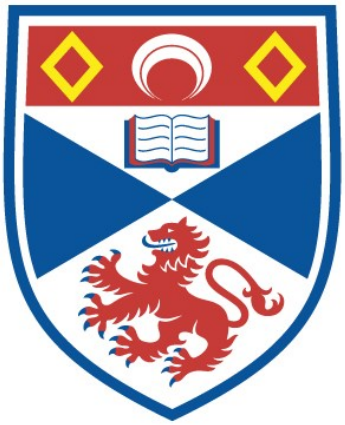
STUDIES ON THE ORGANIC ACID METABOLISM OF PLANTS IN RELATION TO THE FLOODING TOLERANCE OF THEIR ROOTS

Peter David Tyler

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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STUDIES ON THE ORGANIC ACID METABOLISM OF PLANTS IN RELATION TO THE FLOODING TOLERANCE OF THEIR ROOTS

by

Peter David Tyler, B.Sc.

A Thesis submitted to the University of St. Andrews

for the Degree of Doctor of Philosophy

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Department of Botany

University of St. Andrews

DECLARATION

I hereby declare that the following Thesis is based upon work done by me, that the Thesis is my own composition and that it has not been previously presented for a Higher Degree.

The research was carried out in the Department of Botany at the University of St. Andrews, under the supervision of Dr. R.M.M. Crawford.

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CAREER

I graduated from the University of Nottingham in July 1966 with Class II Division 1 Honours in Botany. I was awarded a Science Research Council Postgraduate Studentship for three years research at the University of St. Andrews.

I matriculated at the University of St. Andrews in October 1966 and was admitted as a research student under Ordinance General No. 12 and later as a candidate for the degree of Ph.D. under Resolution of the University Court 1967 No. 1.

CERTIFICATE

I certify that Peter David Tyler has spent nine terms of research under my direction, that he has fulfilled the conditions of Ordinance General No. 12 and Resolution of the University Court 1967 No. 1, and that he is qualified to submit the accompanying Thesis in application for the Degree of Doctor of Philosophy.

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The author wishes to record his gratitude to Dr. R.M.M. Crawford of the Department of Botany, University of St. Andrews, for supervising the work presented in this Thesis and for his encouragement and enthusiasm throughout the project.

He is also pleased to acknowledge the Science Research Council, for the award of a Postgraduate Studentship, and Professor J.A. Macdonald, in whose Department the work was carried out.

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PREFACE

The study of flood tolerance in plants has been mainly concerned with the descriptive ecology of wetland species, rather than with the actual mechanisms which enable these species to tolerate flooded conditions. The nature of the tolerance to flooding shown by some species has not been fully explained, whereas their distribution, performance and competitive effects have been investigated (Parker 1950, Hunt 1951, Lazenby 1955, Ingram 1967), and the effects of nutrient status and oxygen supply considered (Bergman 1959, Webster 1962a & 1962b, Gore & Urquhart 1966, Sparling 1967).

There has been little comparison of the physiology of flood tolerant species with that of flood intolerant species, especially with respect to possible mechanisms by which the former group are favourably adapted to their environment. The nature of most metabolic processes is such that their study involves sensitive analytical biochemical techniques. Within recent years it has become possible to apply such techniques to several aspects of a plant's metabolism and thus measure the changing levels of metabolites and trace the particular pathways concerned. A biochemically based investigation of flood tolerance has therefore become feasible, and the present investigation has attempted to determine if the ecological differences of flood tolerant and intolerant plants are matched by corresponding differences in their metabolic processes.

INTRODUCTION

GROWTH RESPONSES OF DRY-LAND SPECIES TO WATERLOGGING

Most dry-land species are unable to compete against those of natural marsh communities under flooded conditions, and they may show early visible signs of flooding injury. Symptoms such as wilting of the shoots, chlorosis and death of the leaves and death of the roots have been observed in tobacco, sunflower and tomato plants (Kramer 1951, Kramer & Jackson 1954). The initial wilting, which at first sight would have been expected more of plants deprived of, rather than overburdened with, water, results from a sudden decrease in permeability of the roots. The death of root and shoot tissue follows a general reduction of absorption and growth processes (Loehwing 1934), although in roots there may be additional decay from fungal infection (Stolzy, Letey, Klotz & Labanauskas 1965).

The experimental simulation of flooding effects has been much investigated, mainly from the aspect of inadequate oxygen supply. Studies on Zea mays indicate that a reduced oxygen supply rather than a build-up of carbon dioxide is responsible for reduced growth of the young plants under poorly aerated soil conditions (Unger & Danielson 1965). With pea plants in water culture the extent of root elongation depends on the availability of oxygen

(Geisler 1965), and reduced oxygen supply to the roots significantly reduces the amount of dry weight in Avocado seedlings (Labanauskas, Stolzy, Zentmeyer & Szuszkiewicz 1968). Reduction of the oxygen concentration to 8.5% and 3.5% retards the development of lateral roots and root hairs in wheat, broad bean and vegetable marrow (Ranson & Parija 1955), and barley root growth is considerably diminished below 9.5% oxygen (Vlamis & Davis 1943). Sugar cane root growth decreases gradually with each decrease in applied oxygen concentration between air and 3.0% oxygen, with a sharp decrease below 3.0% to virtual cessation of growth at 0.0% (Banath & Monteith 1966), and root growth is stopped at 0.5% oxygen in tomato, soybean and tobacco (Hopkins, Specht & Hendricks 1950). On the other hand, there is no noticeable injury to the rhizomes of water plants such as Nuphar advenum, Nymphaea tuberosa, Peltandra virginica, Scirpus validus and Typha latifolia, when the material is surrounded by gas mixtures of air, 10, 4.6, 3.0, 1.5, 1.0, 0.4 and 0.1% oxygen, or even purified nitrogen (Laing 1940a).

The effects of decreases oxygen and increase carbon dioxide are difficult to distinguish, although those of increased carbon dioxide concentrations do not seem so marked. For instance, cotton root growth remains at an optimum up to 15% carbon dioxide when the oxygen concentration is maintained at 21%, and is only reduced by concentrations between 30 and 45% carbon dioxide (Leonard & Pinckard 1946). On the other hand, pea plants maintained at 21% oxygen showed decreased root length at 8% carbon dioxide (Geisler

1967). In aerated and non-aerated water cultures of tomato, where plants in the latter conditions show increasing limitation of growth and water absorption, the differences were produced by insufficient oxygen in the solution and were developed before the concentration of carbon dioxide reached a possibly toxic level (Erickson 1946). In aerated nutrient solution tomato shows increased vegetative growth of roots, stems and leaves, and increased fruit production (Durrell 1941), and in similar culture wallflower shows considerable increase in dry weight of the leaves (Knight 1924).

A reduction in available oxygen and increase in carbon dioxide can be caused by factors other than flooding, and the negligible permeability of ice for these gases would account for the mortality of winter cereal under an ice crust (Rakitina 1965) Similarly, the asphalt covering and soil compaction around street planting increases the carbon dioxide in the soil air to 4-8%, decreases that of oxygen to 13-14%, restricts the formation of physiologically active roots and leads to premature leaf-fall (Rakhteenko & Kochanovskii 1965).

COMMUNITY RESPONSES TO VEGETATION WATERLOGGING

Vegetation communities where the habitat is subject to natural flooding are commonly found within the British Isles. The term 'swamp' is generally reserved for the extreme type of site where

waterlogging is more or less permanent and the summer water table remains above the surface of the soil (Tansley 1949). 'Marsh' and 'fen' depict wet areas, such as lake margins, low river banks and flood plains of rivers, where the summer water table is at, or near, the soil surface. Marsh is found on mainly mineral soil whereas fen is developed on wet, usually alkaline, peat (Tansley 1953). The term 'bog' is best restricted to Sphagnum dominated acid peat characteristic of upland sites.

In a non-flooded habitat the composition of the soil atmosphere is generally the same as that of the atmosphere above the soil. Thus the oxygen and nitrogen concentrations will be approximately 20% and 79% respectively in both atmospheres, and the carbon dioxide concentration between 0.1 and 1.5% in soil and at 0.03% in air (Table 1).

Table 1. Composition of the air in soils, % by volume.
(From Russell 1961)

Soil	oxygen	carbon dioxide
Arable land, fallow	20.7	0.1
Arable, uncropped, sandy soil	20.6	0.16
Grassland	18.4	1.6
Pasture land	18-20	0.5-1.5

In a flooded site, however, waterlogging reduces the amount

of pore space and hence affects the aeration of the soil (Daubenmire 1947). Furthermore, whereas the diffusion of gases in a non-flooded, aerated soil maintains the atmosphere similar to that above the soil, the diffusion of gases, such as oxygen, through water is approximately 10,000 times slower than through air (Martin 1968a, Boulter, Coult & Henshaw 1963). There is therefore a very profound effect on the soil atmosphere whenever waterlogging occurs and one of the long accepted features of marsh vegetation is that the roots and other submerged parts are growing in a medium with a much lower oxygen concentration than is normal for land plants (Conway 1940, Poel 1960, Bouldin 1968). If there is no lateral movement of water through the site then the oxygen concentration suffers the greatest reduction. In the flush of a Sutherland valley bog oxygen was present to a depth of 16-18 cm, but not detected below 6 cm in the stagnant regions (Armstrong & Boatman 1967), and experimental swards of herbage plants under stagnant water were killed more readily than those under running water Davis & Martin 1949). Although waterlogging also affects the root environment through other factors such as decreased rate of carbon dioxide removal, and accumulation of toxic substances such as hydrogen sulphide (Webster 1962b) and ferrous ions (Martin 1968b), it is the decrease in aeration and therefore reduction in availability of oxygen that is the major influence on the vegetation of flooded soils. The flooding may be for several months during the winter, or for shorter periods within the wet season, but nevertheless the plants of marsh communities must be able

to withstand these periods of imposed anaerobic conditions to survive and compete successfully.

Not all flooded habitats are necessarily alike from the point of view of root physiology. The differences between a stagnant waterlogged site and one continually flushed with water will be reflected in the different composition of the vegetation communities developed there, and base poor flooded sites will support different vegetation from base rich ones. However, since the important factor in the effect of waterlogging upon vegetation is the decrease in available oxygen, the experimental study of the physiological response to flooding has here been confined to that of deficient oxygen supply to the roots.

Where marsh and fen communities develop, the vegetation can often be rich and luxuriant, indicating that the species are well suited to the wet habitat (Plate I). Species common in both marsh and fen include the helophyte class of Raunkiaer's Life Forms, which are those plants with perennating buds lying in mud (Raunkiaer 1937). Although the soil may vary in its nutrient status, often becoming rich in mineral nutrients, the common factor in marsh and fen vegetation is tolerance of the predominant waterlogging. The ability of these helophyte species to tolerate what may be prolonged periods of flooding enables them to compete successfully against the species unable to withstand waterlogging of the soil, and to flourish in these areas to the exclusion of

the latter. Plate II shows the predominance of Iris pseudacorus* and Filipendula ulmaria at the margin of Loch Clunie.

Flooded and unflooded soils may be developed on entirely different sites in respect of factors such as mineral composition and physical nature of the substrate, and the study of community responses to waterlogging can probably be best made in a sand dune area, where dry areas (dunes) alternate with wet areas (slacks) and yet both habitats are developed on the same substrate. The dominant feature of the dune habitat is the dry nature of the substrate and the complete lack of any waterlogging (Salisbury 1952). While the plants of sand dune communities may be well adapted to the light, well-drained soil, their inability to tolerate any waterlogging is shown by their exclusion from the 'slacks' between the dune ridges. Dune slacks are the damp or wet hollows developed between dune ridges, and are characterised by a high water table and frequent flooding (Turrill 1953). The slacks therefore represent a contrast to the dunes in terms of the wet or dry nature of the habitat, even though both sites may be in close proximity to one another and developed on the same type of substrate. The vegetation of the wet slacks is very different from that of the dry dunes (Plates IIIA & IIIB), and is most closely related to marsh vegetation. In those slacks where the water table is never more than 1 metre below the surface, typical marsh

* Nomenclature from Clapham, Tutin & Warburg (1962).

vegetation is found, including Juncus spp., Filipendula ulmaria and Glyceria maxima. The water table may descend to 1-2 metres in slacks whose levels have been raised by erosion of the adjacent dunes, with introduction of less typical marsh species, such as Salix repens. If this raising of the slack level contiues, the water table descends still deeper and the slack vegetation is replaced by characteristic dune vegetation (Chapman 1964). The inability of species of the dune community to compete successfully in the slacks and their exclusion from these wetter areas by species of marsh communities is evidence of a lack of tolerance on their part to waterlogging of the soil. It is therefore both convenient and instructive to investigate the responses of species from adjacent sand dune and slack communities to experimental flooding.

The evidence above shows that waterlogging can have an immediate and retarding effect on the growth and reproduction of several species, and these effects will be reflected in the distribution of those species and the composition of vegetation communities developed at the site. The main cause of injury to plants upon waterlogging is withdrawal of an adequate supply of oxygen. Marsh plants, however, survive and flourish under these conditions. That some plants have an ability to grow when the oxygen supply to their roots is greatly reduced must indicate the possession of morphological or physiological adaptations or both (Bergman 1959).

MORPHOLOGICAL ADAPTATIONS AND FLOOD TOLERANCE

The view has been held for some time that the submerged tissues of marsh plants are supplied with oxygen via the aerial parts (Conway 1940, Sifton 1945 & 1957, Russell 1952). Several of these plants have a linked system of air spaces which achieves continuity between leaf, shoot and root, and in the root the proportion of air space to cell may be as high as 60% (Conway 1937). In a survey of plants from wet and dry habitats, the specific gravity of the roots, which is an approximate measure of the air-content and expresses the degree of aeration, was found to indicate the prevalance of air spaces in the roots of marsh plants and their absence in dry-soil plants (Table 2).

It is suggested that with the entry of oxygen to the aerial parts and its transport to the roots, these latter organs of flood tolerant plants are able to survive in a virtually anaerobic medium (Raalte 19[illegible], Grable 1966). The carbon dioxide of root tissue respiration would be transported in a reverse direction, and not accumulate in the waterlogged parts (Sifton 1945).

The mechanism of this gas transport along the intercellular air spaces has been investigated, and appears to be one of simple diffusion (Evans & Ebert 1960, Heide, Boer-Bolt & Raalte 1963). The importance of the aerial portions has been confirmed, for [illegible]

after removal of the leaves the supply of oxygen is cut off and the protective effect lost (Soldatenkov & Hsien-Tuan 1961). Where studies have included a comparison of normally flood tolerant species with intolerant species, the size of the intercellular air spaces may be a factor affecting the ability to withstand waterlogging. Thus in rice the gas spaces in the roots constitute 5-30% of the tissue and rice thrives under flooded conditions, whereas in barley the corresponding figure is below 1% and in this species poor root growth and even death result from waterlogging (Barber, Ebert & Evans 1962).

Table 2. Specific gravity of the roots of (a) marsh plants, (b) meadow plants, and (c) dry-soil plants. (From Iversen 1949)

		<0.8	0.8-0.89	0.9-0.99	>1.0
(a)	Iris pseudacorus	+			
	Phragmites communis	+			
	Ranunculus flammula	+			
(b)	Juncus bufonius	+			
	Caltha palustris	+	+		
	Ranunculus repens		+	+	
(c)	Bellis perennis			+	+
	Festuca rubra				+
	Achillea millefolium				+

In addition to the diffusion of oxygen from the aerial parts of waterlogged plants to the root tissue, there is evidence that in some bog plants there is diffusion of oxygen out of the roots into the surrounding medium (Armstrong 1964 & 1967a). The diffusion of oxygen into waterlogged soils is regarded as an additional protection, rendering the medium immediately around the roots less anaerobic (Armstrong 1967b). Furthermore, the existence of interspecific and intervarietal differences in oxygen diffusion rate from the roots (Armstrong 1967a & 1969) could account to some extent for the interspecific and intervarietal differences in performance under flooded conditions. Armstrong (1967c) has suggested a classification series, from plants such as Menyanthes trifoliata with considerable root oxidising power and internal oxygen supply, through plants able to receive sufficient internal respiratory oxygen but unable to oxidise even mildly reduced soil, to those which are completely soil-oxygen dependent.

The relation between oxygen diffusion from aerial parts to the submerged organs and survival and growth in waterlogged habitats has been confirmed for Equisetum limosum (Barber 1961), Molinia caerulea (Webster 1962a), and Spartina alterniflora (Teal & Kanwisher 1966). In Menyanthes trifoliata there is diffusion down the stelar air passages to the root tissues and some evidence of its passage into the surrounding medium (Vallance & Coult 1951) and in this species the morphological and functional adaptations

have considerable ecological significance in its ability to flourish in marsh conditions (Coult & Vallance 1958, Coult 1964).

There are a smaller number of reported instances of other morphological changes to facilitate oxygen supply to waterlogged roots, as in the development of a root mat at the soil surface in rice (Alberda 1954), and in the pneumatophores of Avicennia nitida and stilt roots of Rhizophora, both mangrove plants (Scholander, van Dam & Scholander 1955). The development of cortical air spaces in the roots of Zea mays as a result of oxygen scarcity has been reported by McPherson (1939).

All these instances of an apparent morphological adaptation occurring in marsh plants only (Soldatenkov & Chirkova 1963) still do not explain the marked survival properties of these plants in waterlogged conditions. The actual diffusion of oxygen from the shoots to the roots is not questioned, but as this phenomenon is in fact an ubiquitous one and has also been demonstrated in flood intolerant species (Vartapetyan 1964), there are doubts as to whether its beneficial effect alone can ensure flood tolerance in a plant. For instance, in barley the diffusion of oxygen from aerated shoots facilitates the survival of roots kept in an anaerobic medium (Heide, Boer-Bolt & Raalte 1963), and yet barley is not regarded as a natural marsh plant. Similarly, Greenwood (1967) has demonstrated that oxygen diffusion through the stems and roots

of several vegetable seedlings could satisfy all the roots' requirements for oxygen when the plants were grown under anaerobic conditions. If such gaseous diffusion between aerial, aerated shoots and waterlogged, anaerobic roots was the sole operating factor in deciding the survival and growth of a species in flooded conditions, then marsh vegetation would be more varied than it is, with the inclusion of a large number of species at present found restricted to the drier zones outwith the waterlogged marsh area. The functional significance of aerenchyma in plants has in fact been questioned by Williams and Barber (1961), who suggested that the honeycomb structure of aerenchyma is a result of the need for mechanically the greatest possible strength and metabolically the least possible amount of tissue to reduce the oxygen requirement. They further argue that the oxygen resevoir and transport functions of aerenchyma are only incidental to the mechanical and metabolic requirements given above.

Although the diffusion of oxygen from the leaves to the submerged organs during the daylight hours of active photosynthesis may well provide for aerobic respiration in these tissues, nevertheless the availability of oxygen for downward transport during darkness must be limited. As Table 3 shows, there is evidence of a drastic reduction in oxygen concentration within root and rhizome tissues of Nuphar advenum during darkness, so that although the gaseous transport system allows for aerobic respiration during

daylight it does not keep up the supply during darkness, and anaerobic respiration becomes inevitable.

Table 3. Oxygen content of the internal atmosphere of roots and rhizome of Nuphar advenum plants growing in their natural habitat in June. (From Laing 1940b)

	O_2 % by volume	
	dark	sunny
root	1.6	7.9
rhizome	0.6	7.0

It is therefore suggested that, important though it is, the diffusion of gaseous oxygen to the submerged organs of plants plays only a partial role in their survival under flooded conditions. The continued survival and growth of some species under these conditions may also depend upon physiological adaptations.

METABOLIC ADAPTATIONS AND FLOOD TOLERANCE

It is possible to envisage several metabolic adaptations by which plants develop tolerance towards low oxygen and raised carbon dioxide levels. There could be a reduction of the overall metabolic rate, a tolerance of the normally accumulating end-products of anaerobic metabolism, or deviations from the normal metabolic

pathways (Boulter, Coult & Henshaw 1963). There is evidence for a reduction or control of the overall metabolic rate, for while in flood intolerant species there is increased ethanol production and increased activity in alcohol and malic dehydrogenases upon experimental flooding (Crawford 1967b, Crawford & McManmon 1968), no such responses are found in the flood tolerant species. It has also been shown that it is an inability to avoid an excessive rate of glycolysis upon flooding which restricts most species of Senecio to dry sites, and only those species which control this glycolysis exhibit flood tolerance (Crawford 1966). There is probably some tolerance of the end-products of anaerobiosis in the rhizome of Nuphar advenum, for there the oxygen content can fall as low as 2.2% (Laing 1940b) and the tissues must respire at least partially anaerobically. Below 3% oxygen content of the medium, rhizomes of N. advenum in fact form ethanol, and the lower the content of oxygen the greater the formation of alcohol (Laing 1940a). Deviations from the normal metabolic pathways are suggested by the marked difference between the greater activity of catecholase upon experimental flooding in intolerant species than in flood tolerant species (Crawford 1967a).

While the above studies offer a metabolic explanation why most species succumb to flooding, they do not suggest alternative pathways by which flood tolerance is effected. There may be yield of an end-product, such as an organic acid, whose accumulation

during the flooded period can be tolerated. In the rhizome tissue of Iris pseudacorus in winter, the natural period of flooding, accumulations of malic, quinic and shikimic acids have been found (Henshaw, Coult & Boulter 1962). Roots of Salix cinerea become richer in organic acids upon oxygen deficiency, mainly in pyruvate, malate and succinate (Dubina 1961). More recent work with Iris pseudacorus has shown that in the presence of carbon dioxide the rhizome tissue avoids ethanol production by the production of malic acid (Bown, Boulter & Coult 1968). The possible role of organic acids as the natural end-product of anaerobic carbohydrate breakdown in flood tolerant species therefore seems worthy of investigation. Mazelis and Vennesland (1957) have suggested that malic acid in particular could fulfill this role in many plant tissues.

ECOLOGICAL ADVANTAGE OF FLOOD TOLERANCE

The direct relevance of flood tolerance in species to their ability to grow and flourish in waterlogged sites can be seen from their natural distribution. Experimental waterlogging of the heath plants Erica cinerea, Calluna vulgaris and Erica tetralix shows their tolerance to increase in the order listed (Bannister 1964b), and in the field the soils supporting E. cinerea are the driest, those supporting Calluna are wetter, and E. tetralix sites tend to be waterlogged (Bannister 1964a). The inability to invade

marshy habitats without flood tolerance is shown by Pteridium aquilinum, a species restricted to dry sites or very localised, aerated 'islands' within the marsh area (Poel 1961). Mercurialis perennis cannot tolerate flooding and is confined to the better drained, aerated soils in woodlands, but Primula elatior is characteristic of the poorly drained soils which become waterlogged during the spring months (Martin 1968b). Similarly, the bulbs of Endymion nonscriptus cannot tolerate flooding and they are not found in woods below the level of the water table (Knight 1964).

Woody species can also show flood tolerance and invade marshy areas, as with Salix atrocinerea, S. fragilis, S repens, Myrica gale, Alnus glutinosa and Betula pubescens (Armstrong 1968). Salix atrocinerea has experimentally been shown capable of continued root growth in the absence of oxygen, whereas root growth ceases in Pinus banksiana and Picea mariana under such conditions (Wareing 1957). Another pine species, Pinus serotina, is not tolerant of flooding and shows twice as much growth on the better drained site than on the poorly drained one (Graham & Rebuck 1958). Germination studies have shown that in nuttal oak, which is common on sites flooded annually, there is no reduction of the germination percentage of its acorns after submersion in water for periods up to 34 days, whereas in cherry bark oak, which typically grows on sites seldom or never flooded, the germination percentage is significantly lowered by prolonged submersion (Briscoe 1961).

Similarly, the germination of Juncus effusus is greater when the water table is up to the soil surface than when it is 20 cm below the surface (Lazenby 1955).

Species can therefore vary considerably in their tolerance to flooding, and the extent of this ability will be reflected in their ecological amplitude. In the genus Senecio the distribution of some species within wet areas and the exclusion of other species from such sites is a reflection of the former's ability to control anaerobic respiration (Crawford 1966). In the present study a group of naturally occurring marsh plants have been compared, in respect of their organic acid metabolism upon flooding, with other species restricted to dry sites by their lack of flood tolerance.

MATERIALS AND METHODS

COLLECTION, PROPAGATION AND CULTURE OF EXPERIMENTAL MATERIAL

The plant material used in the flooding experiments was grown from seed or vegetative transplants. The initial collections of seed and perennating parts were from natural sites, such as the dune ridges at Tentsmuir for seed of Hieraceum pilosella and Senecio vulgaris, the dunes at West Sands, St. Andrews, for seed of S. jacobaea, the dune slacks at Tentsmuir for transplant material of Carex arenaria, and the margin of Loch of Lowes for transplants of Juncus effusus and C. lasiocarpa (see Fig. 1). Where seed was used, as with all the Senecio species, the supplies were perpetuated under culture in the glasshouse. Species cultivated from vegetative transplants were, where possible, developed as clones by splitting up the young shoots of one plant, as with C. arenaria.

The plants were grown in coarse sand in plastic buckets of 6-7 litre capacity, in a heated glasshouse with a 16-hour daylight regime. Each species was grown in a set of four culture buckets, although the total number of plants per species varied since each culture contained from 3 to 8 plants depending on their size. During an initial period of 3-6 weeks the plants were established in the sand culture under non-flooded conditions. During this

period the plants were watered with Hoagland solution (Thomas, Ranson & Richardson 1956) to supply nutrients and keep the sand moist, without allowing any free liquid to accumulate. Each culture contained a central, polyurethane painted, perforated metal tube, of approximately 1.5 inches in diameter, to allow a visual check on the level of the solution (Plate IVA).

The experimental flooding treatment to these established plants was such that each species underwent 0, 1, 2 and 4 days flooding with Hoagland solution. During the flooding period sufficient solution was added to keep the level just above the surface of the sand (Plate IVB). Immediately upon completion of the flooding treatment, the plants were carefully removed from the sand and the roots cut away from just below the hypocotyl. These roots were then used for the preparation of aqueous root extracts.

There were no visible signs of flooding upon growth during the 4-day flood period, and a further series of plants was allowed to continue growth for an 18-day period to determine if there was any growth response brought about by flooding. The species were classified as flood tolerant and flood intolerant respectively on the basis of increase or decrease in dry weight of the shoots after an 18-day flooded culture compared to an 18-day non-flooded culture. This experimental definition of flood tolerance accounts for certain anomalies when compared with the natural

distribution of the species. For example, Carex arenaria is usually found as a sand dune plant and yet shows no reduction in growth on experimental flooding. From a comparison of the growth response of the species after an 18-day period in flooded and non-flooded conditions, they were described either as 'helophytes' or 'non-helophytes'. The term helophyte is taken from Raunkiaer (1937), by whose definition it is a species with buds submerged in saturated mud or in water. The present investigation is most concerned with the response of the plant to experimental flooding, and the terms helophyte and non-helophyte have been extended to cover, respectively, those species which withstand experimental flooding and those species which do not.

COLLECTION OF FIELD MATERIAL

During the spring of 1967, monthly collections of plant material were made from a sand dune slack at Tentsmuir. The slack chosen was subject to winter flooding and the three collections coincided with the March-May drop in water table and change from flooded to dry conditions. Root material of Erica tetralix, Filipendula ulmaria and Glyceria maxima was dug at each collection, transported back to St. Andrews in mud/earth clods and washed before extracting with water.

For a twelve-month period during 1968, monthly field collections were also made at the margin of Loch Clunie. At this site the winter level of water in the loch causes extensive flooding of a marshy area dominated by Filipendula ulmaria and Iris pseudacorus, yet during the spring the level drops and by summer the area is free of standing water (Plates VA & VB). Within the adjacent shallow region of the loch itself there is an extensive area occupied by Nuphar lutea. At monthly intervals root and rhizome material of N. lutea and I. pseudacorus were collected and transported to St. Andrews for the preparation of root extracts.

INCUBATION OF ROOT TISSUE IN AEROBIC AND ANAEROBIC CONDITIONS

In addition to the experimental flooding and the collection of material growing at naturally flooded sites, some species underwent treatment in completely anaerobic conditions. A comparative experiment was carried out, incubating the root tissues of Senecio viscosus, S. aquaticus and Ranunculus flammula in glucose solution both in aerated containers and under nitrogen.

