Natural soils with specific suppression properties also reduce the density of pigmented hyphae buried in these soils (Wildermuth, 1977) and hyphal lysis is more pronounced in the suppressive soils.

To study the role of soil amoebae in the saprophytic survival of *Ggt* in suppressive and non-suppressive soils, mycelia were buried in soils and associated amoebae were isolated.

The method used

The membrane filter burial technique (Old, 1977b) described earlier was used. Blended hyphal suspensions of *Ggt* were buried in the suppressive WPP and the non-suppressive MB soil. CS was used as control. Soils were collected from the top 0-5 cm of the profile and sieved through a 2 mm sieve. The WPP and MB soils were moistened to -8 kPa and -2 kPa respectively. CS was moistened to 15% moisture (oven dry wt basis). Three replicate membranes were retrieved from each soil at weekly intervals and each membrane was cut in to 4 quarters.

One quarter was used for SEM observations; the second for measuring hyphal density by light microscopy and the other two were used for studying the survival of *Ggt* and the association of amoebae with the fungus. For SEM, membranes were processed using the method described earlier.

To estimate hyphal density, filters were stained with 1% lactophenol cotton blue and examined at 400X. Density was determined using the line intercept method (Rovira *et al.*, 1974). The mean length of mycelium per unit area (L) was calculated by counting the number of intercepts between hyphae and two orthogonal lines on an eyepiece graticule and by using the equation:

$$L = \pi N/2H$$

where, N is the number of intercepts and H is the total length of lines on the graticule.

The third membrane quarter was further divided into 4 approx. 1 mm squares and these were plated on NDY/6 incorporated with 100 µg streptomycin ml^{-1} and 10 µg tetracycline ml^{-1}. Percentage survival of *Ggt* was calculated from the number of pieces showing growth of the fungus after incubation at 25°C for 48 h. The remaining fourth membrane quarter was similarly subdivided and plated on PJA, incubated at 25°C for two weeks and examined for the growth of amoebae.

Amoebae and saprophytic survival of *Ggt* hyphae in suppressive and non-suppressive soil

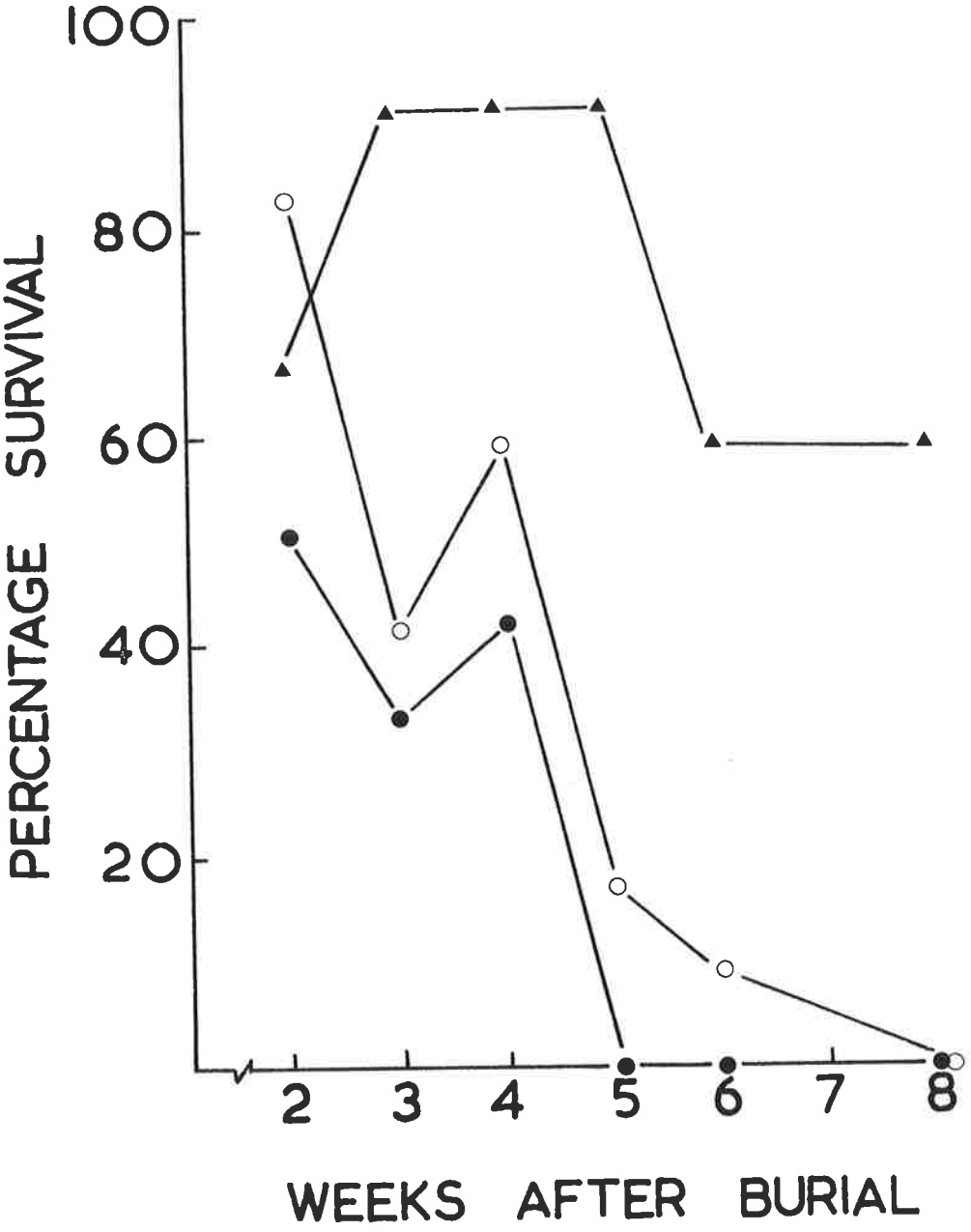
The fungus survived longer in the non-suppressive MB soil than in the suppressive WPP soil (Fig.23). After 4 weeks no membrane

Fig.23: Saprophytic survival of *G.graminis tritici* in suppressive Waite permanent pasture and non-suppressive Murray Bridge soil.

▲—▲ Sterilized coarse sand.

O—O Murray Bridge soil.

●—● Waite permanent pasture soil.


100
80
60
40
20
PERCENTAGE SURVIVAL
2
3
4
5
6
7
8
WEEKS AFTER BURIAL

recovered from the WPP soil had viable fungal hyphae, whereas the fungus survived for up to 6 weeks in the MB soil. In all 3 soils, the density of pigmented hyphae changed with time (Fig.24). Regression analysis showed that the rate of change in density was significantly ($P<0.01$) different in the three soils. For the initial 4 weeks the density increased at a similar rate in all soils but thereafter both MB and CS had progressively higher densities, whereas, density of pigmented hyphae in the WPP soil reduced significantly.

SEM observations of the recovered filters confirm the reduction of hyphal density after 4 weeks in the suppressive WPP soil. After 8 weeks membranes recovered from WPP showed much lower densities (Fig.25a) than those from MB (Fig.25b) and CS (Fig.25c). Extensive erosion of hyphal walls (Fig.25d) was observed in specimens from all soils, though only after 3 weeks of burial in CS. Discrete perforations were observed in hyphae recoved from both WPP and MB soils (Fig.25e). These were quite distinct in their morphology from the general erosion of hyphal walls observed in almost all specimens. The size of perforations varied from 0.6 to 3.5 μm in diameter. No perforations were observed in hyphae recovered from CS. Hyphae recovered from MB soil had fewer perforations and after 8 weeks burial were healthier than those from the WPP soil. However, no quantitative estimate of the number of perforations was made.

Several soil amoebae including mycophagous species were found associated with hyphae recovered from these soils (Table 12). The non-mycophagous *Acanthamoeba* and *Echinamoeba* were obtained from all 3 soils, the mycophagous *Mayorella*, *Saccamoeba*, *Thecamoeba granifera* sub-species *minor* and the leptomyxid amoeba were not isolated from the non-suppressive soil.

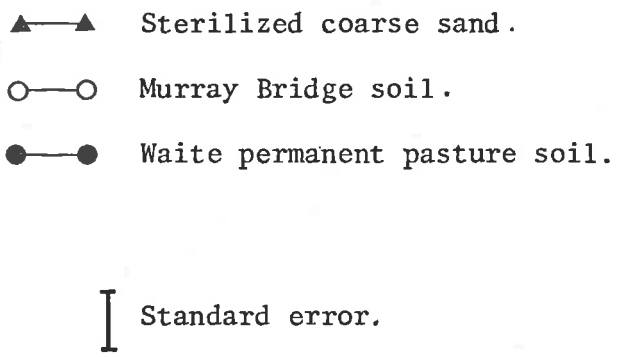


Fig.24: Density of pigmented hyphae of *G.graminis tritici* on millipore filters buried in suppressive Waite permanent pasture and non-suppressive Murray Bridge soil.

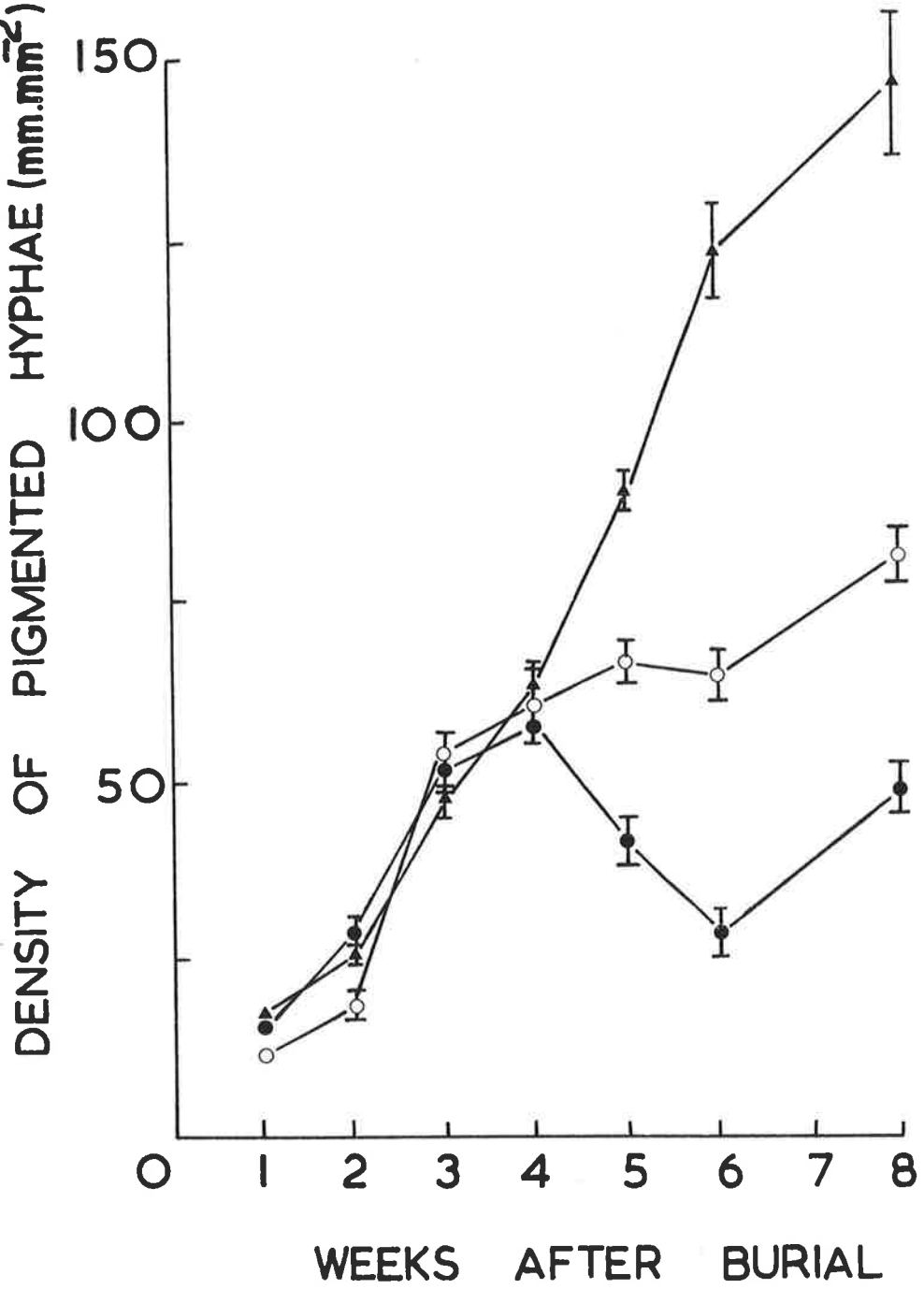

DENSITY OF PIGMENTED HYPHAE (mm.mm^{-2})
150
100
50
0
1
2
3
4
5
6
7
8
WEEKS AFTER BURIAL

Fig.25: Morphology and density of hyphae of *G.graminis tritici* after 8 weeks in suppressive Waite permanent pasture and non-suppressive Murray Bridge soil.

a. Morphology and density of hyphae after 8 weeks in the suppressive WPP soil.

b. Morphology and density of hyphae after 8 weeks in the non-suppressive MB soil.

c. Morphology and density of hyphae after 8 weeks in sterilized coarse sand.

d. Hyphae showing extensive erosion of their walls. These hyphae were recovered from sterilized coarse sand after 6 weeks.

e. Discrete perforations in hyphal walls recovered from the Waite permanent pasture soil.

(Scale bar = 2 µm for all Figs.)

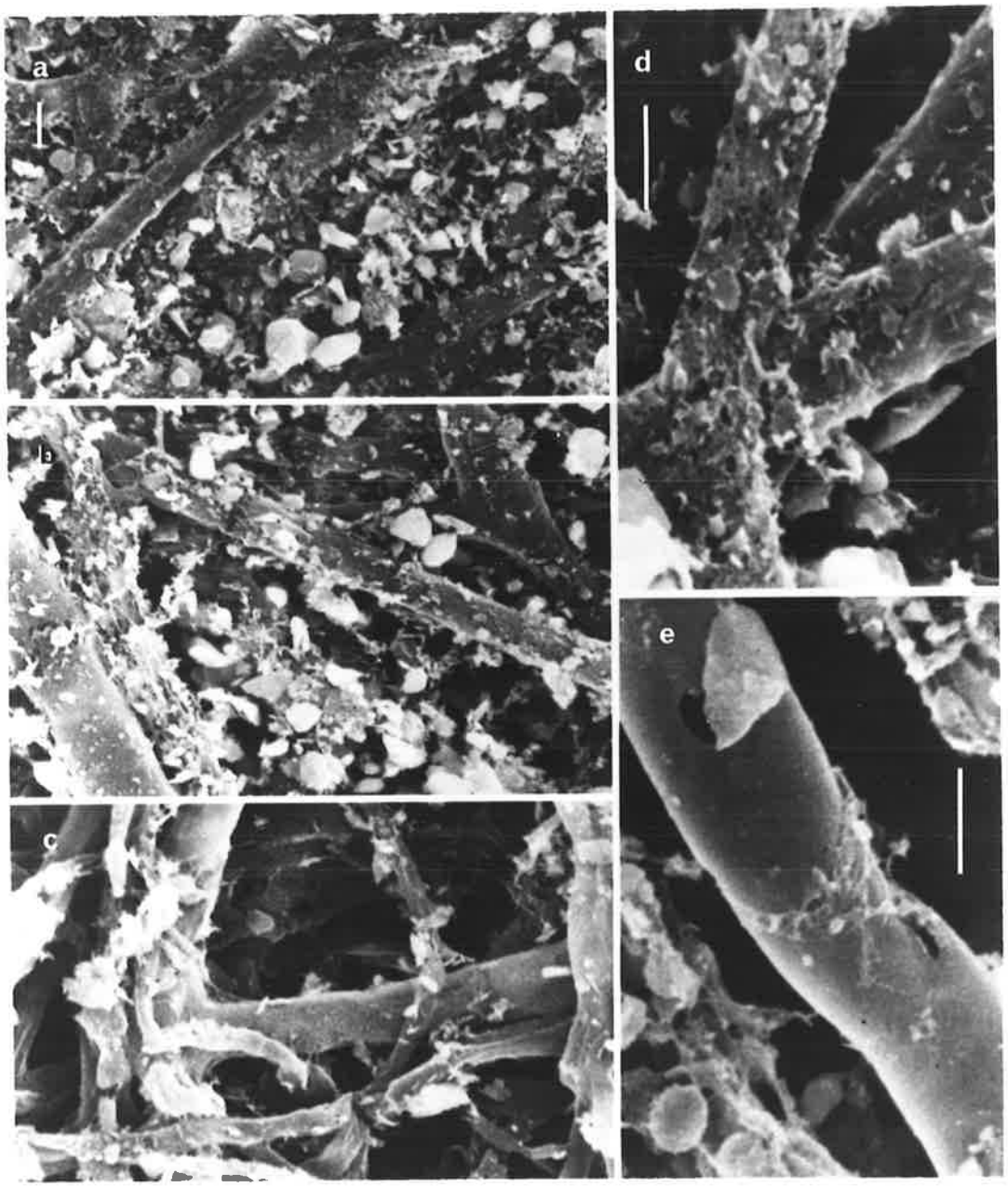

a
b
c
d
e

TABLE 12: Percentage occurrence of amoebae with *Ggt* hyphae buried in soils.

Amoeba	Soil	Weeks after burial						
		1	2	3	4	5	6	8
Mycophagous Leptomyxids	WPP	8	0	17	8	0	17	17
	MB	0	0	0	0	0	0	0
	CS	0	0	0	0	0	8	8
Mayorella spp.	WPP	0	8	0	0	0	8	33
	MB	0	0	0	0	0	0	0
	CS	0	0	0	0	0	0	0
Saccamoeba spp.	WPP	0	0	0	8	0	8	0
	MB	0	0	0	0	0	0	0
	CS	0	0	0	0	0	0	0
T.granifera ssp. *minor*	WPP	0	17	0	0	0	0	0
	MB	0	0	0	0	0	0	0
	CS	0	0	0	0	0	0	0
Non-mycophagous *Acanthamoeba* spp.	WPP	17	75	33	8	8	25	50
	MB	0	0	0	0	25	8	25
	CS	17	0	25	75	100	100	93
Echinamoeba spp.	WPP	8	17	8	0	0	0	25
	MB	0	0	0	0	25	8	58
	CS	0	0	8	0	0	0	0
Platyamoeba spp.	WPP	8	25	17	17	0	17	75
	MB	0	0	0	0	0	0	17
	CS	0	0	0	0	0	0	0
Thecamoeba spp.	WPP	0	25	8	8	0	8	8
	MB	0	0	0	0	0	0	17
	CS	0	0	0	0	0	0	0
Unidentified	WPP	25	58	33	50	8	58	8
	MB	0	0	0	25	50	0	0
	CS	0	0	0	0	0	0	0
Vampyrellids	WPP	0	25	0	17	0	58	33
	MB	0	0	0	0	17	8	25
	CS	0	0	0	0	0	0	0

Saprophytic survival of *Ggt* was reduced in the suppressive WPP soil. In both WPP and MB soils there was an increase in the density of pigmented hyphae during the first 4 weeks but thereafter, hyphal density in the WPP soil was reduced significantly although both MB and CS had progressively higher densities.

In Garrett's (1938) experiments, two heavy textured clay loams encouraged a high rate of inoculum decline in contrast to a majority of infertile soils. Although this is thought to be due to greater numbers of microorganisms rather than to physical effects of aeration etc. and pH, humus content or soil classification (Zogg, 1959); soil nutrient levels, on the other hand, including those of N (Garrett, 1938; Butler, 1953, 1959; Chambers and Flentje, 1969; Scott, 1969; Weste and Thrower, 1971) and P (Chang, 1939; Butler, 1961) are known to influence the saprophytic survival of the fungus. Since the MB soil is lighter in texture and hence lower in nutrients, a second non-suppressive soil from the permanent continuous wheat rotation (CW) was included in a similar experiment using the membrane filter burial technique. Both WPP and CW are red-brown earths with similar pH, textural and nutritional (C, N and P) characteristics (Steward and Oades, 1972).

Amoebae and saprophytic survival of *Ggt* hyphae in WPP, CW, MB and CS

Ggt was buried in these soils and five replicate membranes were retrieved at 2,4,6,8,12,14 and 20 weeks after burial. At recovery, densities of both hyaline and pigmented hyphae were estimated, survival of the fungus studied and amoebal association with the fungus determined.

Though only for two more weeks in the CW soil, *Ggt* in general, survived longer in the non-suppressive soils than in the suppressive WPP soil (Fig.26a). In the WPP soil, the fungus survived for less than 4 weeks. Among the non-suppressive soils, *Ggt* survived for up to less than 12 weeks in the light-textured MB soil, whereas for less than 6 weeks in the heavier CW soil.

In all soils there was a reduction in the density of total hyphae over time (Fig.26b); although the rate of decline was significantly ($P<0.01$) different. For hyaline hyphae, the rate of decline of their density was similar in the WPP and CW soils and very little hyphae were left on membranes after 6 weeks of burial (Fig.26c). Fitted regression lines of the densities of hyaline hyphae over time was linear in CS and curvilinear with initial faster rates of decline in all other soils.

Densities of pigmented hyphae increased initially and thereafter declined in all soils at significantly ($P<0.01$) different rates (Fig.26d). Interestingly enough, the regression line for the density of pigmented hyphae in the CW soil was linear, whereas those in the other three soils were curvilinear with an initial faster rate of pigmentation. Therefore, the rate of destruction of hyaline hyphae was similar in WPP and CW soils, but that of the pigmented hyphae was significantly higher in the WPP soil than in the CW soil.

Initially, up to 4 weeks, higher associations of mycophagous amoebae were recorded with *Ggt* hyphae recovered from the WPP soil, but none after 8 weeks (Tables 13 and 14). This higher association of mycophagous amocbac coincided with live hyphae of the fungus in the WPP soil and mycophagous amoebae were not isolated after 8 weeks of burial when viable hyphae of the fungus could not be detected. No such relationship

Fig.26: Density and survival of *G.graminis tritici* hyphae in suppressive Waite permanent pasture and non-suppressive continuous wheat and Murray Bridge soils.

●—● Waite permanent pasture soil.

△—△ Continuous wheat soil.

▲—▲ Murray Bridge soil.

○—○ Sterilized coarse sand.

a. Saprophytic survival of the fungus in soils.

b. Density of total hyphae.

c. Density of hyaline hyphae.

d. Density of pigmented hyphae.