Roots of plants of these species grown under non-flooded glasshouse sand culture were removed, surface sterilised in 0.02% mercuric chloride, rinsed several times in sterile distilled water, and cut into 1-2 cm lengths. Between 1 and 2 g fresh weight of

this root tissue was placed in 100 ml conical flasks containing 40 ml of 2.5% glucose solution. The flasks were clamped in a mechanical shaker within a water bath maintained at 28°C. Air from an electric pump was bubbled through one set of flasks to maintain aerobic conditions, while nitrogen from a cylinder was bubbled through a second set to maintain strictly anaerobic conditions. After incubation in this way for varying periods up to 6 hours, the roots were removed from the flasks, rinsed in distilled water and extracted with water.

An extended incubation experiment of 12 hours duration was carried out with roots from Senecio spp. grown under flooded glasshouse sand culture for one month. The aqueous extracts were here used for the determination and comparison of ethanol production in a helophyte and a non-helophyte under these anaerobic conditions.

PREPARATION OF AQUEOUS ROOT EXTRACTS

All root tissues were extracted in water by the same method, irrespective of whether taken from experimentally flooded plants, directly from natural sites, or from glucose incubation flasks. After washing in chilled distilled water, the roots (3-10 g fresh weight except in the incubation experiments where quantities were slightly smaller) were cut into approximately 2 cm lengths to

aid maceration, and extracted with 100-150 ml cold distilled water in a Waring Blendor. The extract was passed through muslin then centrifuged at 9,000 r.p.m. for 20 minutes at 2°C. The supernatant taken after centrifugation was the aqueous root extract used for organic acid assay after either perchloric acid deproteinisation or ion exchange purification.

A check was made that this cold water method was extracting all the organic acids from the root tissues. Some non-flooded Senecio viscosus root material was divided into two parts, one part for extraction in chilled water as above, the other part for extraction in hot water. As Table 4 shows, the small differences between the amounts of malic, lactic and oxaloacetic acids determined in the two extracts indicate that the cold water method was the better of the two.

Table 4. Organic acids of non-flooded Senecio viscosus roots, expressed in μ mole / g fresh weight.

	malate	lactate	oxaloacetate
cold water extract	3.0	0.87	0.50
hot water extract	2.6	0.82	0.38

The efficiency of the cold water extraction method was also checked by further extraction of a residue in boiling 95% ethanol.

As Table 5 shows, there was no shikimic acid left in the tissue after cold water extraction, that could be further extracted by boiling ethanol.

Table 5. Shikimic acid content of Nuphar lutea rhizome, expressed in µg / g fresh weight.

	shikimate
cold water extract	31.4
95% ethanol extract of residue	NIL

DEPROTEINISATION OF ROOT EXTRACTS

10 ml portions of the aqueous root extracts were deproteinised by the addition of 10 ml perchloric acid (6% w/w). After centrifuging at 3,000 r.p.m. at room temperature for 20 minutes, the precipitated protein was removed by transferring the supernatant to a cooled beaker in an ice bath. To neutralise the unused perchloric acid, two drops of methyl orange indicator were added, then, while stirring magnetically in the ice bath, potassium carbonate solution (approximately 5M) was added drop-wise until there was evolution of carbon dioxide and the indicator turned salmon-pink. After standing for 10 minutes in the ice bath, the deproteinised extract was decanted from the precipitated potassium perchlorate.

PURIFICATION OF ROOT EXTRACTS BY ION EXCHANGE RESIN

The remainder of the aqueous extract was purified by ion exchange resin, to remove proteins which would interfere with later enzymic analysis, and to remove sugars which would reduce the sensitivity of chromatographic analytical techniques. For this purpose 1 g of strongly basic anion exchange resin Amberlite IRA 400 (Cl) was prepared in the carbonate form with 200 ml of sodium carbonate solution (2% w/v), and packed in a column using a 25 ml burette plugged at the bottom with glass wool. The resin was washed with about 200 ml distilled water until the effluent was acidic to phenol red, then the aqueous root extract passed through the column at an approximate rate of 5-10 ml per minute. After washing the column with a further 200 ml distilled water, the anions were eluted with 200 ml of 1N ammonium carbonate solution. The effluent, containing the organic acids in their ammonium salt form, was then evaporated to dryness three times in a rotary film evaporator to remove excess ammonium carbonate. The residue was taken up in 10 ml distilled water, and this solution used for organic acid micro-estimation.

ORGANIC ACID ASSAY OF THE PURIFIED ROOT EXTRACTS

The quantities of root tissue available for preparing the

extracts were in some experiments less than 2 g, and very rarely over 10 g. While a method of gradient elution down a silica gel or ion exchange column can allow a relatively rapid quantitative estimation of several organic acids in pome fruits (Hulme & Wooltorton 1958), the amounts of acids in these root extracts were too small to allow such a technique to be used. The micro-estimations which were necessary were therefore carried out enzymically, for malic (Appendix p.ii), lactic (p.iii), oxaloacetic (p.iv), citric (p.vi) and succinic acids (p.ix), and microcolorimetrically for shikimic acid (p.xi). The techniques used were not only accurate at the level required but also specific for the particular acid being estimated.

A method of paper chromatography, suitable for detecting small quantities of organic acids, was devised and also used (Appendix p.xiii). The use of this technique was not intended for following changes in levels of particular acids but rather as a quick and reliable scan to check that no otherwise undetected acid occurred in the tissues.

DETERMINATION OF ETHANOL IN THE ROOT EXTRACTS

The extracts used for measuring ethanol production under anaerobic incubation were prepared from 3 to 4 g of root tissue.

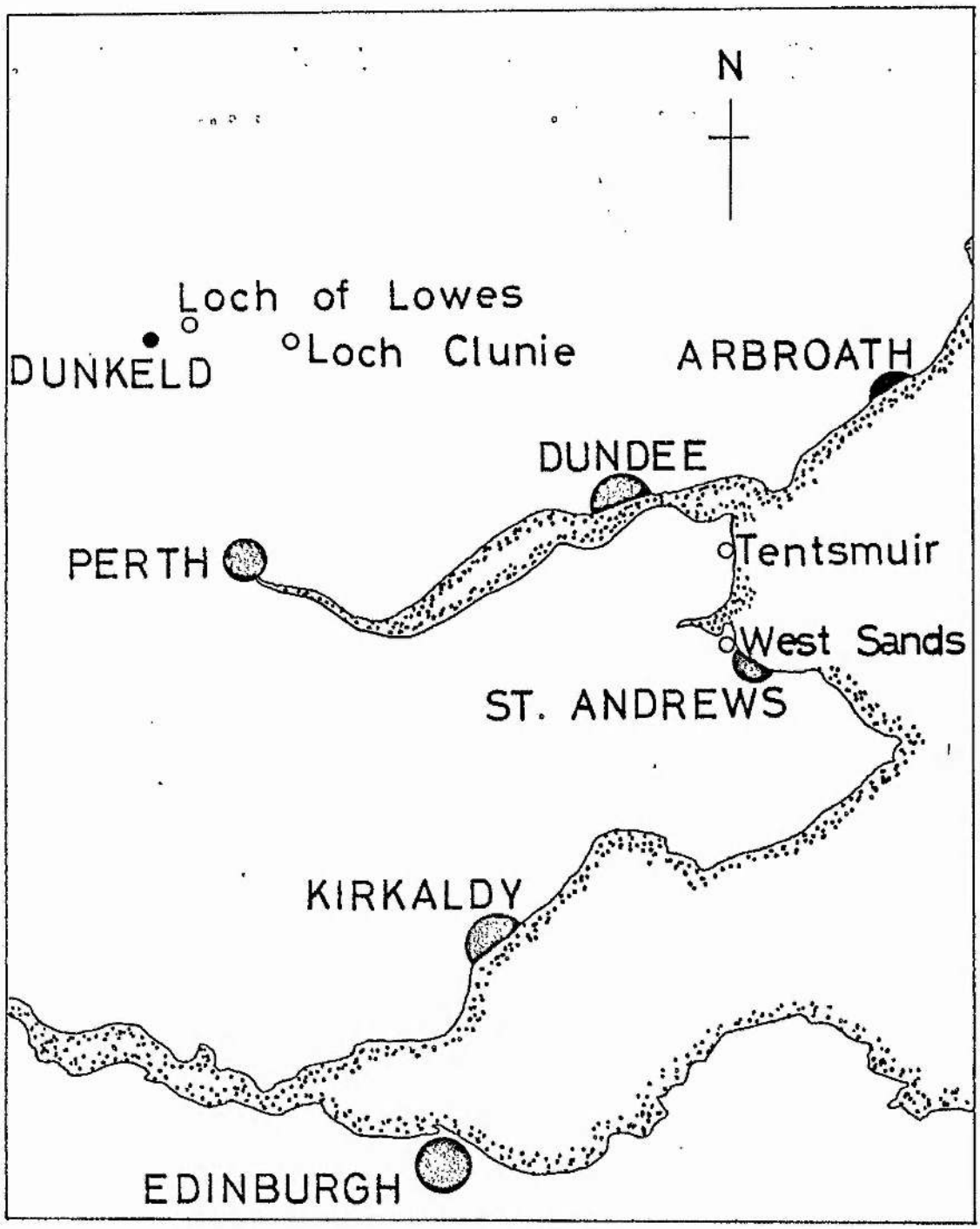
The micro-estimations necessary to detemine the ethanol in these extracts were also carried out enzymically (Appendix p.xv).

Fig. 1. SITES IN THE VICINITY OF ST. ANDREWS USED FOR THE COLLECTION OF SEED, TRANSPLANTS AND ROOT MATERIAL

Key: ● Towns

○ Sites of collection

Fig. 1


N
Loch of Lowes
Loch Clunie
DUNKELD
ARBROATH
DUNDEE
PERTH
Tentsmuir
West Sands
ST. ANDREWS
KIRKALDY
EDINBURGH
scale : 0 5 10 20
miles

RESULTS

EXPERIMENTAL FLOODING

GROWTH UNDER FLOODED AND NON-FLOODED CONDITIONS

After the 18-day growth period in flooded and non-flooded conditions, some species were shown to have their growth retarded by flooding, while others to have their growth enhanced (Fig. 2). In the former group, the non-helophytes, those species most adversely affected by flooding showed chlorosis of the leaves and death of the roots before the end of the 18-day period. As expected, all the Senecio species normally found on dry, open substrates had their growth retarded by flooding, as did Hieracium pilosella taken from the sand dunes at Tentsmuir. On the other hand, Senecio aquaticus, Ranunculus flammula and Juncus effusus are all species of wet sites and their growth was enhanced by flooding. The response of Carex arenaria, a species normally found growing on fixed sand dunes, is less of an anomaly when it is remembered that this is an experimental definition of the species, and that C. arenaria does in fact invade the damp slacks at Tentsmuir.

Three species not included in Fig. 2 were later included with those used for flooding experiments. Of these, Senecio

jacobaea and S. sylvaticus grow on light, sandy substrates (Clapham, Tutin & Warburg 1962) and were found by experiment to be non-helophytes, whereas Carex lasiocarpa grows in reed-swamp and was found to be a helophyte.

MALIC ACID CHANGES

The changes in root malic acid content during the 0-4 day flooding period are shown in Fig. 3. In the helophyte species (Fig. 3a) the quantity of malate at the onset of flooding varied between 1 μ mole (Senecio aquaticus) and nearly 6 μ mole / g fresh weight (Ranunculus flammula), but the levels in the respective species never dropped below their original level throughout the entire flooding period. After four days of flooding there was more malate present in the roots of helophyte species than there had been before flooding.

In the non-helophytes (Fig. 3b) there was less than 3 μ mole malate / g fresh weight at the onset of flooding, and in every species there was a decrease in amount during the flooding period. In three species there was no malate detectable after four days of flooding.

The increase in malate in helophytes and its decrease in

non-helophytes were both rapidly effected. For most species the change was evident after the first day of flooding, and in the remaining few species by the second day. The levels after four days of flood treatment were therefore in most cases simply extensions of a trend established by the second day of flooding. In a helophyte, Carex arenaria, the increase in malate was shown to continue throughout an eight-day flood period (Fig. 4), and in a similar experiment on a non-helophyte, Senecio viscosus, the drop in malate by four days was followed by a slight increase during the next four days. These longer flooding periods were not sufficiently studied for anything other than tentative conclusions to be drawn. It is probable, though, that along with the flooding disease symptoms shown by Senecio viscosus after about 5-6 days of treatment, such as chlorosis of the leaves and discolouration and death of the roots, there is a gradual metabolic breakdown. Thus a metabolic product such as malic acid could accumulate in the dying tissues of S. viscosus whereas in C. arenaria the flood treatment produced only healthy growth and the malate accumulation there is more likely a true metabolic response.

An overall comparison of the malate increase in flooded helophytes and its decrease in flooded non-helophytes is also shown graphically in Fig. 5, by plotting the ratio of malic acid before

and after the four-day flood period. This comparison between the helophyte species and the non-helophytes shows how clearly the two groups of plants differ in their malic acid metabolism upon flooding.

LACTIC ACID CHANGES

The changes in root lactic acid content during the 0-4 day flooding period are shown in Fig. 6. The quantities of lactic acid were always small - in helophytes never more than 0.1 μ mole acid / g fresh weight, and similarly in non-helophytes except for Senecio squalidus (less than 0.3 μ mole / g fresh weight).

In the helophytes (Fig. 6a) the very small quantities of lactate do not allow any clear interpretation of their changes during the course of flooding, although in all species other than Juncus effusus there was a drop in level from the onset of flooding to the fourth day. Similarly, in the non-helophytes the levels were so small that slight fluctuations do not merit evidence of definite lactate movement. Nevertheless, all the non-helophytes contained slightly more lactate after four days of flooding than they did before flooding.

CITRIC ACID CHANGES

The changes in root citric acid content during the 0-4 day flooding period are shown in Fig. 7. Citric acid was present in most root extracts, in helophytes up to 0.5 μ mole citrate / g fresh weight and in non-helophytes up to 1.0 μ mole. Of the helophytes investigated (Fig. 7a) *Carex arenaria* and *Juncus effusus* increased their citrate content during the four day flood period, *C. lasiocarpa* and *Senecio aquaticus* decreased their citrate content, and in *Ranunculus flammula* there was no change overall. In the non-helophytes (Fig. 7b) the fluctuations in citrate content throughout the four days were even more marked and it is impossible to see any definite pattern of citrate movement although, apart from *Senecio viscosus*, all species either kept the same citrate content after four days flooding or slightly increased this acid.

SUCCINIC ACID CHANGES

The changes in root succinic acid content during the 0-4 day flooding period are shown in Fig. 8. Succinate levels were small, especially in the helophytes where the highest level recorded was 0.2 μ mole acid / g fresh weight. Apart from this particular species, *Juncus effusus*, the succinic acid content in the helo-

phytes either decreased or remained at the same low level during the four day period (Fig. 8a). In the non-helophytes (Fig. 8b) there was a constant pattern of succinate rise upon flooding, with an approximately three-fold increase by the fourth day. Although quantities of succinate were small compared with those of malate, there was more than 0.5 μ mole acid / g fresh weight in Senecio squalidus and S. viscosus after four days of flooding.

OVERALL ORGANIC ACID CHANGES AND COMPARISON OF THE RELATIVE CONCENTRATIONS OF DIFFERENT ORGANIC ACIDS

In all the helophytes (Figs. 9a to 9e) malic acid was the most predominant, always present above 1 μ mole acid / g fresh weight of root tissue, and even up to 6 μ mole in Ranunculus flammula. Lactic and succinic acids were, if detected, present only in minute amounts. Citric acid was more abundant, but never present above 0.5 μ mole / g fresh weight.

In the flood treated non-helophytes (Figs. 9f to 9k) malic acid was again predominant at the onset of flooding but fell away in quantity by the fourth day. Furthermore, it was never present above 4 μ mole / g fresh weight, and in three species, Hieraceum pilosella, Senecio squalidus and S. sylvaticus, there was no malate detectable after four days of flooding. Lactic acid was again

present in only minute amounts, but succinic acid was in evidence up to 0.5 μ mole / g fresh weight in Senecio squalidus and S. viscosus. Citric acid was again present up to 1.0 μ mole / g fresh weight. Oxaloacetic acid was not detected in any of the extracts.

Paper chromatograms of the root extracts were run as a final check that no major organic acid had been missed out of the series of individual acid analyses. The chromatograms did not indicate the presence of any organic acid other than some of those already assayed, and in most cases supported qualitatively the earlier quantitative findings. Thus, the paper chromatogram of root extracts of the helophyte Carex lasiocarpa showed malic acid and citric acid to be present in detectable amounts during the 0-4 day flood period, but not any of the other organic acids (Fig. 10). This chromatogram gave some evidence that malate persisted throughout the four days, but that citrate disappeared. In a similar chromatogram of the non-helophyte Senecio viscosus, the only acids detected in quantity were again malate and citrate (Fig. 11), but in this species malic acid did not persist throughout the flooding period but disappeared after the first day.

ORGANIC ACID MOLECULAR RATIOS AFTER FLOODING

The malic and succinic acid levels were shown above to follow

different patterns upon flooding in the two groups of species. In the helophytes flooding was accompanied by a rise in malate and a drop in succinate. In the non-helophytes the pattern was reversed, with a drop in malate and a rise in succinate. This differential response between the helophytes and non-helophytes is shown graphically in Fig. 12, by plotting the molecular ratio of malate to succinate after four days of flooding. In the helophytes large values are recorded, the smallest being a 25-fold ratio in Juncus effusus. In the non-helophytes the ratio is always less than 5, with three species recording zero values. This molecular ratio clearly differentiates between the flooding response of the two groups of species, for it shows how the internal metabolic controls of helophytes and non-helophytes differ upon experimental flooding, in respect of a molecule for molecule rise in malate and drop in succinate in the former group and the reverse intthe latter group. Thus it is not so much the quantitative changes in, say, malic acid, between different species which are important but more the relative amount of one acid to another in respect to the plant's behaviour during a period of flooding.

A similar illustration results from plotting the malate: citrate molecular ratio (Fig. 13), where again a rise in malate in helophytes and drop in non-helophytes is not accompanied by similar changes in citrate levels. The molecular ratio reflects

this response, although since the citrate levels were overall rather constant in the two groups, the values of the ratio are not as strikingly different as are those of the malate:succinate molecular ratio. In the non-helophytes the ratio is still always less than 5, and in the helophytes it lies, except for Ranunculus flammula, between 10 and 25.

Fig. 2. GROWTH OF HELOPHYTES AND NON-HELOPHYTES IN LOW (LWT) AND HIGH (HWT) WATER TABLE CONDITIONS AS MEASURED BY THE DRY WEIGHT OF THE SHOOTS AFTER 18 DAYS

Key:

S. vi. *Senecio viscosus*

S. v. *S. vulgaris* (dune race)

H. p. *Hieraceum pilosella*

S. q. *S. squalidus*

S. aq. *S. aquaticus*

C. a. *Carex arenaria*

R. f. *Ranunculus flammula*

J. e. *Juncus effusus*

Fig. 2

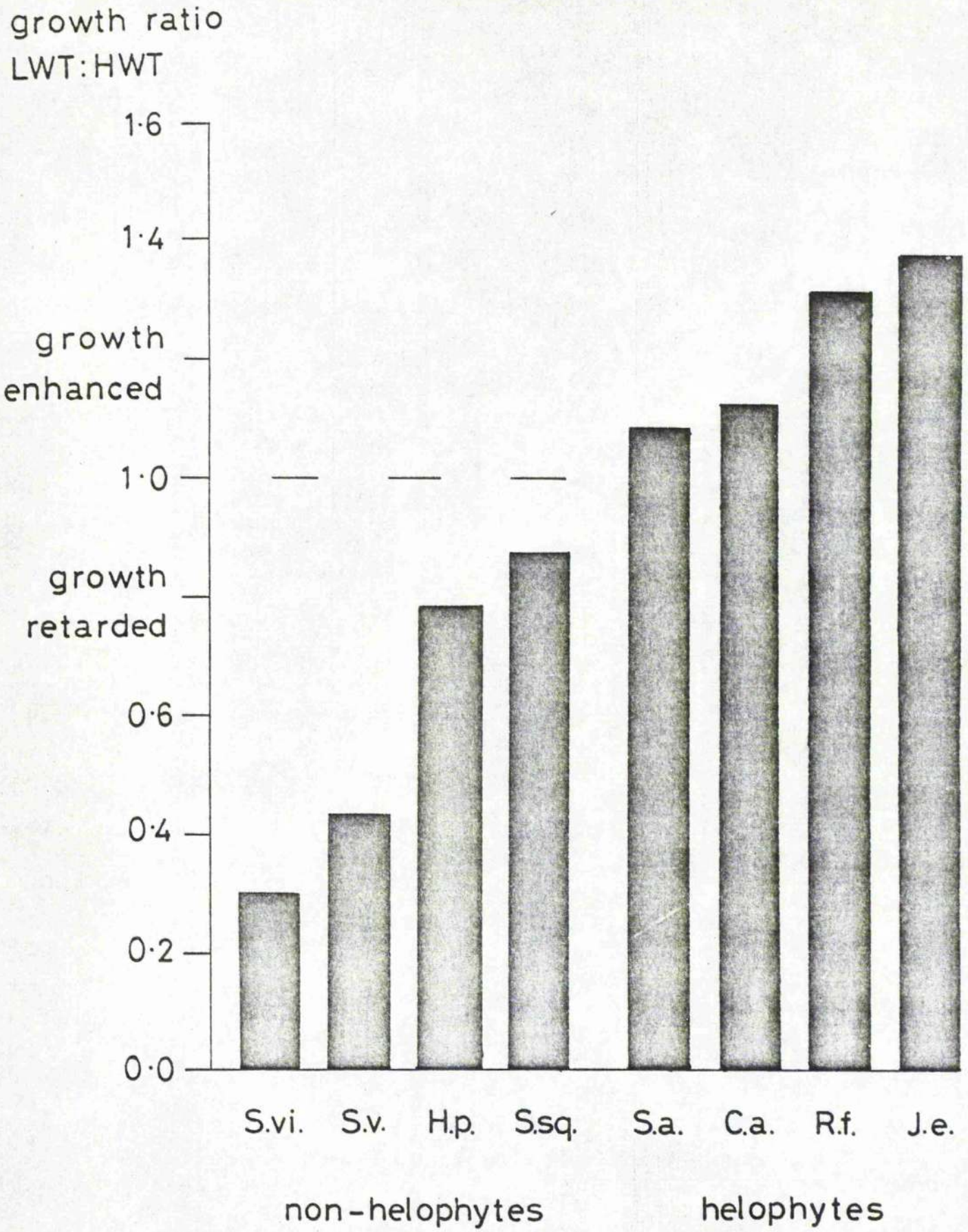

growth ratio
LWT:HWT
1·6
1·4
growth
enhanced
1·0
growth
retarded
0·6
0·4
0·2
0·0
S.vi.
S.v.
H.p.
S.sq.
S.a.
C.a.
R.f.
J.e.
non-helophytes
helophytes

Fig. 3a and 3b. CHANGES IN ROOT MALIC ACID CONTENT INDUCED BY FLOODING IN HELOPHYTES AND NON-HELOPHYTES

Key: a Helophytes

- ● *Carex arenaria*
- ▲ *C. lasiocarpa*
- ■ *Juncus effusus*
- ○ *Ranunculus flammula*
- △ *Senecio aquaticus*

b Non-helophytes

- ● *Hieraceum pilosella*
- ▲ *S. jacobaea*
- ■ *S. squalidus*
- ○ *S. sylvaticus*
- △ *S. viscosus*
- □ *S. vulgaris* (dune race)

Fig. 3a

(helophytes)

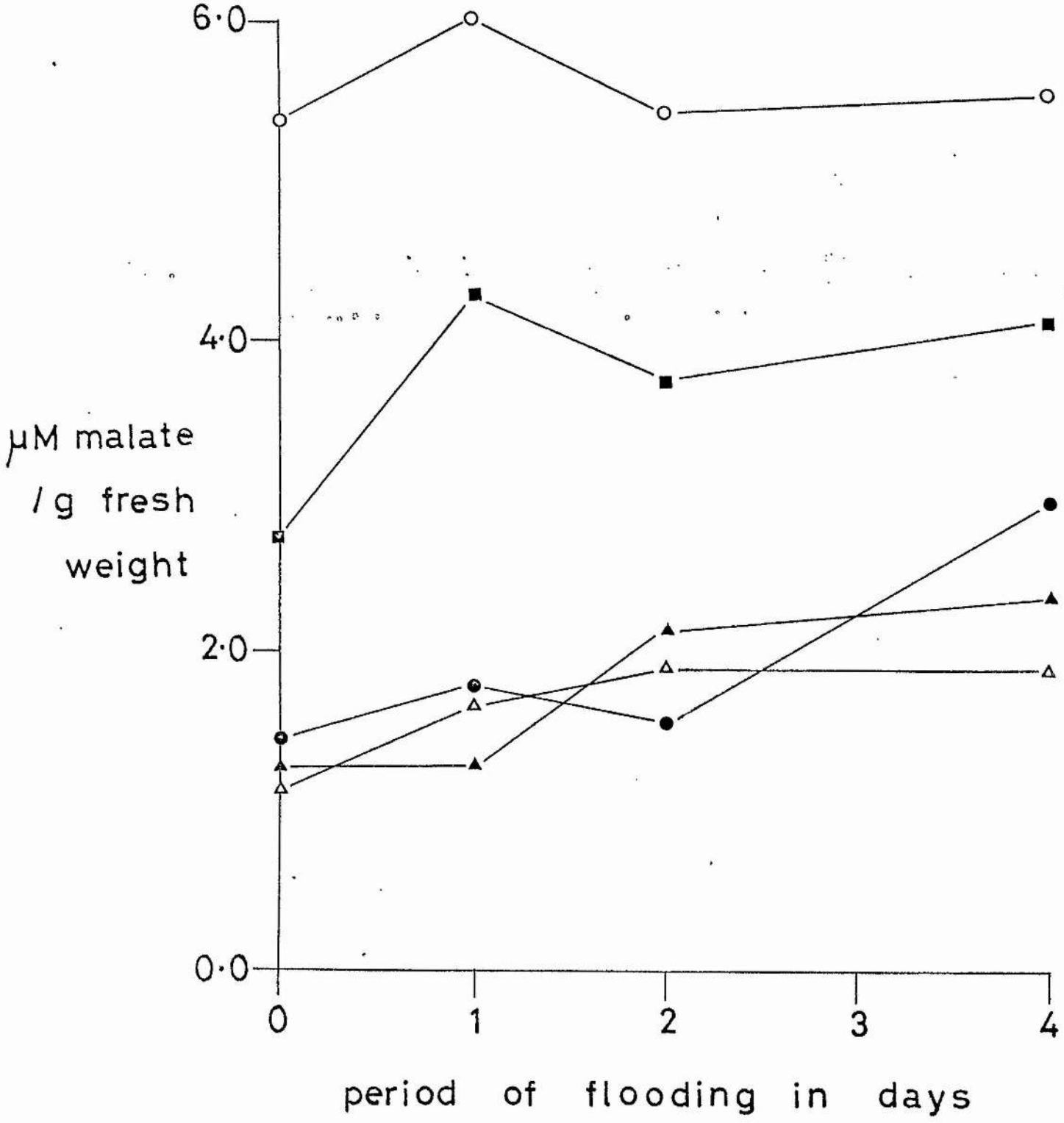


Fig. 3b

(non - helophytes)