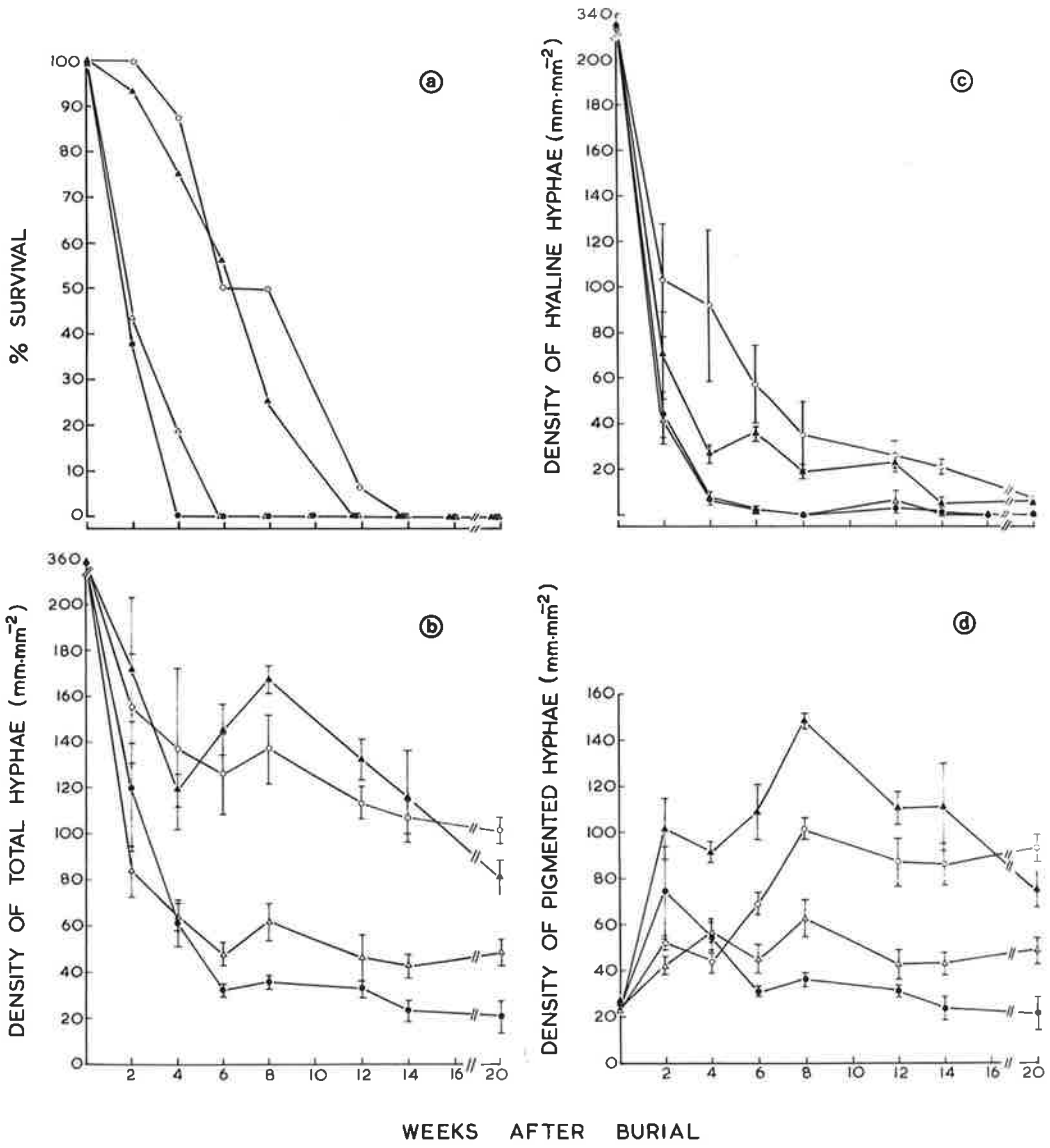

a
% SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
c
DENSITY OF HYALINE HYPHAE (mm·mm⁻²)
340
200
180
160
140
120
100
80
60
40
20
b
DENSITY OF TOTAL HYPHAE (mm·mm⁻²)
360
200
180
160
140
120
100
80
60
40
20
0
d
DENSITY OF PIGMENTED HYPHAE (mm·mm⁻²)
160
140
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
20
WEEKS AFTER BURIAL

TABLE 13: Percentage occurrence of amoebae with *Ggt* hyphae buried in suppressive (WPP) and non-suppressive (CW, MB, CS) soils.

Amoeba	Soil	Weeks after burial						
		2	4	6	8	12	14	20
Mycophagous								
Leptomyxids	WPP	25	25	0	6	0	0	0
	CW	0	0	0	0	0	0	0
	MB	0	0	0	12	6	0	0
	CS	0	0	0	0	18	0	18
Mayorella spp.	WPP	0	12	0	6	0	0	0
	CW	12	0	0	6	0	6	12
	MB	0	18	0	0	0	0	0
	CS	0	0	0	0	0	0	0
Saccamoeba spp.	WPP	0	6	0	0	0	0	0
	CW	0	0	0	0	0	0	0
	MB	0	0	6	0	0	6	0
	CS	0	0	0	0	6	0	25
Thecamoeba granifera ssp. *minor*	WPP	25	25	0	12	0	0	0
	CW	25	0	6	0	12	6	37
	MB	37	0	6	31	0	0	12
	CS	0	0	0	6	43	25	37
Non-mycophagous								
Acanthamoeba spp.	WPP	31	12	6	0	0	57	37
	CW	12	0	6	31	0	6	43
	MB	81	81	62	100	75	68	81
	CS	100	100	81	68	93	93	100
Echinamoeba spp.	WPP	25	18	0	12	0	28	0
	CW	12	0	12	6	0	18	18
	MB	6	43	43	18	12	25	6
	CS	0	25	12	31	0	25	25
Platyamoeba spp.	WPP	37	25	6	6	0	21	6
	CW	12	0	6	31	18	6	25
	MB	81	50	81	81	31	68	75
	CS	0	31	25	6	0	6	6
Thecamoeba spp.	WPP	6	12	0	0	0	0	0
	CW	0	0	0	12	0	6	12
	MB	0	0	0	12	0	0	0
	CS	0	0	0	6	12	50	0
Vampyrellids	WPP	12	25	0	6	0	21	0
	CW	18	0	0	0	18	6	0
	MB	81	87	62	81	62	62	37
	CS	0	0	6	0	0	0	0
Unidentified	WPP	37	43	6	6	0	42	75
	CW	43	0	31	37	43	56	50
	MB	62	87	62	68	62	62	68
	CS	6	0	56	68	43	43	43

TABLE 14: Summary of amoebae isolated from *Ggt* hyphae buried in suppressive (WPP) and non-suppressive (CW, MB and CS) soils. (Percentage of 1 sq.mm millipore membrane plated).

Soil	Weeks after burial													
	2		4		6		8		12		14		20	
	A[a]	M[b]	A	M	A	M	A	M	A	M	A	M	A	M
WPP	19	25	20	27	1	0	5	3	0	0	20	0	13	0
CW	13	18	0	0	6	10	13	0	9	15	11	5	20	18
MB	35	10	35	0	32	3	40	10	25	25	29	2	28	4
CS	10	0	15	0	18	0	18	3	21	40	24	10	25	31

[a] = all amoebae
[b] = mycophagous amoebae.

was observed in the non-suppressive CW and MB soils. A lower percentage of mycophagous amoebae, compared to that in the WPP soil, was found associated with the fungus in the CW and MB soils throughout the experimental period of 20 weeks; even though no viable *Ggt* was recovered from after 6 weeks in CW and 12 weeks in MB soil.

These results suggest that soil type may be of some importance but by itself can not fully explain the reduced saprophytic survival of the fungus in the suppressive WPP soil. As in other soils (Garrett, 1938; Zogg, 1959), the saprophytic survival of the fungus in these soils is related to cropping history and greater number of microorganisms which may directly or indirectly be influenced by the soil physical factors, since the biological and physical settings of the soil are inseparable when considering the behaviour of soil microbes (Stotzky and Rem, 1966). The differential survival and reduction of hyphal densities of the fungus in the otherwise physically similar WPP and CW soils further suggest that soil suppressiveness was associated with the reduced survival of the fungus in the WPP soil. Furthermore, the abundance and activities of mycophagous amoebae coincided with the decline in densities and survival of the fungal hyphae in the suppressive WPP soil. In TAD soils also, intensified development and activity of microflora cause reduced survival of inoculum and result in fewer infective particles (Gerlagh, 1968; Pope and Jackson, 1973; Vojinovic, 1973).

Saprophytic survival of *Ggt* in sterile soils amended with suspensions of mycophagous amoebae

Mycelia of *Ggt* were buried in sterile (autoclaved for 1 h for 3 consecutive days at 121^{o}C) WPP and MB soils containing a mixture of

suspensions (about 10 ml plate^{-1}) of three mycophagous amoebae, *Saccamoeba* sp., *Gephyramoeba* sp. and the unidentified leptomyxid. In an initial experiment, *Thecamoeba granifera* sub-species *minor* was included with these three amoebae, but as the *Fusarium*-like fungus associated with this amoeba almost always overgrew *Ggt* on NDY/6 plates, this was excluded from the test. Controls were maintained with sterilized soils containing suspensions of the food bacterium, *Klebsiella* sp., sterilized soil only and unsterile WPP and MB soil. Three replicate membranes were retrieved 1,2,4 and 8 weeks after burial and 1 mm squares were plated on NDY/6.

Results show that there was a slight decrease in the survival of the fungus after 2 and 4 weeks of burial in sterile WPP soil in the presence of the amoebae (Table 15). In the non-suppressive soil however, there was no loss of viability even after 8 weeks in the presence of the amoebae. The bacterium did not affect the survival of the fungus but the fungus failed to survive in either of the sterile soils after 8 weeks. Reduction in the saprophytic survival as before, was observed in the unsterile WPP and MB soils.

Steam sterilization alters both the organic and mineral fractions of soils (Eno and Popenoe, 1964; Sonneveld, 1979). The first colonisers can increase in previously sterilized soil to high levels because of the release of nutrients and reduced competition (Johnson and Curl, 1972). In fumigated soil however, there is a slower recolonisation by *Ggt* as measured by its incidence on wheat roots, compared to that in the untreated soil (Warcup, 1976). Ludwig and Henry (1943) reinfested sterile soil with *Ggt*; when recontaminated by additions of small amounts of field soil, the total microflora including some antagonists of the take-all fungus quickly increased in density to a higher level than in

TABLE 15: Saprophytic survival of *Ggt* in sterile suppressive (WPP) and non-suppressive (MB) soils amended with suspensions of mycophagous amoebae (% survival).

Weeks after burial	Soils							
	WPP				MB			
	Unstr.[a]	Str.[b]	Kleb.[c]	Mix.[d]	Unstr.	Str.	Kleb.	Mix.
1	85	100	70	95	80	100	100	100
2	80	0	100	85	100	95	100	100
4	50	45	100	55	80	0	100	100
8	5	0	100	95	90	0	60	100

[a] = Unsterilised
[b] = Sterilised
[c] = Sterilised soil + suspension of *Klebsiella* sp.
[d] = Sterilised soil + mixed suspension of *Saccamoeba* sp., *Gephyramoeba* sp. and the unidentified leptomyxid.

unsterile soil. Effects of altered physical and chemical status of the sterile soils and absence of other microorganisms may have a bearing on the interactions between the take-all fungus and the mycophagous amoebae tested; since the precarious balance of microbial interactions in many cases only work in biologically balanced soils (Baker and Cook, 1974; Bowen and Rovira, 1976).

Ability of mycophagous amoebae to invade infected wheat roots

Darbyshire and Greaves (1971) found that the bacteriophagous soil amoeba *Acanthamoeba palestinensis* (Reich, 1933) invaded the epidermis and outer cortical tissues of pea roots when inoculated with a *Pseudomonas* sp. Since no amoebae were found inside roots inoculated only with *A.palestinensis*, the invasion was explained as a passive entry of trophozoites inside roots following the food bacterium. A report on the presence of *Leptomyxa reticulata* Goodey, 1915 var. *humuli* McLennan, 1930 inside tissues of hop plants was published by McLennan (1930). Tuzet and Roquerol (1961, 1962) also recorded the presence of amoebae inside roots of rice plants.

Besides killing ectotrophic hyphae on live roots, invasion of infected wheat roots by mycophagous amoebae in natural soil may enable amoebae to perforate and lyse the fungal hyphae inside host residues and thereby reduce the saprophytic inoculum. An attempt was therefore made to test if mycophagous amoebae were capable of invading infected wheat roots.

Four weeks old seminal roots of wheat infected with *Cgt* were obtained from a bioassay and about 2 cm pieces were incubated at 25^{o}C with a mixed

AS suspension of *Gephyramoeba* sp., *Saccamoeba* sp., *Thecamoeba granifera* sub-species *minor* and the unidentified leptomyxid. After 3 weeks roots were surface sterilised with one of the following sterilants, washed twice in SDW and plated on PJA for growth of amoebae. Alternatively, infected wheat roots were obtained from a bioassay in which a mixture of suspensions of these amoebae were added to CS and wheat was grown for 4 weeks. Pieces of these roots were surface sterilised and plated as previously. Surface sterilants used were:

1). Actidione 200 µg ml^{-1} for 5 min;

2). $HgCl_2$ 0.1% solution for 5 min;

3). $AgNO_3$ 0.1% solution for 5 min and washed with 0.5% NaCl;

4). C_2H_5OH 70% solution for 5 min;

5). Milton solution (sodium hypochlorite 1% and sodium chloride 16.5%, Milton Pharmaceutical Company, Sydney) full strength for 5 min.

Controls were washed with SDW only.

The unidentified leptomyxid was isolated from roots treated with all of the sterilants (Table 16) and *Saccamoeba* sp. was absent only in 0.1% $AgNO_3$ treated roots. *T.granifera* sub-species *minor* and *Gephyramoeba* sp. were not isolated from roots treated with 70% C_2H_5OH, 0.1% $HgCl_2$ or 0.1% $AgNO_3$. Since the *Saccamoeba* sp. is non-cystic, the results can not be explained on the basis of higher resistance of cysts towards these chemicals. The results suggest that mycophagous amoebae were able to invade wheat roots infected with the take-all fungus.

TABLE 16: Percent recovery of amoebae from surface sterilised wheat roots infected with take-all.

Sterilant	Amoeba recovered			
	Unidentified Leptomyxid	*Gephyramoeba* sp.	*T.granifera* ssp. *minor*	*Saccamoeba* sp.
Actidione	100	60	90	60
Milton	10	5	5	5
C_2H_5OH	10	0	0	10
$HgCl_2$	16	0	0	5
$AgNO_3$	33	0	0	0
Control (SDW)	100	85	95	95

Discussion

The majority of saprophytic inoculum of *Ggt* originates from previously infected host tissue (Garrett, 1956, 1970); although the possibilities of free-living mycelium has certainly been indicated (Fellows and Ficke, 1939; Warcup, 1957). While alive in plant debris, it is able to respond to the presence of growing roots by limited growth in the rhizosphere (Brown and Hornby, 1971; Pope and Jackson, 1973; Wildermuth, 1977). The fungus can also grow in soil in the absence of wheat roots (Brown and Hornby, 1971; Wildermuth *et al.*, 1979). Thus, root-inoculum contact (Garrett, 1936) is not always necessary for colonisation of roots and the fungus can colonise wheat roots from a zone 9-14 mm away from roots in 28 days (Wildermuth, 1977). The rate of growth of hyphae through soil was calculated to be about 0.4 - 0.6 mm day^{-1}, which is less than the rate of ectotrophic growth on roots (2.0 - 4.4 mm day^{-1}, Wildermuth, 1977).

Growth of *Ggt* is initiated by hyaline hyphae which may later become pigmented. After burial, within one week, hyphae became pigmented in all soils, the rate of pigmentation varying with the soil. Pigmentation of hyphae protects them in soils and the fungus present in naturally-infested soil apparently survives longer than in artificially-colonised straw because of the presence of dark hyphae (MacNish, 1976; Smith, 1979). Cunningham (1975) also found that dark hyphae had strong saprophytic capacity. This pigment, melanin or melanin-like compounds in fungal cell-walls, imparts resistance to microbial lysis (Lockwood, 1960; Lingappa *et al.*, 1963; Bartnicki-Garcia and Reyes, 1964; Kuo and Alexander, 1967). Melanin extracted from hyphae of *Ggt* is known to inhibit specific lysis of the fungus by *Streptomyces lavendulae* (Waksm. and Curt.) Waksm and Henr. and less lysis has been observed in darker isolates (Tschudi and Kern, 1979).

Of the two soils, WPP and CW, with similar physical and chemical properties used in my experiments, the suppressive WPP soil showed a faster rate of destruction of pigmented hyphae than the CW soil. The density of hyaline hyphae, on the other hand, was reduced at a similar but much faster rate than that of the pigmented hyphae in both of these soils. One difference between these soils, therefore lies in the rate of destruction of pigmented hyphae which are responsible for the survival of the fungus. Considering the growth of the fungus from an inoculum source in soil to the host root, propagules with large reserves may grow several centimetres to the root (Wildermuth, 1977; Brown and Hornby, 1971) and this period of pre-colonisation is apparently the time when competition with *Phialophora* takes place (Deacon, 1976), and *Pseudomonas fluorescens* Migula restricts its growth (Cook and Rovira, 1976). The demonstration that soil amoebae can produce cellulase and chitinase makes them capable of lysing hyaline hyphae of *Ggt* (Tracey, 1955). On the other hand, to my knowledge, soil amoebae are the only group of organisms capable of lysing melanised fungal propagules (Old, 1977a; Anderson and Patrick, 1978, 1980).

The WPP soil showed a higher association of mycophagous amoebae with *Ggt* hyphae for the initial 4 weeks when the fungus was alive in this soil. The walls of many hyphae were perforated. Similar perforations caused by mycophagous amoebae have been recorded before in hyphal walls of the fungus recovered from a TAD soil (Homma *et al.*, 1979).

B. POPULATIONS OF AMOEBAE IN SUPPRESSIVE AND NON-SUPPRESSIVE SOILS

Several of the theories proposed to explain TAD invoke the development of an antagonistic microflora which suppresses the fungus. Vojinovic (1973) reported selective stimulation by infected wheat roots of rod-shaped non-sporing bacteria in TAD soils; Cook and Rovira (1976) and Smiley (1979) found a highly specific group of fluorescent pseudomonads; Zogg and Jaggi (1974) reported an actinomycete with intense hypholytic activity; and Slope *et al.* (1978) reported the occurrence of *Phialophora radicicola* Cain var. *graminicola* Deacon predominantly in TAD soils. Brown *et al.* (1973) suggested that TAD operated through changes in the soil microflora which modified the root environment and limited the disease nutritionally.

These reports suggest that the diversity and magnitude of microbial populations in suppressive and non-suppressive soils are different and an attempt was therefore made to study the composition and population of mycophagous and other amoebae in suppressive and non-suppressive soil.

The dilution culture technique originated by Cutler (1920) and developed by Singh (1946a, 1955) has been widely used for estimating numbers of naked amoebae in soils (Heal, 1971). Singh (1946a) estimated the method to be 64-73% efficient. Darbyshire *et al.* (1974) introduced the microtiter dilution plate as a modification to the dilution culture method of Singh (1955); this technique is rapid, requires fewer plates and is suitable for soil protozoa in general. Using a selective medium, Pussard *et al.* (1979) estimated the population of the strictly mycophagous *Thecamoeba granifera* sub-species *minor* and compared the efficiency of this 'soil weights technique' with that of the dilution

culture method of Singh (1955). They considered their technique appropriate for amoebae like *T.granifera* sub-species *minor*, which have a population density of less than 100 amoebae g^{-1} dry wt of soil (Alabouvette *et al.*, 1981) and which are strictly mycophagous in nature. The mycophagous amoebae reported in this thesis and elsewhere (Old, 1977a; Anderson and Patrick, 1978, 1980) are able to feed on several other organisms in addition to fungi.

Soils

Of the seven major 'great soil groups' of the Australian wheat growing zones, solonised brown soils cover the widest areas, nearly 27% (McGarity, 1975). Two wheat fields, one from Balaklava (Avon) and the other from near Murray Bridge (MB), South Australia, were selected to represent two sub-groups of this major soil group. The third soil was the red-brown earth, WPP (Northcote, 1981). The WPP soil is suppressive to *Ggt* (Wildermuth, 1977) and the other two are non-suppressive (Table 4, page 18). Classification, particle size distribution, pH, cropping history and moisture characteristic curve of each soil have been recorded earlier (pages 10 and 11).

Fields were subdivided into grids; approx. 10m x 10m for Avon and MB and 2m x 2m for WPP, and about 2 kg of soil from the top 0-5 cm was collected from each of the 20 intercepts. Samples were stored in sealed plastic bags at room temperature. Soil moisture contents of the soils at sampling were:

Avon - 1.7%
MB - 0.6% (oven dry wt. basis).
WPP - 2.2%

Consideration of methods

One aim of the present investigation was to determine the populations of different genera of soil amoebae and thereby calculate the population of mycophagous genera in these soils. The modified soil dilution technique of Cutler (1920) (Cutler *et al.*, 1922) offers the opportunity of both examining and estimating the population of different genera on agar plates. However, as Singh (1955) pointed out, Cutler's method has three disadvantages: (1) insufficient replications; (2) likelihood of missing some of the protozoan species when the whole surface of the Petri dish has to be examined, and (3) the random selection of food organisms which may happen to grow on the plate - this is particularly relevant as many soil bacteria are not only inedible to protozoa but produce secretions toxic to them (Singh, 1945). With Singh's (1955) ring method, however, identification of amoebae is difficult when they are present inside a thick bacterial suspension; on the other hand, identification is relatively easier on a clear agar surface as can be obtained with Cutler's (1920) method.

The soil dilution method of Cutler *et al.* (1922) was therefore used with modifications. A suspension of the food bacterium, *Klebsiella* sp., was spread on PJA and five replicates of each of the 10-fold dilutions were plated. Two-fold and four-fold dilution series are however, more accurate than ten-fold series (Singh, 1946a, 1955; Stevens, 1958), and Singh (1946a) recommended the use of 15 levels of two-fold series with 8 replicates at each level. This would involve the examination of 120 plates per soil sample if the dilution culture method (Cutler *et al.*, 1922) is used.

In mineral soils protozoa can be suspended in soil dilutions by shaking (Singh, 1946a; Darbyshire and Greaves, 1967) but in litter or organic soils protozoa may be trapped in the larger organic particles. Heal (1971) found that estimates of numbers of amoebae in litter increased four-fold with light homogenising which disintegrated the litter. To study the distribution of amoebae in various fractions of the WPP soil, 2 g each from three samples was suspended in 100 ml AS and sieved through a 60 mesh sieve. Several particles larger than 500 µm in size were plated on PJA + *Klebsiella* plates at a rate of two particles plate^{-1}. The remaining soil suspension was allowed to settle for about 5 min and 0.5 ml of the supernatant was plated on further PJA + *Klebsiella* plates. Portions of the sediment were similarly plated using a wire loop. Ten replicate plates of each treatment were examined for amoebae after two weeks at 25^{o}C. *Acanthamoeba* and *Platyamoeba* were isolated from all fractions; the unidentified leptomyxid was absent from the 60 mesh fraction and *Thecamoeba* and the unidentified vampyrellid amoeba were not isolated from the soil suspension (Table 17). In determining the populations of amoebae, the soil suspensions were therefore, agitated for about 2-3 min using a 'Vortex' shaker and immediately plated with a wide-mouthed pipette to include all fractions.

Pussard *et al.* (1979) used neomycin (10-100 µg ml^{-1}) and rifamycine (100 µg ml^{-1}) for the selective isolation of a strictly mycophagous amoeba . In my studies, concentrations of 10,50,100 or 200 µg ml^{-1} of neomycin did not have any selective effect on either mycophagous or non-mycophagous amoebae as a group. The selective effect of rifamycine was studied by using 10,50,100 and 200 µg ml^{-1} of this antibiotic incorporated PJA medium to assess the population of mycophagous and other amoebae

TABLE 17: Isolation of amoebae from various fractions of the WPP soil (Percentage of fragments plated).

Amoeba	Fraction								
	60 Mesh			Soil suspension			Sediment		
	A*	B	C	A	B	C	A	B	C
Acanthamoeba spp.	75	100	100	80	100	60	100	80	100
Platyamoeba spp.	62	33	100	20	40	80	20	60	20
Thecamoeba spp.	12	17	17	0	0	0	20	40	0
Leptomyxid	0	0	0	20	20	0	40	20	20
Vampyrellid	12	33	0	0	0	0	20	40	40

*soil sample.

from three samples of the WPP soil by the modified soil dilution culture technique (Cutler *et al.*, 1922).

Five g soil was suspended in 50 ml AS in a conical flask, shaken and 1 ml of this suspension was diluted with 9 ml AS in a test tube using a wide-mouthed pipette and the tube shaken. In this way, the original soil was serially diluted to 10^{-1}, 10^{-2} and 10^{-3}. Immediately after agitation, 50 µl of each dilution was plated on each of five replicate PJA + *Klebsiella* plates incorporated with rifamycine. Control plates were without antibiotic. Plates were examined for amoebae after 2 weeks at 25°C. Populations were estimated by the most probable number technique (Halvorson and Ziegler, 1933) using the values given by Taylor (1962) to correspond to the number of plates showing positive growth. Populations were expressed in terms of number g^{-1} dry wt of soil.

Results show that all concentrations of rifamycine significantly reduced the populations of total amoebae (Table 18). Highest populations of mycophagous amoebae were obtained in the control and 50 µg ml^{-1} treatment of the antibiotic; but this decreased significantly in all other concentrations.

It appears that rifamycine does not have a selective effect on all mycophagous amoebae. However, the combined effect of neomycin and rifamycine in 1% malt extract agar medium as used by Pussard *et al.* (1979) was not studied.

Non-selective media give wider range of species but smaller numbers than selective media (Dixon, 1937; Stout, 1956) and it is assumed that

TABLE 18: Population estimates of amoebae from the WPP soil obtained by plating samples on rifamycine incorporated PJA medium.

Amoeba	Rifamycine ($\mu g\ ml^{-1}$)				Control
	10	50	100	200	
Mycophagous	338	549	249	83	541
Others	5947	1696	810	703	8175
Total	6285	2245	1059	786	8716

Least significant difference:

	Total amoebae	Mycophagous amoebae
P = 0.05	944.4	124.6
P = 0.01	1440.5	190.0

non-selective media provide representative samples of the species active in soil (Heal, 1970); the use of a solid agar medium, however, select amoebae rather than free-swimming ciliates.

Populations of amoebae in the soils

Populations of different genera in these soils were estimated by the modified soil dilution technique (Cuter *et al.*, 1922) described earlier. In determining populations by genus, *Gephyramoeba* and the unidentified leptomyxid were grouped together as leptomyxids and included with *Saccamoeba* and *T.granifera* sub-species *minor* to calculate the population of mycophagous amoebae. A number of species of *Mayorella*, apparently distinguishable by trophozoite dimensions, were recorded in some soil samples. So far only one species of this genus has been found to be mycophagous and as it was not possible to distinguish the mycophagous species on soil plates, Mayorellas were not included in the mycophagous group. *T.granifera* sub-species *minor*, due to the relative ease of its identification, could be recorded separately from other *Thecamoeba* species.

Data on the mean generic populations are presented in Table 19; populations per sample are provided in Appendix IIA. The populations of both mycophagous and other amoebae were significantly ($P<0.01$) higher in the suppressive WPP soil than in the non-suppressive MB and Avon soils; whereas, population differences between the non-suppressive soils were not. The cumulative population of leptomyxids, *T.granifera* sub-species *minor* and *Saccamoeba* was between 40 and 1796 in the WPP soil, 0-540 in the Avon soil and 0-180 in the MB soil. The frequency of occurrence of the mycophagous genera in the soil samples was higher in

TABLE 19: Populations of various genera of amoebae in WPP, Avon and MB soils (mean of 20 samples).

Amoeba	Number g^{-1} dry wt of soil		
	WPP	Avon	MB
Acanthamoeba spp.	1360	1280	467
Echinamoeba spp.	4609	380	677
Leptomyxids	99	105	48
Mayorella spp.	19	18	39
Platyamoeba spp.	807	518	651
Saccamoeba spp.	220	4	2
Thecamoeba spp.	92	52	52
T.granifera ssp. *minor*	71	14	23
Unidentified	6002	280	443
Vampyrellids	200	53	132
Total	13280	2709	2537
Mycophagous	394	124	73

Least significant difference

	Mycophagous	Total
P = 0.01	189.95	5899
P = 0.05	132.81	4124

the suppressive WPP soil than in the non-suppressive soils (Fig.27). The non-mycophagous genera, except for *Mayorella*, on the other hand, were distributed throughout the majority of samples of all three soils.

Populations of *Ggt* in the survey soils

Being a root-inhabiting fungus, *Ggt* occupies the host tissue during its parasitic phase (Garrett, 1956, 1970) and usually saprophytic inoculum originates in this manner (Lucas, 1955). Natural inoculum in soils is capable of causing infection to a wheat crop after two years summer fallow or two crops of oats or clover (Broadfoot, 1934b; Russel, 1934; Glynne, 1935). In some fields, MacNish and Dodman (1973) found that over a year there was only a small drop in the incidence of *Ggt* on stubble. Shipton (1972) found the fungus surviving in soil under a non-susceptible break crop for up to 66 months. A range of grass species, cultivated and wild, can support the growth of the fungus (Russel, 1934; Padwick, 1935; Garrett, 1941) and the susceptibility of a grass species is related to the saprophytic perpetuation of the fungus (Chambers and Flentje, 1968; Chambers, 1971). The fungus is associated especially with organic fragments (Gams and Domsch, 1967) larger than 420 µm (Hornby, 1968), and a seedling baiting technique has been used to estimate the number of infective units (Hornby, 1971).

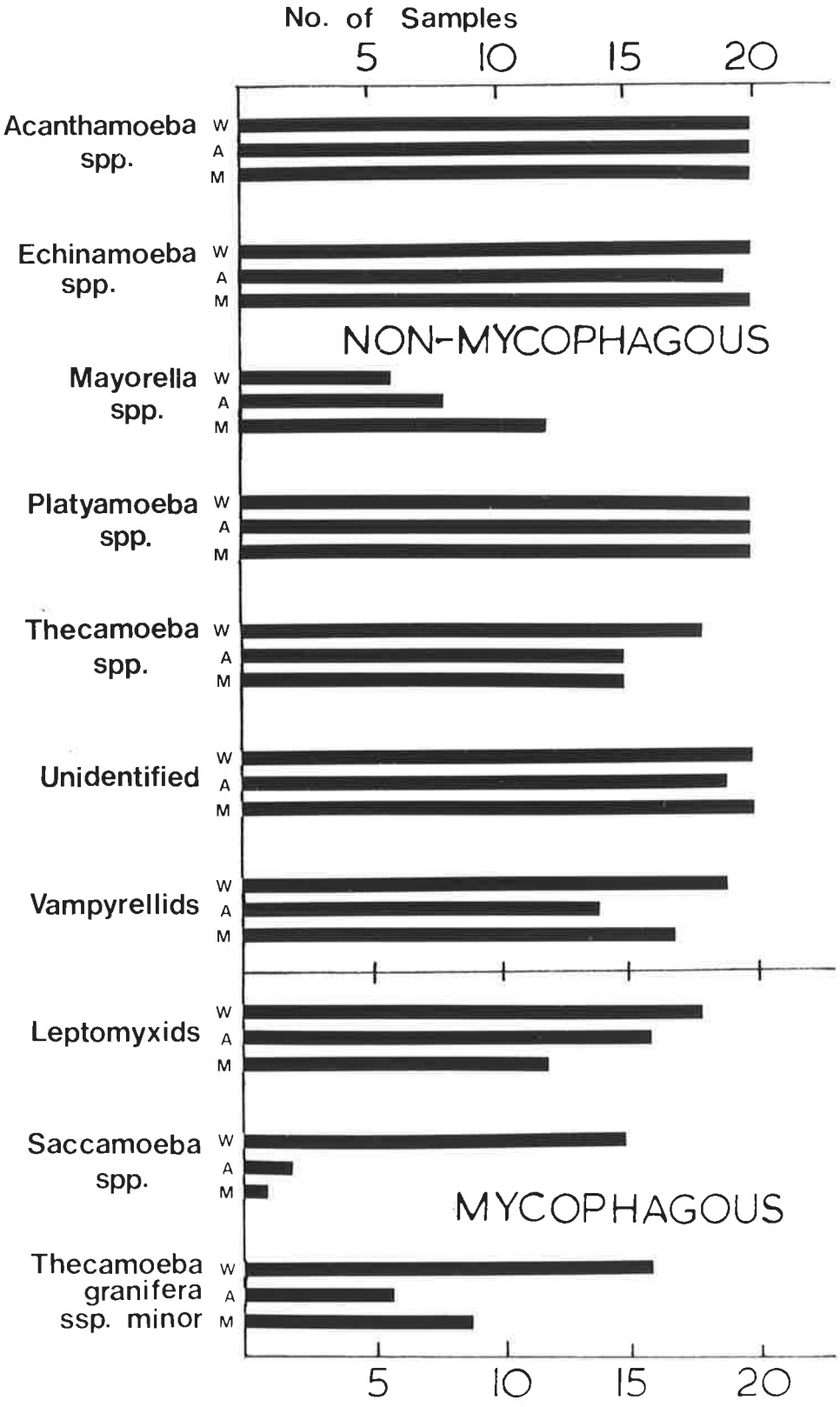
Populations of *Ggt* from one sample of each of the survey soils was assessed using the seedling baiting technique with modifications. Wheat seeds of the cultivar Halberd were washed with distilled water and surfaced sterilised with full strength Milton solution for 10 min. Seeds were washed twice in SDW and placed on moist filter paper in Petri plates and incubated for 60 h at 15^{o}C for germination. PVC conduits

Fig.27: Distribution of genera of amoebae in samples of suppressive and non-suppressive soils.

W = Waite permanent pasture soil.

A = Avon soil.

M = Murray Bridge soil.


No. of Samples
5
10
15
20
Acanthamoeba spp.
Echinamoeba spp.
Mayorella spp.
Platyamoeba spp.
Thecamoeba spp.
Unidentified
Vampyrellids
Leptomyxids
Saccamoeba spp.
Thecamoeba granifera ssp. minor
W
A
M
NON-MYCOPHAGOUS
MYCOPHAGOUS

(2.7 cm internal diameter and 12.5 cm long) were half-filled with CS. About 10 g of a test soil was added on top of the CS in each PVC conduit and one pre-germinated seed was sown. Seeds were covered with a thin layer of CS and watered with tap water. One hundred conduits, distributed among three metal trays were used for each soil sample. After 4 weeks, plants were harvested, roots washed and examined for *Ggt*. The percentage of infected seedlings in these soils were:

WPP - 2.0
MB - 1.0
Avon - 3.0.

Of the three soils, WPP had not had a wheat crop for the last 27 years. Although some grasses can maintain the fungus (Russel, 1934; Padwick, 1935; Garrett, 1941), the population does not reach a high level due to the low competitive saprophytic ability (Garrett, 1970) of the fungus. Avon had a direct drilled wheat crop followed by pasture in 1979 and MB soil had wheat in 1979 after two years of pasture preceded by barley (page 10).

The WPP soil is finer in texture and has a higher water-holding capacity than the Avon and MB soils. The higher populations of both mycophagous and other amoebae in this soil may be partly due to these physical factors.

Effect of soil moisture on amoebal activity

The activity of mycophagous and other amoebae at five different soil moisture tensions was studied in the WPP and MB soils using the membrane filter burial technique (Old, 1977b) described earlier. Activity was the ability of amoebae to move through the 5.0 µm pores of the nuclepore filters which could be detected by their isolation from the internal millipore filter.

Soils passed through a 2 mm sieve and moistened to approx. -1 kPa were placed in plastic conduits (6.5 cm long and 5.5 cm internal diameter). Millipore filters deposited with homogenised hyphal suspensions of *Ggt* were buried in moist soils and suctions of 1,5,10, 100 and 1500 kPa were applied for two weeks.

Five replicate membranes were retrieved from each treatment. Each membrane was cut into 8 approx. 1 sq mm pieces and four of these were plates on PJA + *Klebsiella* plates and incubated for the growth of amoebae. The rest of the membrane squares were plated on NDY/6 medium incorporated with 100 µg streptomycin ml^{-1} and 10 µg tetracycline ml^{-1} and the percentage survival of *Ggt* was calculated from the number of pieces showing growth of the fungus after 48 h at 25°C.

Amoebal activity was detected within the range of -1 kPa to -100 kPa in the WPP soil and between -5 kPa to -100 kPa in the MB soil (Table 20). No amoebae grew from membranes recovered from either soil maintained at -1500 kPa tension. Recovery of mycophagous amoebae was affected at -100 kPa in the WPP soil although not statistically significant. *Ggt* survived over a range of soil moisture tensions in both soils (-1 to -100 kPa, Table 20). In the MB soil, the fungus survived equally well at all tensions except at -1500 kPa; whereas, in the WPP soil, maximum survival was recorded at -100 kPa and a significant ($P<0.01$) reduction was observed at all other tensions. Survival of *Ggt* was apparently inversely correlated with activity of mycophagous amoebae in both soils and an increase in recovery of the mycophagous amoebae almost always corresponded with a reduced survival of the fungus (Table 20).

TABLE 20: Association of amoebae with *Ggt* hyphae and survival of the fungus in the WPP and MB soils maintained at various soil moisture tensions for 2 weeks.

Moisture tension (kPa)	Amoebal association*				*Ggt* survival*	
	All amoebae		Mycophagous			
	WPP	MB	WPP	MB	WPP	MB
-1	40.0A**	0.0 ***	13.7C	0.0 ***	40E	15H
-5	37.0A	29.3B	20.2C	6.3D	5F	35H
-10	34.0A	28.0B	19.1C	17.8D	30E	25H
-100	26.0A	28.0B	1.9C	7.1D	100G	50H
-1500***	0.0	0.0	0.0	0.0	0	0

* Percentage of 1 sq mm millipore membrane plated

** Values followed by the same letter in a column do not differ significantly (P = 0.01) according to χ^2 test

*** Not included in analysis.

Water retention of soils is governed by soil texture (Salter and Williams, 1965). The WPP soil with its heavier sandy-loam texture retains more available moisture than the sandy MB soil at all tensions. However, the differences in the recovery of amoebae at suctions of -5 to -100 kPa were not statistically significant between these soils.

Darbyshire (1975) found that at a suction of -50 kPa, no multiplication of the ciliate (*Colpoda steini* Maupas) population occurred and largest populations were obtained in saturated soils. Cook and Homma (1979) found that mycophagous amoebae, however, were unable to perforate fungal propagules in saturated soils, and perforations occurred between moisture tensions of -5 and -20 kPa. My results show that mycophagous and other amoebae were active in both soils at higher tensions of up to -100 kPa and they were detectably inactive at near saturation in the coarse textured MB soil.

Survival of amoebae under extremes of moisture stress

To study the effects of moisture stress on their viability, seven different amoebae were deposited on sterile filter papers (Page, 1967a) and kept in the laboratory for one year. Equal amounts of AS suspensions of the test amoebae were deposited on wedges of sterile filter paper, placed inside sealed Petri plates and stored at room temperature and humidity. To test their viability, 10 wedges of filter paper for each amoeba were placed on PJA + *Klebsiella* medium, moistened with a drop of AS and examined for amoebal growth after two weeks at 25^{o}C.

Three out of seven amoebae, *Acanthamoeba polyphaga*, *Platyamoeba stenopodia* and the unidentified leptomyxid survived these conditions for one year (Table 21). The non-cystic *Saccamoeba* and an unidentified amoeba (Un.1); and cystic *Gephyramoeba* and the unidentified vampyrellid did not survive. These results show that cysts of different amoebae react differently towards environmental conditions (Bodenheimer and Reich, 1933; Page, 1967a). Taylor (1960) recovered viable *Amoeba* spp. from dried cultures. Ability to withstand desiccation and temperature fluctuations would enable an organism to be air-borne and ensure a wider distribution of the species (Gislen, 1940; Pennak, 1953). Puschkarew (1913) estimated that there were about 2.5 viable protozoan cysts per cubic m of air and obtained 13 species, mainly small amoebae, by exposing containers of sterile media to air. Schlichting (1961, 1969) obtained three different species of amoebae from the atmosphere using similar techniques.

Populations of amoebae in suppressive and non-suppressive soils with similar soil texture

Further data on populations of soil amoebae were obtained from two other plots with similar texture from the rotation experiment at the Waite Institute. Of these, the pasture-pasture-wheat (PPW) is suppressive and the continuous wheat (CW) is non-suppressive to take-all (Wildermuth, 1977). Both are red-brown earths and were under wheat. Plots were sampled as previously and populations of mycophagous and other amoebae assessed from 10 samples. The population levels of both mycophagous and other amoebae were significantly ($P<0.05$) higher in the suppressive PPW soil than in the non-suppressive CW soil (Table 22). Mycophagous amoebal populations ranged from 40 - 1555 g^{-1} dry wt in the PPW soil and 0 - 134 g^{-1} dry wt in the CW soil.

TABLE 21: Viability of various amoebae after one year at room temperature and humidity.

Amoeba	Growth on PJA + *Klebsiella*
Cystic species	
Acanthamoeba polyphaga	+
Gephyramoeba sp.	-
Platyamoeba stenopodia	+
Unidentified leptomyxid	+
Unidentified vampyrellid	-
Non-cystic species	
Saccamoeba sp.	-
Unidentified amoeba (Un.1)	-

TABLE 22: Generic populations of amoebae in suppressive (PPW) and non-suppressive (CW) wheat soils. (Mean of 10 samples).

Amoeba	Mean number g^{-1} dry wt of soil	
	CW	PPW
Acanthamoeba spp.	1108	1719
Echinamoeba spp.	522	491
Leptomyxids	21	53
Mayorella spp.	21	51
Platyamoeba spp.	434	364
Saccamoeba spp.	12	77
Thecamoeba spp.	35	65
T.granifera ssp. *minor*	29	63
Vampyrellids	25	60
Unidentified	666	2106
Total population	2873	5049*
Mycophagous	62	193*

*Significantly ($P<0.05$) higher than the corresponding values in CW soil.

Suitability of the survey soils as a medium for amoebal growth

In an attempt to study the effect of soil type on the growth and multiplication of amoebae, three mycophagous amoebae, the unidentified leptomyxid, *Saccamoeba* sp. and *T.granifera* sub-species *minor* were re-introduced into sterilised WPP, Avon and MB soils and their populations monitored over a period of 8 weeks. Soils sieved through 2 mm sieve were moistened to -8 kPa. One hundred g of moist soil was dispensed in 250 ml conical flasks and autoclaved at 121°C for 1 h. Stock cultures of these amoebae were suspended in AS and 0.5 ml of the mixed suspension containing approx. 32000 ml^{-1} of the unidentified leptomyxid, 5600 ml^{-1} of *Saccamoeba* sp. and 260 ml^{-1} of *T.granifera* sub-species *minor* was added to each flask. The flasks were shaken to distribute the suspension, sealed with 'Alfoil' and incubated in darkness at 25°C. Populations of the three amoebae were assessed from three replicate flasks for each soil at 0,1,2,4 and 8 weeks after reintroduction.

Factorial analysis of the data showed that the populations of *Saccamoeba* and *T.granifera* sub-species *minor* declined in all soils over the period of 8 weeks (Table 23). Populations of the unidentified leptomyxid showed an initial increase followed by a gradual decline in all three soils. Populations of all amoebae declined at a significantly higher rate in the MB soil and this was followed by the Avon and the WPP soil. The population of the accompanying food organisms, *Klebsiella* sp. and the *Fusarium* sp. were not studied during these periods.

Discussion

The number of amoebae g^{-1} dry wt of soil varied from 900 to 55400 in the three soils. This is somewhat lower than the range of 1000 to

TABLE 23: Populations of three mycophagous amoebae after their reintroduction into sterilised suppressive (WPP) and non-suppressive (MB and Avon) soils.

Amoeba	Soil	Population (No. g^{-1} dry wt of soil)				
		Initial	Weeks after reintroduction 1	2	4	8
Unidentified Leptomyxid	WPP	3276	38685	32300	45070	15211
	Avon	4698	32314	32314	17403	5952
	MB	3150	6596	9824	3992	1978
Saccamoeba sp.	WPP	7605	6544	4337	6572	197
	Avon	7137	635	117	57	0
	MB	10105	350	142	0	0
T.granifera ssp. *minor*	WPP	293	61	17	256	167
	Avon	86	16	0	31	0
	MB	77	0	0	0	14

Factorial analysis:

Variance ratios	Unidentified leptomyxid	*Saccamoeba* sp.	*T.granifera* ssp. *minor*
Soil	20.339**	4.606*	9.946**
Time	10.822**	7.849**	3.262*
Soil x Time	2.842*	1.164ns	1.047ns

**significant at P = 0.01
* significant at P = 0.05
ns,non-significant.

1690,000 g^{-1} dry wt obtained by others using similar techniques (Heal, 1971). As soil samples were stored at room temperature and humidity for up to 18 months before population estimation, the effects of storage on the populations of soil amoebae were studied. Fresh samples were collected from approx. the same sites of the MB soil and populations were assessed from two of these samples using the same technique. There was no significant difference in amoebal populations between these and those of the old samples collected from the same sites (Table 24). Similar results were obtained by Fantham and Paterson (1924) and Cutler and Dixon (1927) who obtained viable amoebae from soils stored for three years and longer.