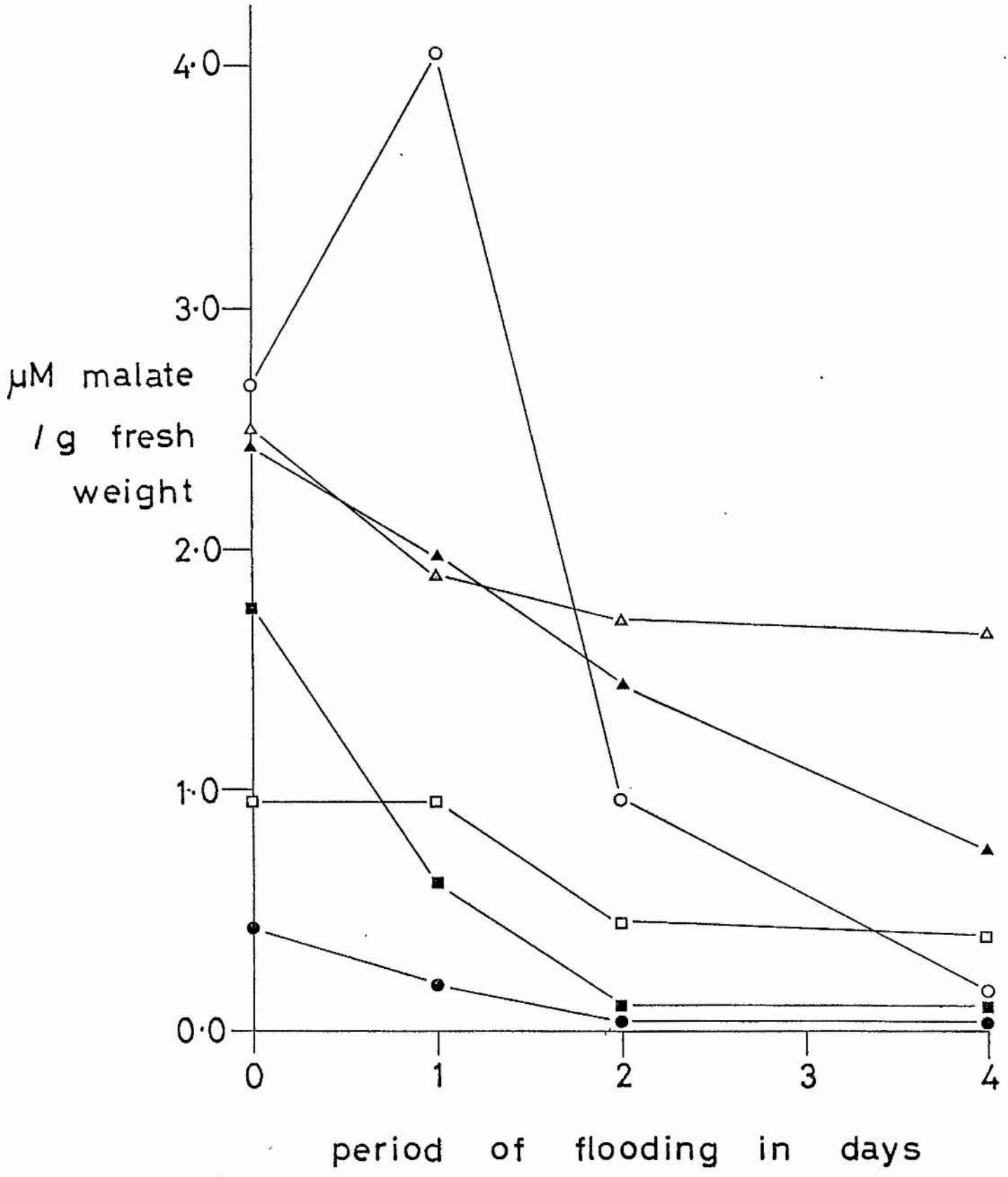


Fig. 4. CHANGES IN ROOT MALIC ACID CONTENT INDUCED BY AN 8-DAY FLOODING PERIOD IN A HELOPHYTE AND A NON-HELOPHYTE

Key: ● Carex arenaria (helophyte)

▲ Senecio viscosus (non-helophyte)

Fig. 4

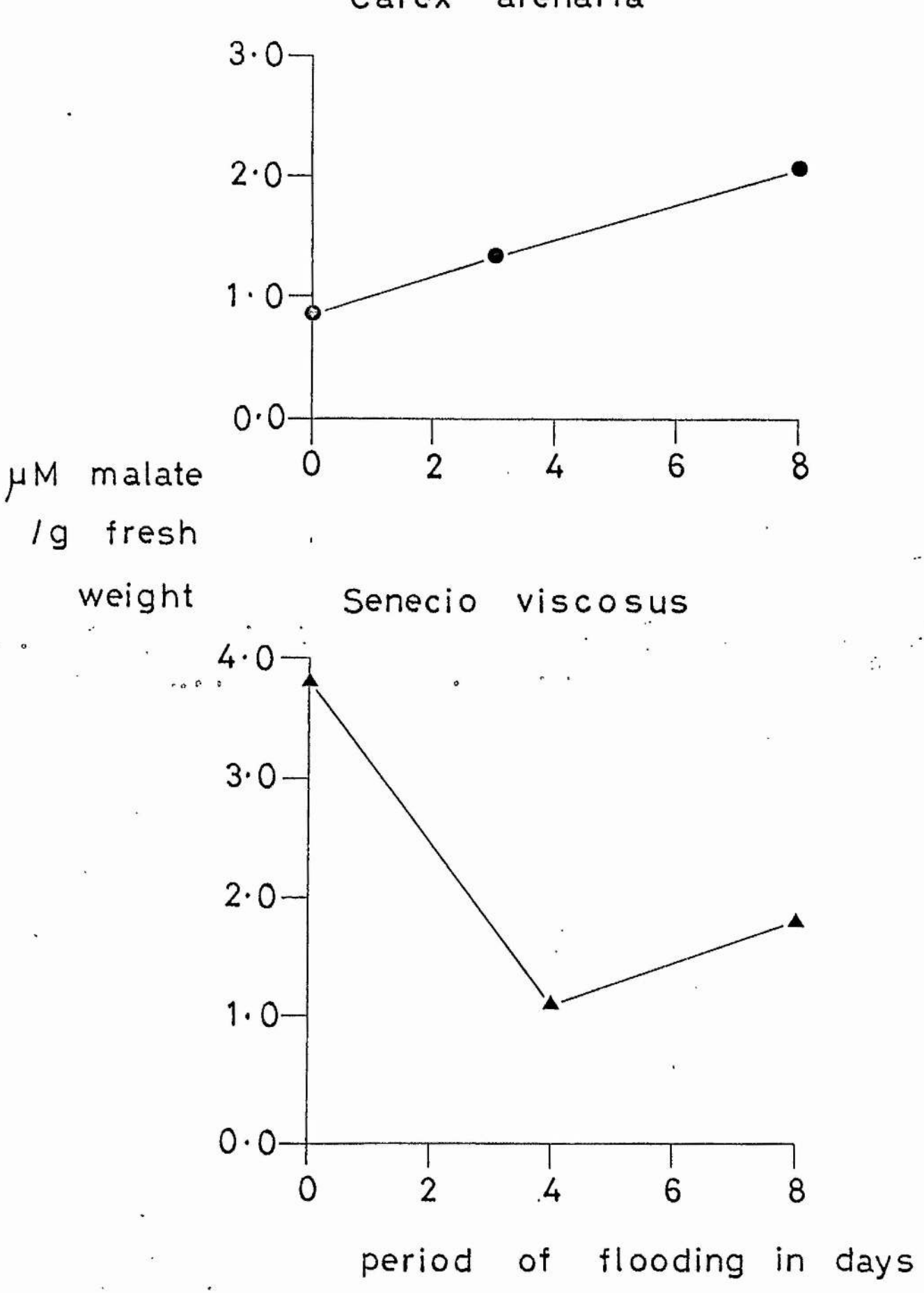

Carex arenaria
3·0
2·0
1·0
0·0
0
2
4
6
8
µM malate /g fresh weight
Senecio viscosus
4·0
3·0
2·0
1·0
0·0
0
2
4
6
8
period of flooding in days

Fig. 5. RATIO OF MALIC ACID CONTENT OF ROOTS OF HELOPHYTES AND NON-HELOPHYTES BEFORE AND AFTER FLOODING FOR 4 DAYS

Key: H. p. Hieraceum pilosella

S. sq. Senecio squalidus

S. s. S. sylvaticus

S. j. S. jacobaea

S. v. S. vulgaris (dune race)

S. vi. S. viscosus

R. f. Ranunculus flammula

J. e. Juncus effusus

S. a. S. aquaticus

C. l. Carex lasiocarpa

C. a. C. arenaria

Fig. 5

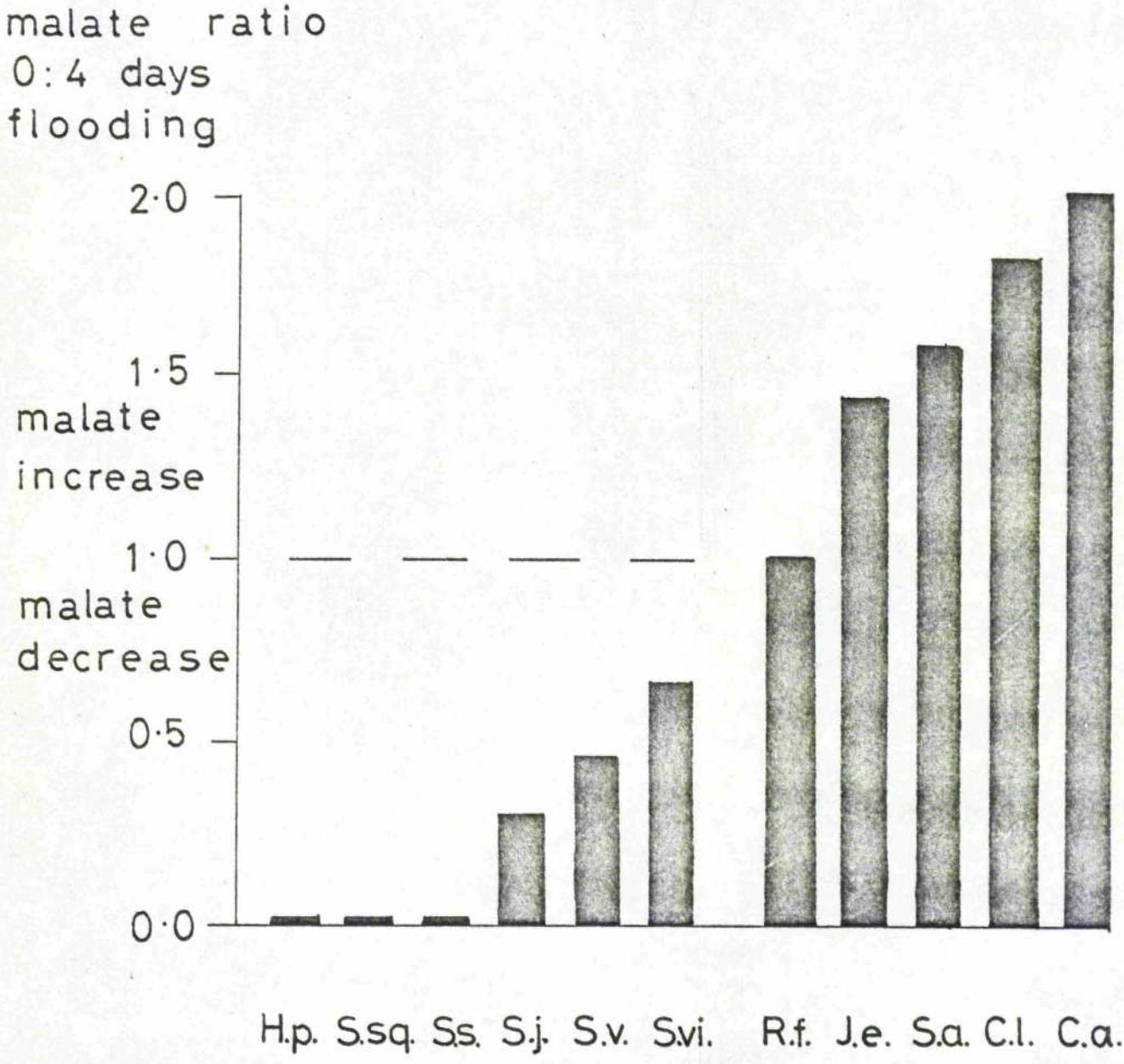

malate ratio
0:4 days
flooding
2·0
1·5
malate
increase
1·0
malate
decrease
0·5
0·0
H.p.
S.sq.
S.s.
S.j.
S.v.
S.vi.
R.f.
J.e.
S.a.
C.l.
C.a.
non-helophytes
helophytes

Fig. 6a and 6b. CHANGES IN ROOT LACTIC ACID CONTENT INDUCED BY FLOODING IN HELOPHYTES AND NON-HELOPHYTES

Key: a Helophytes

- ● *Carex arenaria*
- ▲ *C. lasiocarpa*
- ■ *Juncus effusus*
- ○ *Ranunculus flammula*
- △ *Senecio aquaticus*

b Non-helophytes

- ● *Hieraceum pilosella*
- ▲ *S. jacobaea*
- ■ *S. squalidus*
- ○ *S. sylvaticus*
- △ *S. viscosus*
- □ *S. vulgaris* (dune race)

Fig. 6a

(helophytes)

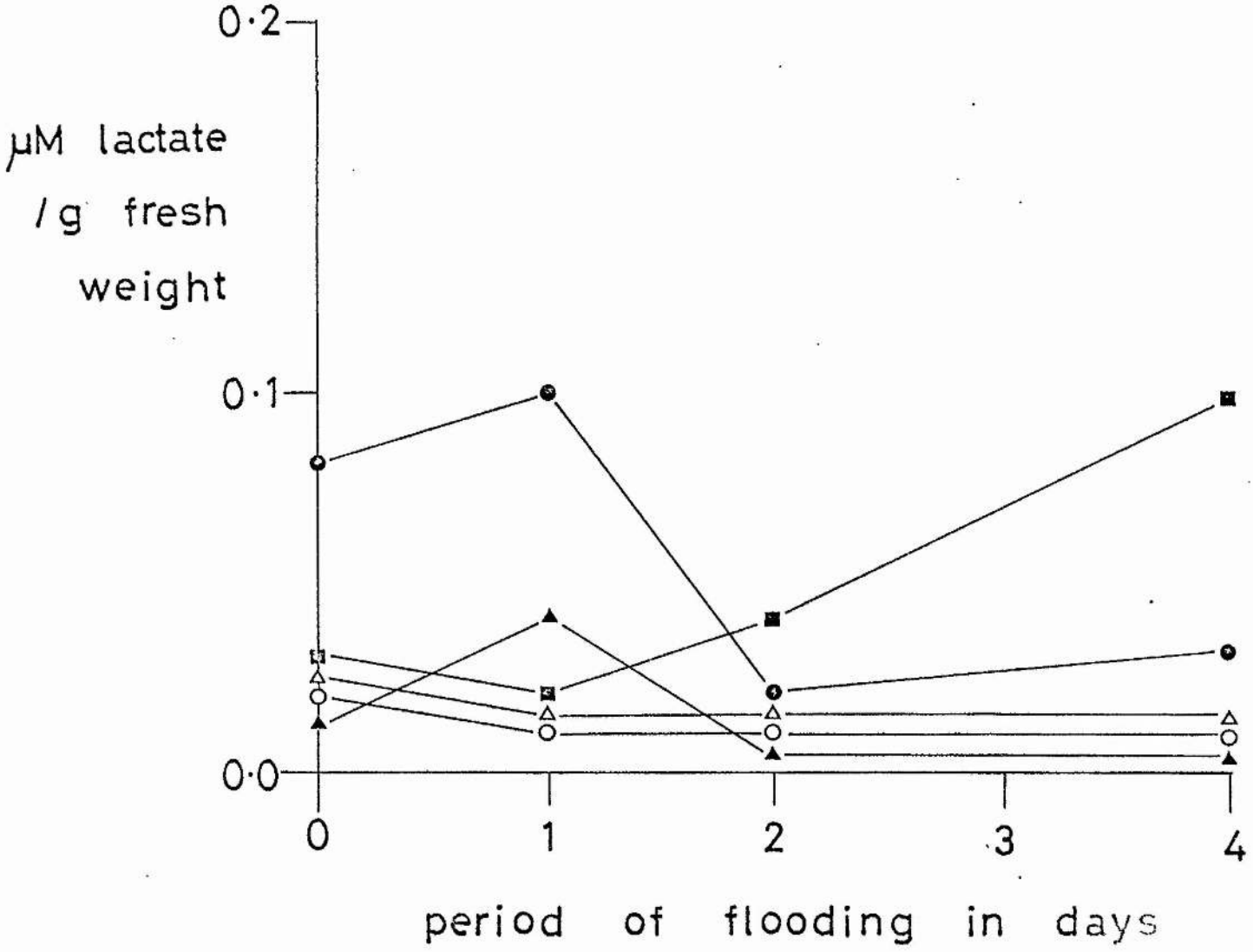


Fig. 6b

(non - helophytes)

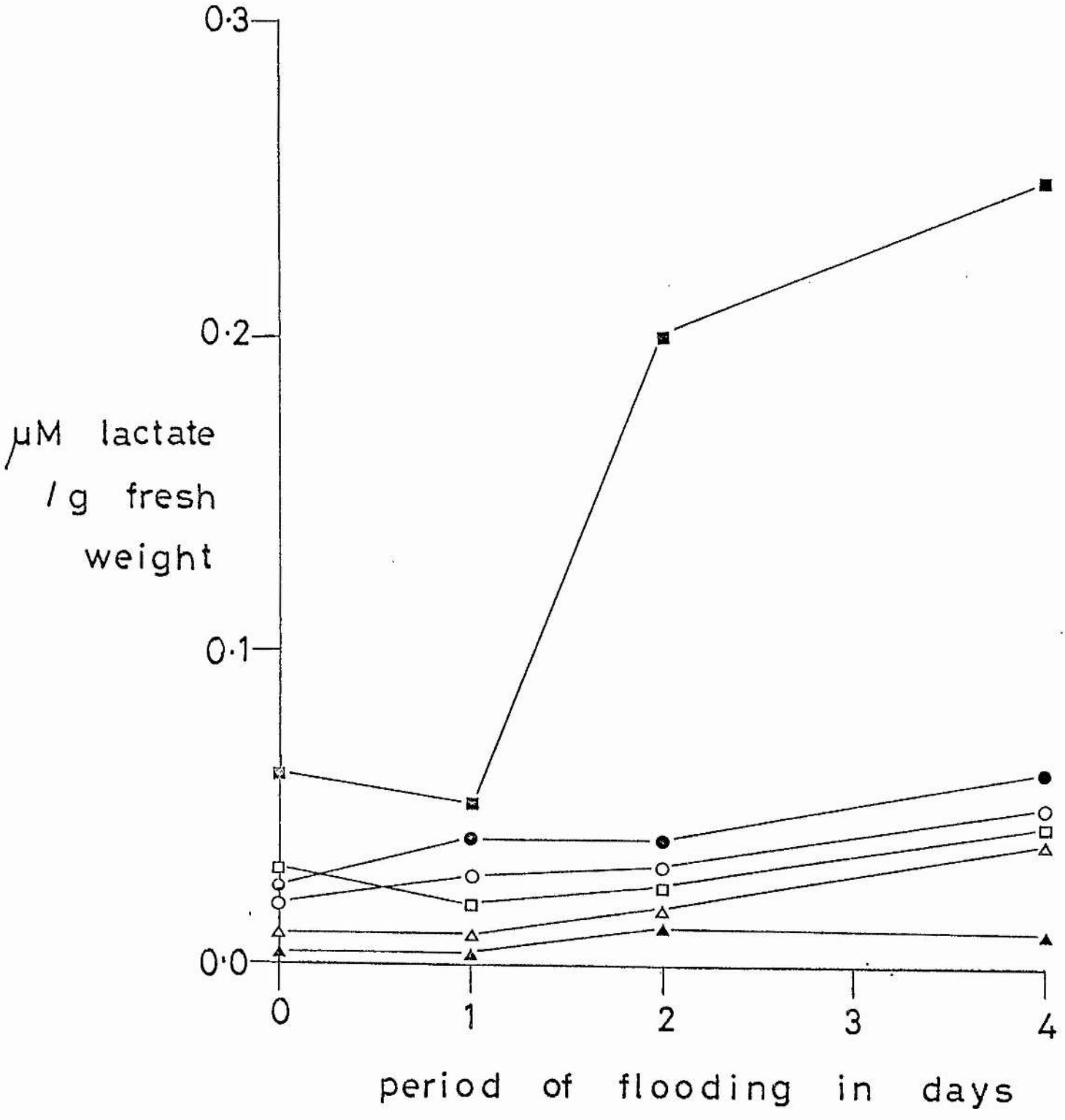


Fig. 7a and 7b. CHANGES IN ROOT CITRIC ACID CONTENT INDUCED BY FLOODING IN HELOPHYTES AND NON-HELOPHYTES

Key: a Helophytes

- ● *Carex arenaria*
- ▲ *C. lasiocarpa*
- ■ *Juncus effusus*
- ○ *Ranunculus flammula*
- △ *Senecio aquaticus*

b Non-helophytes

- ● *Hieraceum pilosella*
- ▲ *S. jacobaea*
- ■ *S. squalidus*
- ○ *S. sylvaticus*
- △ *S. viscosus*
- □ *S. vulgaris* (dune race)

Fig. 7a

(helophytes)

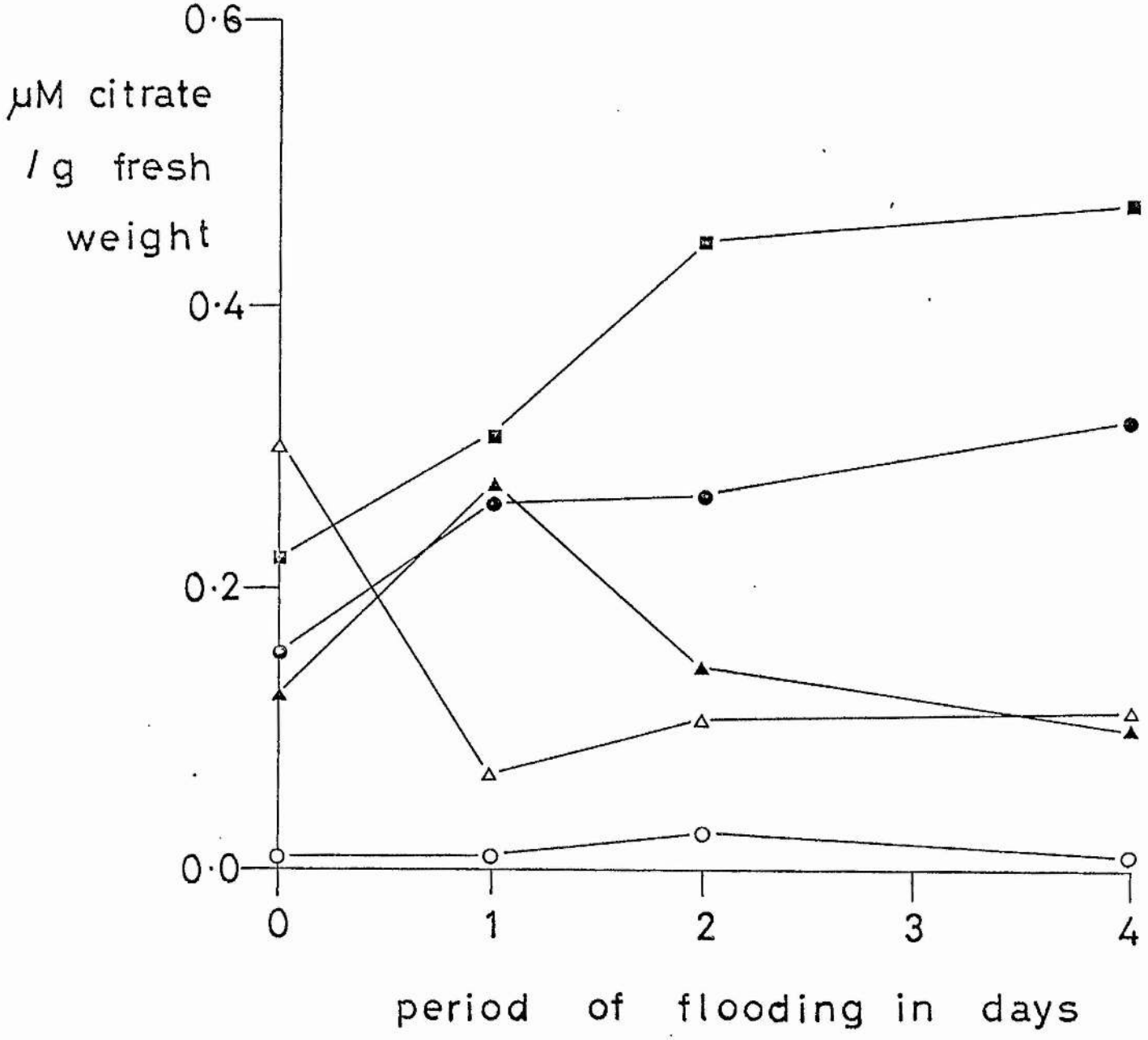


Fig. 7b (non-helophytes)

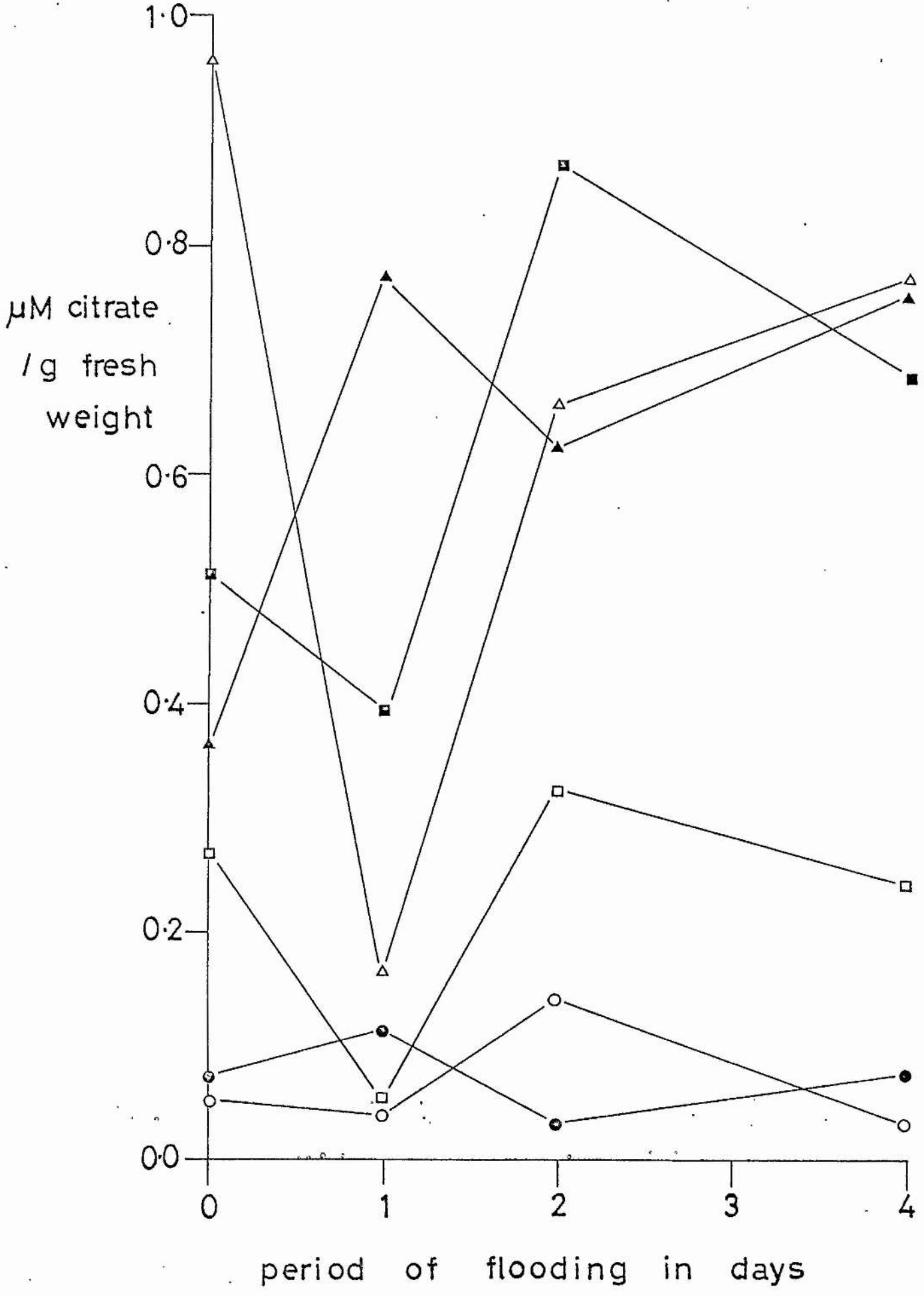


Fig 8a and 8b. CHANGES IN ROOT SUCCINIC ACID CONTENT INDUCED BY FLOODING IN HELOPHYTES AND NON-HELOPHYTES

Key:

a Helophytes

- ● *Carex arenaria*
- ▲ *C. lasiocarpa*
- ■ *Juncus effusus*
- ○ *Ranunculus flammula*
- △ *Senecio aquaticus*

b Non-helophytes

- ● *Hieraceum pilosella*
- ▲ *S. jacobaea*
- ■ *S. squalidus*
- ○ *S. sylvaticus*
- △ *S. viscosus*
- □ *S. vulgaris* (dune race)

Fig. 8a

(helophytes)

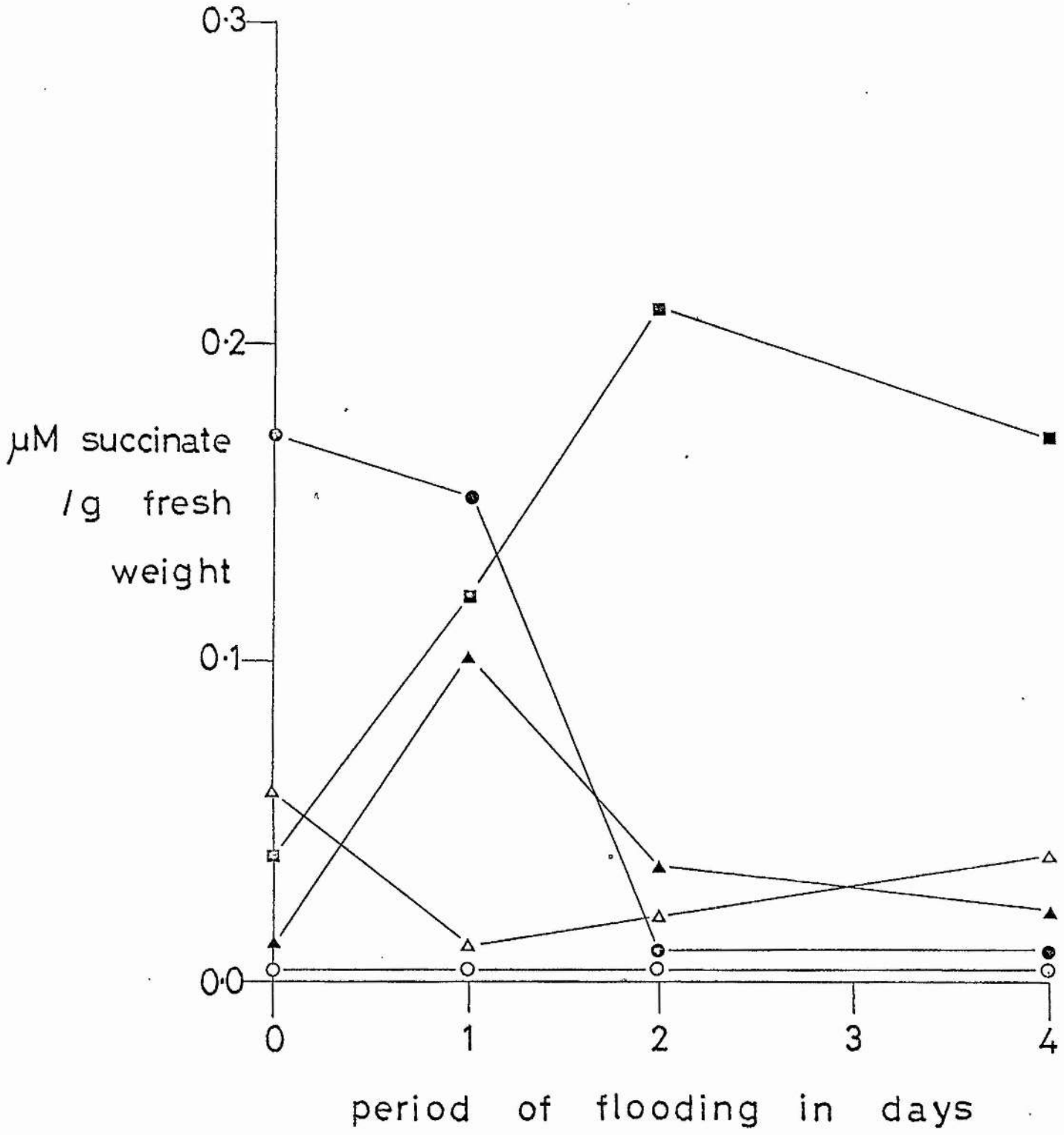


Fig. 8b

(non - helophytes)

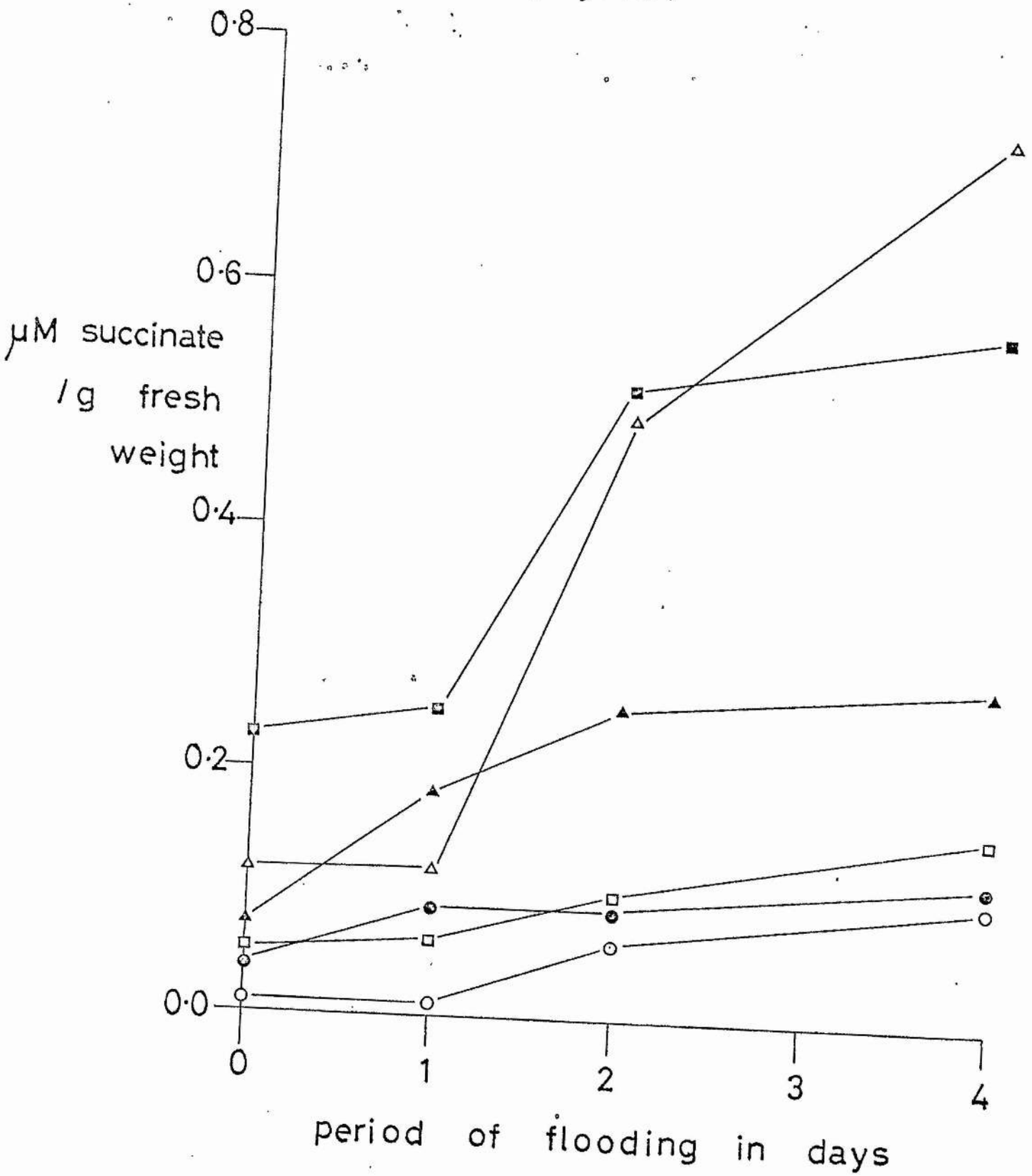


Fig. 9a to 9k. CHANGES IN ROOT ORGANIC ACID CONTENT INDUCED BY FLOODING IN HELOPHYTE AND NON-HELOPHYTE SPECIES

Key: ● malic acid

▲ lactic acid

■ citric acid

X succinic acid

Fig. 9a *Carex arenaria*

9b *C. lasiocarpa*

9c *Juncus effusus*

9d *Ranunculus flammula*

9e *Senecio aquaticus*

9f *Hieraceum pilosella*

9g *S. jacobaea*

9h *S. squalidus*

9i *S. sylvaticus*

9j *S. viscosus*

9k *S. vulgaris* (dune race)

Fig. 9a

Carex arenaria

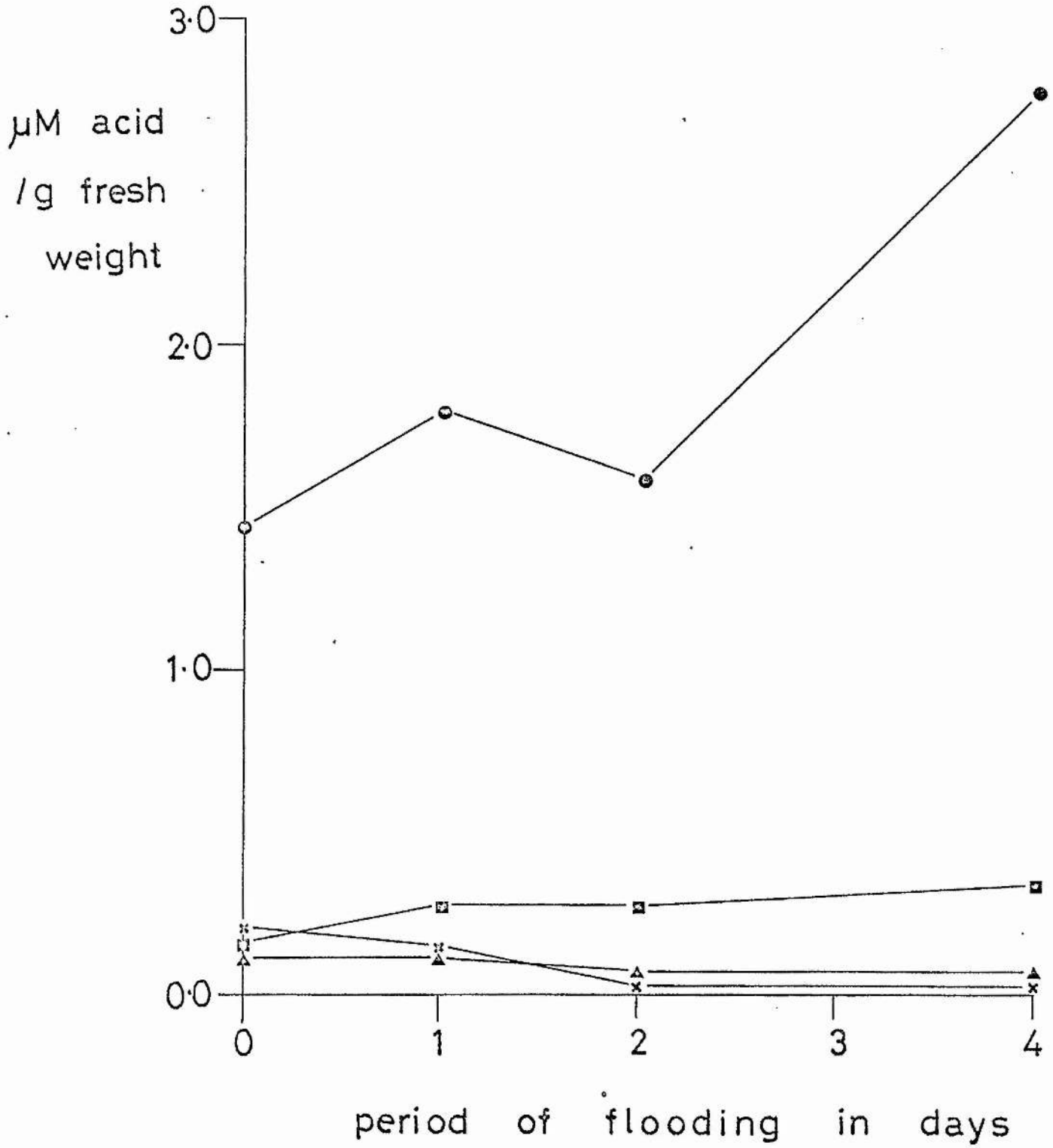


Fig. 9b

Carex lasiocarpa

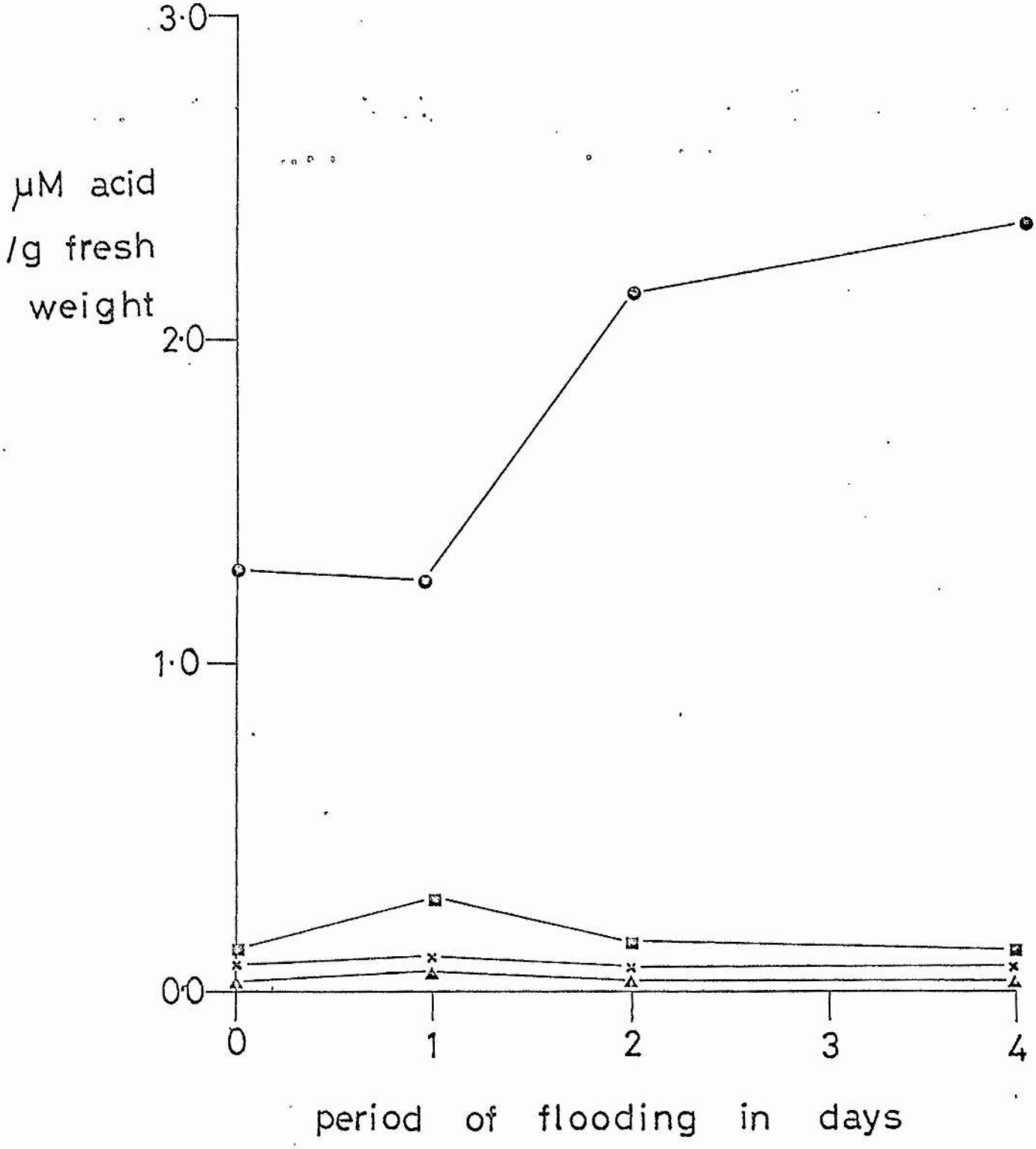


Fig. 9c

Juncus effusus

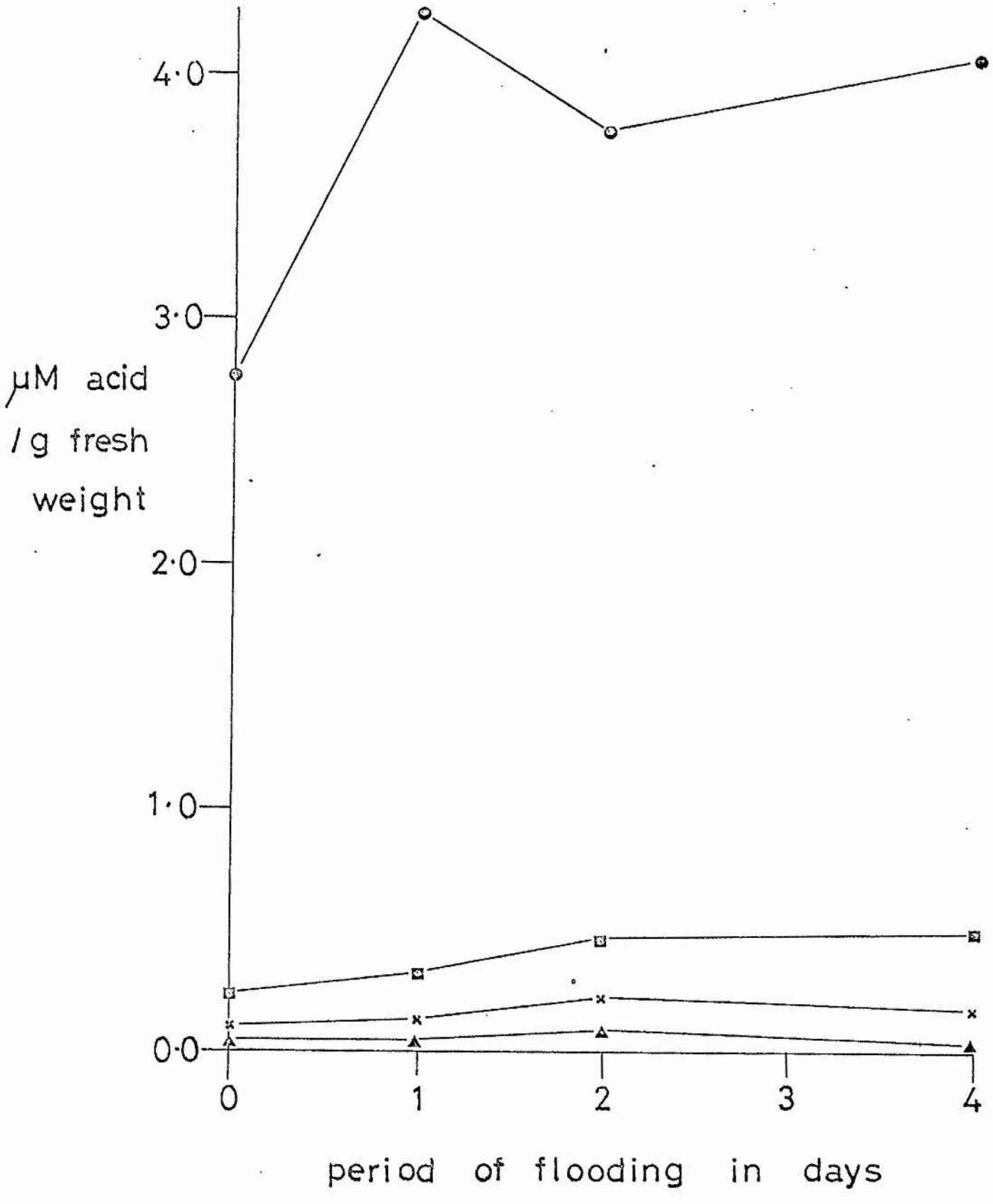


Fig. 9d

Ranunculus flammula

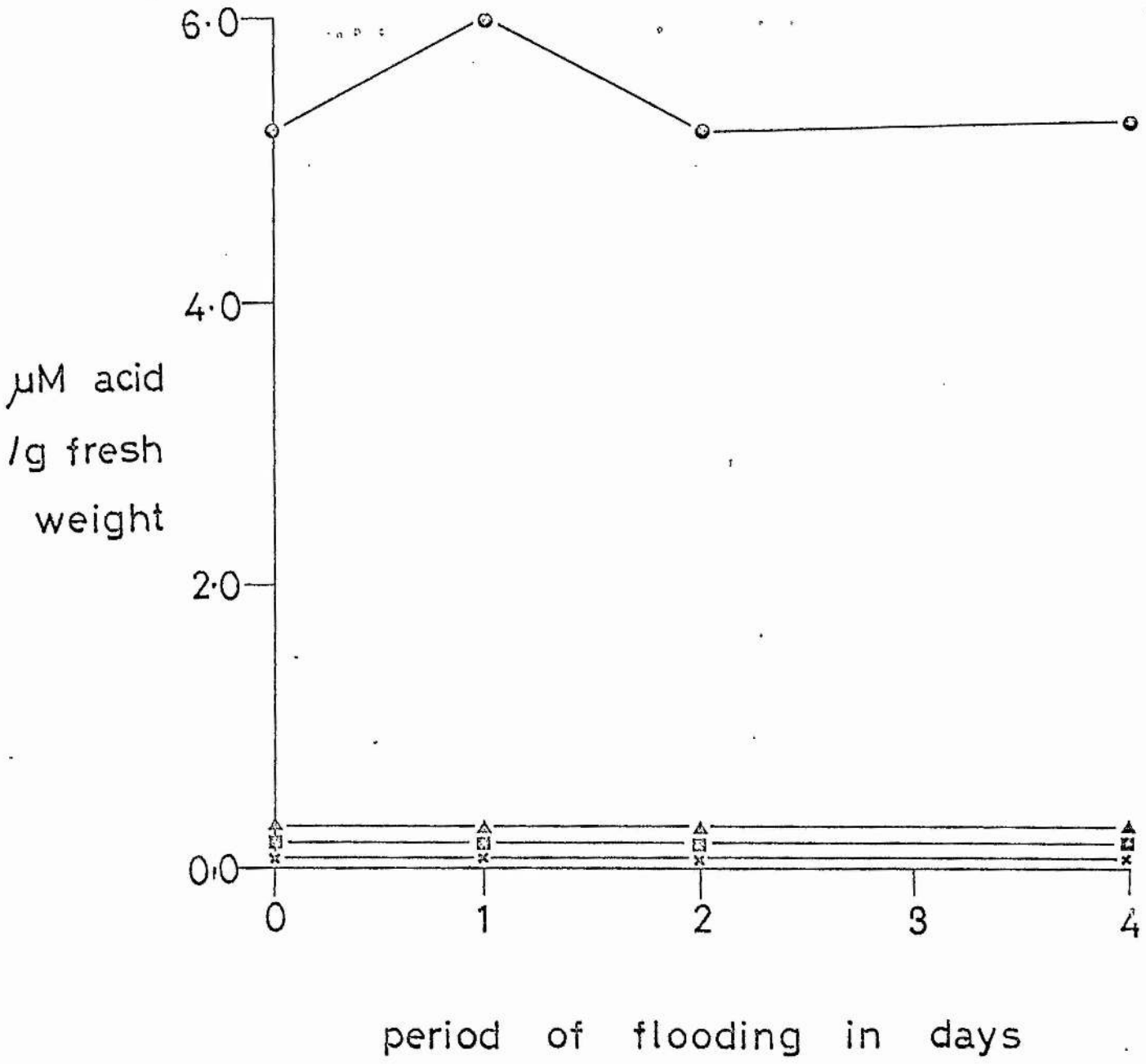


Fig. 9e

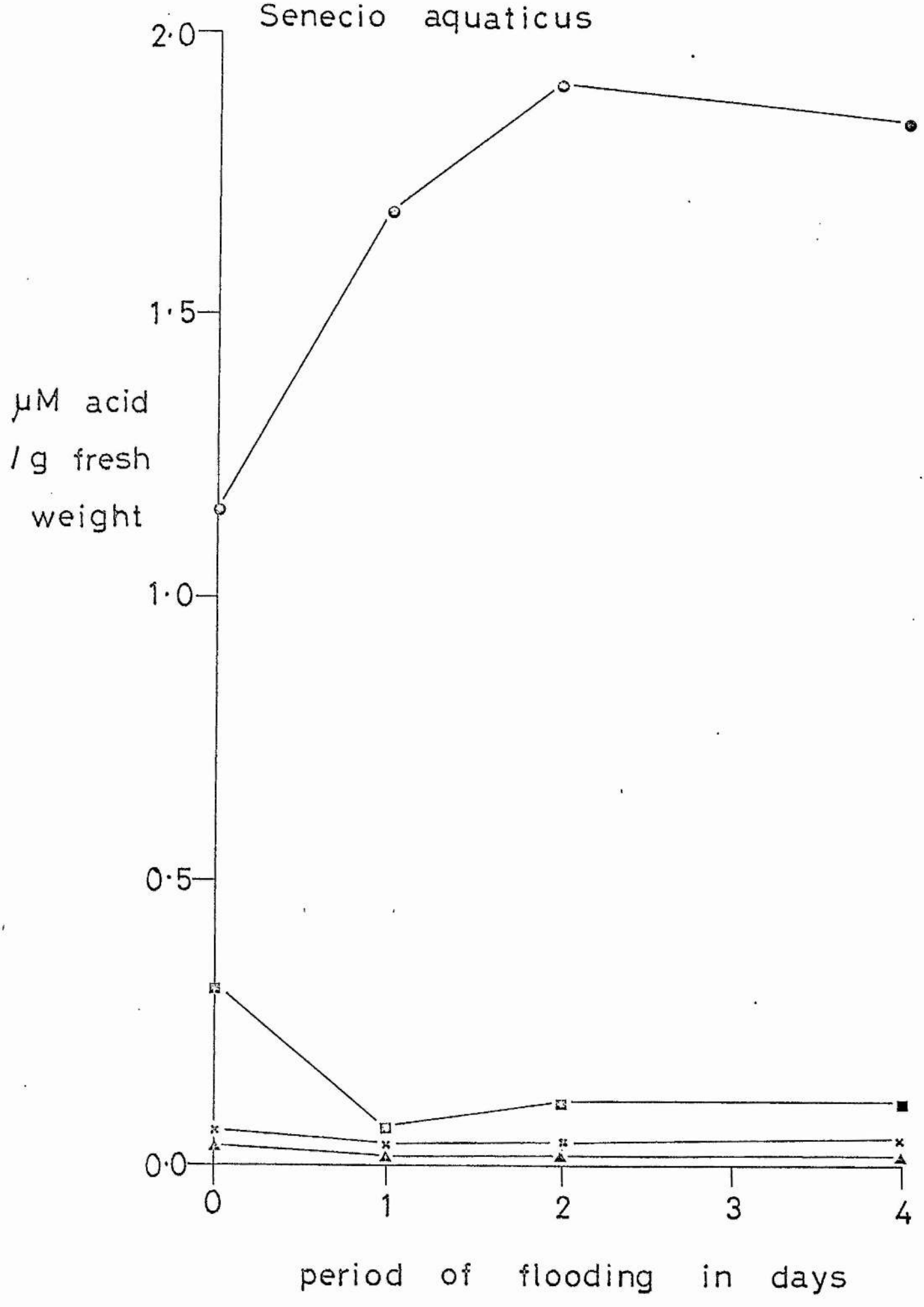

Senecio aquaticus
2·0
1·5
1·0
0·5
0·0
µM acid / g fresh weight
0
1
2
3
4
period of flooding in days