In these studies, the total population was calculated by adding the populations of various genera which could be easily identified. This did not take into account the number of species of each genus in these soils; also in many samples, the unidentified group included more than one type of amoeba. The method therefore, underestimated the total population but was useful in determining the populations of different genera and my estimates on the population of the mycophagous *T.granifera* sub-species *minor* are comparable to those obtained by Alabouvette *et al.* (1981) using selective techniques.

Populations of mycophagous amoebae are generally lower than some of the other common soil amoebae like *Acanthamoeba*. The population of *T.granifera* sub-species *minor* in soils is usually less than 100 individuals g^{-1} and higher densities (15,000 to 30,000 g^{-1}) are obtained in heat-treated and fungus-enriched soils (Alabouvette *et al.*, 1981). Using the most probable number technique, Anderson and Patrick (1980)

TABLE 24: Populations of soil amoebae in freshly collected and 18 month old samples of MB soil.

Amoeba	Sample 1		Sample 2	
	Fresh	Old	Fresh	Old
Acanthamoeba spp.	440	460	220	440
Echinamoeba spp.	520	280	540	340
Leptomyxids	90	0	0	40
Mayorella spp.	40	0	0	40
Platyamoeba spp.	186	660	136	280
Thecamoeba spp.	40	90	90	0
Unidentified	280	90	186	220
Vampyrellids	80	80	90	0
Total	1676	1660	1262	1360
Mycophagous	90	0	0	40

estimated the population of the mycophagous *Vampyrella lateritia* Leidy, 1879 in various garden soils as between 1 and 13 g^{-1} dry wt of soil. The cumulative population of the mycophagous leptomyxids, *T.granifera* sub-species *minor* and *Saccamoeba* was between 40 and 1796 g^{-1} dry wt in the WPP soil; 0 - 540 g^{-1} dry wt in the Avon soil and 0 - 180 g^{-1} dry wt in the MB soil.

Terrestrial protozoa are regarded as freshwater species that invaded land with varying degrees of success (Stout, 1963; Schonborn, 1964). The same species is often found in both aquatic and terrestrial habitats and many species of amoebae in soil are distinctly smaller in size than their aquatic forms (Fantham and Paterson, 1926; Chaudhuri, 1929). Their small size allows them to exploit restricted spaces in soil where moisture is retained (Jongerius, 1957; Bamforth, 1973). The effects of soil moisture on amoebae have been studied by a number of workers (Koch, 1916; Cutler *et al.*, 1922; Cutler and Dixon, 1927; Losina-Losinsky and Martinov, 1930; Bonnet, 1964; Nicoljuk, 1964; Volz, 1972) and the general conclusion is that soil amoebae are active within a range between 20% to 60% of the water holding capacity of most soils. Active amoebae were detected between moisture tensions of -5 and -100 kPa in two soils of different textures in my studies.

Results presented here suggest that soil texture, water holding capacity and soil type are not a major influence on population levels of mycophagous and other amoebae. Stout (1968) failed to determine any direct influence of soil texture on the amoebal fauna and Cutler and Crump (1935) could not correlate changes in amoebal populations of field soils with temperature and moisture, although such environmental factors had some effect on the growth and activity of other soil protozoa.

It is almost impossible to correlate the distribution of protozoan species with major soil types or most macroenvironmental factors (Darbyshire, 1975).

When reintroduced into sterilised survey soils with the accompanying food organisms, the mycophagous amoebae produced larger final populations in the WPP soil than in the other two soils. Perhaps the single important factor which regulates the soil amoebal population is the quantity of available food organisms (Sandon, 1927; Singh, 1945, 1946a, 1948). Although physical characters of soil like texture, water holding capacity and soil type do not directly influence amoebal fauna (Sandon, 1927; Stout, 1968), the effects of these on the food organisms may be important in the regulation of population build up in these soils.

Bryant *et al.* (1982) studied interactions between populations of *Acanthamoeba polyphaga* and *Pseudomonas paucimobilis* Holmes *et al.* 1977 in soil microcosms with fluctuating moisture content. They found that in constantly moist soil amoebal grazing limited bacterial numbers, whereas, in soil alternately wetted and dried, this effect was intermittent; although the amoebal population was almost entirely encysted in dry soil, their total population was not affected by three dryings.

Smiley (1979) recovered highly antagonistic Pseudomonads from a suppressive wheat-monoculture soil but not from a non-suppressive soil. Slope *et al.* (1978) found that *Phialophora radicicola* var. *graminicola*, the fungus inhibitory to *Ggt*, was abundant on wheat after grass where it seemed to delay the onset of take-all by one year. Higher populations of mycophagous amoebae in the suppressive soils support the general contention that in suppressive soils there is an abundance of

organisms which are antagonistic, inhibitory, and/or competitors of *Ggt* and this results in the reduced saprophytic survival of the fungus (Pope and Jackson, 1973; Wildermuth, 1977).

C. ASSOCIATION OF AMOEBAE IN WHEAT RHIZOSPHERES IN SUPPRESSIVE AND NON-SUPPRESSIVE SOILS

In 1904 Hiltner coined the term 'Rhizospäre' to describe the zone of soil within the sphere of influence of legume roots. This original concept has been extended considerably and now includes non-leguminous plants, microbes and a very wide range of both inorganic and organic materials released from roots (Rovira and McDougall, 1967; Darbyshire and Greaves, 1973; Rovira and Davey, 1974; Rovira, 1979). The extent of the rhizosphere, which is related to the release and diffusion of components from the root, is dependant on soil conditions, plant species and their age and various other environmental conditions (Papavizas and Davey, 1961; Rovira and Davey, 1974; Brown, 1975).

Generally the rhizosphere supports a higher population of micro-organisms and this is due to the 'rhizosphere effect' which is defined as the ratio of the number of microorganisms in the rhizosphere soil to the number in the corresponding root-free soil (R/S ratio). Starky (1938) and Linford (1942) were the first to demonstrate the presence of protozoa in rhizospheres of plants. Rouatt *et al.* (1960) determined the R/S ratio for the major groups of soil microorganisms in the rhizosphere of spring wheat and these are presented below:

Organism	Number g^{-1} dry wt soil		R/S ratio
	Rhizosphere soil (R)	Non-rhizosphere soil (S)	
Bacteria	$1200 \times 10^{6*}$	53×10^{6}	23:1
Actinomycetes	$46 \times 10^{6*}$	7×10^{6}	7:1
Fungi	$12 \times 10^{5*}$	1×10^{5}	12:1
Protozoa	$24 \times 10^{2*}$	10×10^{2}	2:1
Algae	5×10^{3}	27×10^{3}	0.2:1

*Significantly (P=0.01) higher than the non-rhizosphere soil.

Geltzer (1963) reported a large accumulation of *Hartmannella* sp., *Amoeba* sp., *Mayorella* sp., *Naegleria* sp. and an unidentified small amoeba in rhizospheres of wheat, flax and peas. Darbyshire (1966) found that the populations of both flagellates and amoebae were higher in the rhizosphere soil of *Lolium perenne* L. than those in interrow soil. Large differences between rhizosphere and unplanted soil populations of amoebae, flagellates and bacteria were found during flowering of both an annual (*Sinapsis alba* L.) and a perennial plant (*Trifolium repens* L.) by Darbyshire and Greaves (1967). From an early stage in plant growth, the populations of these organisms were significantly higher in the rhizosphere soil, whereas ciliates were only found in low numbers in both rhizosphere and unplanted soils. Biczók (1956) reported that certain species of protozoa were only found in the rhizosphere; however, Darbyshire and Greaves (1967) concluded that the protozoan genera found in the rhizosphere and unplanted soil were identical and no genus was specific to the rhizosphere of mustard, clover or ryegrass.

Accumulation of large numbers of an organism in the rhizosphere is an indication that these play a role in the inter-relationships between plant roots, the soil environment and other microorganisms in the immediate vicinity of the roots. The role of soil amoebae as rhizosphere organisms is largely unknown. Elliot *et al.* (1979) found that in the rhizosphere of *Boutelona gracilis* (H.B.K.) Lag. ex Steud., a species of grass, gnotobiotic associations of amoeba (*Acanthamoeba* sp.) and bacteria (*Pseudomonas* sp.) mineralised significantly more (50-100%) NH_4-N than those with bacteria alone. Similar higher mineralisation of inorganic phosphorus and ammonium nitrogen in microcosms containing amoebae and bacteria were reported by Coleman *et al.* (1977).

Several of the hypotheses advanced to explain suppression propose the development and/or increased activities of microflora antagonistic to *Ggt* in wheat rhizospheres of suppressive soils (Vojinovic, 1972; Pope and Jackson, 1973; Cook and Rovira, 1976; Hornby and Brown, 1977). Soil amoebae have been found associated with *Ggt* in its saprophytic phase (Homma *et al.*, 1979) and it is likely that both these organisms occupy similar ecological niches. In the case of *Ggt*, the two types of existence, saprophytic and parasitic, are inter-related. Soil amoebae to be closely associated with *Ggt* in its parasitic phase, will have to compete with several other microorganisms in the rhizosphere, which is considered as a zone of high microbial activities (Rovira, 1956; Rouatt *et al.*, 1960; Rovira and Davey, 1974).

Association of mycophagous and other amoebae in wheat rhizospheres and rhizoplanes in suppressive and non-suppressive soils was determined during two wheat crops and that of the harvested wheat rows during an inter-crop fallow using one non-suppressive and two suppressive soils. Soil moisture and the occurrence of *Ggt*, assessed throughout this period, were correlated with the populations of mycophagous and other amoebae to determine the inter-relationships between these variables.

Association of soil amoebae with wheat roots - a survey

As a preliminary experiment, the association of soil amoebae with wheat roots was studied using 9 - 13 weeks old plants obtained from six widely separated locations in South Australia. The sample sites were selected at random by the S.A. Department of Agriculture for a survey on the severity of wheat diseases, and plants with adhering soil were obtained from them. Details on the cropping history and soil characteristics of the sample sites are presented in Table 25.

TABLE 25: Cropping history and soil characteristics of the six soils.

Area (Hundred of)	Soil type	Cropping history	Available P (ppm)	K	Total N (ppm)	pH	Salinity ms - 25°C (conductivity) 1/5
Pinkawillinie	sandy loam	PWPWP*	17	360	0.049	7.9	0.112
Stirling	sandy loam	WPPPB	38	550	0.136	8.5	0.282
Kapunda	clay loam	PPBW	40	476	0.133	6.2	0.242
Carina	sandy loam	PBWPB	29	350	0.053	8.6	0.147
Yaninee	sandy loam	PBWWP	44	715	0.105	8.4	0.188
Minnipa	sandy loam	WPOWP	43	440	0.074	8.6	0.186

*P = pasture; W = wheat; B = barley; O = oat
(Courtesy - Dr. A. Mayfield, S.A. Dept. of Agriculture).

Roots were washed clear of adhering soil particles and about 2 cm pieces of the seminal roots cut from an area 1 cm away from the seed. These pieces were washed twice in SDW and plated on PJA + *Klebsiella* plates. Four replicates, each with two pieces of root, were incubated at 25°C for two weeks and examined for amoebae. Several different amoebae were isolated from these samples. Most genera, except *Acanthamoeba* and the leptomyxids, were present in wheat rhizoplanes in all sites (Table 26).

It is evident that soil amoebae are a part of the microbial component of the wheat root surface in the soils studied.

Population dynamics of amoebae in suppressive and non-suppressive soils

Wheat rhizosphere populations of mycophagous and other amoebae were estimated during the 1981 season and for up to 10 weeks after sowing in 1982 from two suppressive and one non-suppressive soil of the permanent rotation trials at the Waite Agricultural Research Institute. For the inter-crop fallow, amoebal populations were estimated from the soil of the harvested wheat rows.

Selection of soils and sampling

The selection of the three soils was based on their suppressive properties; the pasture-pasture-wheat (PPW) rotation has a high degree of suppressiveness, the pea-wheat (PeW) rotation with a moderate suppressiveness and the continuous-wheat (CW) without any detectable suppressiveness to the disease in pot bioassays (page 18, Table 4).

TABLE 26: Percent recovery of amoebae from wheat roots collected from six locations in South Australia.

Amoeba	SITES					
	Stirling	Yaninee	Carina	Minnipa	Kapunda	Pinka-willinie
Acanthamoeba spp.	50	0	0	25	12.5	12.5
Platyamoeba spp.	37.5	75	100	87.5	87.5	37.5
Thecamoeba spp.	50	37.5	100	100	50	100
Leptomyxids	37.5	37.5	0	25	50	25
Vampyrellids	100	12.5	12.5	25	37.5	25
Unidentified	62.5	87.5	87.5	37.5	100	50

A number of methods are available for sampling and estimating population of organisms in rhizosphere soils and these have been reviewed by Johnson and Curl (1972), Darbyshire and Greaves (1973), Rovira and Davey (1974); but because of the heterogeneous nature of the rhizosphere no precise and simple sampling method will suffice under every plant and soil condition. I used the traditional plate count method of Katznelson (1946) with modifications.

Each plots was subdivided into grids, approx. 2m x 2m and wheat plants in a row with soil to a depth of about 10 cm were lifted using a spade from three randomly selected intercepts for each plot at each sampling. Roots were shaken, often after breaking large soil clods by hand, to discard the surplus soil from around roots. The firmly adhering soil together with roots constituted the rhizosphere soil. Populations of amoebae were estimated from two samples of each rotation using the modified soil dilution technique described earlier. As before, *Gephyramoeba* and the unidentified leptomyxid were grouped together and included with *Saccamoeba* and *Thecamoeba granifera* sub-species *minor* to calculate the population of mycophagous amoebae. Often suspensions were lightly homogenised to break large fragments of roots.

The PPW and PeW rotations are maintained in more than one plot so that each crop is represented in one plot each year and wheat rhizosphere and rhizoplane samples in 1982 for these rotations came from plots adjacent to the ones used in 1981 samplings. During the intercrop fallow, samples from the top 0 - 10 cm layer of harvested wheat rows were collected from three randomly selected intercepts of each plot using a 4 cm internal diameter soil auger. Samples were collected at monthly intervals and populations of amoebae determined.

Populations of mycophagous and other amoebae in the PPW, PeW and CW soils

Populations of amoebae were determined from rhizosphere soil of the three rotations throughout the 1981 wheat season and for up to 10 weeks after sowing in 1982. The non-suppressive CW soil was however, not included in determinations until 8 weeks after sowing in the 1981 season. Data on the populations of various genera of amoebae are presented in Appendix IIB.

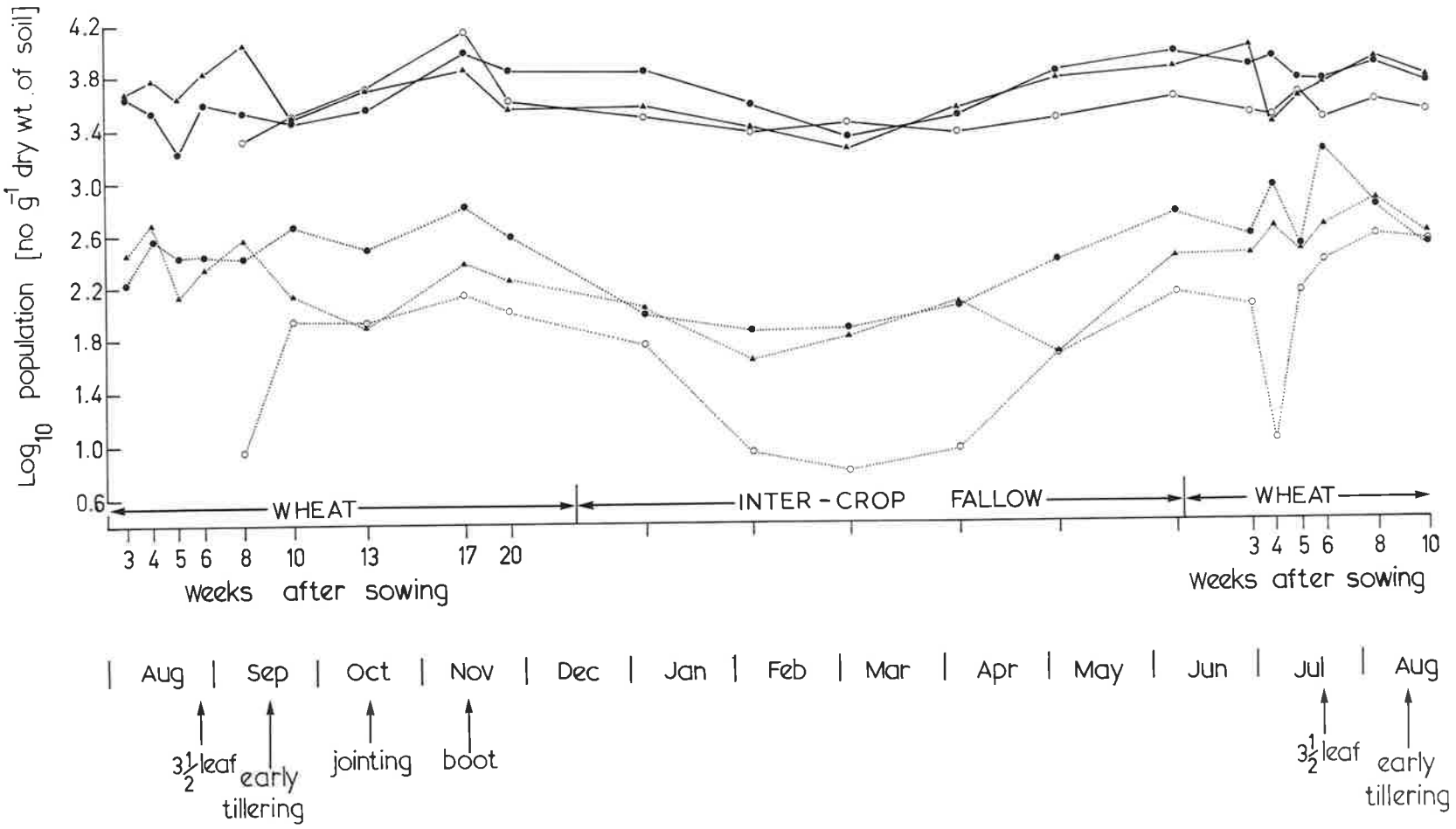
Populations of mycophagous amoebae were consistently higher in the rhizospheres of plants from the suppressive PPW and PeW plots than in the non-suppressive CW (Fig.28). Populations of all other amoebae were also higher in the suppressive soils throughout most part of the growing season except during the boot stage of the crop in 1981, when it was higher in the CW than in the other two soils (Fig.28). In the suppressive PPW soil, population peaks of mycophagous amoebae were observed at boot stage in 1981 and 3½ leaf stage in 1982 season. In PeW soil, relatively smaller peaks could be detected at early tillering and boot stages. In the non-suppressive CW soil however, there was no apparent peak and the populations of mycophagous amoebae were generally higher during the entire crop season starting from the tillering phase. Populations of all other amoebae reached their peaks during the boot stage in all soils.

After harvesting, there was a drop in the populations of mycophagous amoebae in all soils; population g^{-1} in these soils were 824 and 92 in PPW, 172 and 112 in PeW and 108 and 92 in CW, three weeks before and three weeks after harvest respectively. However, samples collected after harvest can not be regarded as rhizosphere samples.

Fig.28: Populations of soil amoebae during two wheat crops and the inter-crop fallow in the suppressive pasture-pasture-wheat (●), pea-wheat (▲) and non-suppressive continuous-wheat (○) soil.

..... Mycophagous amoebae.

——————— All other amoebae.


Log10 population [no g-1 dry wt of soil]
4.2
3.8
3.4
3.0
2.6
2.2
1.8
1.4
1.0
0.6
WHEAT
INTER-CROP
FALLOW
WHEAT
3 4 5 6 8 10 13 17 20
weeks after sowing
3 4 5 6 8 10
weeks after sowing
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
3½ leaf
early tillering
jointing
boot
3½ leaf
early tillering

The recovery of mycophagous amoebae was drastically reduced during the inter-crop fallow in the non-suppressive soil (Fig.28); in comparison, populations were much higher in the suppressive PPW and PeW soils during this period. Population of all other amoebae was slightly reduced in all soils during the inter-crop fallow although, the reduction was far less compared to that of the mycophagous amoebae. Lowest populations of both mycophagous and other amoebae were observed during February-March period in all soils.

Amoebae in wheat rhizoplane in suppressive and non-suppressive soils

The association of mycophagous and other amoebae on the rhizoplanes of wheat grown in suppressive PPW and PeW and non-suppressive CW soils was determined throughout the 1981 season and for up to 10 weeks after sowing in 1982.

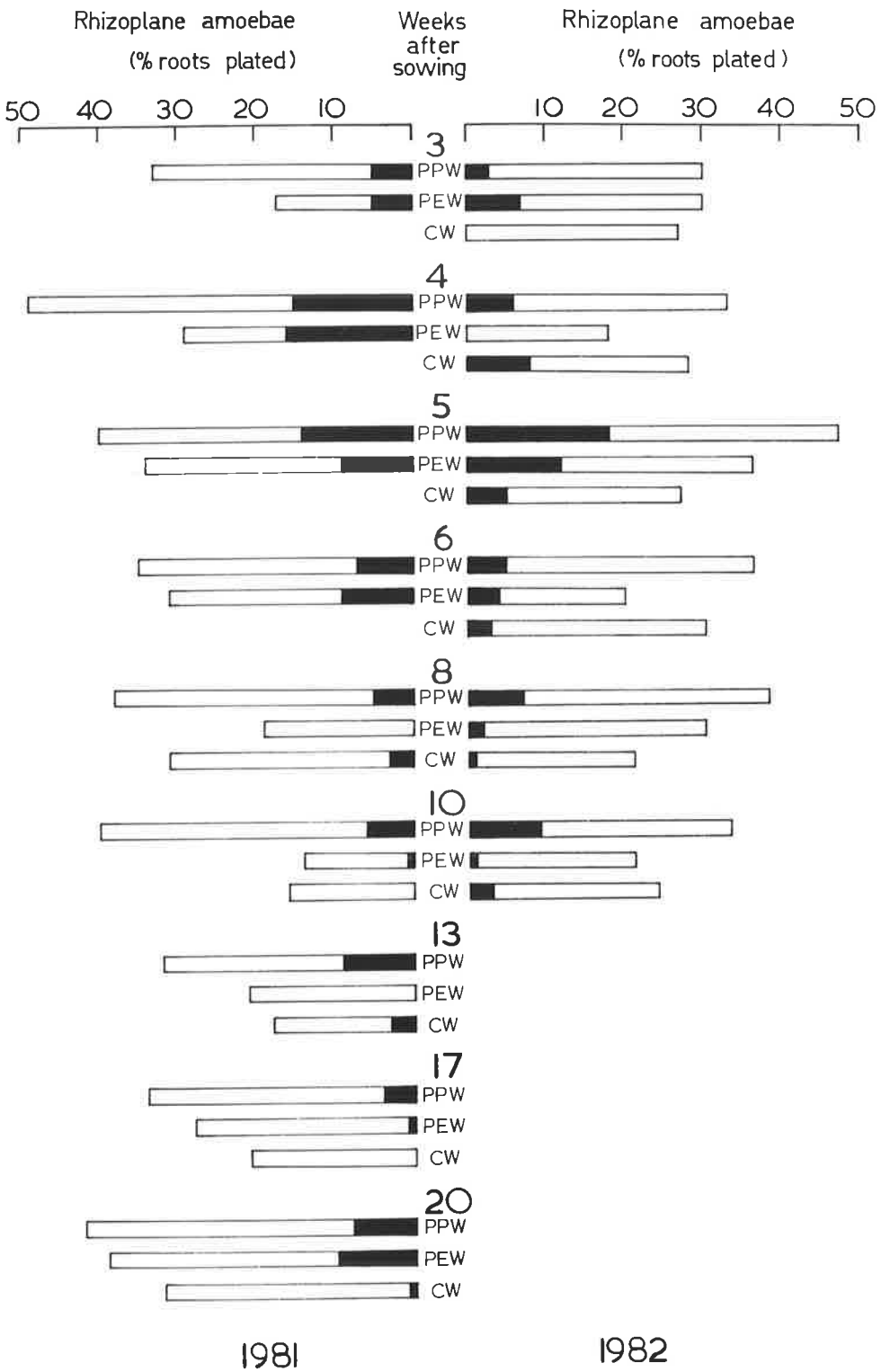
About 2-3 cm pieces of the seminal roots from an area 1 cm away from the seed were plated on PJA + *Klebsiella* plates using the root plating method described earlier. Ten replicate pieces of root were plated from each soil at each sampling and the association of amoebae was determined by recording the amoebal genera on the PJA plates after an incubation of two weeks at 25^{o}C.