Fig. 9f

Hieraceum pilosella

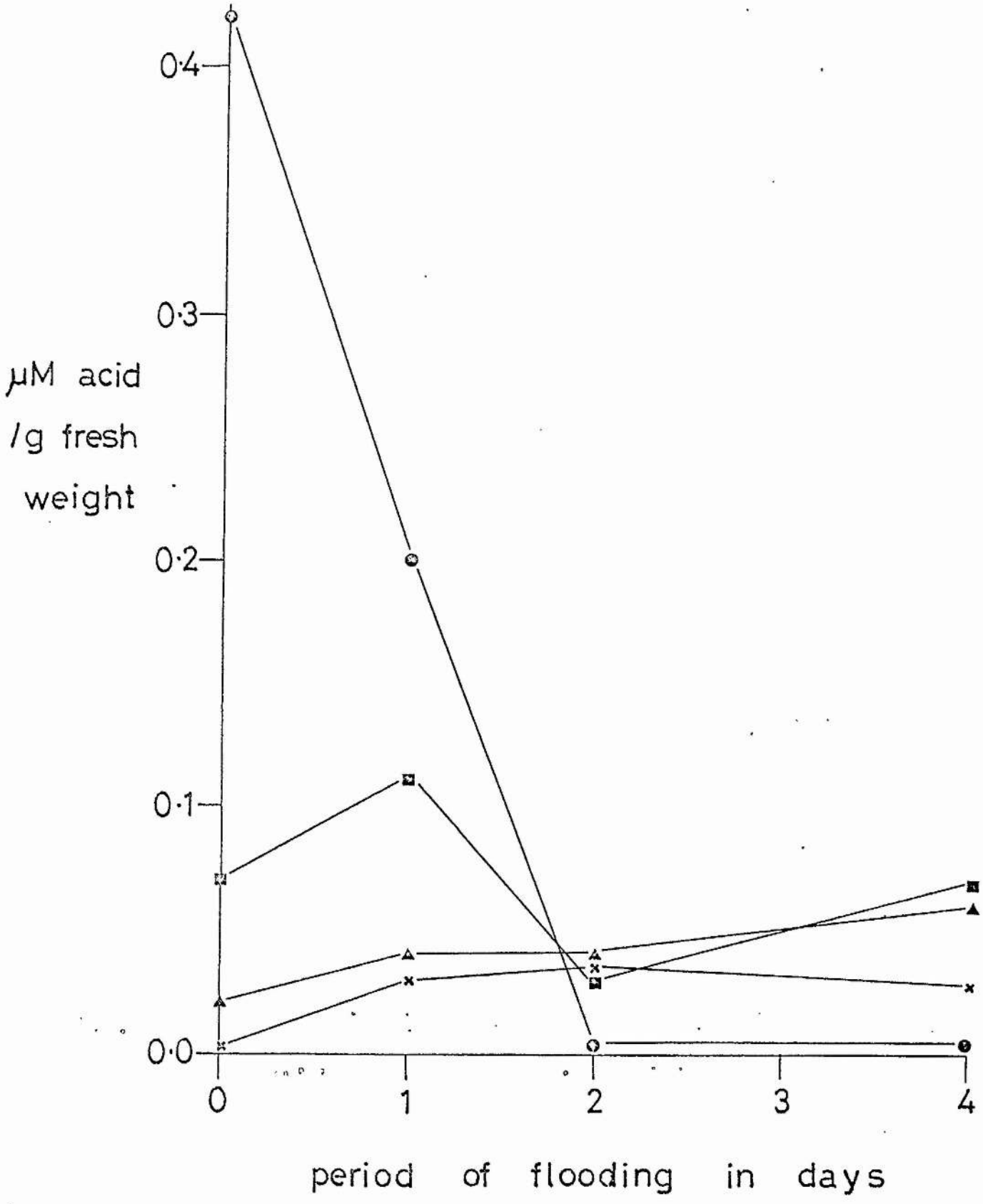


Fig. 9g

Senecio jacobaea

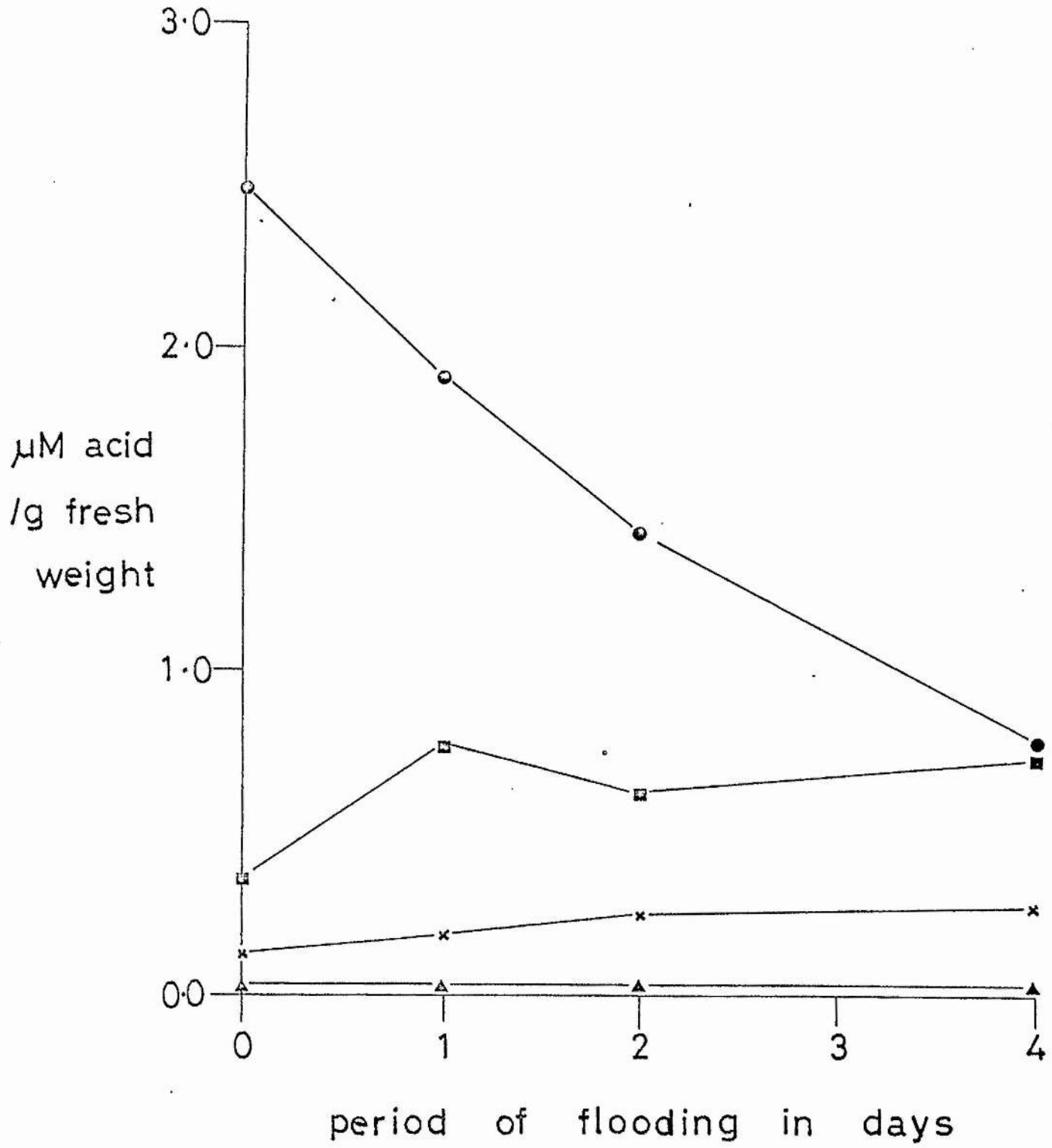


Fig. 9h

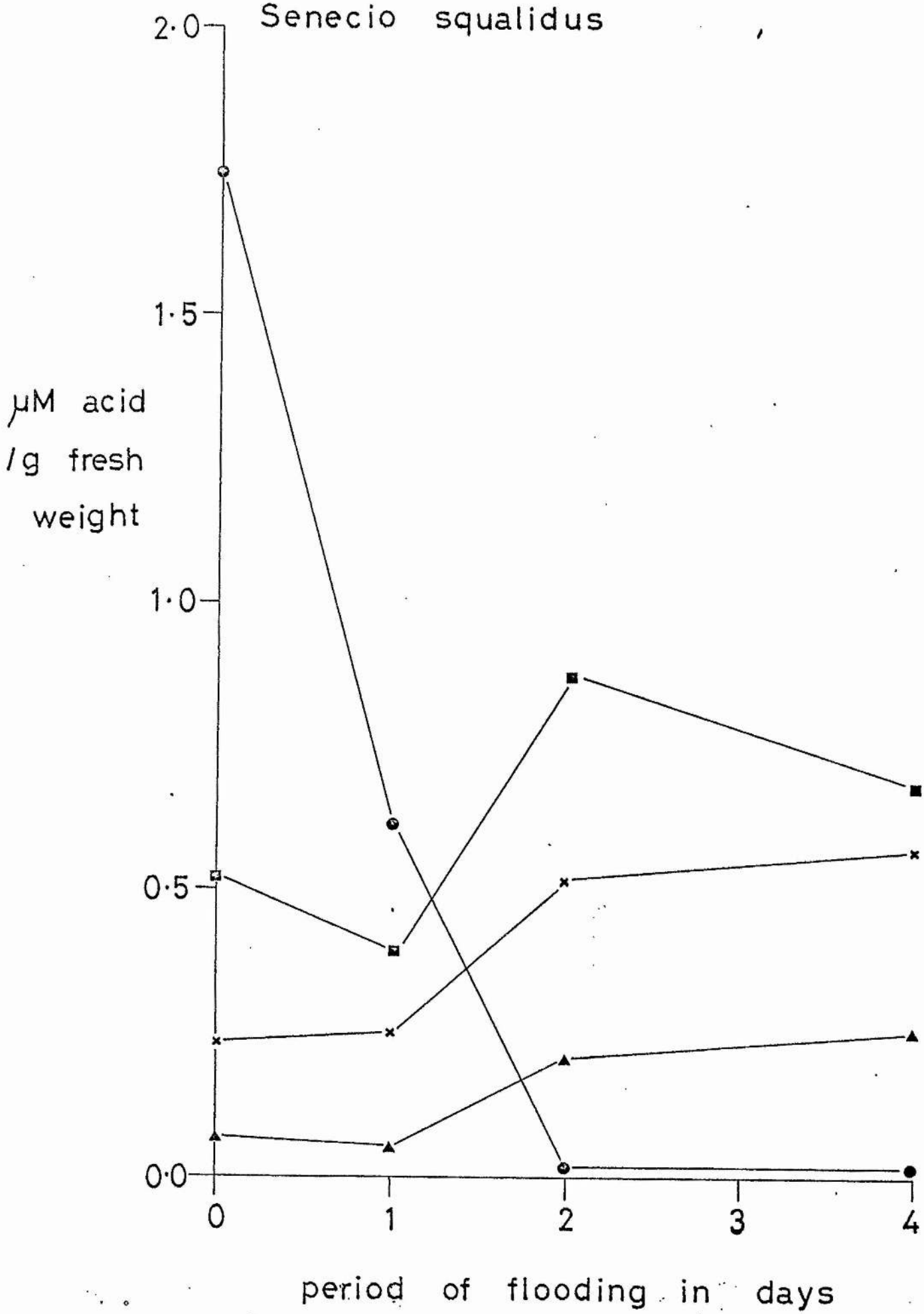

Senecio squalidus
2·0
1·5
1·0
0·5
0·0
µM acid /g fresh weight
0
1
2
3
4
period of flooding in days

Fig. 9i

Senecio sylvaticus

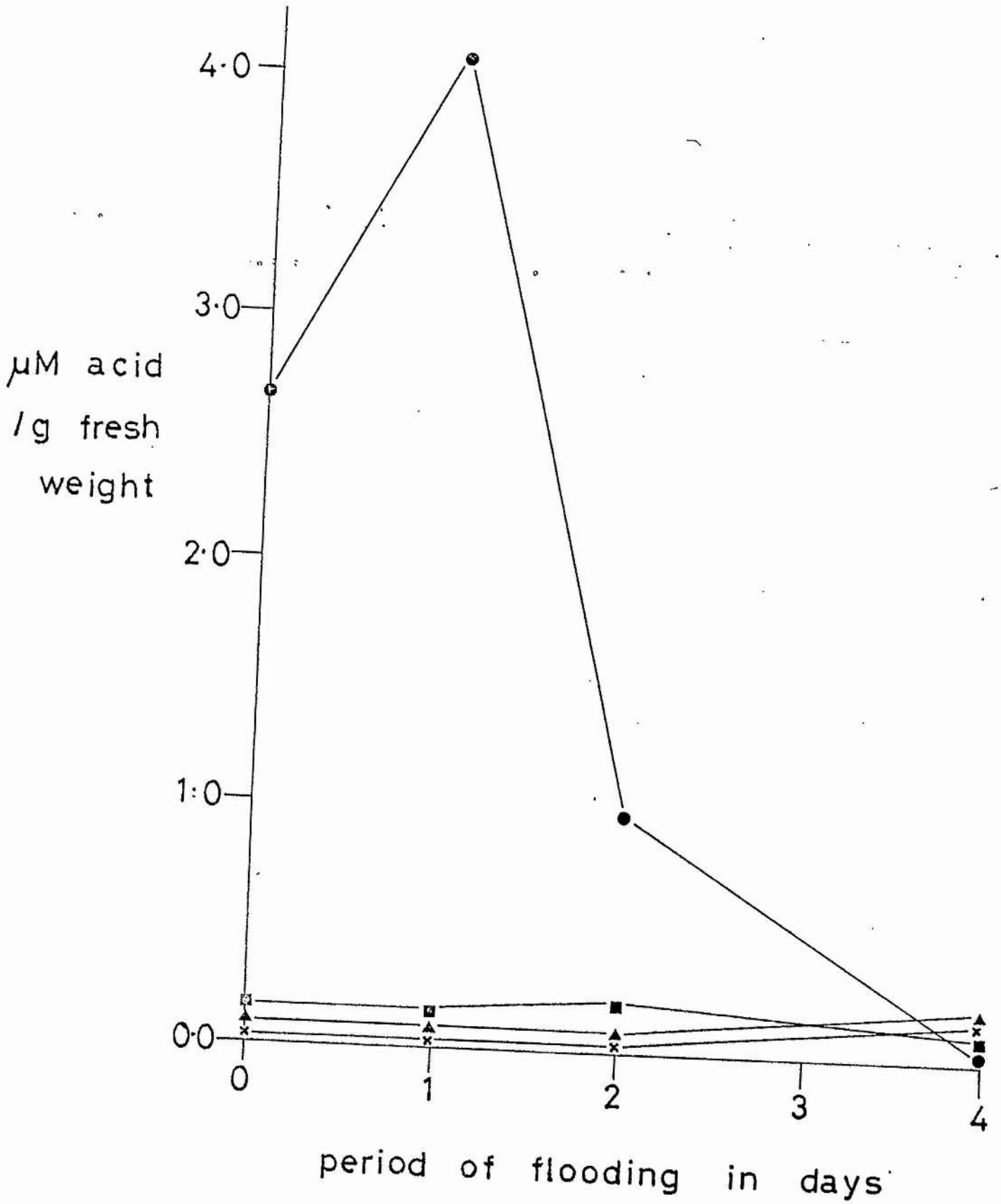


Fig. 9 j

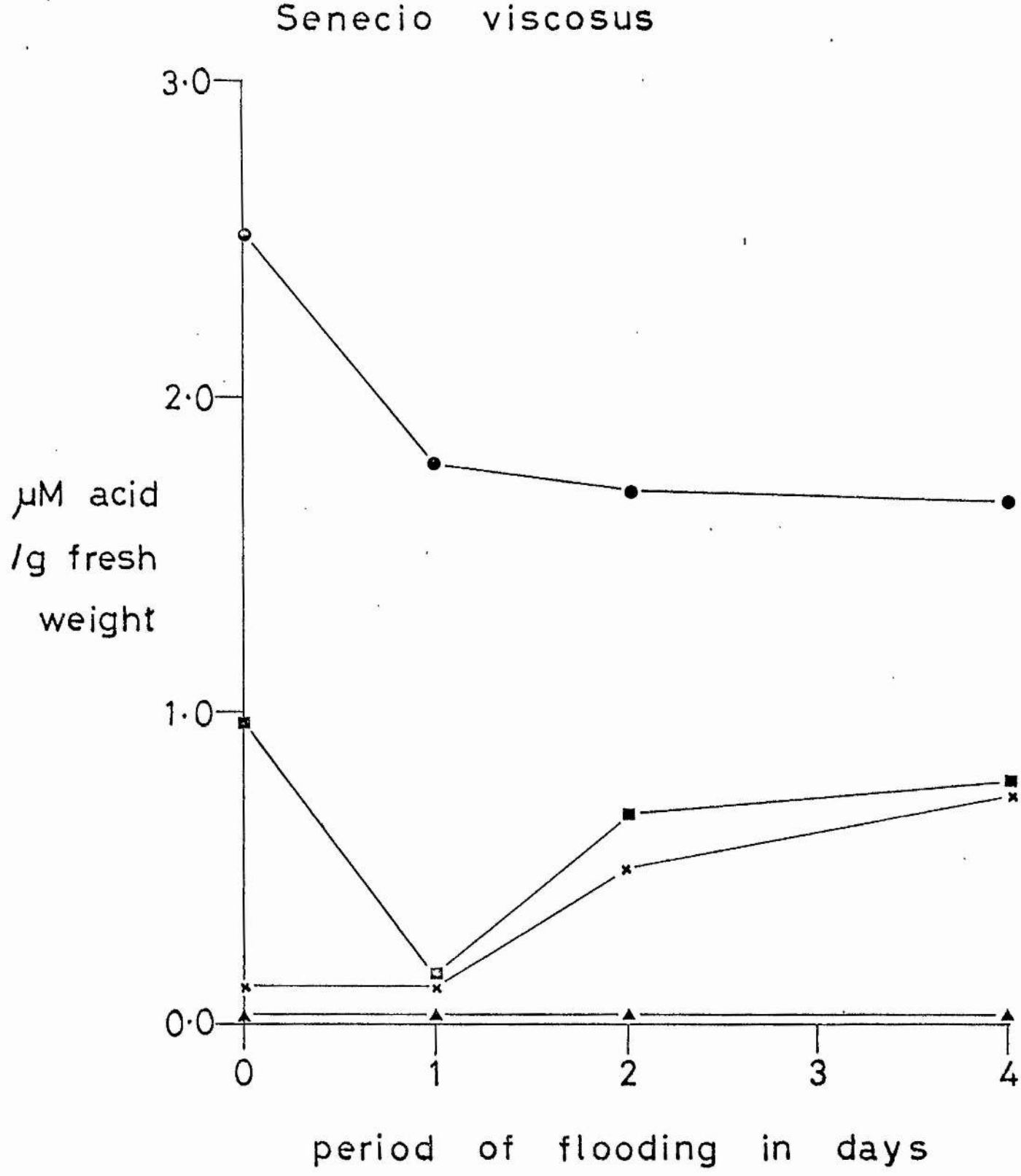

Senecio viscosus
3·0
2·0
1·0
0·0
µM acid /g fresh weight
0
1
2
3
4
period of flooding in days

Fig. 9k

Senecio vulgaris (dune race)

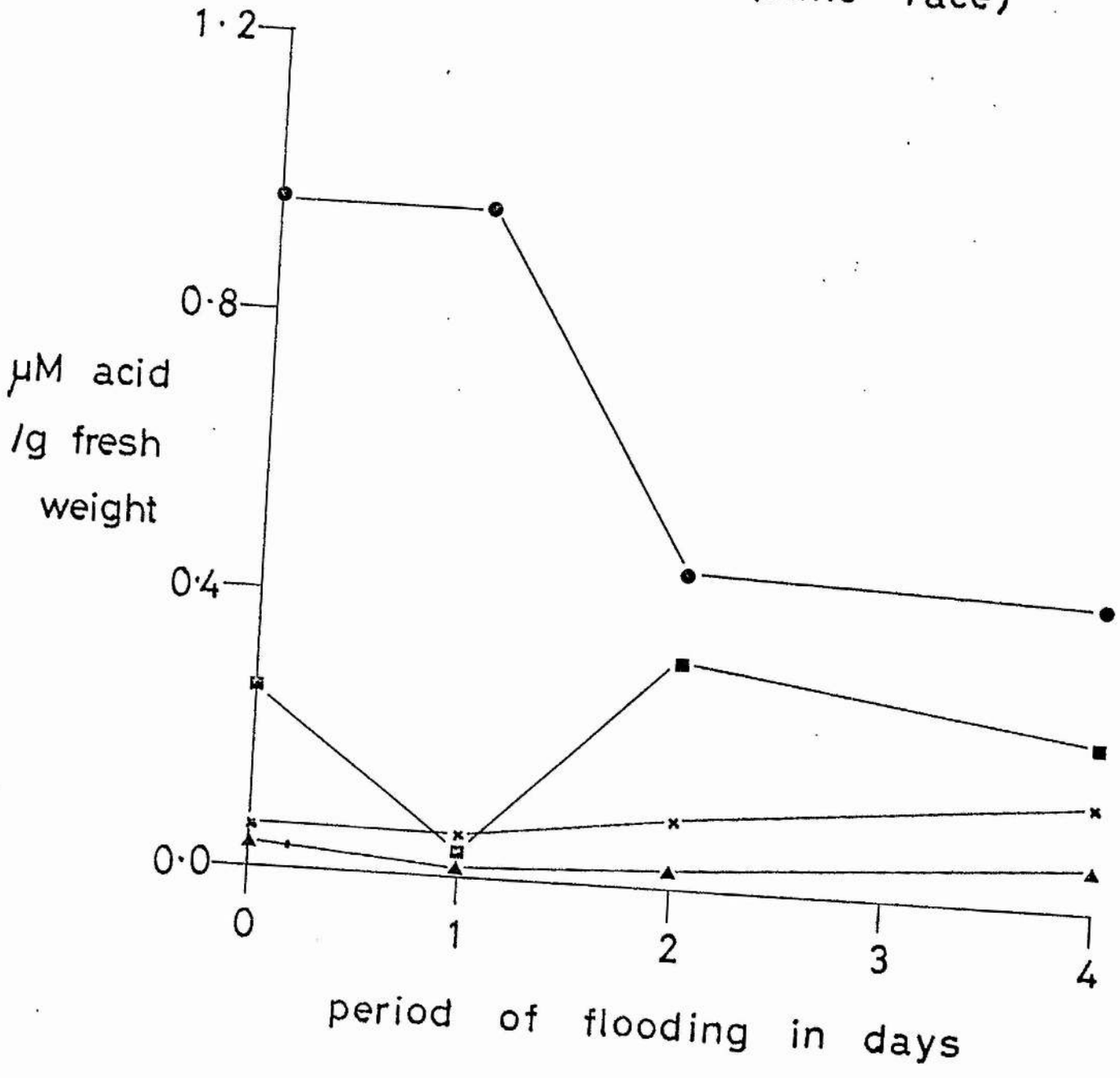


Fig. 10. COPY OF PAPER CHROMATOGRAM OF ROOT EXTRACTS OF FLOOD TREATED CAREX LASIOCARPA

Paper: Whatman no. 1.

Solvent: propanol / eucalyptol / formic acid / water
50 / 50 / 20 / 5

Indicator: aniline-glucose.

Key: X and X' - mixtures containing 10 µg and 20 µg respectively of each of the following organic acids: succinic, lactic, malic, citric and shikimic.

0
1
2
4

} number of days of flooding treatment.

su - succinic acid

l - lactic acid

m - malic acid

c - citric acid

sh - shikimic acid

Fig. 10 solvent front

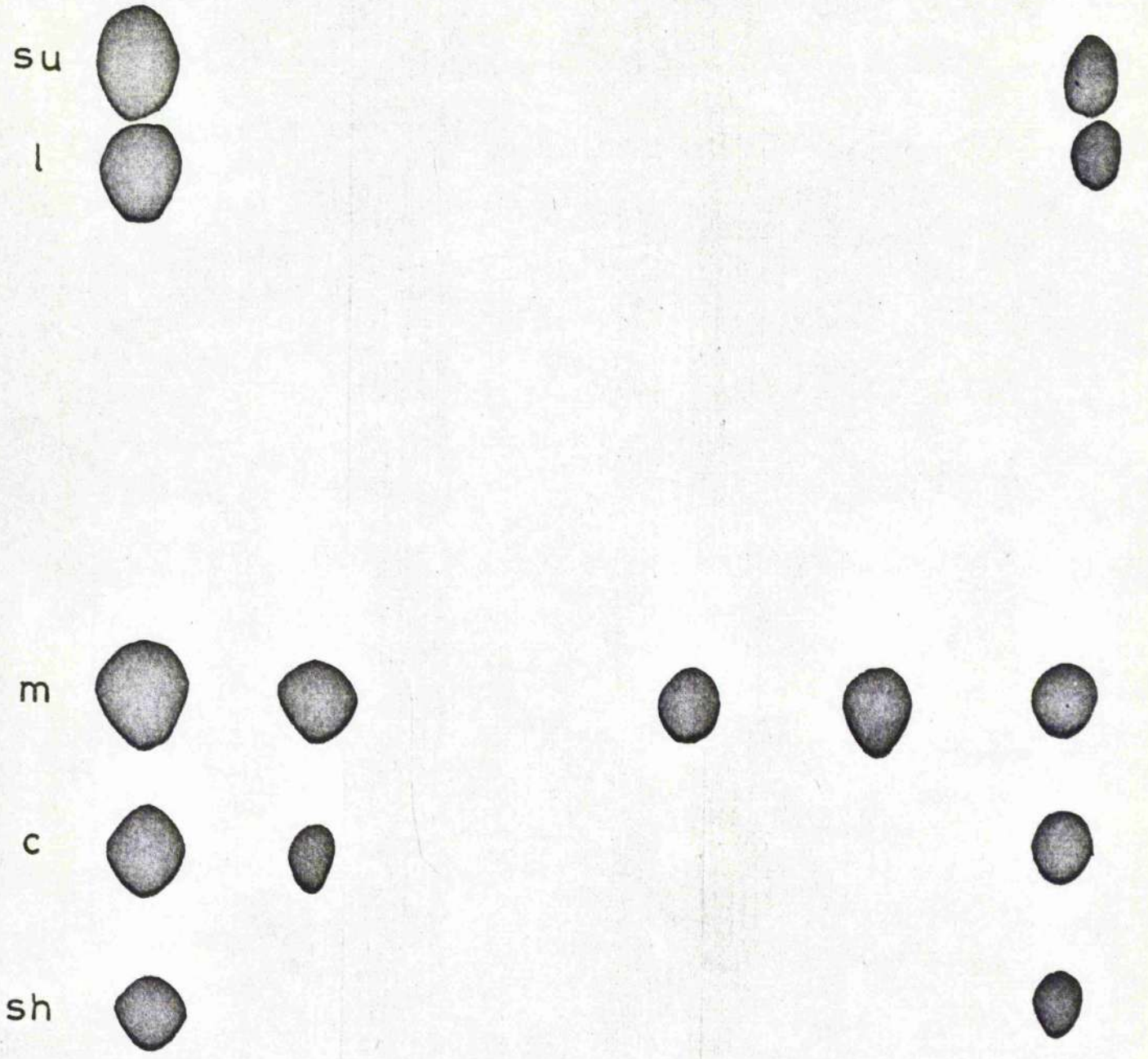


Fig. 11. COPY OF PAPER CHROMATOGRAM OF ROOT EXTRACTS OF FLOOD TREATED SENECIO VISCOSUS

Paper: Whatman no. 1.