Results on the occurrence of various genera on wheat rhizoplanes in these soils are presented in Appendix IIC. Figure 29 summarises the association of mycophagous and other amoebae with wheat roots in PPW, CW and PeW soils during the 1981 and 1982 seasons. There was generally a higher association of mycophagous amoebae with wheat roots in the suppressive PPW and PeW soils than in the non-suppressive CW soil (Fig.29). The PPW soil, with its higher degree of suppressiveness,

Fig.29: Wheat rhizoplane amoebae during two crops in suppressive (PPW and PeW) and non-suppressive (CW) soils.

Mycophagous amoebae

All other amoebae.


Rhizoplane amoebae
(% roots plated)
Weeks after sowing
Rhizoplane amoebae
(% roots plated)
50 40 30 20 10
10 20 30 40 50
3
PPW
PEW
CW
4
PPW
PEW
CW
5
PPW
PEW
CW
6
PPW
PEW
CW
8
PPW
PEW
CW
10
PPW
PEW
CW
13
PPW
PEW
CW
17
PPW
PEW
CW
20
PPW
PEW
CW
1981
1982

showed a consistently higher rhizoplane population of both mycophagous and other amoebae with peaks around the 3½ leaf stage of the crop (4-5 weeks after sowing). Both PeW and CW soils showed a more or less similar degree of association of amoebae other than mycophagous during the initial period of up to 10 weeks after sowing but thereafter, more amoebae were found in wheat rhizoplanes in the PeW than in the CW soil.

Correlation between soil moisture, population of *Ggt* and amoebae in the suppressive and non-suppressive soils

Correlations between populations of amoebae, *Ggt* and soil moisture were calculated in an attempt to determine the basis for the higher populations of mycophagous and other amoebae in the suppressive PPW and PeW soils.

At each sampling, the presence of *Ggt* was assessed and soil moisture determined from the three samples of each plot (Table 27). During the 1981 crop season, roots were examined for take-all and 2 cm pieces from infected roots were plated on NDY/6 incorporated with 100 µg streptomycin ml^{-1} and 10 µg tetracycline ml^{-1}. For the 1982 season and the inter-crop fallow however, bulk soil was used in the seedling baiting technique (Hornby, 1971) described earlier to assess the presence of the fungus. Portions of infected roots from the 1982 samples were also plated to compare the two techniques. Little correlation between the two techniques was found and the seedling baiting technique always gave a higher assessment of the fungus.

Data on the soil moisture, *Ggt* recovery and populations of mycophagous and other amoebae were correlated by calculating Pearson's correlation coefficients. Results show that at the 1% level of

TABLE 27: Soil moisture ang *Ggt* populations during two wheat seasons and inter-crop fallow in suppressive (PPW and PeW) and non-suppressive (CW) soils.

	Weeks after sowing Wheat season, 1981									Soil moisture (%)[a] Inter-crop fallow						Weeks after sowing Wheat season, 1982					
Soil	3	4	5	6	8	10	13	17	20	Jan	Feb	Mar	Apr	May	Jun	3	4	5	6	8	10
PPW	20.5	21.1	20.4	18.0	15.2	16.2	8.6	14.8	4.9	3.1	3.1	2.0	7.0	16.6	17.0	18.4	17.7	18.1	16.2	13.7	17.0
PeW	19.2	20.2	18.8	15.6	15.2	11.3	7.3	11.3	4.2	2.5	3.0	2.4	4.1	16.3	17.0	16.5	15.9	16.9	14.7	12.4	15.3
CW	No determination				13.5	11.6	6.4	13.6	3.6	2.5	2.9	2.3	5.0	15.9	16.9	17.0	16.7	17.4	14.8	13.5	15.7
										Ggt population (%)[b]											
PPW	6.6	0.0	0.0	6.6	6.6	6.6	0.0	0.0	0.0	15.3	7.7	7.7	34.1	15.8	45.6	26.4	58.5	35.3	43.0	75.6	70.0
PeW	26.0	0.0	0.0	6.6	6.6	0.0	13.3	6.6	0.0	1.5	10.9	12.6	12.9	9.6	29.7	12.4	26.9	20.6	40.0	33.4	39.2
CW	No determination				6.6	6.6	0.0	13.3	0.0	3.1	13.8	12.9	9.5	11.3	33.2	24.9	29.4	30.4	35.3	42.6	38.1

[a] = Oven dry wt basis
[b] = Percentage of infected roots for 1981 season and percentage of inoculum units in bulk soil, as detected by the seedling baiting technique, for 1982 season and inter-crop fallow.

probability, populations of mycophagous amoebae correlated with soil moisture and *Ggt* recovery in the non-suppressive CW soil and only with soil moisture in the suppressive PeW soil (Table 28). In the suppressive PPW soil however, no correlation was observed between soil moisture and amoebal populations.

These results indicate that the population build-up of the mycophagous amoebae in the CW and PPW soil was dependant on the population of *Ggt*. This is also supported by the fact that during the inter-crop fallow, when population of the fungus was detectably lower in these soils, a lower population of the mycophagous amoebae occurred in these soils. The reduction in the population of mycophagous amoebae was higher in the non-suppressive CW soil than in the PPW soil, which in turn also showed a generally lower population of *Ggt* during the inter-crop fallow (Table 27). Geltzer (1963) also found that amoebae multiplied in the rhizospheres of six plant species only when there was an active development of the food organisms, bacteria in that case, on them.

Nicoljuk (1964) found the largest numbers of individuals and species in irrigated soils and suggested that protozoan populations are greatly influenced by soil moisture. Darbyshire (1966) found highest populations of amoebae and flagellates in warm and moderately wet rhizosphere soils. In my studies, the lack of a higher positive correlation between soil moisture and populations of both mtcophagous and other amoebae in the suppressive PPW indicates that soil moisture alone can not explain the higher populations of these amoebae in this soil throughout the growing seasons.

TABLE 28: Correlation coefficients between populations of mycophagous and other amoebae, soil moisture and *Ggt* recovery in the suppressive (PPW and PeW) and non-suppressive (CW) soils.

	Population of Mycophagous amoebae	Population of other amoebae	Soil moisture (%)
Population of other amoebae			
PeW	0.43 (P=0.03)*		
PPW	0.36 (P=0.06)		
CW	0.26 (P=0.16)		
Soil Moisture (%)			
PeW	0.68 (P=0.01)	0.53 (P=0.09)	
PPW	0.23 (P=0.16)	-0.002 (P=0.49)	
CW	0.54 (P=0.01)	0.10 (P=0.36)	
Ggt recovery (%)			
PeW	0.29 (P=0.11)	-0.02 (P=0.45)	0.25 (P=0.14)
PPW	0.40 (P=0.04)	0.25 (P=0.14)	0.17 (P=0.23)
CW	0.57 (P=0.01)	-0.07 (P=0.38)	0.69 (P=0.002)

*Denotes the probability level of significance.

Darbyshire *et al.* (1977) studied the inter-relationships of bacteria, protozoa and the take-all fungus associated with the roots of growing barley and of the post-harvest stubble. During the growth of the crop, the bacterial and the trophic protozoan populations gradually increased in all soils near the roots and these increases were accompanied by changes in the levels of NH_4 and NO_3 nitrogen.

Rovira and Wildermuth (1981) reported a massive proliferation of Gram-negative, non-sporing, rod-shaped bacteria on wheat roots following infection by *Ggt* and proposed that this was the forerunner to TAD. Plant roots in general, stimulate certain groups of micro-organisms and several reports suggest that some spore-forming *Bacillus* spp. and Gram-positive cocci are depressed in the rhizosphere (Clark, 1940; Lochhead, 1940). Conversely, Gram-negative, non-sporing bacteria such as *Agrobacterium* and *Pseudomonas* spp. may account for as much as 40 to 50% of the rhizosphere populations of bacteria (Vagnerova *et al.*, 1960; Rouatt and Katznelson, 1961). Therefore, the suggestion that the proliferation of a certain type of bacterium in the rhizosphere is the forerunner to TAD appears to be inconclusive.

Data on the populations of amoebae in this section could not be statistically analysed for significance due to the lack of adequate replications. Even with two replicates in each plot, a total of 90 plates had to be examined to estimate amoebal populations at each sampling. The results however, indicate that the populations of myco-phagous amoebae are consistently higher in the suppressive soils than in the non-suppressive soil studied. More detailed investigations are needed to test these interesting findings on the association of myco-phagous amoebae in wheat rhizospheres of suppressive and non-suppressive soils and their inter-relationships with other soil microorganisms, especially bacteria and plant pathogenic fungi.

D. REDUCTION OF TAKE-ALL BY MYCOPHAGOUS AMOEBAE IN POT BIOASSAYS

Suppression of the take-all disease of wheat is one of the best-known and most-studied cases of a naturally occurring biological control of a soil-borne plant pathogen (Baker and Cook, 1974; Walker, 1975; Cook, 1981; Papavizas and Lewis, 1981; Rovira and Wildermuth, 1981). Although the precise mechanism of control is still uncertain, the demonstration that the suppressive factors are sensitive to heat (Gerlagh, 1968; Shipton *et al.*, 1973; Cook and Rovira, 1976) and some forms of nitrogen (Brown *et al.*, 1973) and also that suppression can be re-established by the addition of 1% antagonistic soil to fumigated or steamed soil (Shipton *et al.*, 1973; Wildermuth, 1977) or partly re-established by the re-colonization of fumigated soil by air-borne contaminants (Baker and Cook, 1974; page 206) suggests that the suppressive factors are microbial in nature. This has led to the formulation of several hypotheses to explain suppression (Hornby, 1979). Rovira and Wildermuth (1981) summarised these hypotheses as follows:

1. Increased microbial activity associated with the pathogen and the infected host (Gerlagh, 1968; Vojinovic, 1972, 1973; Shipton, 1975).
2. Increased microbial activity associated with the pathogen in the soil away from the root (Zogg and Jaggi, 1974).
3. Changes in the virulence of the pathogen with the involvement of virus-like particles (Lapierre *et al.*, 1970).
4. Development of a microflora in the rhizosphere of wheat growing in suppressive soil which inhibits the trophic growth of the fungus to the wheat root (Pope and Jackson, 1973).

5. Selective stimulation by infected wheat roots of rod-shaped, non-sporing bacteria (Vojinovic, 1972) or a highly specific group of fluorescent Pseudomonads (Cook and Rovira, 1976).
6. A change in the general micro-flora of TAD soil which alters the ratio of NH_4^+-N to NO_3^--N in the rhizosphere (Hornby and Brown, 1977).
7. An effect upon the emergence of hyphae from the infectious propagule and subsequent darkening of the hyphae (Wildermuth *et al.*, 1979).

The lack of understanding of the mechanisms of suppression has not barred workers from attempting to control the disease using specific antagonists or competitors of the take-all fungus (Cook, 1981; Papavizas and Lewis, 1981). Successful biological control of *Ggt* in the field was obtained by introducing *Bacillus mycoides* Flügge (Campbell and Faull, 1979); *Gaeumannomyces graminis* (Lemaire *et al.*, 1977); *G.graminis* var. *graminis* and *Phialophora radicicola* Cain (Wong and Southwell, 1980). Up to 46% control of take-all in field plots were obtained by introducing hypo-virulent strains of *Ggt* (Lemaire *et al.*, 1971, 1976; Tivoli *et al.*, 1974).

Apart from these field studies, Rovira and Wildermuth (1981) demonstrated the inhibition of *Ggt* on agar and protection of wheat roots by cultures of fluorescent Pseudomonads, and Sivasithamparam *et al.* (1975) discovered a volatile factor inducing transmissible self-perpetuating lysis of *Ggt*. However, the relevance of inhibition of the fungus on agar plates is open to question and Sivasithamparam and Parker (1978) could not show any correlation between the antagonism

towards *Ggt* on agar by five isolates each of actinomycetes, bacteria and fluorescent Pseudomonads and the ability of these organisms to protect wheat seedlings in sterile sand with added *Ggt*. Cook (1981) considers that *Pseudomonas fluorescens* introduced with wheat seed at sowing has potential to reduce damage from take-all in field.

The possibility of using mycophagous amoebae for the control of soil-borne plant pathogens has been indicated (Homma *et al.*, 1979; Old and Patrick, 1979). Experiments were conducted to test if mycophagous amoebae could effectively reduce the severity of take-all and thereby protect wheat seedlings during the initial four weeks of plant growth in a controlled environment.

Effect of mycophagous amoebae on the severity of take-all

A preliminary experiment to determine the effects of mycophagous amoebae on the severity of take-all was conducted using Penrose's (1980) technique for scoring the susceptibility of wheat cultivars to *Ggt*. Plastic pots were filled with 350 g CS and five 10 mm diameter discs of a potato dextrose agar culture of *Ggt* were placed on the surface of the CS. Discs of potato dextrose agar medium only were used in control pots. One pre-germinated seed of wheat was placed on each agar disc and one of the following treatments applied:

a. A suspension (1 ml) of one of the mycophagous *Saccamoeba*, unidentified leptomyxid, *Gephyramoeba* or *Thecamoeba granifera* sub-species *minor*, prepared in AS was poured on top of each wheat seed.

b. A mixed suspension (1 ml) of all four mycophagous amoebae was poured on top of each wheat seed.

c. Sieved (2 mm) WPP soil (1g) was sprinkled on each wheat seed.

d. AS suspension (1 ml) of *Klebsiella* sp. poured on top of each wheat seed.

e. AS suspension (1 ml) of the accompanying organisms of *T.granifera* sub-species *minor* poured on top of each wheat seed.

f. Only AS (1 ml) poured on top of each wheat seed.

Seeds were covered with 50g CS and an additional 50 ml of distilled water was added to each pot. Plants were harvested after two weeks, rated for disease and plant heights determined.

Of the four mycophagous amoebae used, three reduced the disease rating and increased plant height significantly ($P<0.01$) compared with the *Ggt* treatment (Table 29). Greatest protection was obtained with *Gephyramoeba* or with the mixture of amoebae. The accompanying organisms of *T.granifera* sub-species *minor*, consisting of a *Fusarium* sp. and bacteria, also significantly reduced the disease rating, whereas, the food bacterium, *Klebsiella* sp. did not. Since the accompanying organisms were present in suspensions of *T.granifera* sub-species *minor* only, the reduction of disease severity by *Gephyramoeba* and *Saccamoeba* is independant of the effects of the accompanying organisms.

Reduction of take-all severity by soil cultures of mycophagous amoebae

The contribution of mycophagous amoebae in disease suppression can be better understood if their effects can be directly compared with that of a suppressive soil. While addition of AS suspensions of mycophagous amoebae serves the purpose of demonstrating their effect on the disease,

TABLE 29: Effect of suspensions of mycophagous amoebae on the severity of disease caused by *Ggt* (mean of 25 plants treatment^{-1}).

Treatment	Disease rating	Plant height (cm)
*L*Ggt* + WPP soil	1.8	11.3
L*Ggt* + *Gephyramoeba* sp.	2.1	11.2
L*Ggt* + *T.granifera* ssp. *minor*	2.6	10.8
L*Ggt* + *Saccamoeba* sp.	2.8	9.7
L*Ggt* + Unidentified leptomyxid	3.1	9.9
L*Ggt* + Mixture of all four amoebae	2.2	11.4
L*Ggt* + *Klebsiella* sp.	3.5	9.7
L*Ggt* + Accomp. org.†	2.6	10.5
L*Ggt*	3.3	9.9
PDA disc	0.0	13.9
Least significant differences		
P=0.01	0.35	1.03
P=0.05	0.25	0.72

*L = Live

†Accomp. org. = Accompanying organisms of *T.granifera* ssp. *minor*.

the effects of a suppressive soil with its added advantage of supplying plant nutrients can not be simply compared with that of AS suspensions. Mycophagous amoebae were therefore cultured in sterilized soils for use in pot bioassays.

Soil cultures of mycophagous amoebae

Samples of both WPP and Avon soils were sieved and moistened to -8 kPa. Moist soils were dispensed in glass Petri plates, wrapped with 'Alfoil' and autoclaved at 121°C for 1 h for three consecutive days. Small amounts of AS suspension from PJA cultures of each of the four mycophagous amoebae were added to sterilized soil and incubated at 25°C in darkness. Suspensions of accompanying organisms of *T.granifera* subspecies *minor* and the food bacterium, *Klebsiella* sp., were added to sterilized soils and similarly incubated for controls. After two weeks, growth of amoebae were tested by plating small amounts of soil cultures on PJA + *Klebsiella* plates. All four amoebae were isolated from both Avon and WPP soils, although only cultures in Avon soil were used in the bioassay. Since the viability of amoebae decreased after about four weeks in the Avon soil (page 133, Table 23), only fresh (15-20 days old) cultures were used.

Effect of soil cultures of mycophagous amoebae on the severity of take-all

The pot bioassay (Shipton *et al.*, 1973) used to test soil suppressiveness against *Ggt* was used except that CS was used instead of fumigated soil. Ground oat grain inoculum of *Ggt* was used. Three controls with dead (autoclaved oat grain inocula) *Ggt* in which either sterilized Avon soil or soil culture of accompanying organisms + *Klebsiella* sp. was

added were compared with live *Ggt* and live *Ggt* plus the suppressive WPP soil or soil cultures of the mycophagous amoebae, or a mixture of all four amoebae. Soil cultures of the accompanying organisms of *T.granifera* sub-species *minor* and *Klebsiella* sp., were also used with live *Ggt*. Inoculum of *Ggt* was added at the rate of 0.2% and the suppressive soil/soil culture at the rate of 1%. Five wheat seeds were sown per pot, covered with CS and 50 ml distilled water was added. Plants were harvested after four weeks, rated for disease, heights measured and dry wt of tops determined (Table 30). Portions (about 2 cm) of roots from the mixture of amoebae treatment were plated on PJA + *Klebsiella* plates to determine the association of amoebae with wheat roots.

Soil cultures of *Gephyramoeba*, *Saccamoeba* and *T.granifera* sub-species *minor* significantly ($P<0.01$) reduced disease severity and increased plant height (Fig.30) and top dry wt (Table 30). The reduction of disease and the increase in top dry wt in plants where the mixture of amoebae were used was comparable to the effect of the suppressive WPP soil and there was no significant difference between these two treatments. Soil cultures of the accompanying organisms also significantly reduced disease rating and increased height and top dry wt of plants. Similarity in disease ratings in live *Ggt* treatments with and without sterile Avon soil shows that the soil as such did not have any detectable effect on the fungus or the host. All four amoebae were found associated with roots; of the number of pieces of roots plated, 98% had the unidentified leptomyxid, 83% had *Gephyramoeba*, 90% had *T.granifera* sub-species *minor* and 87% had *Saccamoeba*.

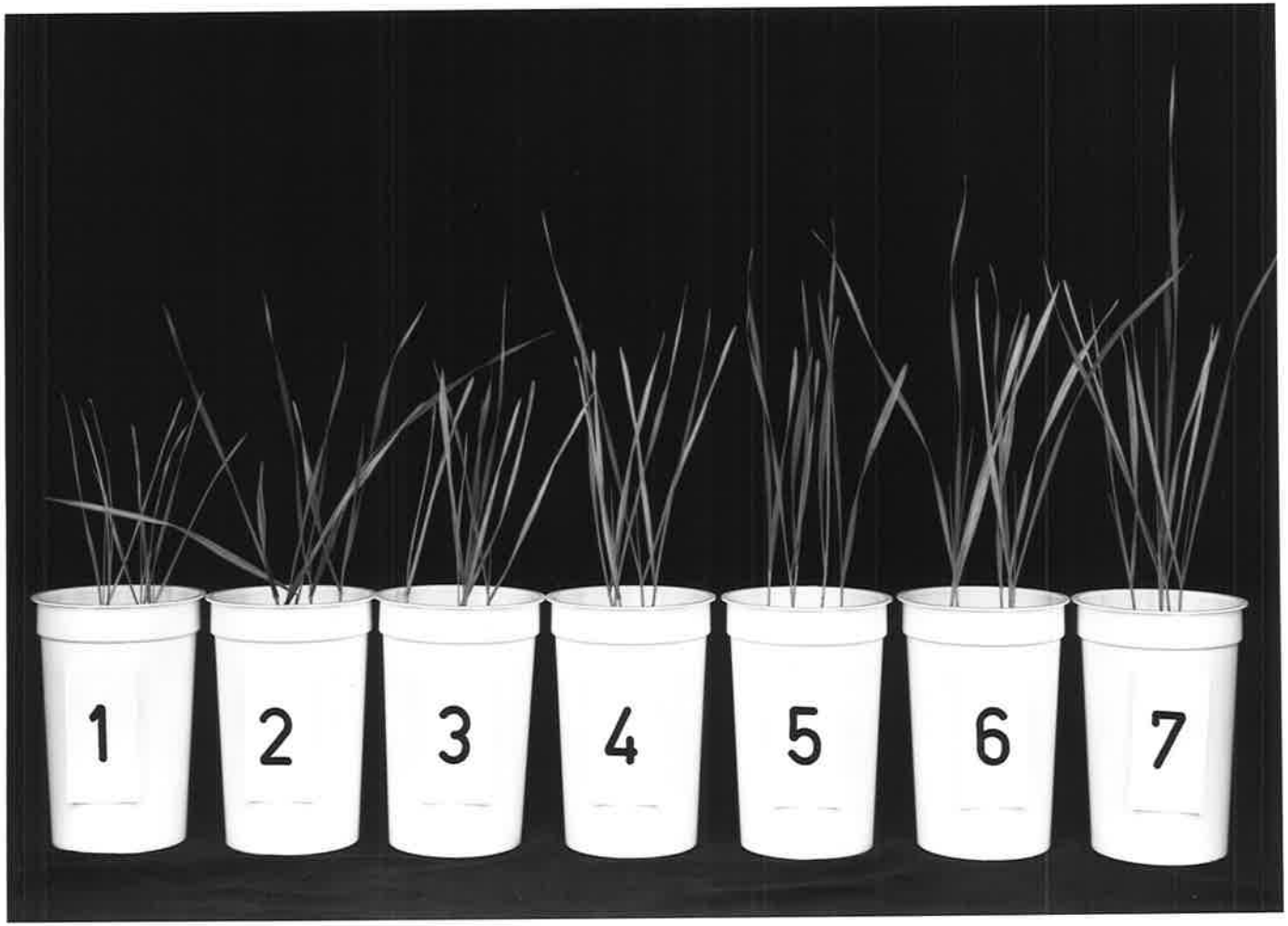
TABLE 30: Disease rating, height and top dry wt of plants grown in sterile coarse sand with soil cultures of amoebae and 0.2% *Ggt* inoculum (mean of 25 plants treatment^{-1}).

Treatment	Disease rating	Plant height (cm)	Top dry weight (mg)
*LGgt + WPP soil	1.9	15.9	25.8
LGgt + *Gephyramoeba* sp.	2.2	14.7	24.6
LGgt + *T.granifera* ssp. *minor*	2.0	15.3	23.0
LGgt + *Saccamoeba* sp.	2.8	13.9	22.3
LGgt + Unidentified leptomyxid	3.8	13.3	19.4
LGgt + Mixture of all four amoebae	1.9	15.6	28.7
LGgt + Accomp. org.	2.2	15.9	23.6
LGgt + Str. Avon soil	4.2	12.7	16.6
LGgt	4.2	12.7	16.3
DGgt + Str. Avon soil	0.0	18.9	29.4
DGgt	0.0	19.5	32.2
Least significant differences			
P=0.01	0.71	2.37	4.82
P=0.05	0.50	1.65	5.37

*L = Live; D = Dead; Accomp. org. = Accompanying organisms of *T.granifera* ssp. *minor* + *Klebsiella* sp.

Fig.30: Growth of wheat after 28 days in sterilized coarse sand inoculated with *G.graminis tritici* and soil cultures of mycophagous amoebae or the suppressive Waite permanent pasture soil.

1. Live *Ggt*.
2. Dead *Ggt*.
3. Live *Ggt* + suppressive soil.
4. Live *Ggt* + *Saccamoeba* sp.
5. Live *Ggt* + *Thecamoeba granifera* sub-species *minor*.
6. Live *Ggt* + *Gephyramoeba* sp.
7. Live *Ggt* + Mixture of four mycophagous amoebae.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Effect of initial population size of mycophagous amoebae on the severity of take-all

The effect of large initial populations of the mycophagous amoebae on the severity of take-all was tested in a pot bioassay using two population levels. Five and 50 ml of AS suspension of individual amoeba was added to 400g CS containing 0.2% ground oat grain inocula of *Ggt*; this gave initial population levels of approx. 2 g^{-1} and 20 g^{-1} for *Gephyramoeba* and 40 g^{-1} and 400 g^{-1} of CS for *Saccamoeba* and *T.granifera* sub-species *minor*. The mixed amoebae treatments consisted of approx. 27 g^{-1} and 270 g^{-1} of CS of these amoebae. The unidentified leptomyxid was not used in this experiment due to its inability to reduce the disease severity in the previous tests.

Higher population levels of all amoebae caused a further reduction in the disease rating (Table 31), however, this was not statistically significant in any treatment. Only in the mixed amoebae treatment, a higher initial population resulted in a significant ($P<0.01$) increase in the height and top dry wt of plants. Top dry wt of plants was also significantly increased with an increase in the initial population of the accompanying organisms. All mycophagous amoebae, however, were effective in reducing disease rating and increasing plant heights and top dry wt when compared to plants in the live *Ggt* treatments.

The accompanying organisms of *Thecamoeba granifera* sub-species *minor*

A significant reduction in the disease rating and increase in the plant height and top dry wt was obtained in bioassays using either AS suspensions or soil cultures of the accompanying organisms of *T.granifera* sub-species *minor* (Tables 29,30,31). The accompanying organisms are a

TABLE 31: Disease rating, height and top dry wt of plants grown in sterile coarse sand with two population densities of mycophagous amoebae and 0.2% *Ggt* inocula (mean of 25 plants treatment^{-1}).

Treatment	Amount of suspension added (ml)	Plant height (cm)	Disease rating	Top dry weight (mg)
*L*Ggt* + WPP soil		14.7	2.0	26.8
L*Ggt* + *Gephyramoeba* sp.	5	17.0	2.4	29.2
	50	17.2	1.8	28.8
L*Ggt* + *T.granifera* ssp. *minor*	5	17.1	2.3	24.8
	50	18.1	1.6	26.8
L*Ggt* + *Saccamoeba* sp.	5	16.8	2.6	29.1
	50	17.1	2.4	27.1
L*Ggt* + Mixture of all three amoebae	5	18.2	1.8	27.9
	50	19.8	1.5	33.7
L*Ggt* + AS	5	12.9	4.3	17.4
	50	12.6	4.9	16.9
L*Ggt* + Accomp. org.	5	14.1	2.5	22.0
	50	14.8	2.1	25.8
D*Ggt* + AS	5	17.4	0.0	29.4
	50	18.1	0.0	30.0
Least significant difference				
P=0.01		1.69	0.62	4.78
P=0.05		1.20	0.52	3.39

* L = Live; D = Dead; Accomp. org. = Accompanying organisms of *T.granifera* ssp. *minor* + *Klebsiella* sp.

mixture of bacteria and a fungus which produces sickle-shaped two-septate conidia and has been tentatively identified as a *Fusarium* sp. Several bacteria , including *Bacillus* (Campbell and Faull, 1979) and fluorescent *Pseudomonas* (Cook and Rovira, 1976; Rovira and Wildermuth, 1981) and some actinomycetes (Zogg and Jaggi, 1974; Zogg, 1976; Tschudi and Kern, 1979) have hypholytic properties and species of *Bacillus* and *Pseudomonas* have been shown to reduce the severity of take-all in pot and field experiments. The presence of fluorescent pseudomonads in the culture of the accompanying organisms of *T.granifera* sub-species *minor* was examined using a selective medium.

PJA cultures of the accompanying organisms were flooded with SDW and the suspension was serially diluted in a 10-fold dilution series with SDW. A drop (0.1 ml) of each suspension was spread on Petri plates poured with NPCC medium (Simon *et al.*, 1973, Appendix I) prepared at pH 7.2. Plates were incubated at 30°C and examined for yellow-green or blue-white fluorescence in and around the colonies under ultraviolet radiation.

Some fluorescent pseudomonads, as detected by the presence of a yellow-green fluorescence in and around the colonies, were present in the accompanying organisms of *T.granifera* sub-species *minor* and these may have contributed to the reduced severity of take-all. However, as *Gephyramoeba* and *Saccamoeba* are maintained with a *Klebsiella* sp. and this bacterium itself did not have any detectable effect on the disease (Table 29), the effects of both of these amoebae on take-all severity are independant of the effects of the accompanying orgamisms of *T.granifera* sub-species *minor*.

Discussion

Three mycophagous amoebae, *Gephryamoeba*, *Saccamoeba* and *T.granifera* sub-species *minor*, were able to protect wheat roots against take-all during the initial four weeks of plant growth. Higher level of protection was obtained when these amoebae were used in combination and at higher population densities.

Anderson and Patrick (1978) reported that populations of *Chalara elegans (=Thielaviopsis basicola)* and *C.sativus* may be reduced in pot experiments by additions of cysts of vampyrellid amoebae. Old (unpublished, 1979) observed significantly less pre-emergence seedling blight when seeds inoculated with *C.sativus* were first inoculated with cysts of *A.impatiens*.

However, the protection offered by mycophagous amoebae in controlled bioassays does not imply that such protection is operative under field conditions. Faull (1978) found that 25% of the isolates of saprophytic bacteria were antagonistic to *Ggt* on agar plates, 6% were effective in sand, 3% effective in soil and only 1% effective in field trials. Some of the mycophagous amoebae can be grown with a food bacterium on low-nutrient media and stored at low temperatures in diatomaceous earth granule cultures for up to one year. Field trials with cultures of amoebae either introduced before sowing or with seeds can be expected to provide a better assessment of their practical value as biological control agents.

Old and Patrick (1979) reviewed the suitability of mycophagous amoebae, especially vampyrellids, as biological control agents. They considered that the factors that would tend to limit their use would be

their limited growth at higher soil moisture tensions, the possibility that soil amoebae themselves may constitute a biohazzard (Culbertson, 1971) and their apparent lack of specificity in selection of fungi as food. Another aspect is the ability of soil amoebae to invade plant tissues (McLennan, 1930; Darbyshire and Greaves, 1971) which may result in pathogenic symptoms in host plants. However, observations so far have suggested that the invasion follows the penetration of plant tissues by their food organisms (Darbyshire and Greaves, 1971).

V. GENERAL DISCUSSION

Several aspects of mycophagy by soil amoebae have been investigated in some detail in this study. While *Arachnula*, *Theratromyxa*, *Vampyrella*, *Thecamoeba* and *Cashia* were known as mycophagous genera, the inclusion of *Gephyramoeba*, *Saccamoeba*, *Mayorella* and the unidentified leptomyxid has extended this list of genera with mycophagous species to nine. More research is needed to fully appreciate the proportion of genera of soil amoebae which can feed on fungi.

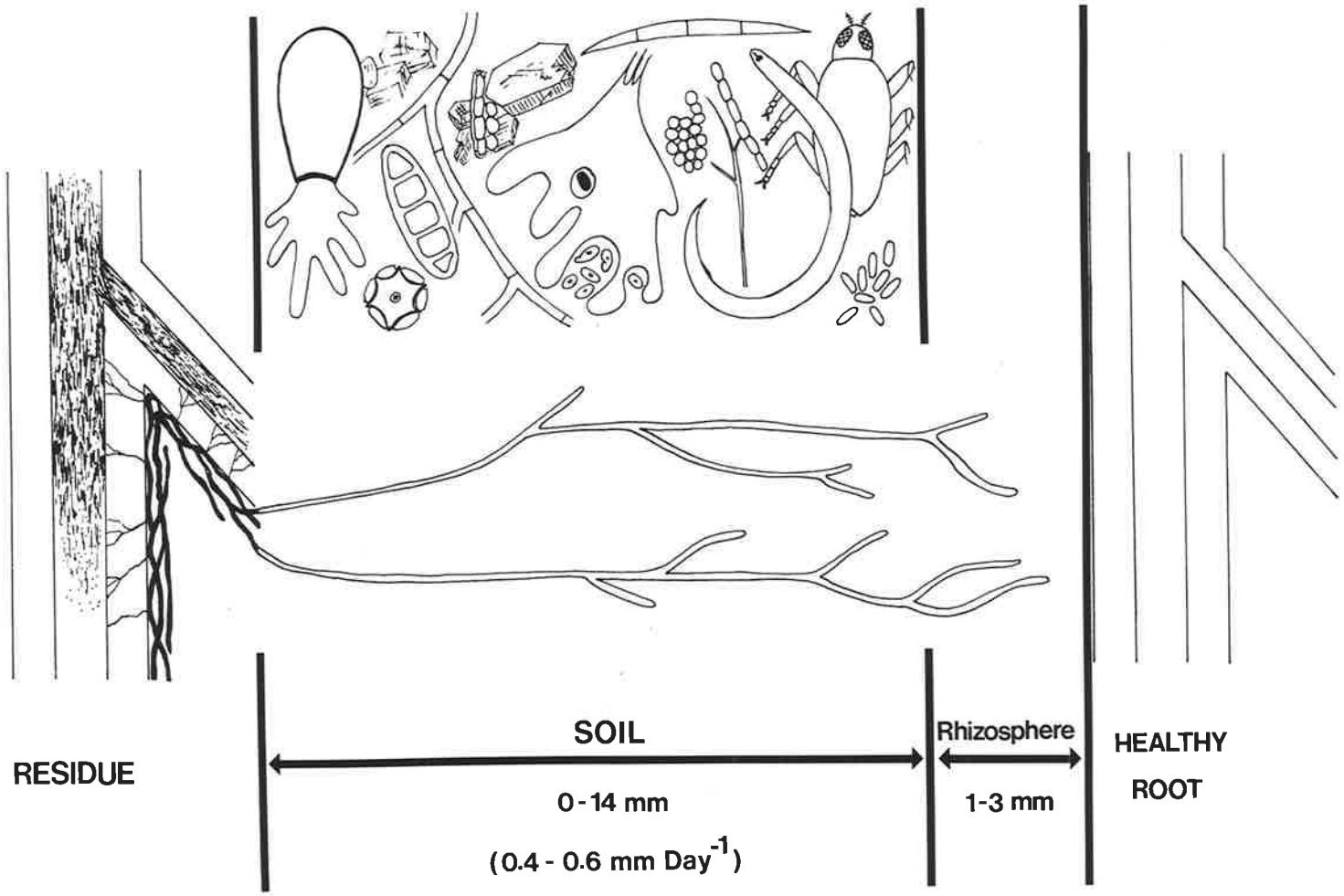
Many investigators have considered that the presence of perforations in the fungal cell-walls is a positive indication of mycophagy by amoebae. While many amoebae may cause perforations, the results reported here show that perforation may not be detectable in all amoeba-fungal interactions. Although, it is generally believed that amoebae gain access to the cell cytoplasm of fungi by dissolving portions of the wall, subsequent feeding by amoebae may often lead to distortion and collapse of the fungal propagule thereby rendering the perforations undectectable. This type of distortion has been observed in hyaline hyphae of *Ggt* after feeding by trophozoites of the *Saccamoeba* sp. In case of the unidentified leptomyxid feeding on mature chlamydospore of *Phytophthora cinnamomi*, delicate protoplasmic threads were seen traversing the space between the digestive vacuolar membrane and the chlamydospore wall. However, whether these protoplasmic threads are capable of dissolving holes in the chlamydospore wall is not known. Only formless debris is extruded from a digestive vacuole at the end of the feeding period. Time-lapse transmission electron microscopic studies may help in understanding the exact mechanism of chlamydospore penetration by this amoeba.

Of particular interest is the ability of many mycophagous amoebae to perforate and lyse melanised fungal propagules. It is believed that this process of perforation is, at least, partly enzymatic (K.M.Old, personal communication). Melanins have been regarded as resistant to enzymatic degradation (Bull, 1970). The mechanisms of perforation and lysis of melanised walls of fungi by mycophagous amoebae deserve further attention.

The take-all suppressive soils studied here showed a higher population of mycophagous and other amoebae irrespective of the texture and water-holding capacity of the soil. The basis for the development of a higher number of individuals in these soils is not known. While large populations of food organisms have been considered essential for higher amoebal populations in some soils (Sandon, 1927; Singh, 1945, 1946a, 1948), a definite relationship between these has not been shown (Bryant *et al.*, 1982).

Figure 31, based on a conceptual model of the plant rhizosphere (Bowen, 1979), shows a hypothetical case of wheat root colonisation by *Ggt* from an infected host residue. Microbial interaction with *Ggt* can be envisaged in the three processes, as suggested by Bowen (1979), *viz:* germination of the propagule, movement towards the root and growth on the root surface. Being a root-inhabiting fungus, pathogenic activities during the parasitic phase of *Ggt* establishes a prior possession of the host tissue before the death of the host. Once the host-tissue is dead, potential saprophytic invaders may challenge its earlier monopoly (Garrett, 1956, 1970). The threat to the saprophytic survival of the fungus increases apparently as ageing and decomposition of the saprophytic inoculum proceeds; thus the smaller fragments lose their infectivity more quickly (Hornby, 1975).

Fig.31: A diagrammatic representation of the process of growth of *G.graminis tritici* hyphae from an infected host residue through soil to infect a healthy host root.


RESIDUE
SOIL
0 - 14 mm
(0.4 - 0.6 mm Day^{-1})
Rhizosphere
1-3 mm
HEALTHY
ROOT

Invasion of *Ggt*-infected host residues by saprobic microbes may therefore result in the reduction of the saprophytic inoculum even prior to germination of the propagule and mycophagous amoebae have been isolated from surface sterilised infected wheat roots in my experiments.

A higher association of mycophagous amobae with *Ggt* hyphae in a suppressive soil and the presence of perforations in hyphal walls suggest that soil amoebae are a part of the microbial component responsible for the reduction of saprophytic survival of the fungus in the permanent pasture soil.

As emphasized by Skou (1981), contact between host root and *Ggt* is established by trophic growth of the hyphae towards roots rather than by chance; the importance of antagonistic microorganisms during this phase of pre-colonisation can be easily understood. It is during this period that *Phialophora* spp. and *Pseudomonas fluorescens* were reported to have restricted the growth of the fungus (Deacon, 1976; Cook and Rovira, 1976). In my studies, hyphae became pigmented at a faster rate in the suppressive pasture soil, presumably as a result of a response to competing microorganisms (Deacon, 1973). While many organisms, including mycophagous amoebae, can lyse hyaline hyphae, only mycophagous amoebae have been shown to perforate and lyse pigmented hyphae of the fungus. Growth of *Ggt* in soil is initiated by hyaline hyphae which later become pigmented (Wildermuth, 1977); mycophagous amoebae would therefore, be likely organisms to control the fungus at this pre-colonisation stage.

The zone of root exudates (rhizosphere?) is determined by the distances volatile and dissolved exudates diffuse from roots (Bowen and

Rovira, 1976) and often some form of selective antagonism and inhibition between organisms occurs within this zone (Rovira, 1956; Rouatt *et al.*, 1960; Rovira and Davey, 1974). The fact that there is a higher association of mycophagous amoebae with wheat roots and a higher rhizosphere population of soil amoebae occur in suppressive soils indicates that these organisms are also effective in this selective zone of root exudates. Reduction of the severity of take-all in pot bioassays by mycophagous amoebae further supports this fact. However, whether this reduction in disease severity results from a reduction in the ectotrophic growth of the fungus or by the perforation and lysis of pigmented runner hyphae on the root surface, or both, is not known.

Rovira and Wildermuth (1981), proposing a mechanism for the development of transferrable suppression with wheat monoculture, stated that there is a proliferation of non-sporing rod shaped bacteria on *Ggt* lesions of wheat roots; a small proportion of these bacteria build up on new roots and hyphae with successive wheat crops and result in less take-all. Although the development of suppression was not studied in this work, the scheme proposed by Rovira and Wildermuth (1981) can be used to envisage the role of mycophagous amoebae in suppression in the pasture soil. As with other antagonists of *Ggt*, mycophagous amoebae associate themselves with the fungus in infected host residues, reduce the saprophytic survival of the fungus, lyse both pigmented and hyaline hyphae during the pre-colonisation and parasitic phase of the fungus and result in the reduction in disease severity.

In proposing this role of mycophagous amoebae in suppression, at no stage is it intended to claim that they are the only organisms responsible for suppression. The data on associated organisms with

Thecamoeba granifera sub-species *minor* would substantiate this. Other studies also show that several organisms can reduce the severity of take-all at least in pot experiments and therefore attempts to explain suppression on the basis of specific antagonists may well be futile (Hornby, 1979). It is likely that several different mechanisms are functioning simultaneously. This observation is also supported by the apparent lack of a high degree of specificity of suppressive soils (Baker and Cook, 1974; Wildermuth, 1977).

One practical aspect of the ability of mycophagous amoebae to reduce take-all severity is the hope for their future use as biological control agents against the disease. Two approaches are possible, firstly, modifications in soil management practices to augment mycophagous amoebae, and secondly, the introduction of the organisms to soils to suppress the disease. Since natural soils often seem to resist attempts to alter their microbial balance by introducing large amounts of pure cultures of antagonists (Garrett, 1956), enhancement of activities and populations of mycophagous amoebae by modifying soil environment appears to be a better choice. For this further research on the effects of soil environmental conditions on these organisms would be needed.

VI. APPENDICES

APPENDIX I

Formulae for media and saline solutions

NDY/6 (Warcup, 1955)

$NaNO_3$	0.33 g
KH_2PO_4	0.16 g
$MgSO_4$, $7H_2O$	0.08 g
KCl	0.08 g
$FeSO_4$, $7H_2O$	0.001g
Difco Yeast Extract	0.08 g
Sucrose	5.0 g
Agar (Davis)	10.0 g
Distilled water	1 l

V-8 Juice agar (Miller, 1955)

V-8 juice	200 ml
$CaCO_3$	3.0 g
Agar	20.0 g
Distilled water	800 ml

2% Malt agar

Malt extract (Difco)	20 g
Agar	12 g
Distilled water	1 l

Czapek-Dox agar (Proprietary medium,
Difco Laboratories, Detroit, U.S.A.).

49 g of the following mixture in 1 *l* of distilled water

Saccharose (Difco)	30 g
$NaNO_3$	2 g
K_2HPO_4	1 g
$MgSO_4$, $7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4$, $7H_2O$	0.01g
Bacto agar	15 g

Tryptone-Glucose-Yeast agar
(TGY, Cavender and Raper, 1965)

Bacto tryptone	5 g
Glucose	1 g
Yeast extract	5 g
Agar	12 g
Distilled water	1 *l*

Prescott and James' Agar (PJA)

One ml of each of the following stock solutions in 997 ml of glass-distilled water makes Prescott and James' solution (Prescott and James, 1955).

Bacto agar is added at a rate of 1% to make PJA.

Stock solution A

$CaCl_2$,$2H_2O$	0.433 g
KCl	0.162 g
Glass-distilled water	100 ml

Stock solution B

K_2HPO_4	0.512 g
Glass-distilled water	100 ml

Stock solution C

$MgSO_4$, $7H_2O$	0.280 g
Glass-distilled water	100 ml

Modified Neff's amoeba saline (AS, Page, 1967)

A separate stock solution of each of the following components is made by dissolving the amounts given in 100 ml glass-distilled water. Ten ml of each of the stock solution are mixed and the volume is made up to 1 *l* with glass-distilled water.