Solvent: propanol / eucalyptol / formic acid / water

50 / 50 / 20 / 5

Indicator: aniline-glucose.

Key: X' - mixture containing 20 µg of each of the following organic acids: succinic, lactic, malic, citric and shikimic.

0
1
2
4
} number of days of flooding treatment

su - succinic acid

l - lactic acid

m - malic acid

c - citric acid

sh - shikimic acid

Fig. 11 solvent front

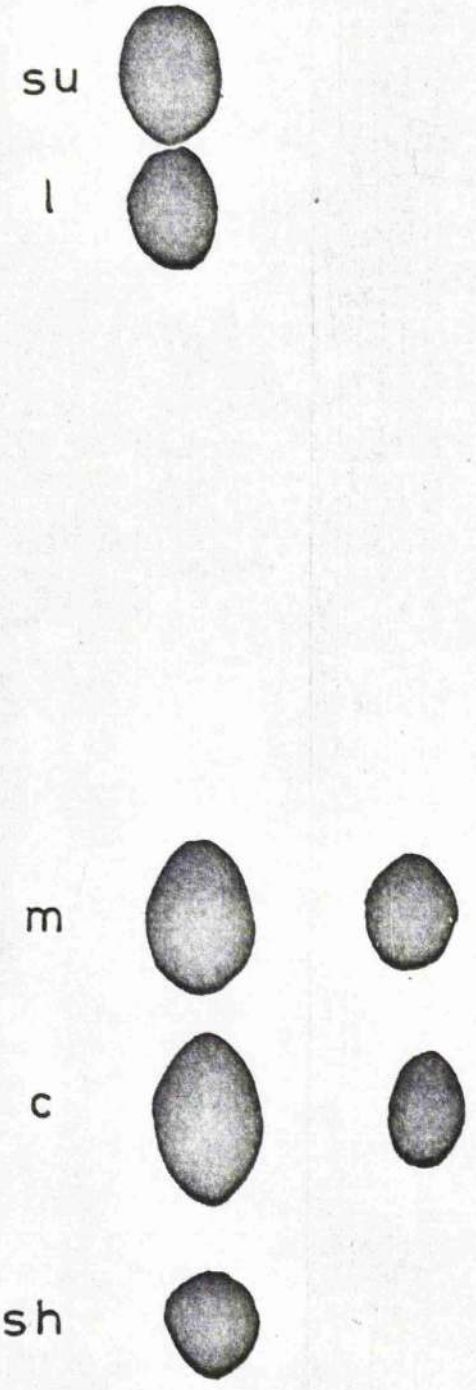


Fig. 12. RATIO OF MALIC:SUCCINIC ACID CONTENT OF ROOTS AFTER FLOODING FOR 4 DAYS

Key: H. p. *Hieraceum pilosella*

S. sq. *Senecio squalidus*

S. s. *S. sylvaticus*

S. vi. *S. viscosus*

S. j. *S. jacobaea*

S. v. *S. vulgaris* (dune race)

J. e. *Juncus effusus*

S. a. *S. aquaticus*

C. l. *Carex lasiocarpa*

C. a. *C. arenaria*

R. f. *Ranunculus flammula*

Fig. 12

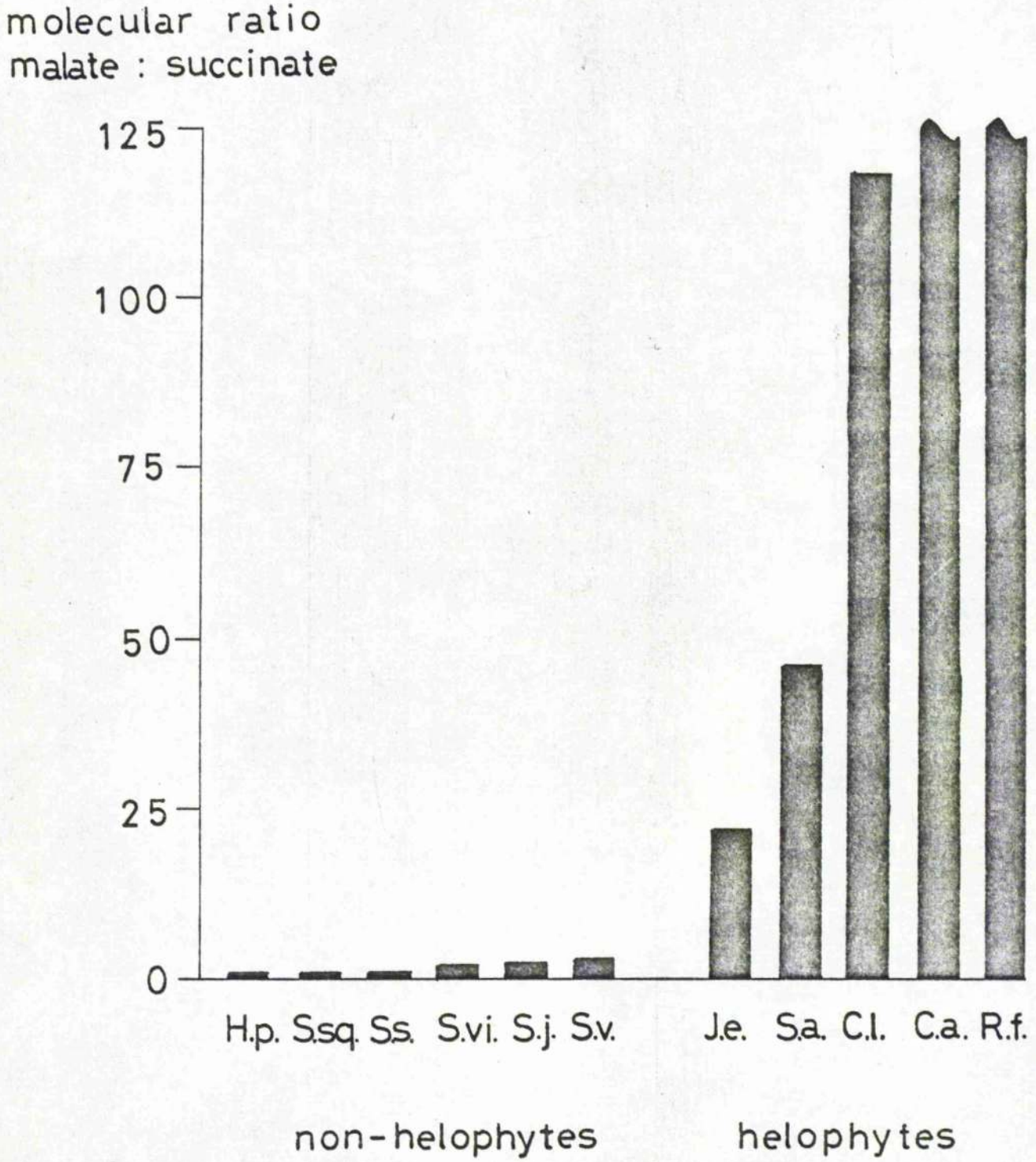

molecular ratio
malate : succinate
125
100
75
50
25
0
H.p.
S.sq.
S.s.
S.vi.
S.j.
S.v.
J.e.
S.a.
C.l.
C.a.
R.f.
non-helophytes
helophytes

Fig. 13. RATIO OF MALIC:CITRIC ACID CONTENT OF ROOTS AFTER FLOODING FOR 4 DAYS

Key: H. p. *Hieraceum pilosella*

S. sq. *Senecio squalidus*

S. s. *S. sylvaticus*

S. j. *S. jacobaea*

S. v. *S. vulgaris* (dune race)

S. vi. *S. viscosus*

J. e. *Juncus effusus*

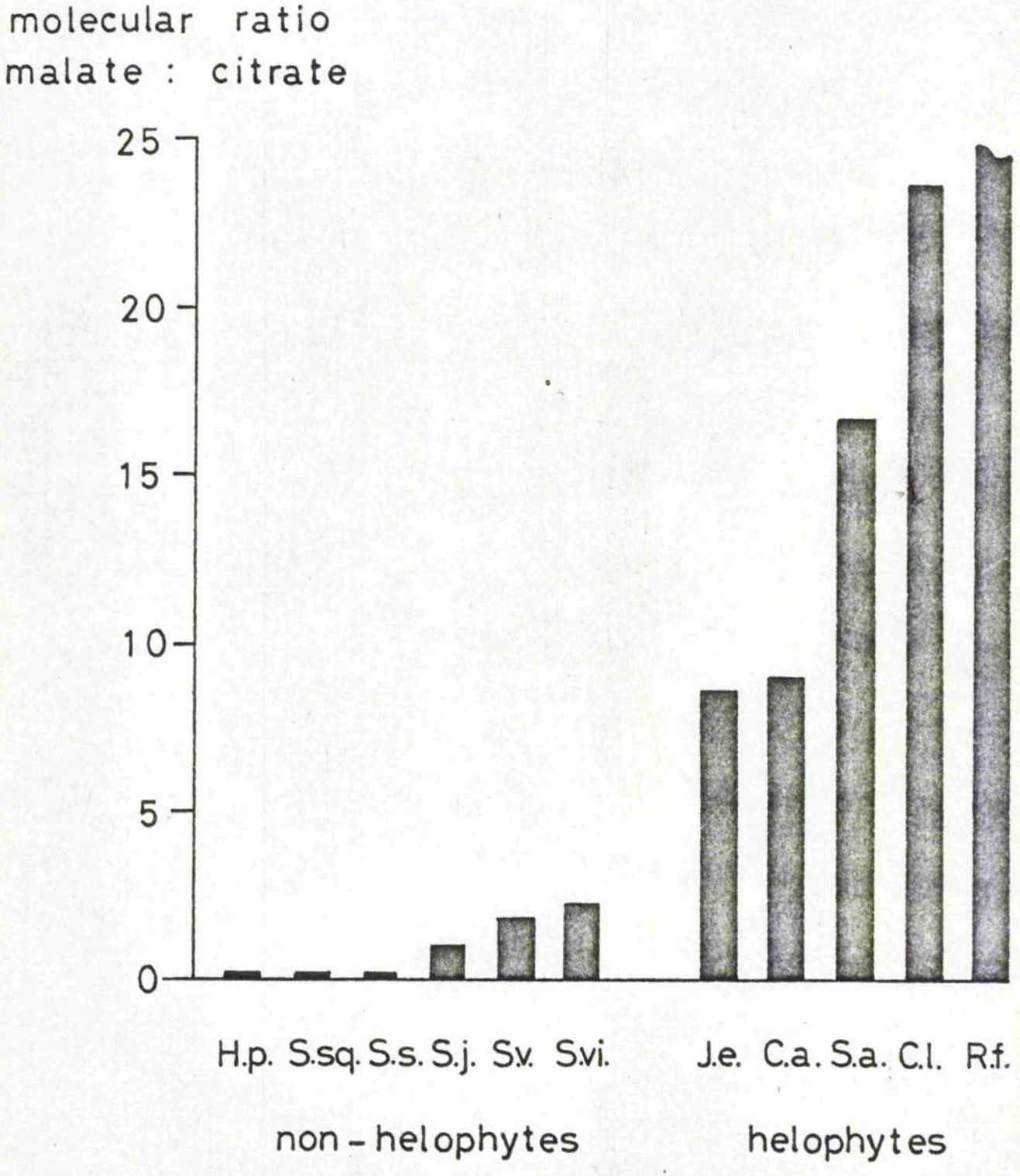
C. a. *Carex arenaria*

S. a. *S. aquaticus*

C. l. *C. lasiocarpa*

R. f. *Ranunculus flammula*

Fig. 13


molecular ratio
malate : citrate
25
20
15
10
5
0
H.p.
S.sq.
S.s.
S.j.
S.v.
S.vi.
J.e.
C.a.
S.a.
C.l.
R.f.
non - helophytes
helophytes

RESULTS

NATURAL FLOODING

MALIC ACID CHANGES

The root extracts of the three helophyte species, Filipendula ulmaria, Erica tetralix and Glyceria maxima, collected in March from the flooded dune slack at Tentsmuir all contained malic acid (Fig. 16). There was over 5 μ mole malate / g fresh weight in one species, F. ulmaria, yet by May the level in all three species had dropped to below a detectable amount. The presence of malate in the root tissues during natural flooding, and its disappearance with the drop in water table to non-flooded conditions in May, is in agreement with the experimental finding that flooding in helophytes is accompanied by an increase in malic acid in the roots.

Malic acid was present throughout all the months of the year in the roots and rhizome of Nuphar lutea growing in Loch Clunie. The quantities of malate present varied up to 0.2 μ mole acid / g fresh weight (Fig. 14). It is important to note that this fresh-water macrophyte is continually submerged by water, with the roots and rhizome in the bottom mud. The plant is therefore living in totally different conditions from those of the plants studied at Tentsmuir, where the flooding was only intermittent. The root

and rhizome tissues of an aquatic plant therefore fulfill quite different roles from those of the roots of dune slack plants, and the large fleshy rhizome of N. lutea probably functions as an important food storage organ. The organic acids of the rhizome could be carbohydrate food reserves adapted to the continual submersion, and their turn-over could be linked to particular growth phases, such as leaf formation, flowering, or fruiting. There is in fact a malate peak in June, the time of flowering.

Iris pseudacorus is also a plant adapted to continual submersion, although in this case where it was sampled growing at the margin of Loch Clunie, there was some drying out of the substrate during the summer months (Plate VA and VB). The malic acid content of the roots and rhizome of this I. pseudacorus material also showed changes during the March to November period (Fig. 15), ranging from less than 0.1 μ mole malate / g fresh weight to nearly 0.5 μ mole. It is unfortunate, however, that no measure was made of the monthly change in water table at the collecting site, where standing water in winter contrasted with dry conditions in August and a rising water table in October and November. The malic acid changes that have been recorded show that, apart from the low root content in September, there is considerable parallel between the malate levels of root and rhizome tissue, even if no clear-cut pattern with flooded conditions has been established. It is feasible that the malic acid metabolism

of these tissues is influenced by the flooding of the substrate, but a more comprehensive study is needed to establish any connection.

SHIKIMIC ACID CHANGES

Shikimic acid was detected only in the roots and rhizomes of Iris and Nuphar, the macrophyte species capable of living in aquatic [redacted] habitats. However, when shikimic acid was present in these tissues it was usually in large quantities, and the paper chromatogram of I. pseudacorus rhizome extracts (Fig. 17) shows the high concentration of shikimate when compared with other organic acids. The quantitative micro-estimations showed that between March and November there was always between 1.0 and 1.4 μ mole shikimate / g fresh weight in the rhizomes, and up to 0.6 μ mole in the roots (Fig. 18). A steady drop in shikimate from March to May was followed by a steady rise between June and November, in both tissues. The shikimic acid content of the roots and rhizomes of Nuphar lutea also showed fluctuations during the December to September period, although only up to the 0.2 μ mole acid / g fresh weight level (Fig. 19) and furthermore there was no steady pattern of increase or decrease for any spell of time during these months.

Shikimic acid, for all its predominance in the root and rhizome tissues of I. pseudacorus and N. lutea, was not detected in any other extract, either from experimental or natural material. The role of this acid in the metabolism of these two species will be discussed later.

CITRIC ACID CHANGES

Although the paper chromatogram of the rhizome extracts of Iris pseudacorus (Fig. 17) did not indicate the presence of citric acid, the more sensitive and specific enzymic method of assay was able to measure small quantities in these and the root extracts. The levels of citrate throughout the March to November period were very small - always less than 0.1 μ mole acid / g fresh weight - although a steady drop from March to June followed by a steady rise from July to November was recorded in the rhizome extracts (Fig. 20). The fluctuations in the root citrate levels were all within the 0.02 to 0.05 μ mole acid / g fresh weight range, and did not warrant any strong interpretation

LACTIC AND SUCCINIC ACID CHANGES

Since citric acid had been measured enzymically in Iris

pseudacorus after not being detected chromatographically, it was thought worthwhile to also assay enzymically for lactic and succinic acids. Neither of these acids could be detected in either the root or rhizome extracts of Iris pseudacorus.

Fig. 14. MALIC ACID CONTENT OF ROOT AND RHIZOME TISSUE OF NUPHAR LUTEA GROWING UNDER NATURAL CONDITIONS AT LOCH CLUNIE DECEMBER 1967 TO SEPTEMBER 1968

Key: ○ N. lutea rhizome

△ N. lutea root

Fig. 14

Nuphar lutea

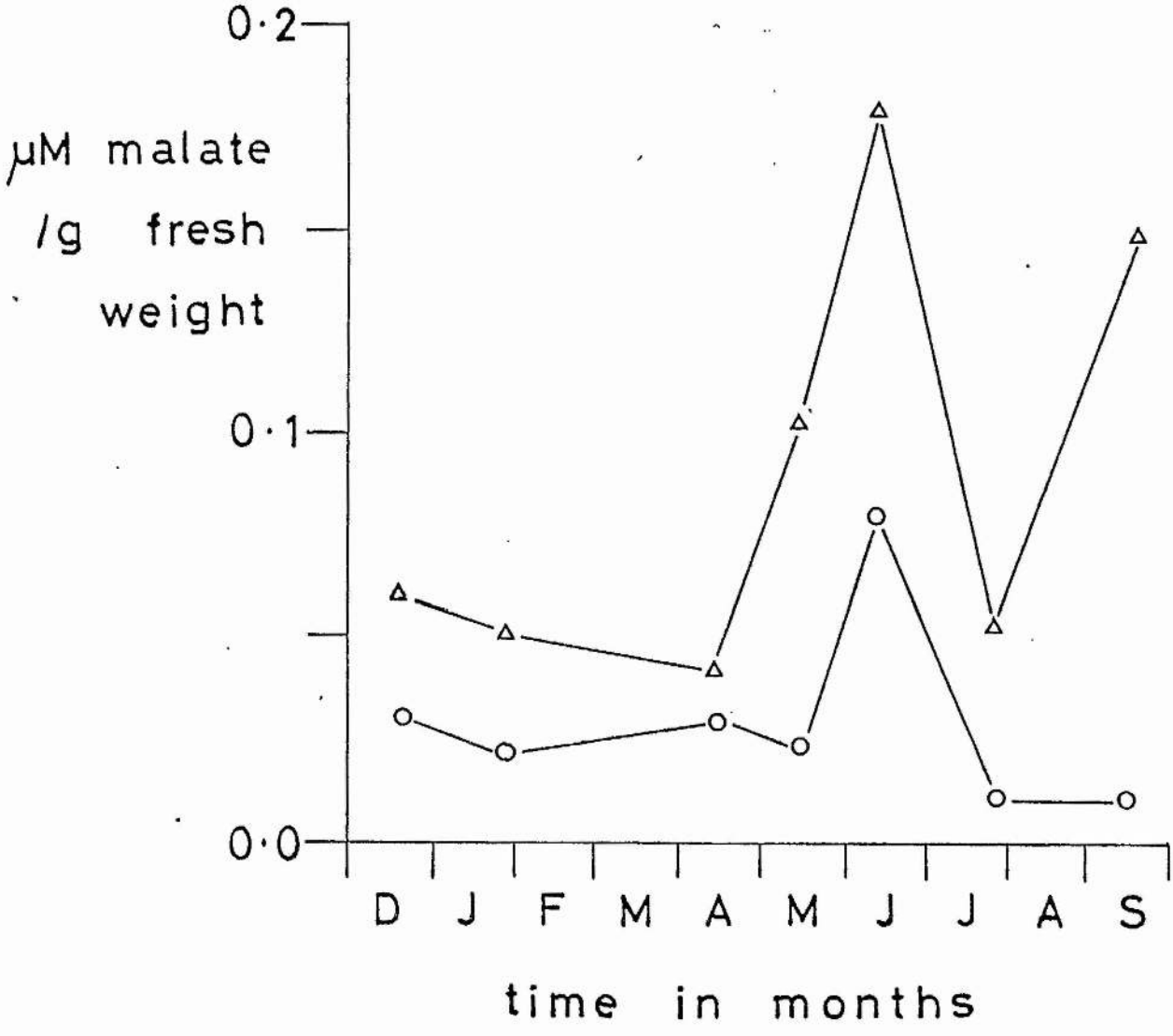


Fig. 15. MALIC ACID CONTENT OF ROOT AND RHIZOME TISSUE OF IRIS PSEUDACORUS GROWING UNDER NATURAL CONDITIONS AT LOCH CLUNIE, MARCH TO NOVEMBER 1968

Key: ○ I. pseudacorus rhizome

△ I. pseudacorus root

Fig. 15

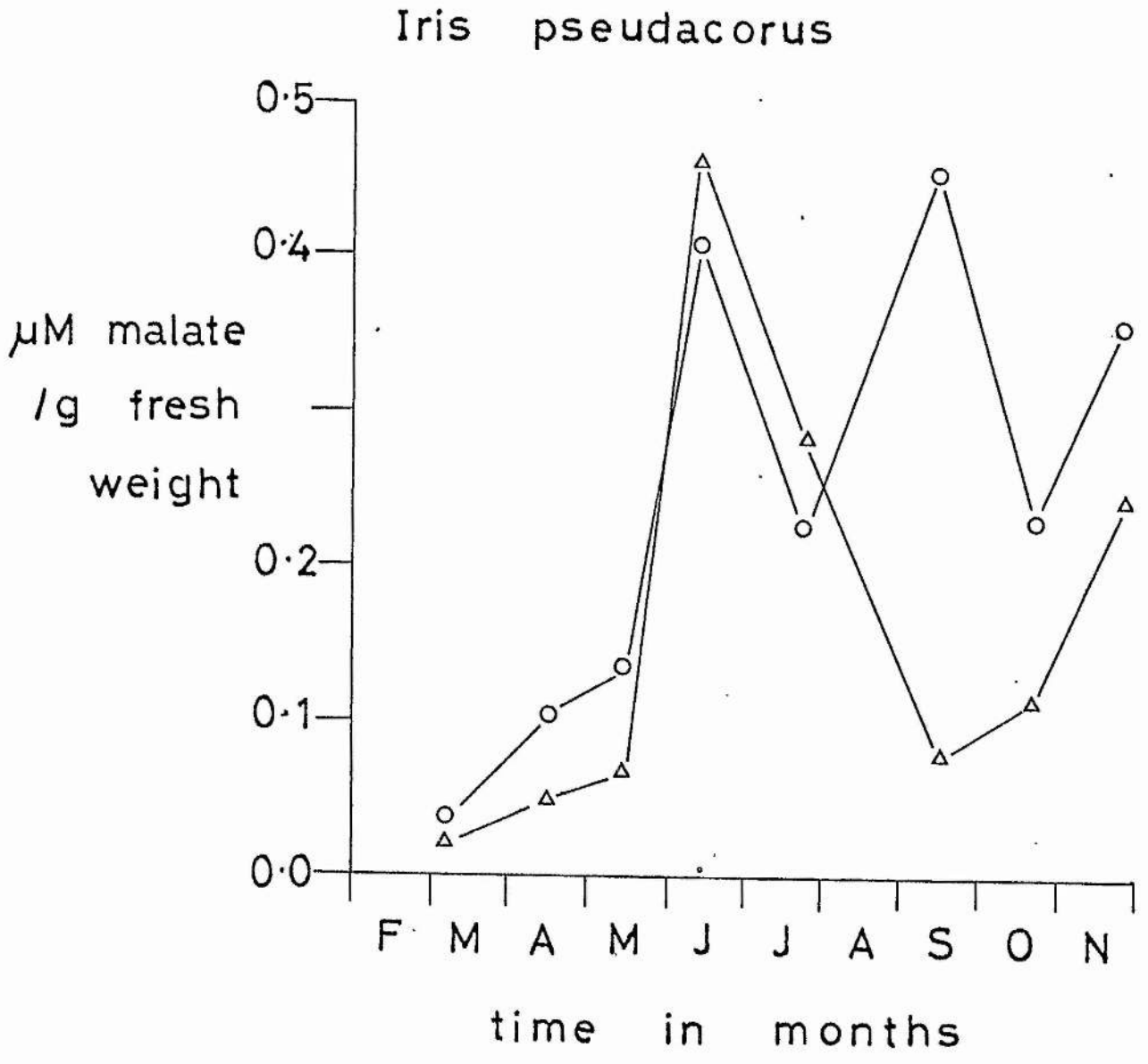

Iris pseudacorus
0·5
0·4
0·2
0·1
0·0
µM malate /g fresh weight
F M A M J J A S O N
time in months

Fig. 16. ROOT MALIC ACID CONTENT OF HELOPHYTES GROWING UNDER NATURAL CONDITIONS IN A DUNE SLACK AT TENTSMUIR, FIFE.

Key: ■ Filipendula ulmaria

● Erica tetralix

▲ Glyceria maxima

Fig. 16

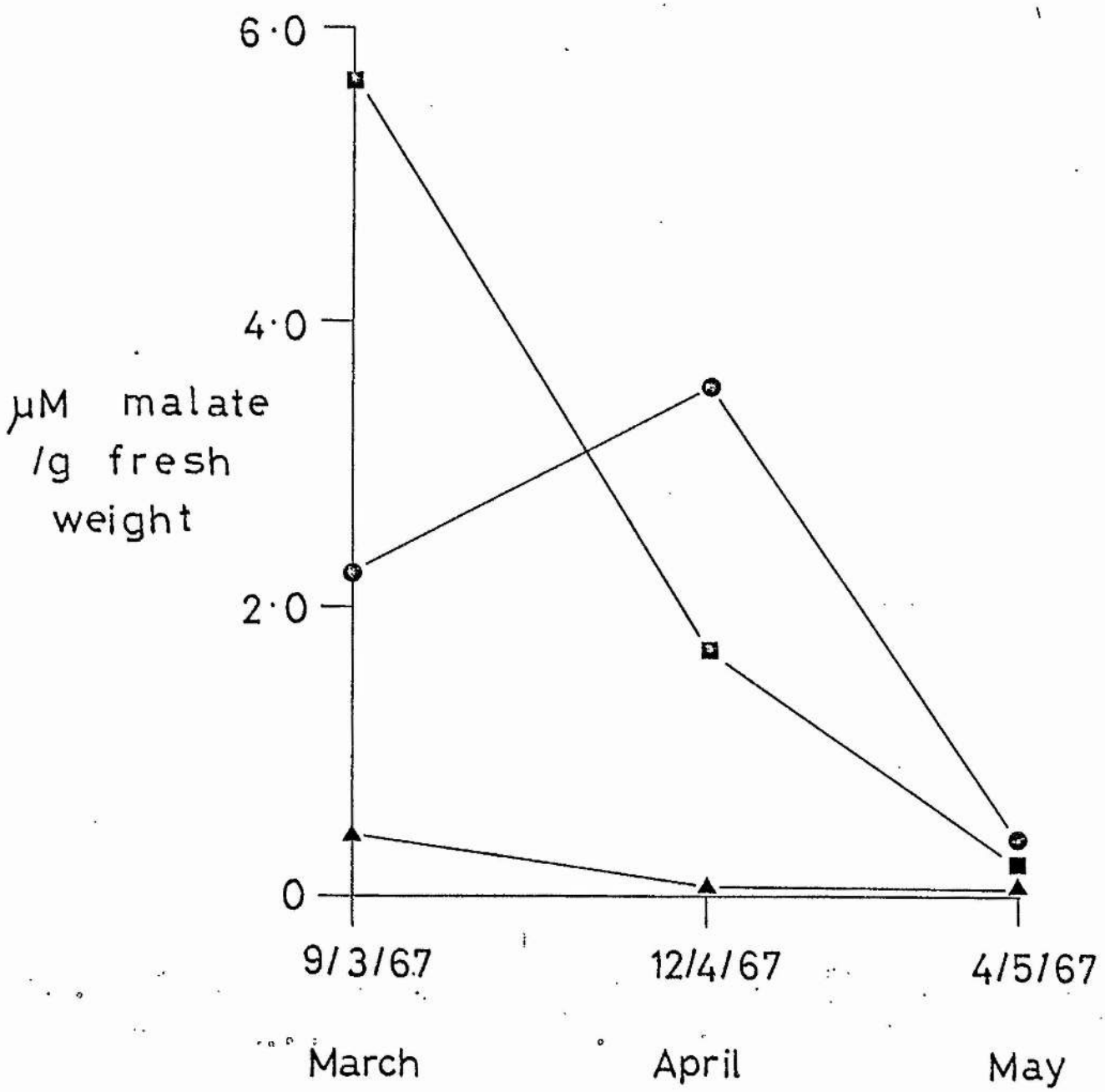

6·0
4·0
2·0
0
µM malate /g fresh weight
9/3/67
12/4/67
4/5/67
March
April
May

Fig. 17. COPY OF PAPER CHROMATOGRAM OF RHIZOME EXTRACTS OF IRIS PSEUDACORUS

Paper: Whatman no. 1.