1). NaCl	1.2 g
2). $MgSO_4$, $7H_2O$	0.04 g
3). Na_2HPO_4	1.42 g
4). $CaCl_2$, $2\ H_2O$	0.04 g
5). KH_2PO_4	1.36 g

NPCC medium (Simon *et al.*, 1973)

The basal medium:

Difco Proteose Peptone No.3	20 g
Oxoid Ionagar No.1 (Oxoid purified agar)	12 g
Glycerol	8 ml
K_2SO_4	1.5 g
$MgSO_4$, $7H_2O$	1.5 g
Distilled water	940 ml

pH adjusted to 7.2 with 0.1N NaOH before autoclaving for 15 min and following antibiotics are added before pouring plates:

Penicillin G (Benzopenicillin)	75 units ml^{-1}
Novobiocin (Albamycin)	45 µg ml^{-1}
Cycloheximide (Actidione)	75 µg ml^{-1}
Chloramphenicol (Chloromycetin)	10 µg ml^{-1}

APPENDIX IIA: Generic populations of amoebae in samples of the Waite permanent pasture, Avon and Murray Bridge soil.

Amoeba \ Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
									Waite permanent pasture											
Acanthamoeba spp.	980	980	660	980	2600	980	1580	1580	980	340	2600	1580	920	980	460	660	1580	4800	980	980
Echinamoeba spp.	10800	18400	7000	980	18400	2800	3400	340	280	1400	440	2200	920	1580	520	440	5600	10800	280	5600
Leptomyxids	40	90	40	0	340	90	80	0	40	146	40	260	40	80	90	146	90	136	146	90
Mayorella spp.	40	40	0	0	40	0	0	0	0	90	0	0	0	90	0	0	0	0	0	90
Platyamoeba spp.	260	440	136	220	540	220	136	280	1580	2200	220	340	1580	3400	540	340	660	1900	186	980
Saccamoeba spp.	40	660	460	0	260	220	40	40	280	0	146	146	146	0	0	0	90	1580	40	260
Thecamoeba spp.	260	146	90	40	146	90	90	146	136	40	40	0	90	40	40	0	40	280	40	90
T.granifera minor	40	90	40	40	80	90	40	40	80	186	40	0	40	40	0	0	0	80	40	460
Unidentified amoebae	1400	10800	18400	5600	32000	7000	18400	420	920	340	220	7000	280	4400	136	136	2600	3400	2200	4400
Vampyrellid amoebae	260	40	260	460	980	460	146	0	80	80	340	146	40	220	40	146	40	40	90	146
										Avon										
Acanthamoeba spp.	220	340	460	10800	2600	660	660	980	460	980	280	1580	660	920	660	980	660	440	340	920
Echinamoeba spp.	146	36	540	146	340	40	186	80	136	280	280	80	36	0	4400	220	220	186	80	186
Leptomyxids	90	0	136	40	80	36	90	440	40	0	460	220	0	146	40	40	90	80	90	0
Mayorella spp.	0	40	40	0	0	0	40	0	0	0	40	0	0	90	40	40	40	0	0	0
Platyamoeba spp.	440	1580	146	460	146	1580	440	280	136	220	540	1580	136	80	340	280	980	460	460	90
Saccamoeba spp.	0	0	0	0	0	0	0	0	0	0	40	0	0	0	0	0	40	0	0	0
Thecamoeba spp.	40	90	40	90	40	40	0	0	90	146	0	40	146	136	40	40	40	0	40	0
T.granifera minor	40	0	0	40	0	40	90	0	0	0	40	0	0	0	0	0	40	0	0	0
Unidentified amoebae	146	186	340	136	420	280	520	80	186	80	136	1580	280	80	340	280	280	0	80	186
Vampyrellid amoebae	0	40	0	40	36	0	0	90	0	146	90	40	146	90	146	90	36	40	36	0
										Murray Bridge										
Acanthamoeba spp.	660	440	460	980	220	220	460	980	340	660	660	220	460	340	660	460	90	460	146	440
Echinamoeba spp.	2600	340	280	1900	280	440	340	1580	540	980	340	440	260	660	440	340	440	280	540	520
Leptomyxids	90	40	0	136	0	0	90	36	0	90	40	0	0	40	90	90	146	0	0	90
Mayorella spp.	90	40	90	90	40	0	90	40	40	90	0	0	40	0	40	0	0	0	0	90
Platyamoeba spp.	136	280	146	186	260	980	920	440	220	340	280	146	540	2600	540	980	2600	660	340	440
Saccamoeba spp.	0	0	0	0	0	0	0	0	0	0	0	0	40	0	0	0	0	0	0	0
Thecamoeba spp.	90	0	40	90	40	146	40	40	0	40	0	40	40	0	40	146	0	90	80	90
T.granifera minor	40	0	40	40	0	40	40	0	40	0	0	90	0	0	90	40	0	0	0	0
Unidentified amoebae	980	220	540	540	40	136	1580	80	40	280	260	280	1580	440	660	540	280	90	220	80
Vampyrellid amoebae	260	0	260	90	40	90	136	146	136	260	0	40	146	90	146	460	220	80	40	0

APPENDIX IIB: Population (no. g^{-1} dry wt) of soil amoebae during two wheat crop and inter-crop fallow in suppressive (PPW and Pew) and non-suppressive (CW) soil.

Amoeba	Pasture-pasture-wheat (PPW) soil																					
	Weeks after sowing, 1981 season									Inter-crop fallow						Weeks after sowing, 1982 season						
	3	4	5	6	8	10	13	17	20	Jan	Feb	Mar	Apr	May	June	3	4	5	6	8	10	
Acanthamoeba spp.	3020	2242	418	2744	825	1546	1279	2347	1177	2982	1372	1142	1924	1907	915	1570	3159	1257	1885	2777	2158	
Echinamoeba spp.	0	267	274	21	1968	752	1499	2946	1345	289	423	254	146	647	580	539	607	390	715	729	301	
Leptomyxids	81	168	85	188	127	111	41	52	162	0	41	20	43	0	48	0	133	78	140	0	78	
Mayorella spp.	188	82	280	106	74	212	98	52	71	66	0	120	0	0	228	441	212	225	101	163	192	
Platyamoeba spp.	73	583	135	180	365	283	262	258	483	116	515	387	322	1343	1144	1852	1288	427	858	370	470	
Saccamoeba spp.	0	57	141	45	76	313	74	338	347	0	21	20	0	87	192	110	303	223	154	162	82	
Thecamoeba spp.	25	178	141	82	160	256	191	108	178	41	20	20	69	77	169	293	559	457	667	1134	331	
T.granifera minor	81	149	56	48	70	110	191	281	21	92	20	45	139	209	292	248	368	0	1336	466	422	
Unidentified amoebae	704	204	392	658	280	178	339	3638	4311	4025	1403	224	569	1979	5301	1999	2321	2405	870	2187	1543	
Vampyrellid amoebae	50	107	75	225	94	0	128	238	76	66	0	20	139	77	81	0	0	0	0	23	142	
	Pea-wheat (PeW) soil																					
Acanthamoeba spp.	2055	953	3203	7704	3620	2105	2687	2357	1650	1847	1484	839	2502	1779	1904	5750	642	1276	2099	2138	2469	
Echinamoeba spp.	0	81	0	240	4245	124	1554	1037	793	238	422	284	112	298	506	407	523	337	293	647	665	
Leptomyxids	60	175	111	100	109	50	43	0	76	46	20	66	70	23	21	23	47	0	52	182	23	
Mayorella spp.	210	194	24	106	53	45	21	73	112	0	0	20	20	0	105	53	71	252	46	20	0	
Platyamoeba spp.	99	188	267	133	4328	158	285	186	222	218	250	248	294	525	1434	910	511	1215	1137	342	519	
Saccamoeba spp.	74	301	55	110	176	22	0	99	20	20	0	0	67	21	48	167	197	141	126	215	139	
Thecamoeba spp.	216	219	104	76	109	22	70	76	41	46	20	20	20	53	361	155	110	644	205	163	139	
T.granifera minor	115	56	55	23	76	73	43	146	76	46	20	0	20	0	169	179	178	135	238	253	206	
Unidentified amoebae	4668	4390	738	163	127	352	226	3158	553	1200	443	164	291	2508	2218	1784	582	589	891	4059	1654	
Vampyrellid amoebae	22	223	24	153	47	22	73	45	96	20	20	66	41	212	72	155	23	0	0	53	47	
	Continuous-wheat (CW) soil																					
Acanthamoeba spp.	No determination				948	1414	641	949	1162	1303	1154	2725	1083	1129	1288	674	1537	872	645	1758	783	
Echinamoeba spp.	"				473	1403	2853	8222	1421	346	422	217	139	713	193	711	408	423	434	254	211	
Leptomyxids	"				23	22	0	43	0	20	0	0	21	23	24	24	24	24	171	20	0	
Mayorella spp.	"				84	67	126	23	67	0	0	0	21	0	78	135	108	109	105	101	53	
Platyamoeba spp.	"				280	203	288	567	788	328	515	271	294	559	673	590	450	1513	684	729	1139	
Saccamoeba spp.	"				23	45	69	23	41	0	20	20	0	23	24	72	48	112	46	248	100	
Thecamoeba spp.	"				107	45	159	104	41	20	0	20	97	23	210	141	78	211	46	254	177	
T.granifera minor	"				0	50	48	75	67	41	20	0	21	0	87	54	0	72	23	107	258	
Unidentified amoebae	"				196	125	1464	4365	778	1354	373	202	662	487	1877	807	336	1187	1091	1191	746	
Vampyrellid amoebae	"				128	0	63	130	41	20	0	20	0	23	111	0	0	0	0	52	23	

APPENDIX IIC: Wheat rhizoplane amoebae (percentage root plated) during two crops in suppressive (PPW and PeW) and non-suppressive (CW) soil.

Amoeba	Pea-wheat (PeW) soil WEEKS AFTER SOWING 1981 season									1982 season					
	3	4	5	6	8	10	13	17	20	3	4	5	6	8	10
Acanthamoeba spp.	10	10	30	20	20	20	0	20	100	10	10	30	10	80	40
Echinamoeba spp.	0	0	0	0	80	40	30	30	30	30	10	50	0	30	20
Leptomyxids	20	50	30	0	0	0	0	0	10	40	0	40	0	0	0
Mayorella spp.	20	0	20	60	0	0	0	0	0	30	0	10	30	0	0
Platyamoeba spp.	0	10	70	40	50	30	60	70	60	80	50	70	30	80	60
Saccamoeba spp.	20	60	60	70	0	10	0	10	40	30	0	50	40	0	20
Thecamoeba spp.	0	20	40	10	0	0	0	10	10	0	10	10	0	0	0
T.granifera minor	10	50	0	20	0	0	0	0	50	0	0	30	0	20	0
Unidentified amoebae	60	100	90	70	20	30	90	90	90	80	90	70	70	90	60
Vampyrellid amoebae	30	0	0	20	10	10	30	50	0	0	10	0	20	0	10
	Pasture-pasture-wheat (PPW) soil														
Acanthamoeba spp.	70	40	20	10	70	60	60	80	90	50	40	40	60	50	20
Echinamoeba spp.	0	30	0	20	30	60	30	60	70	40	10	10	50	30	10
Leptomyxids	0	30	30	0	10	40	0	10	10	30	0	60	20	10	10
Mayorella spp.	60	60	70	40	40	0	0	0	10	10	0	20	0	0	10
Platyamoeba spp.	20	40	20	60	50	70	40	60	70	60	70	90	80	80	50
Saccamoeba spp.	10	70	90	50	20	20	50	30	20	20	20	80	20	40	50
Thecamoeba spp.	10	30	30	40	10	20	10	0	0	10	50	40	50	10	20
T.granifera minor	40	50	20	20	30	0	40	0	50	10	40	40	10	20	30
Unidentified amoebae	90	100	100	90	80	60	70	90	100	90	80	90	70	100	80
Vampyrellid amoebae	30	40	20	20	40	70	20	10	0	10	20	0	0	40	50

continued/...

APPENDIX IIC: continued

Amoeba	Continuous-wheat (CW) soil, WEEKS AFTER SOWING														
	1981 season									1982 season					
	3	4	5	6	8	10	13	17	20	3	4	5	6	8	10
Acanthamoeba spp.	No determination				30	0	0	10	60	60	40	10	40	10	20
Echinamoeba spp.	"				80	30	40	30	40	20	30	10	50	20	20
Leptomyxids	"				20	0	0	0	0	0	20	0	0	0	0
Mayorella spp.	"				0	0	0	0	0	10	0	0	0	0	0
Platyamoeba spp.	"				90	60	30	50	90	70	60	90	40	60	50
Saccamoeba spp.	"				10	0	10	0	0	50	50	50	30	10	30
Thecamoeba spp.	"				0	0	0	0	10	0	0	10	0	0	10
T.granifera minor	"				0	0	20	0	10	0	10	0	0	0	0
Unidentified amoebae	"				40	30	50	100	100	60	70	100	90	90	80
Vampyrellid amoebae	"				40	40	30	20	20	0	0	0	50	20	30

APPENDIX III: Publications

CHAKRABORTY S. and OLD K.M. (1982). Mycophagous soil amoeba : interactions with three plant pathogenic fungi. *Soil Biology and Biochemistry* 14: 247-255.

CHAKRABORTY S., OLD K.M. and WARCUP J.H. (1983). Amoebae from a take-all suppressive soil which feed on *Gaeumannomyces graminis tritici* and other soil fungi. *Soil Biology and Biochemistry* 15: 17-24.

CHAKRABORTY S. and WARCUP J.H. (1983). Soil amoebae and saprophytic survival of *Gaeumannomyces graminis tritici* in a suppressive pasture soil. *Soil Biology and Biochemistry* 15: (in press).

CHAKRABORTY S. and WARCUP J.H. (1983). Population of mycophagous and other amoebae in take-all suppressive and non-suppressive soils. *Soil Biology and Biochemistry* 15: (in press).

CHAKRABORTY S. (1983). Population dynamics of amoebae in soils suppressive and non-suppressive to wheat take-all. *Soil Biology and Biochemistry* 15: (in press).

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MYCOPHAGOUS SOIL AMOEBA: INTERACTIONS WITH THREE PLANT PATHOGENIC FUNGI

S. CHAKRABORTY

Department of Plant Pathology, Waite Agricultural Research Institute, The University of Adelaide, Glen Osmond, South Australia 5064

and

K. M. OLD

Division of Forest Research, C.S.I.R.O., Canberra, A.C.T. 2600, Australia

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Summary—An amoeba of the order Leptomyxida was isolated from wheat take-all decline soil and was found to attack and lyse hyphae and spores of *Gaeumannomyces graminis* var. *tritici* and *Phytophthora cinnamomi*. The amoeba enveloped portions of hyphae of both fungi and penetrated the cell walls by means of fine holes. One-week old chlamydospores and hyphal swellings of *P. cinnamomi* were also attacked in this way, protoplast lysis being completed within 1 h. Hyphal fragments which could be ingested by the amoeba were lysed leaving amorphous cell debris. Three-week old chlamydospores of *P. cinnamomi* were enclosed within large food vacuoles and completely digested in about 20 h. Pigmented conidia of *Cochliobolus sativus* were transported across the substratum for up to several hours but were not perforated or lysed.

INTRODUCTION

Since 1977 there have been a series of reports concerning the mycophagous activities of soil amoebae. Particular interest has been shown in the genera *Arachnula*, *Vampyrella* and *Theratromyxa* which belong to the family Vampyrellidae in the classification of the protozoa proposed by Honigberg *et al.* (1964), (Old, 1977; Old and Darbyshire, 1978; Old and Oros, 1980; Anderson and Patrick, 1978, 1980; Homma *et al.*, 1979). A number of other genera have also been found to have mycophagous species notably *Thecamoeba* (Esser *et al.*, 1975; Allabouvette *et al.*, 1979; Pussard *et al.*, 1979) and *Hartmannella* (C. Palzer, personal communication). As early as 1936, Dreschler reported that the testate amoebae, *Geococcus vulgaris* and *Arcella vulgaris* attacked oospores of *Pythium ultimum*. The mode of attack varies with amoebal species. *Arachnula impatiens* Cienk. was shown by Old (1977, 1978) to penetrate the walls of conidia of *Cochliobolus sativus* (Ito et Kurib.) Drechs. ex Dastur by erosion of an annular depression into the conidium surface. This disc of wall material is dislodged, allowing the spore contents to extrude into the amoebal trophozoite. The closely related *Theratromyxa* encompasses whole spores within digestive cysts, the spore wall being penetrated by many small holes each *ca.* 0.5 μm dia (Old and Oros, 1980; Anderson and Patrick, 1980). *Mayorella* species have been shown to engulf up to five conidia of *C. sativus* and retain them within the trophozoite for many hours. Extruded spores are often lysed but little information is available on the feeding mechanism of this genus (Anderson and Patrick, 1980). Pussard *et al.* (1979) have described in detail the penetration of hyphae and microconidia of *Fusarium oxysporum* by *Thecamoeba granifera* ssp. *minor*. They regard this species as being strictly mycophagous, whereas Old and Darbyshire (1978) showed that *A. impatiens* has an extremely broad range of potential prey including bacteria, algae, protozoa and nematodes.

The significance of mycophagous amoebae as agents for control of soil-borne fungal propagules is unknown. Old and Patrick (1979) reviewed available knowledge, particularly with respect to vampyrellids and recommended further research on the effects of amoebal groups on populations of soil borne pathogens. A pathogen of particular interest in many temperate regions is *Gaeumannomyces graminis* (Sacc.) v. Arx & Olivier var. *tritici* Walker, the cause of take-all disease of cereals. The phenomenon of take-all decline, that is, the observed reduction of disease after repeated wheat crops has been well documented and has attracted the attention of plant pathologists and soil microbiologists for more than 15 yr. A number of theories as to its cause have emerged and were summarized by Hornby (1979); several of these invoke the gradual development of an antagonistic microflora which suppresses the pathogen. The demonstration that mycophagous amoebae are widely distributed in soils offers the opportunity to test whether they could have a role in take-all decline (Homma *et al.*, 1979).

During a search for mycophagous amoebae in a soil which has been demonstrated to be suppressive to *G. graminis* var. *tritici* (G. B. Wildermuth, unpublished Ph.D. Thesis, University of Adelaide, 1977) an amoebal species has been regularly isolated which differs markedly from previously-described mycophagous amoebae, both in its morphology and feeding mechanism. We describe this unidentified amoeba

and the manner of its feeding on propagules of three soil-borne plant-pathogenic fungi, *G. graminis* var. *tritici*, *C. sativus* and *Phytophthora cinnamomi* Rands.

MATERIALS AND METHODS

The amoeba was isolated from soil collected from a permanent rotation trial plot at the Waite Agricultural Research Institute, Glen Osmond, Australia. The plot has been under continuous pasture for over 20 yr and the soil has been suppressive to *G. graminis* var. *tritici*. Soil was collected from the top 0–5 cm of the profile and moistened to − 8kPa. Moist soil was placed into 15 cm Petri plates to a depth of 7 to 8 mm. Mycelial mats of *G. graminis* var. *tritici*, obtained from 1 week-old cultures grown in 0.4% Malt Extract broth at 25°C were washed twice with sterile distilled water (SDW), placed in silk cloth bags (5 × 5 cm) and buried in moist soil (J. H. Warcup, personal communication). After 3 weeks at 25°C, mycelial mats were recovered and homogenized to form a suspension which was streaked on 1.0% Bacto agar in Prescotts and James (1955) solution (PJA). Amoebae which migrated from the mycelium on to the PJA medium were isolated from other amoebae by repeated transfers of small blocks of agar bearing single individuals, and gradually freed from bacterial contamination (Culbertson, 1971). Isolated amoebae were maintained on PJA bearing *Aerobacter aerogenes* as a food bacterium. Stock cultures of *G. graminis* var. *tritici*, *C. sativus* and *P. cinnamomi* were maintained on Czapek–Dox agar and cornmeal agar respectively. Mycelium of *G. graminis* to be used in feeding trials was grown on 0.4% malt extract broth for 1 week, washed twice with SDW and rinsed in sterile Neffs amoeba saline solution (AS) (Page, 1976). Mycelium was either left intact or homogenized.

Conidia of *C. sativus* were harvested from Czapek–Dox cultures by flooding plates with SDW and lightly rubbing with a sterile loop. Suspensions of conidia were filtered through sterile cheese cloth and washed twice in SDW. Mycelium and chlamydospores of *P. cinnamomi* were produced by inoculating V8 juice liquid medium containing 20 μg cholesterol ml^{-1} (D. D. Darling, unpublished information) with approximately 50 small pieces of agar bearing *P. cinnamomi* and incubating for up to 3 weeks at 25°C in continuous light. Thalli were harvested, washed in sterile AS and either homogenized or left intact. Feeding trials were conducted, using the three fungi in 9 cm dia Petri plates containing the fungal substrate suspended in sterile AS. The saline covered the base of the Petri plates to a depth of approx. 5 mm. At the outset of each trial, six or seven 12 mm dia glass coverslips were placed into each Petri plate.

Stock cultures of the amoeba were flooded with sterile AS and suspensions of amoebal cysts were drawn up into sterile Pasteur pipettes. Drops of these suspensions were added to the fungal substrate and the cultures incubated for 4–5 days at 25°C. Detailed observations of the interactions between the amoebal trophozoites and their prey were made by transferring coverslips bearing these organisms to Cruikshank chambers (Sterilin Ltd, Teddington, Middlesex, England) and examining by phase contrast microscopy (PCM). After feeding for 3 to 14 days, coverslips were airdried and mounted on metal stubs for scanning electron microscopic (SEM) examination. Other specimens, including intact thalli of *P. cinnamomi* and spore suspensions of *C. sativus* that had been incubated with the amoeba were fixed in 3% glutaraldehyde in phosphate buffer overnight at 4°C, dehydrated in a graded ethanol series and critical-point dried in a Polaron unit using liquid CO_2. Before SEM examination, all specimens were coated with a layer of gold palladium in a Polaron E5100 sputter coating unit (Polaron Equipment Ltd, Herts., England). Specimens were examined with a JEOL JSMU3 SEM.

RESULTS

Trophozoite morphology

The amoebal trophozoite exists in two distinct forms, a branched flattened form ranging from 80 to 150 μm in length and 28 to 95 μm in breadth; (Figs 1 and 4) and a limax form (Fig. 2). The change between the two forms can be readily observed. The limax form is common in aqueous media which are disturbed and can be found at the interface between the suspension and the air. Amoeba in non-disturbed cultures tend to settle to the bottom of the dish and produce the branched form. The latter form is also common on agar media. Limax forms are narrower at the posterior end than at the anterior end and measure from 50 to 180 μm in length and 20 to 40 μm in width. Both forms are uninucleate, the nucleus as seen by PCM measures 6 to 10 μm in dia (Figs 1, 3, 4) and contains an elongated nucleolus (3 to 5 μm). The protoplast is distinctly divisible into a hyaline ectoplasm and a granular, compact endoplasm (Fig. 3). The branched form contains one to many vacuoles up to 16 μm in dia. The limax form normally has a single large vacuole at the posterior end of the cell (Fig. 2).