Solvent: propanol / eucalyptol / formic acid / water
50 / 50 / 20 / 5.

Indicator: aniline-glucose.

Key: X - mixture containing 10 µg of each of the following organic acids: succinic, lactic, malic, citric and shikimic.

1 - 6/3/68
2 - 16/4/68
3 - 14/5/68
4 - 11/6/68
5 - 25/7/68
6 - 14/9/68

} Date of collection of I. pseudacorus rhizomes from Loch Clunie.

su - succinic acid

l - lactic acid

m - malic acid

c - citric acid

sh - shikimic acid

Fig. 17 solvent front

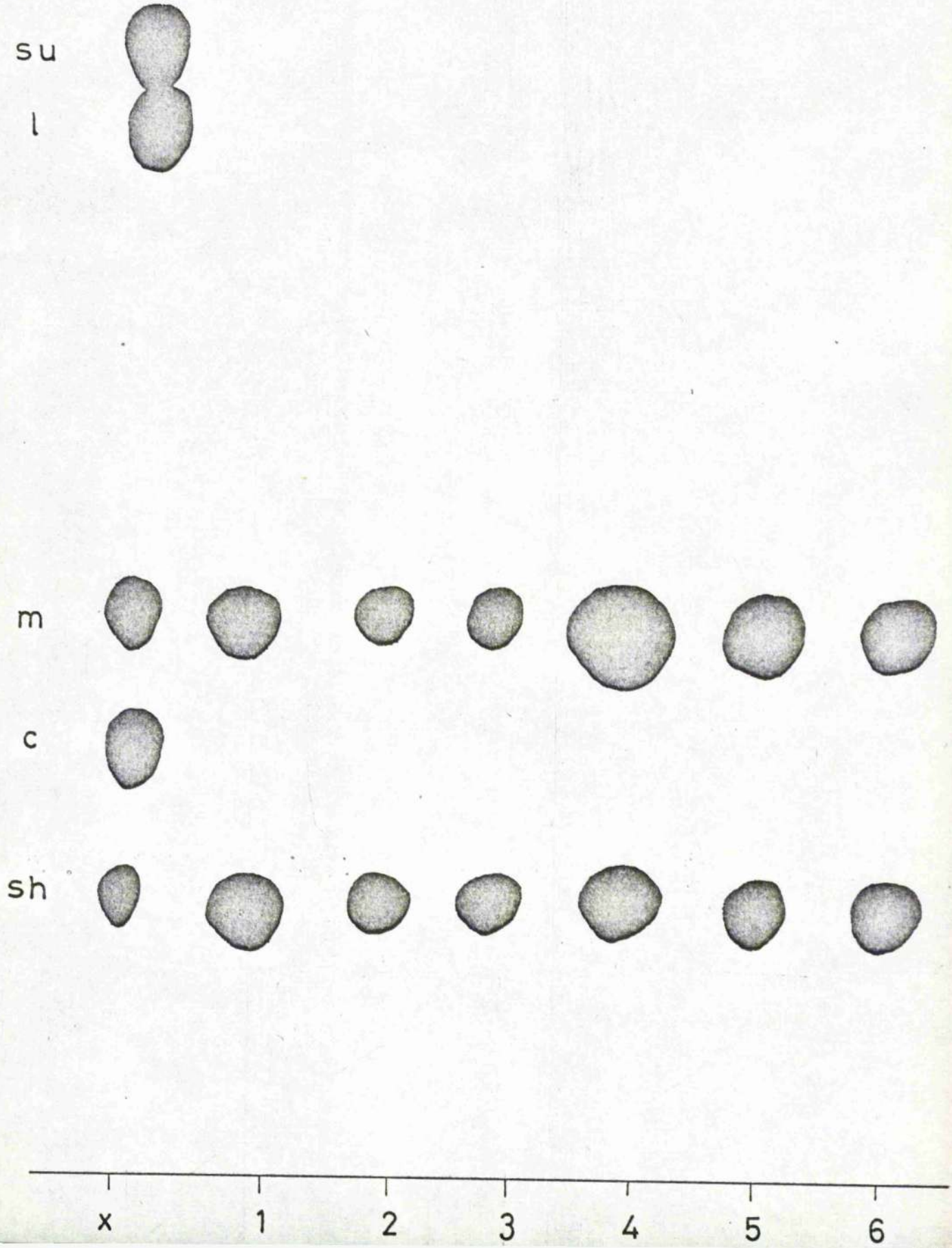


Fig. 18. SHIKIMIC ACID CONTENT OF ROOT AND RHIZOME TISSUE OF IRIS PSEUDACORUS GROWING UNDER NATURAL CONDITIONS AT LOCH CLUNIE, MARCH TO NOVEMBER 1968.

Key: o I. pseudacorus rhizome

Δ I. pseudacorus root

Fig. 18

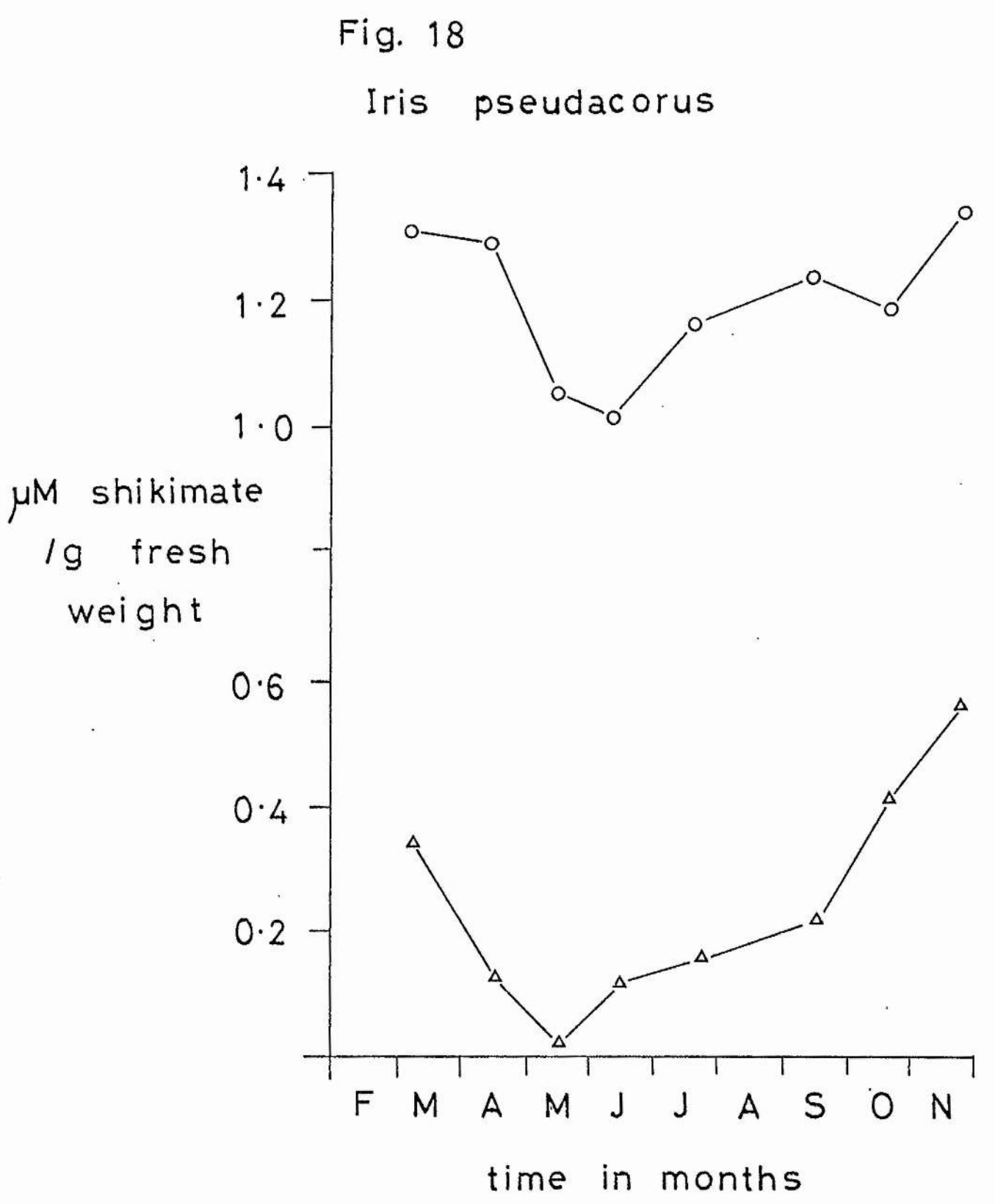

Iris pseudacorus
1·4
1·2
1·0
µM shikimate /g fresh weight
0·6
0·4
0·2
F M A M J J A S O N
time in months

Fig. 19. SHIKIMIC ACID CONTENT OF ROOT AND RHIZOME TISSUE OF NUPHAR LUTEA GROWING UNDER NATURAL CONDITIONS AT LOCH CLUNIE, DECEMBER 1967 TO SEPTEMBER 1968.

Key: ○ N. lutea rhizome

△ N. lutea root

Fig. 19

Nuphar lutea

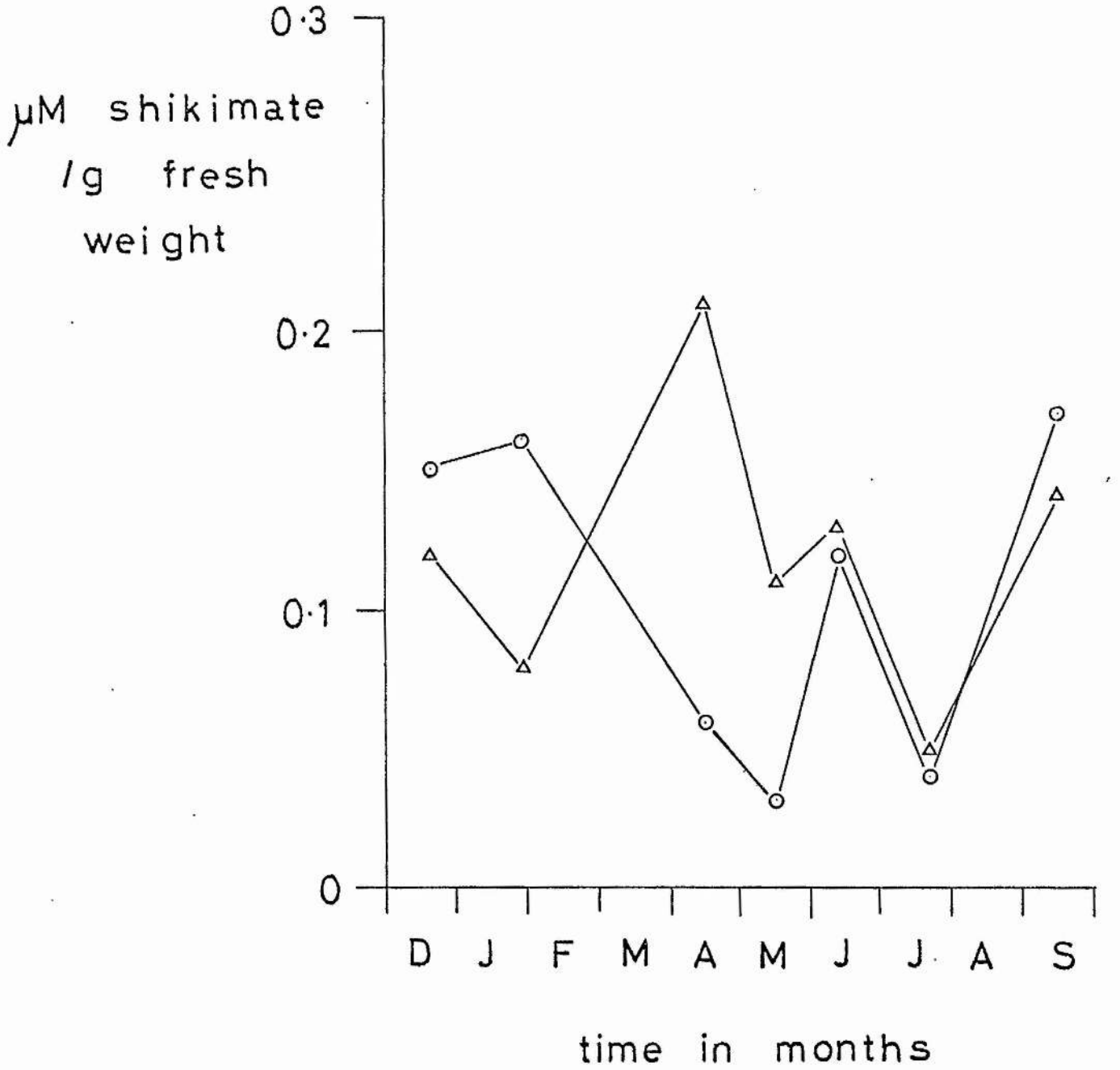
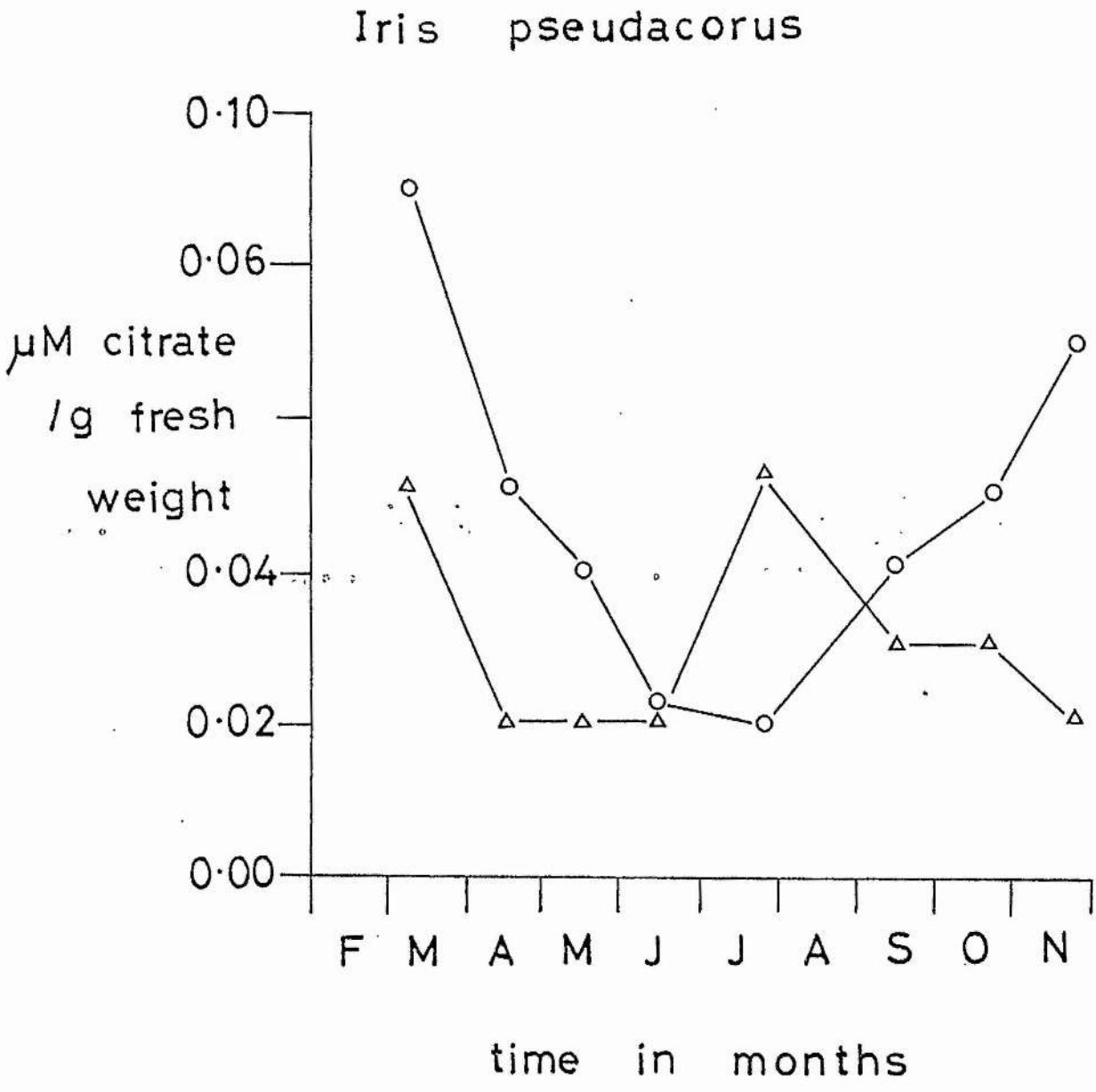


Fig. 20. CITRIC ACID CONTENT OF ROOT AND RHIZOME TISSUE OF IRIS PSEUDACORUS GROWING UNDER NATURAL CONDITIONS AT LOCH CLUNIE, MARCH TO NOVEMBER, 1968.

Key: o Iris pseudacorus rhizome

Δ Iris pseudacorus root

Fig. 20


Iris pseudacorus
0·10
0·06
µM citrate /g fresh weight
0·04
0·02
0·00
F M A M J J A S O N
time in months

RESULTS

INCUBATION OF ROOT TISSUE IN AEROBIC AND ANAEROBIC CONDITIONS

MALIC ACID CHANGES

The root tissue malic acid content of all three species before incubation was between 1.0 and 2.0 μ mole acid / g fresh weight. In aerated incubation, malic acid showed a decrease in Senecio viscosus and S. aquaticus, and a slight rise in Ranunculus flammula (Fig. 21a). In completely anaerobic conditions the malate level in all three species fell, and in S. aquaticus and R. flammula none was detectable after the full 6-hour period (Fig. 21b). There was thus no accumulation of malate as seen in the roots of intact plants under flooding induced anaerobiosis.

LACTIC ACID CHANGES

At the onset of incubation, there was no lactate in the root tissues of any of the three species. Upon aerated incubation, a slight rise to 0.1 μ mole lactate / g fresh weight occurred in S. aquaticus, but in S. viscosus and R. flammula lactate remained undetected (Fig. 22a). Under the anaerobic conditions imposed by bubbling nitrogen through the culture, there was a rise in

lactate in the first three hours in all three species (Fig. 22b). This rise was continued in S. aquaticus and R. flammula, but followed by a drop during the second three hours in S. viscosus.

CITRIC ACID CHANGES

The citric acid content of the helophytes was quite high (between 0.9 and 1.6 μ mole citrate / g fresh weight) before incubation but there was none in the non-helophyte at this stage. During aerobic incubation citric acid decreased in the helophytes, dropping to 0.5 μ mole acid / g fresh weight in R. flammula and disappearing altogether in S. aquaticus (Fig. 23a). In S. viscosus, however, there was a steady build-up of citrate to more than 0.5 μ mole acid / g fresh weight by the sixth hour. Under anaerobic conditions, the citrate contents of S. aquaticus and R. flammula decreased sharply during the first $1\frac{1}{2}$ hours, but recovered and rose slowly during the next $4\frac{1}{2}$ hours (Fig. 23b). The citrate content of S. viscosus remained below the level of detection throughout the entire period of anaerobic incubation.

SUCCINIC ACID CHANGES

All three species contained between 0.6 and 0.8 μ mole succ-

inate / g fresh weight before incubation. Under aerobic conditions there were considerable fluctuations in the succinate levels throughout the six-hour incubation period (Fig. 24a). The succinic acid in both helophytes had fallen after $4\frac{1}{2}$ hours, but whereas that of R. flammula continued to fall to below the level of detection by the sixth hour, that of S. aquaticus rose sharply to the original level. In S. viscosus an initial rise in succinate in the first $1\frac{1}{2}$ hours was followed by a steady drop. Under anaerobic conditions the succinate in S. aquaticus fell steadily and disappeared by the sixth hour, but in both S. viscosus and R. flammula there was a similar pattern of decreasing succinate in the first three hours followed by a recovery to approximately the original levels (Fig. 24b). The six-hour extract of R. flammula was lost before its succinate content could be estimated.

OVERALL ORGANIC ACID CHANGES

The changes in levels of root tissue malic, lactic, citric and succinic acids in the two helophytes and one non-helophyte species during their six-hour incubation in aerobic and anaerobic conditions are shown in Fig. 25. All the individual acid levels were below 2.0 μ mole acid / g fresh weight. Although malate was predominant in the acid metabolism of all three species, it was not present in excessive proportions and was at some stage

in one or other of the species exceeded by lactic, citric and succinic acids. Lactic acid was generally absent or present in only small amounts, never exceeding 0.3 μ mole acid / g fresh weight and often not detected at all. Citric and succinic acids were present in levels intermediate between malic and lactic acids, never exceeding 1.0 μ mole acid / g fresh weight except for the initial level of citrate in R. flammula (1.7 μ mole citrate / g fresh weight). Oxaloacetic acid was not detected in any of the extracts.

There was a general trend for all acids to either remain at approximately constant levels during the six-hour incubation period, or to decrease gradually during that time. Decreasing levels of acids were more evident in the two helophytes, R. flammula (Fig. 25b) and S. aquaticus (Fig. 25c), while in the non-helophyte S. viscosus (Fig. 25a) the levels tended to remain more constant.

It must be emphasised that the organic acid changes detailed above are those occurring in roots which have been detached, surface sterilised and then incubated in glucose solution. These results are therefore not comparable with those of the flooding experiments, where the roots were taken from entire growing plants immediately before extraction with water.

ETHANOL CONTENT UNDER ANAEROBIC CONDITIONS

The changes in root tissue ethanol content in S. squalidus and S. aquaticus during a 12-hour incubation period under nitrogen are shown in Fig. 26. The concentration in both species after three hours was approximately equal at 2.0 μ mole ethanol / g fresh weight, but whereas in S. aquaticus the level dropped slightly during the 12-hour period to 0.5 μ mole, in S. squalidus it rose sharply to reach more than 7.0 μ mole. These results are consistent with those of earlier experiments with other Senecio species (Crawford 1966).

Fig. 21a and 21b. ROOT TISSUE MALIC ACID CONTENT UPON INCUBATION UNDER AEROBIC (AERATED) AND ANAEROBIC (NITROGEN) CONDITIONS

Key: Fig. 21a. Aerated

○ Senecio viscosus

△ Ranunculus flammula

□ S. aquaticus

Fig. 21b. Nitrogen

● S. viscosus

▲ R. flammula

■ S. aquaticus

Fig. 21a

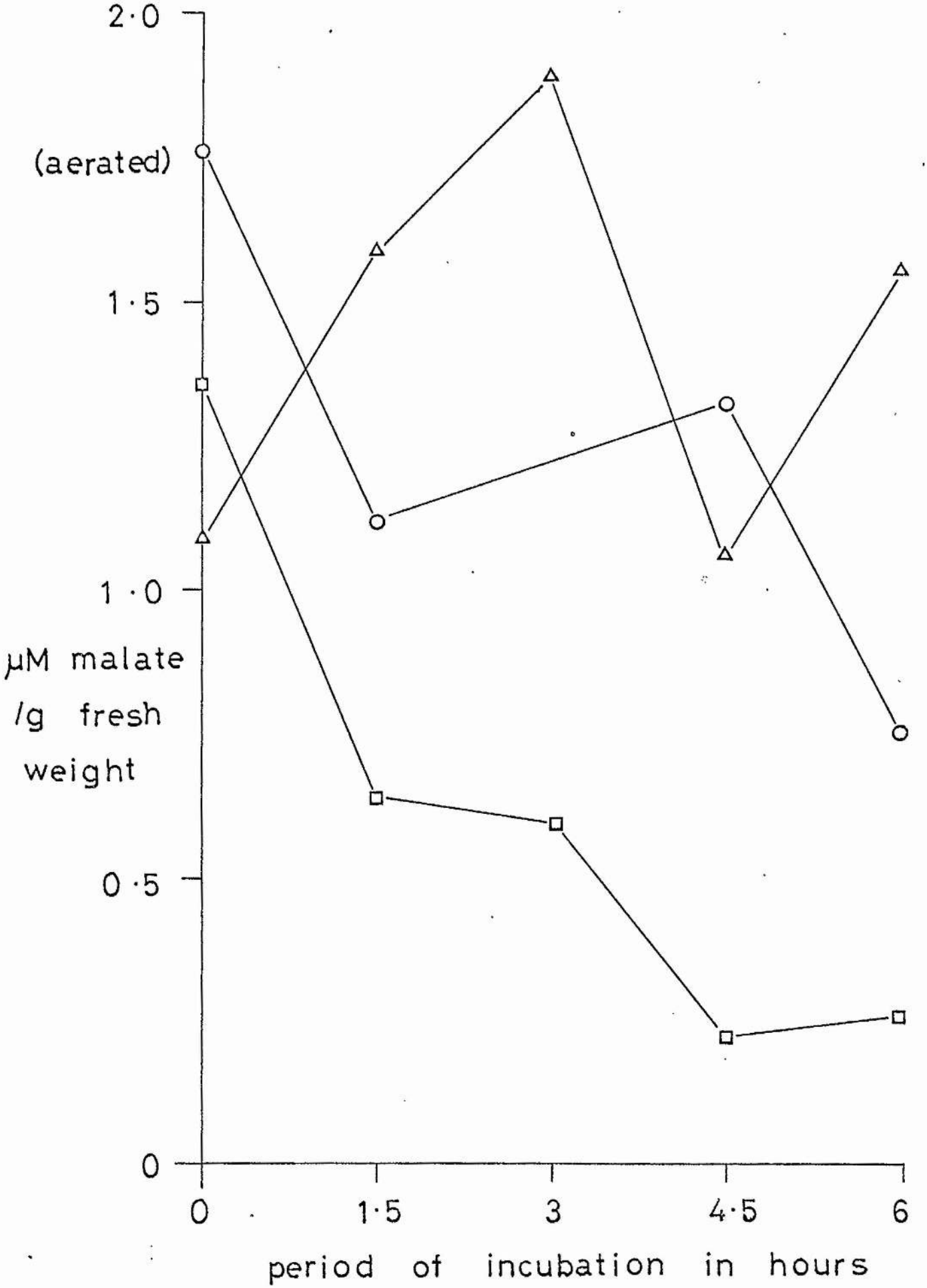

2·0
(aerated)
1·5
1·0
µM malate
/g fresh
weight
0·5
0
0
1·5
3
4·5
6
period of incubation in hours