Locomotion is particularly interesting, and can be as rapid as 5 μm s^{-1} in the branched forms. From the advancing margin of the cell small eruptive pseudopods (type lobosa) are formed by the ectoplasm into which the granular endoplasm flows. As the trophozoite advances the posterior margin forms a variable number of fine filopods (Fig. 4). Similar filopods also are formed when a lobose pseudopod is retracted. Pseudopods can be projected in a number of different directions simultaneously, but ultimately flow in one of these directions. True polyaxial movement has not been observed. Anastomosis between individual trophozoites has not been observed so far even though crowding is common on agar cultures.

During cell division the trophozoite extends linearly with pseudopods adhering to the substratum at both ends. Two nuclei can be seen clearly in such dividing cells. The central portion of the protoplast becomes constricted and finally the two daughter cells separate. The generation time on PJA medium with *A. aerogenes* as a food source varies from 3 to 5 h.

Cyst morphology

The amoeba forms cysts which are usually spherical in shape (Fig. 12), but this can vary if individuals are crowded, or cysts form within the confines of lysed fungal cells. For instance, cysts often form within empty sporangia of *P. cinnamomi* (Fig. 11). Cysts are

Plate 1.

Fig. 1. Large flattened trophozoite of the mycophagous amoeba. Note the anterior lobopodia (L), contractile vacoules (CV) and single nucleus (N).

Fig. 2. Tubular limax amoebae. Note the tapered posterior and contractile vacuole (CV).

Fig. 3. Form intermediate between branched and limax showing the single nucleus (N), hyaline ectoplasm (E) and granular endoplasm (EN).

smooth walled and measure 15–30 μm in dia. The single nucleus can be seen just before excystment. Rarely up to 3 nuclei have been seen at this stage. A single trophozoite invariably emerges from the cyst. Excystment can be stimulated by placing cysts into sterile AS or on PJA bearing *A. aerogenes*.

Feeding mechanism of the amoeba on P. cinnamomi

The amoeba employs two separate mechanisms when feeding on *P. cinnamomi*. Penetration and lysis of hyphae, sporangia and hyphal swellings of the fungus is very rapid and is completed in 35 to 45 min. Mature chlamydospores are digested within large digestive vacuoles over a prolonged period, between 21 to 36 h. These processes are quite distinct and will be described separately.

Rapid penetration of hypha

The trophozoite contacts the hypha, movement ceases and it adheres firmly to the cell surface. If hyphal fragments are encountered these may be dragged along the substratum attached firmly to the posterior filopods. When attacking an intact mycelial thallus the amoeba stretches itself along the hyphal length, attaching at any part (Fig. 5). The trophozoite is extremely thin and extended, eventually engulfing a portion of the hypha. This process takes 10–15 min after which the amoeba contracts and masses around a small section of hypha (Fig. 6). This process of extension and contraction may be repeated 2–6 times. The trophozoite then moves along the hypha, or may leave it altogether. At this stage, it can be seen that the portion of hypha where the trophozoite concentrated is completely devoid of cytoplasm (Fig. 8). The amoeba exhibits considerable mechanical force at this time and free ends of hyphae may be physically distorted to encompass them within the body of the trophozoite. The feeding activity of the amoebae is very sensitive to bright illumination. Photography and observation must be carried out with intermittent or low light intensity or feeding will be slowed or cease altogether.

Sporangia and hyphal swellings or immature chlamydospores of *P. cinnamomi* are dealt with by the amoeba in a similar way to hyphae, except that engulfment of the propagule may be incomplete. A similar series of extensions and contractions of the trophozoite occurs and the contents of the sporangium flow into the amoebal cell. The fungal propagule is rendered completely devoid of contents in 35–45 min after feeding begins and the amoeba either moves away to feed on other cells or may migrate within the sporangium or hyphal swelling and form a cyst (Fig. 12). Scanning electron microscopy of fungal material after prolonged feeding showed that hyphae, and other propagules were perforated by circular holes varying from 1.0 to 3.0 μm dia (Fig. 9). No annular depressions similar to those described by Old (1977) were recorded.

Digestion of mature chlamydospores of P. cinnamomi

Having contacted the chlamydospore the trophozoite completely engulfs it. Formation and expulsion of the contents of numerous small vacuoles within the amoebal protoplast are intense at this stage. This process results in the formation of a large fluid filled vacuole containing the chlamydospore within approximately 20–35 min. This vacuole is the subsequent site of digestion of the spore. During this initial phase of attack the amoeba is particularly light-sensitive and rounds up if illumination is too intense. Once the digestive vacuole is fully formed (Fig. 13) the amoeba is less sensitive. Careful microscopical observation of spores within digestive vacuoles shows that delicate protoplasmic threads traverse the space between the main vacuolar membrane and the spore wall. These are not resolved adequately in Figs 13–16. Contraction of these strands may be the cause of the rotation of the chlamydospore within the vacuole shown in Figs 13–16.

The main activity seen within the amoebal protoplast at this time is the continuous formation of vesicles which develop bordering the digestive vacuole and discharge their contents (Figs 15, 16). At no stage did a cyst wall develop around the trophozoite in the manner described for *Theratromyxa* (Old and Oros, 1980). In fact, the outline of the amoeba was in a state of continuous though gradual change, and the trophozoite could be observed to migrate a small distance on the substratum, by normal pseudopodial movement.

After about 16 to 17 h from formation of the digestive vacuole, the diameter of the vacuole gradually reduces (Fig. 17). The amoeba resumes more active pseudopod formation and the generally spherical shape is lost. The fluid layer between the chlamydospore and the amoebal protoplast disappears completely. At this stage the contents of chlamydospore appear to be disorganized and granular (Fig. 18).

Plate 2.

Fig. 4. Trophozoite of the mycophagous amoeba showing anterior lobopodia (L), posterior filopodia (F) nucleus (N) and the typical branched morphology.

Fig. 5. Amoeba feeding on a hypha of *P. cinnamomi*. The trophozoite has enveloped a length of hypha (arrows).

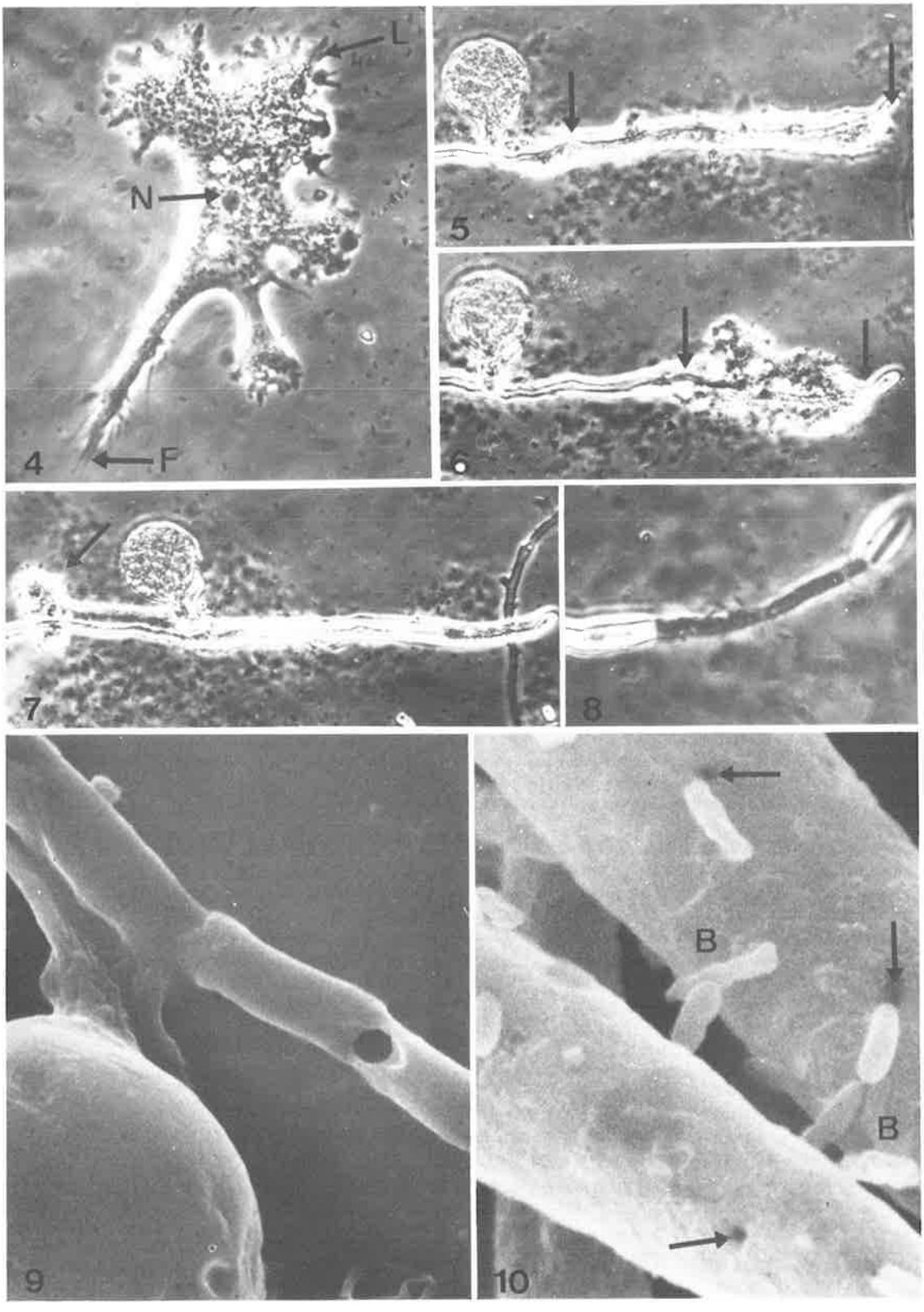
Fig. 6. Trophozoite has concentrated around the subterminal portion of the hypha (arrows).

Fig. 7. Trophozoite has migrated back along the hypha and is concentrating at a new location (arrow). The hyphal tip now lacks protoplasmic contents.

Fig. 8. Detail of the hyphal tip.

Fig. 9. Perforated hypha of *P. cinnamomi*.

Fig. 10. Perforations in hyaline hypha of *G. graminis* var. *tritici* (arrows). The bacteria (B) are *A. aerogenes*.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Typical trophozoite movement resumes and the remains of the digestive vacuole and its contents are conveyed along by the trophozoite. Finally, the digestive vacuole becomes completely disrupted and the remaining fungal protoplasm is taken into the amoebal cytoplasm. As the trophozoite rapidly moves away, a trail of debris, presumably the undigested contents of the digestive vacuole are extruded on to the substratum (Fig. 19).

Feeding of the amoeba on hyphae of G. graminis *var.* tritici

The mode of feeding varies according to the ability of the trophozoite to fully surround the hypha. When intact thalli are used, the amoeba envelops sections of hypha and exhibits the extensions and contractions shown for *P. cinnamomi* hyphae (Figs 5–7). Protoplasmic contents of these hyphal cells are lost and subsequent SEM examination shows that holes varying in size from 0.3 to 2 μm are present in the fungal cell walls. Presumably these are the sites of attack by which the amoeba gains access to the fungal protoplast (Fig. 10). When the fungal thallus is fragmented before feeding trials, the trophozoites ingest whole pieces of hypha. This is commonly achieved by twisting and bending the hypha until it is fully accommodated inside the trophozoite. The trophozoites continue to move across the substratum and in as little as 30–45 min the hyphal fragments are no longer recognizable. Shortly afterwards undigested residues are extruded from the trophozoite.

Interaction of the amoeba with C. sativus

The amoeba commonly encircles conidia of *C. sativus* and the spores frequently adhere to the posterior of the trophozoite, apparently held by the group of filopods. Clumps of 5 or more conidia, short fragments of conidiophores and immature spores accumulate in this position and may be conveyed along by the trophozoite for many hours. Fungal cells are left behind from time to time and others accumulated. However, careful observation of many such trophozoites has never indicated that the amoeba is attacking these spores and digesting their contents. Examination of specimens taken from 14-day old cultures containing conidia and very large numbers of active trophozoites by SEM has not revealed any perforated conidia.

DISCUSSION

The amoeba we describe shares with other species, conclusively shown to be mycophagous, the ability to digest discrete holes in fungal cell walls. But, a particularly interesting characteristic is the ability to employ two distinct feeding mechanisms in attacking different cell types of a single fungus. The criterion which directs the feeding option is the ability of the trophozoite to fully surround the propagule. If this is achieved, often by bending and coiling the hypha by application of considerable force, then digestion of the cells is almost complete in 30 to 60 min. Undigested residues are expelled from the moving trophozoite. If, however, the amoeba attacks an intact thallus and is unable to fully enclose the propagule, then discrete perforations in the wall are made and the protoplast only is ingested. Hyphal walls appear to be otherwise little damaged. These types of feeding have been closely observed for *P. cinnamomi* and hyaline hyphae of *G. graminis* var. *tritici*.

Hyphae of both these fungi and hyphal swellings and chlamydospores in 1 week-old cultures of *P. cinnamomi* are rapidly attacked. Protoplast ingestion commonly takes only 30 to 60 min. When preparations of older chlamydospores (2 to 3 weeks) are exposed to the amoeba the feeding mechanism is modified further. After complete encirclement of the chlamydospore a large digestive vacuole develops and the trophozoite remains almost immobile for a prolonged period (17 to 20 h) during which the entire chlamydospore is digested. It is particularly significant that the mature chlamydospore requires much longer for lysis to be completed than do hyphae or young chlamydospores. Chlamydospores are widely regarded as survival structures, more resistant than hyphae to lysis, but the structural basis for this characteristic has not been discovered. The stimulus to the formation of the large food vacuole by the amoeba may be directly related to the susceptibility of the wall components to enzymatic digestion. This newly-described amoeba, unlike *A. impatiens*, *Theratromyxa* and *Vampyrella* spp (Old, 1977; Old and Oros, 1980; Anderson and Patrick, 1978) is unable to attack conidia of *C. sativus*. Opportunities for digestion of these spores are commonly observed as the trophozoites adhere to conidia and convey them for considerable distances across the substratum. Yet the fungus remains in an apparently viable condition as shown by the presence of intact protoplasts and per-

Plate 3.

Fig. 11. Amoebal cyst within empty *P. cinnamomi* sporangium (arrow).

Fig. 12. Amoebal cysts within lysed swollen hyphae.

Fig. 13. Chlamydospore of *P. cinnamomi* within a digestive vacuole (Figs 13–19 are a sequence of micrographs at the same magnification showing lysis of the chlamydospore).

Fig. 14. Change of amoebal shape and rotation of the chlamydospore.

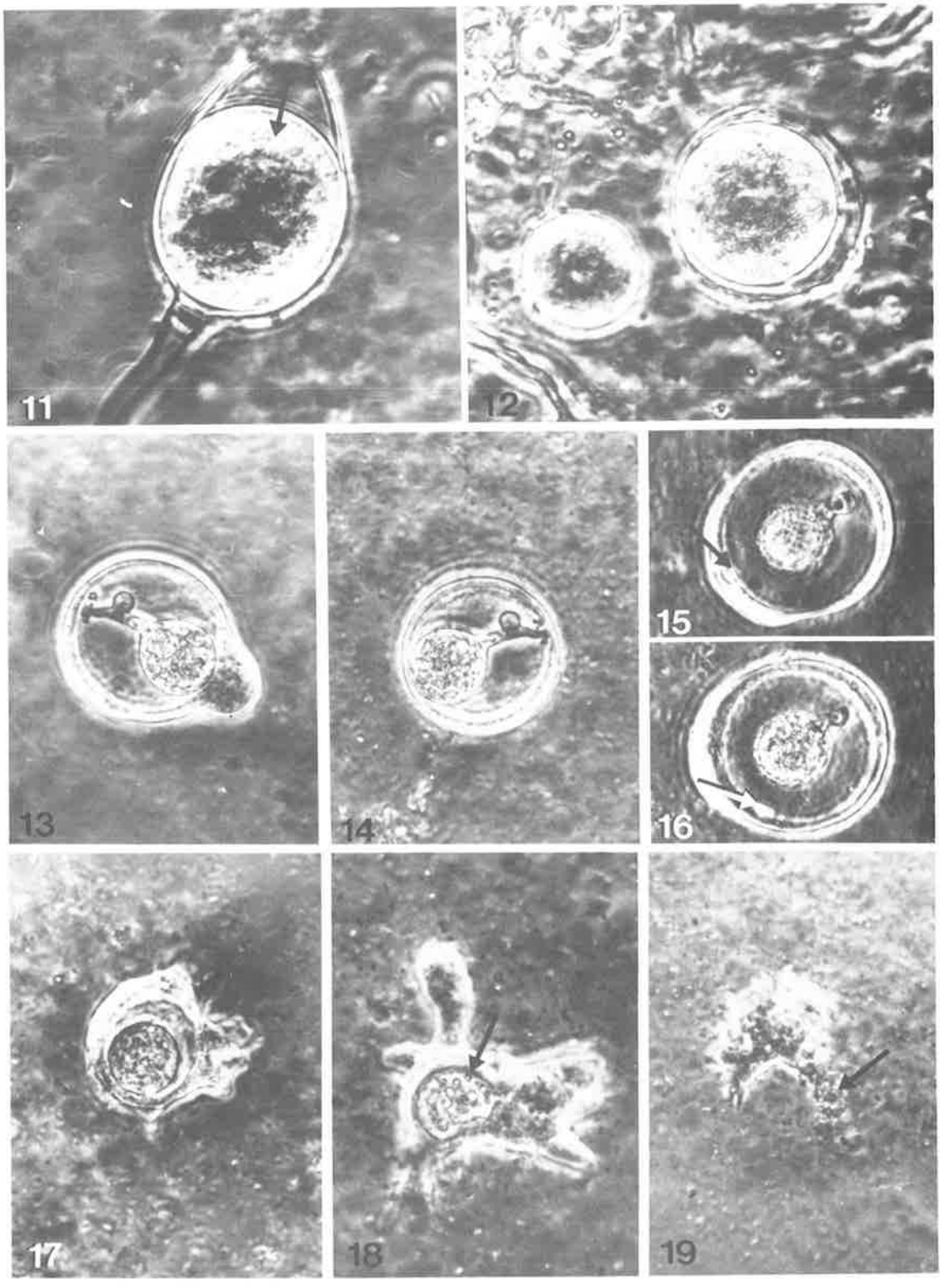
Fig. 15. Digestive vacuole showing a vesicle forming in the amoebal cytoplasm (arrow).

Fig. 16. As Fig. 15 but with a mature vesicle about to discharge into the digestive vacuole (arrow).

Fig. 17. The digestive vacuole is reduced in volume and the amoeba becomes more mobile.

Fig. 18. The chlamydospore (arrow) has lost its structural integrity.

Fig. 19. The trophozoite moves away leaving a trail of fungal cell debris (arrow).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foration-free walls. Movement of fungal spores by amoebae, without apparent damage to them was described by Heal (1963) and has been regularly seen by us for species of *Mayorella*. The walls of *C. sativus* conidia contain deposits of melanin in their outer layers (Old and Robertson, 1970) and may protect the spore from perforation. Melanin has been shown to markedly inhibit the activity of glucanases and chitinases which digest major fungal cell wall components (Bull, 1970). Hyphae of *G. graminis* in old cultures become pigmented with age and an attempt was made to study their susceptibility to attack by the amoeba. Some perforated hyphae were found, but it was not possible to distinguish, by SEM examination, pigmented hyphae from the hyaline hyphae still present in the thallus.

It is extremely difficult to determine the taxonomic affinities of large freshwater and soil amoebae. Many of the early workers who observed these organisms, usually in mixed culture, described them as new species and often erected new genera on the basis of differences in their specimens. The axenic culture of large amoebae is very demanding, and type cultures are largely unavailable for comparison. Zwillenberg (1953) in describing a new species of *Theratromyxa* considered 19 genera as possible synonyms. The genera receiving detailed modern treatment are *Leptomyxa* (Pussard and Pons, 1976a, b), *Gephyramoeba* (Pussard and Pons, 1976c), *Arachnula* (Old and Darbyshire, 1980), *Vampyrella* (Anderson and Patrick, 1978, 1980) and *Theratromyxa* (Sayre, 1973; Anderson and Patrick, 1980; Old and Oros, 1980). The amoeba we describe here does not fit any of the species descriptions which we have encountered, however it clearly has close affinities with *Leptomyxa*, especially *L. flabellata* (Goodey, 1915). In 1976, Pussard and Pons considered that the genus *Leptomyxa* is so different from the other members of the order Proteomyxida that a new order Leptomyxida was necessary. This new order includes amoebae forming thin spreading reticulate trophozoites with polyaxial locomotion. Pseudopods are of indeterminate shape of the type lobosa, the ectoplasm is poorly developed. No flagellate stage is present in the life cycle and no fructifications are known. The order contains two families, Leptomyxidae and Gephyramoebidae, distinguished on the basis of multinucleate trophozoites in the former, and uninucleate trophozoites in the latter family. *Leptomyxa flabellata* Goodey was retained within the same genus as *L. reticulata*, the type species, (Pussard and Pons, 1976b) despite significant differences in trophozoite morphology. In *L. flabellata*, trophozoites are branched rather than reticulate and locomotion is not polyaxial. Another significant characteristic is the production of tubular limax forms when growing in a water film. In all these characteristics our amoeba closely resembles *L. flabellata*. Photomicrographs presented by Pussard and Pons (1976b) clearly show a posterior fringe of filopods as illustrated in Fig. 1. The principal difference appears to be in the nuclear number. *L. flabellata* is reported to have from 1 to 48 nuclei per trophozoite. We have consistently observed a single nucleus in the trophozoite. Up to three nuclei were seen in cysts in our cultures.

As nuclear number is basis for separating the families Leptomyxidae and Gephyramoebidae we are unable to attribute our isolates to the species *L. flabellata*. Further work and comparison of cultures will be needed to name this amoeba with certainty. At present we are confident in placing the amoeba in the order Leptomyxida.

The mycophagous amoeba of the order Leptomyxida described here has been frequently isolated from the take-all suppressive soil and from a number of other sites in South Australia. It is probably found in many other soils and regions in common with other mycophagous genera already studied. Evidence so far suggests that it may be restricted to feeding on hyaline fungal propagules and unable to penetrate pigmented spores. Nevertheless its ability to feed on bacteria would allow populations of this amoebal species to maintain themselves in moist soils as a component of the soil ecosystem that may exert an influence on soil-borne pathogens. It shares many of the attributes discussed by Old and Patrick (1979) and is unlikely to lend itself to ready manipulation as a biocontrol agent. Even so, the study of mycophagy in soil amoebae deserves the closest attention as a factor influencing the population dynamics of soil fungi.

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