Fig. 21 b

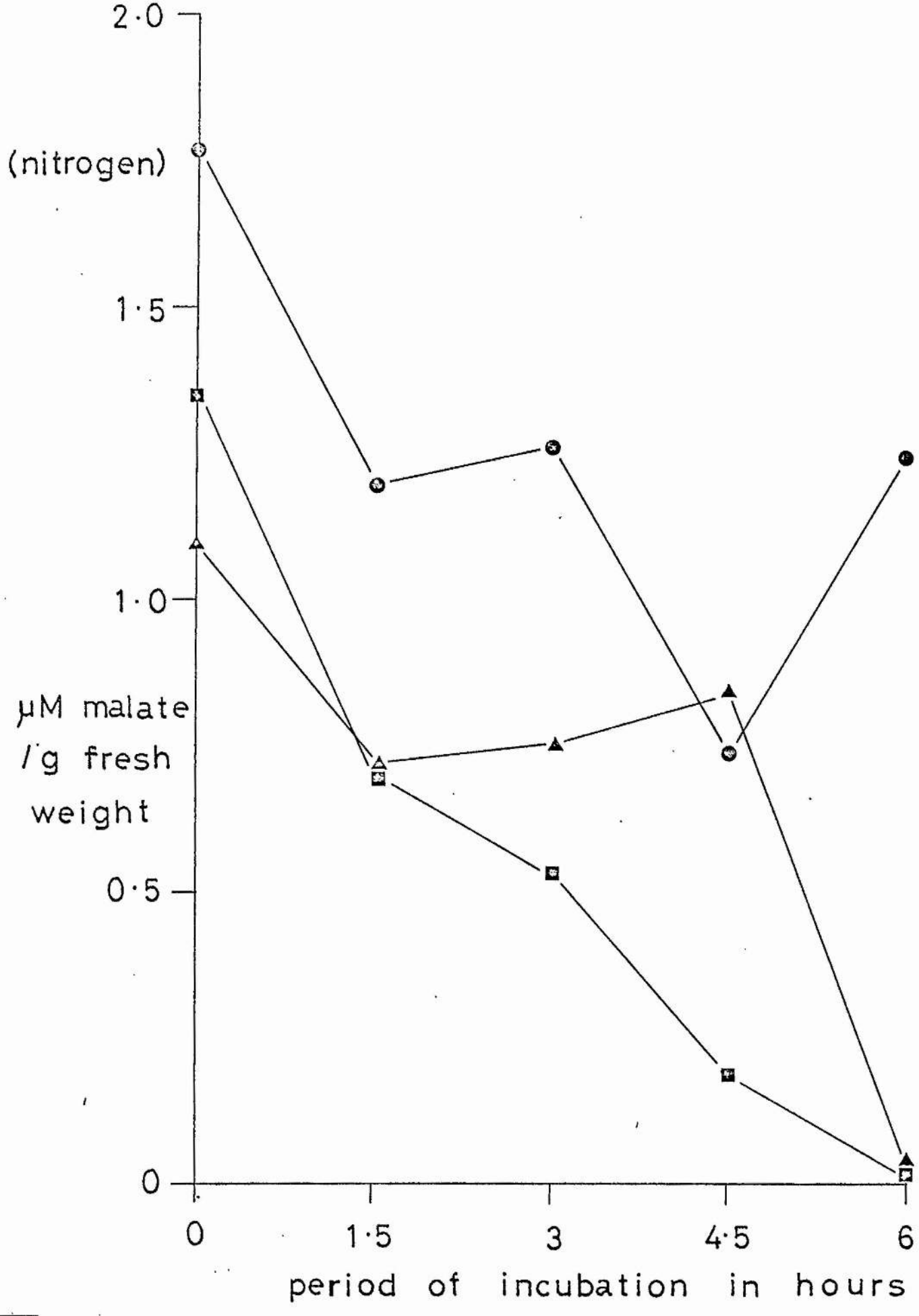

2·0
(nitrogen)
1·5
1·0
µM malate
/g fresh
weight
0·5
0
0
1·5
3
4·5
6
period of incubation in hours

Fig. 22a and 22b. ROOT TISSUE LACTIC ACID CONTENT UPON INCUBATION UNDER AEROBIC (AERATED) AND ANAEROBIC (NITROGEN) CONDITIONS

Key: Fig. 22a. Aerated

- ○ Senecio viscosus
- △ Ranunculus flammula
- □ S. aquaticus

Fig. 22b. Nitrogen

- ● S. viscosus
- ▲ R. flammula
- ■ S. aquaticus

Fig. 22a

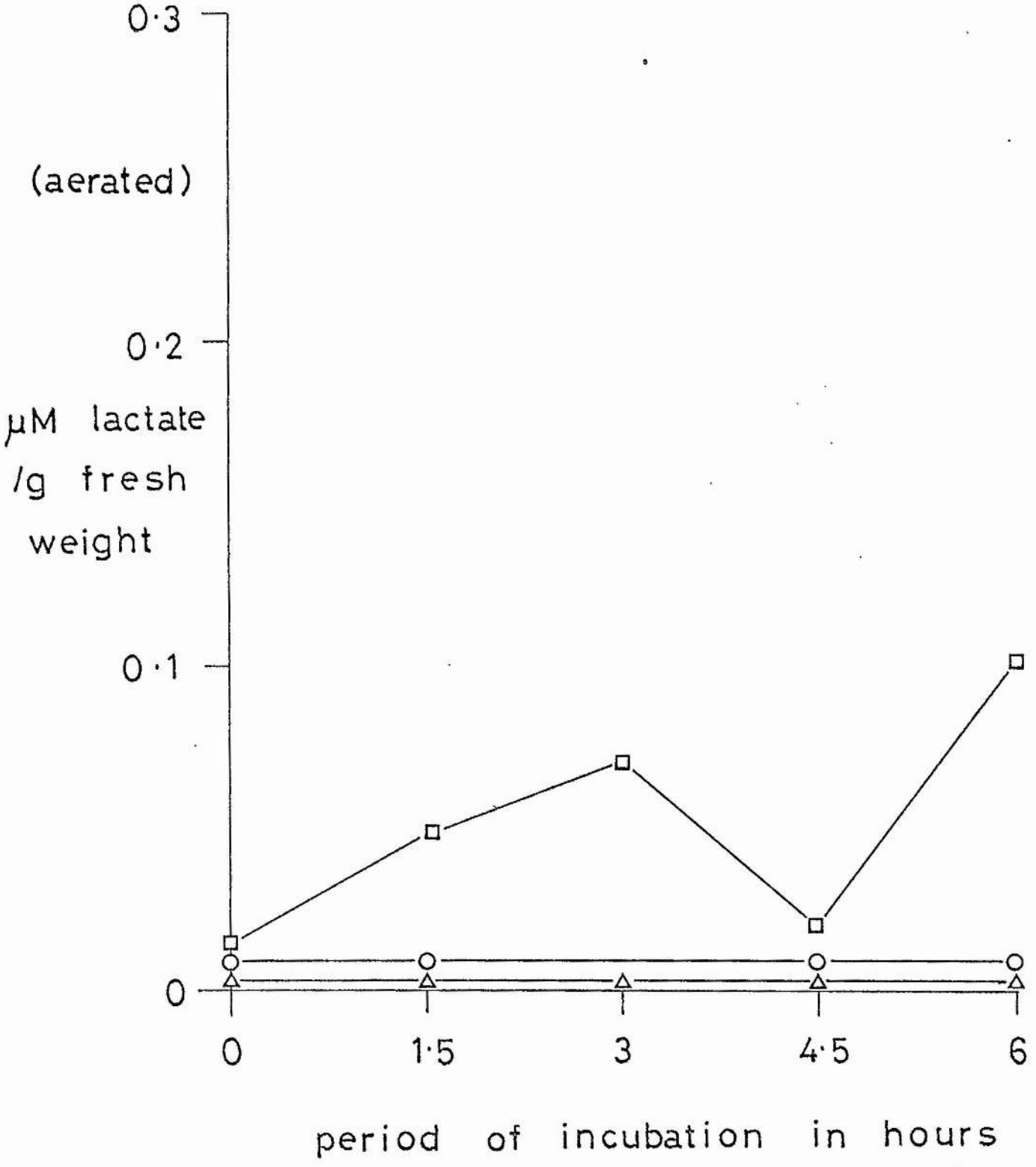

0·3
(aerated)
0·2
µM lactate
/g fresh
weight
0·1
0
0
1·5
3
4·5
6
period of incubation in hours

Fig. 22b

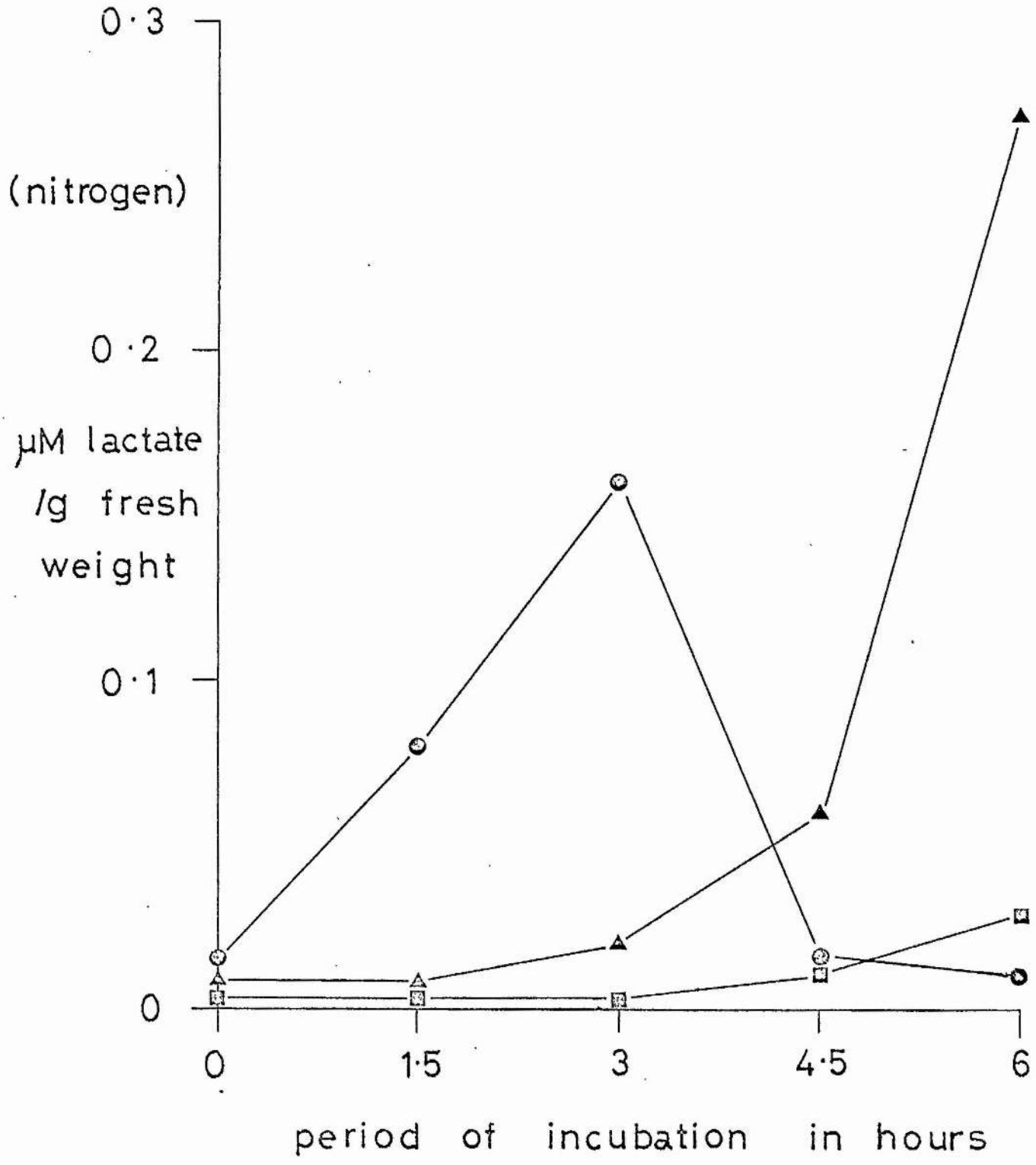

0·3
(nitrogen)
0·2
µM lactate
/g fresh
weight
0·1
0
0
1·5
3
4·5
6
period of incubation in hours

Fig. 23a and 23b. ROOT TISSUE CITRIC ACID CONTENT UPON INCUBATION UNDER AEROBIC (AERATED) AND ANAEROBIC (NITROGEN) CONDITIONS

Key: Fig. 23a. Aerated

○ Senecio viscosus

△ Ranunculus flammula

□ S. aquaticus

Fig. 23b. Nitrogen

● S. viscosus

▲ R. flammula

■ S. aquaticus

Fig. 23 a

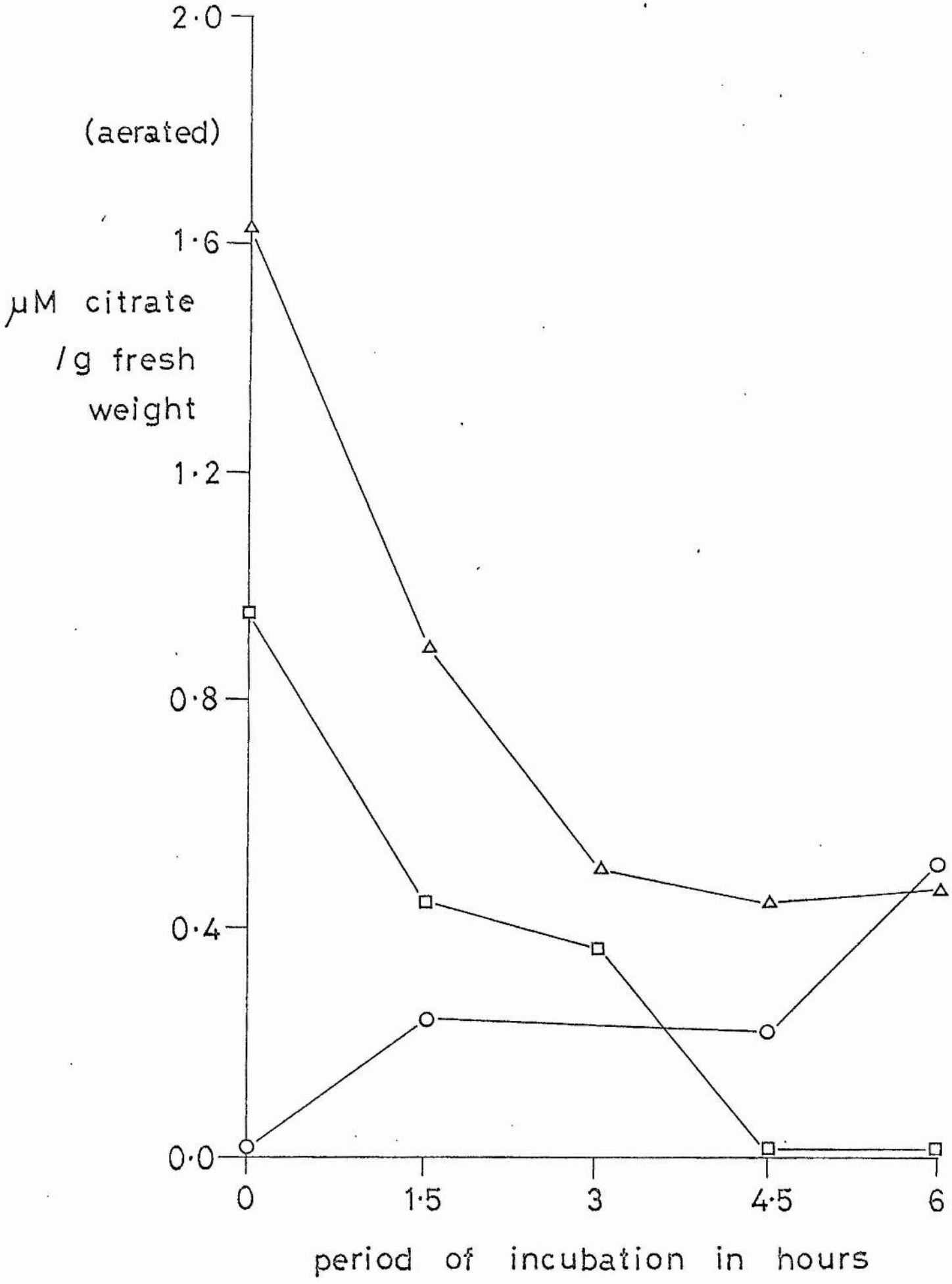

2·0
(aerated)
1·6
µM citrate
/g fresh
weight
1·2
0·8
0·4
0·0
0
1·5
3
4·5
6
period of incubation in hours

Fig. 23 b

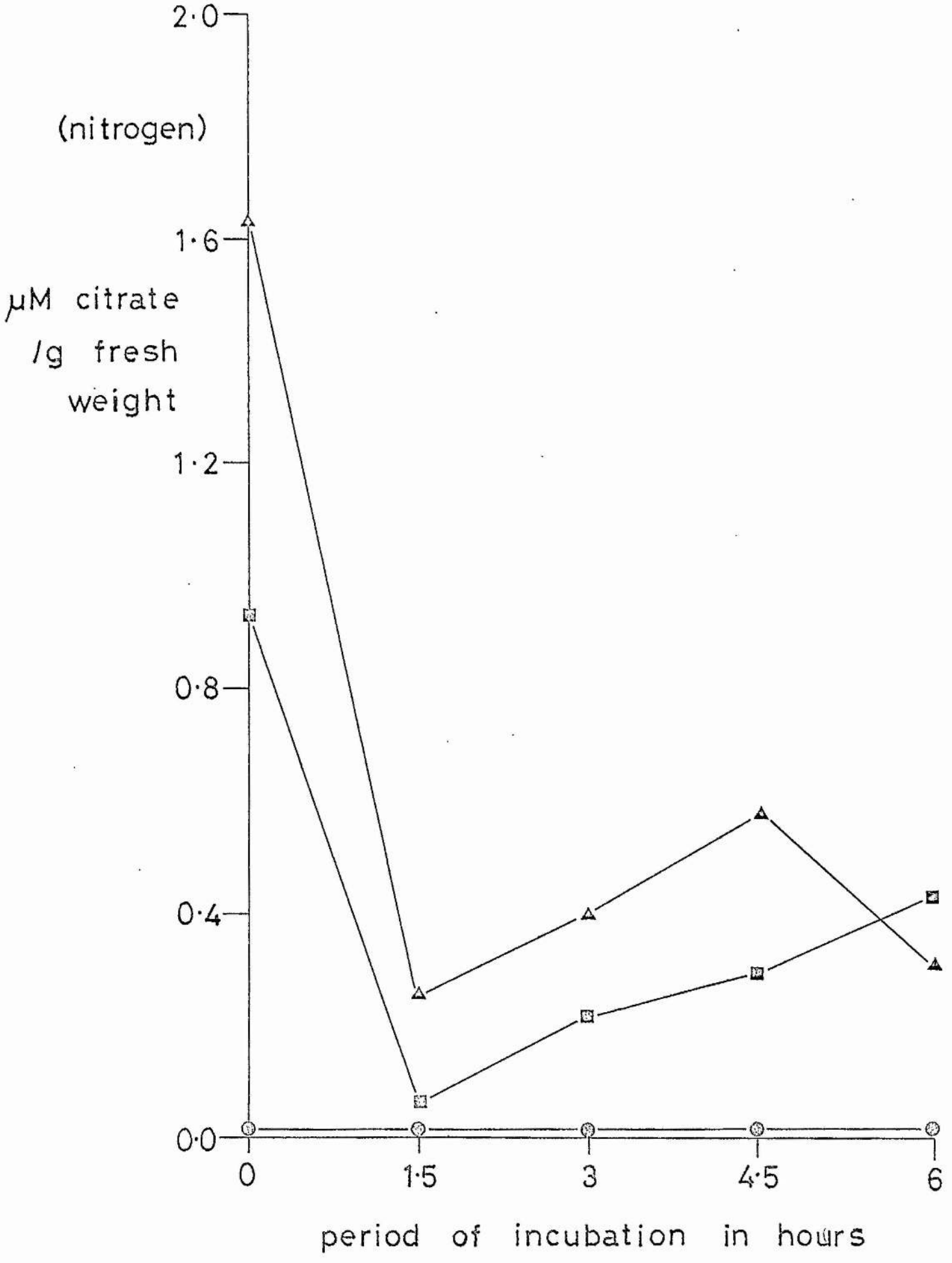

2·0
(nitrogen)
1·6
µM citrate
/g fresh
weight
1·2
0·8
0·4
0·0
0
1·5
3
4·5
6
period of incubation in hours

Fig. 24a and 24b. ROOT TISSUE SUCCINIC ACID CONTENT UPON INCUBATION UNDER AEROBIC (AERATED) AND ANAEROBIC (NITROGEN) CONDITIONS

Key: Fig 24a. Aerated

○ Senecio viscosus

△ Ranunculus flammula

□ S. aquaticus

Fig. 24b. Nitrogen

● S. viscosus

▲ R. flammula

■ S. aquaticus

Fig. 24 a

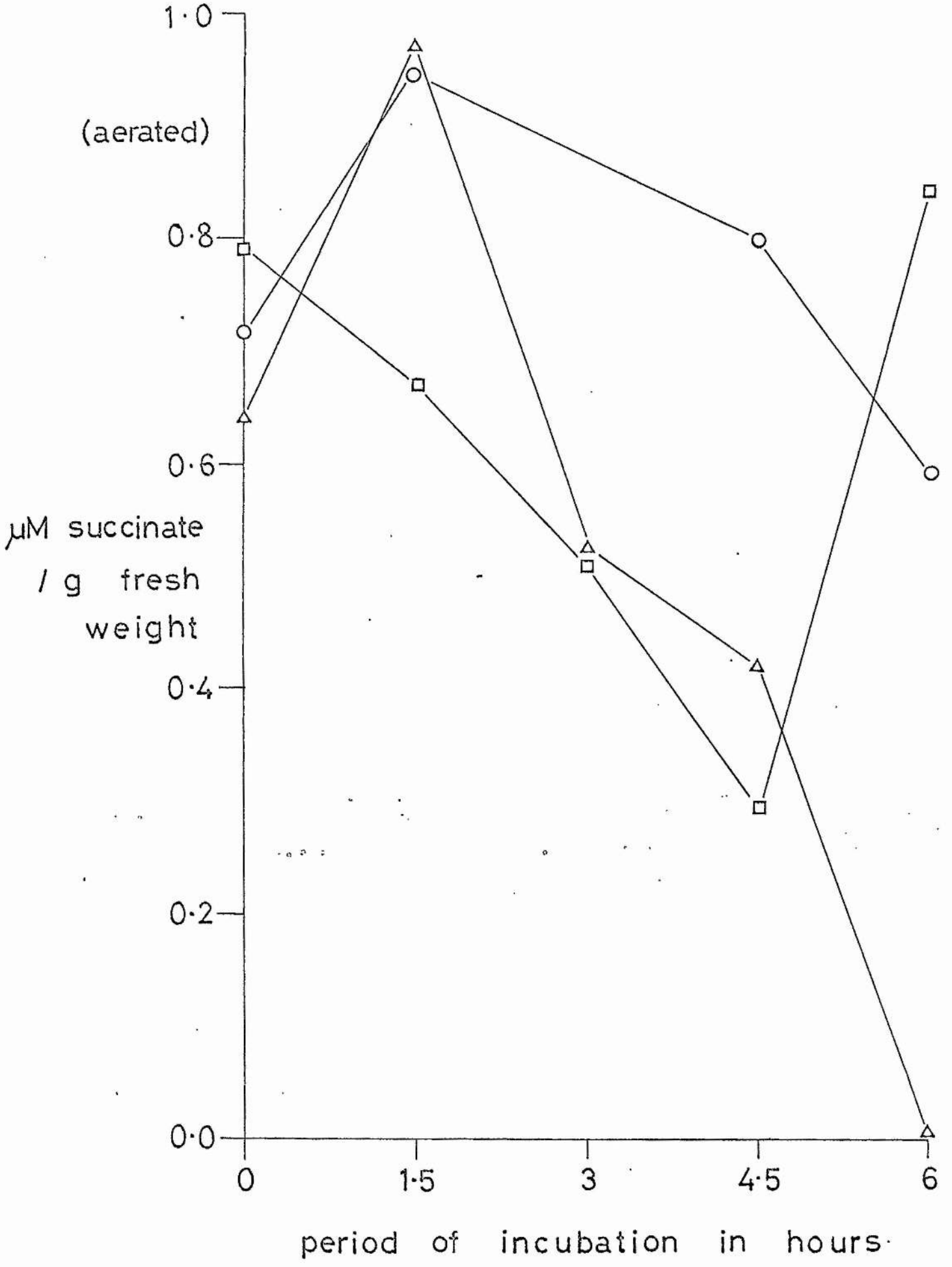

1·0
(aerated)
0·8
0·6
µM succinate / g fresh weight
0·4
0·2
0·0
0
1·5
3
4·5
6
period of incubation in hours

Fig. 24 b

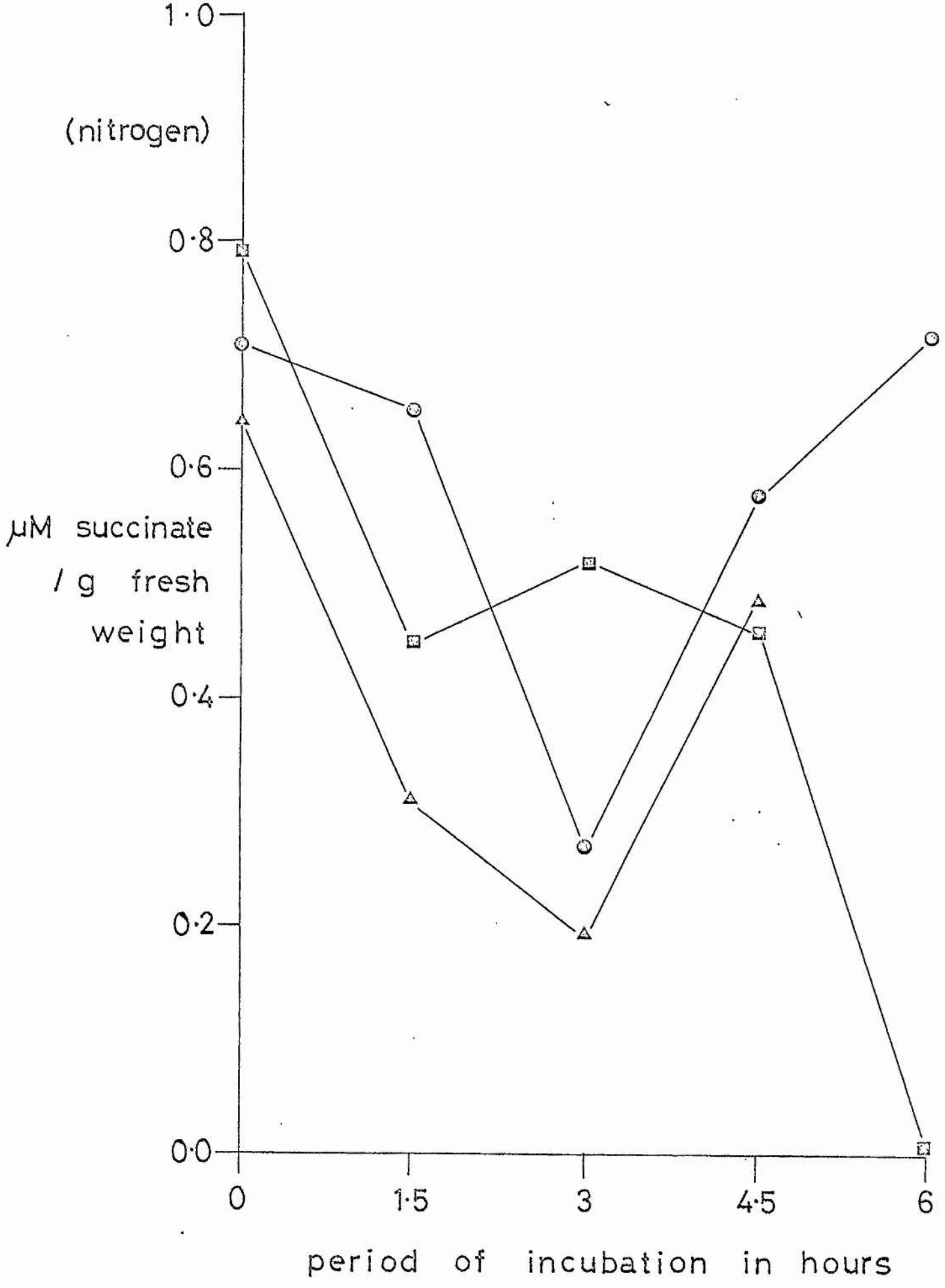

1·0
(nitrogen)
0·8
0·6
µM succinate
/ g fresh
weight
0·4
0·2
0·0
0
1·5
3
4·5
6
period of incubation in hours

Fig. 25a and 25b. CHANGES IN ROOT ORGANIC ACID CONTENT UPON AERATED- AND NITROGEN-INCUBATION OF HELOPHYTE AND NON-HELOPHYTE TISSUE

Key: ○ and ● - malic acid

△ and ▲ - lactic acid

□ and ■ - citric acid

X and X - succinic acid

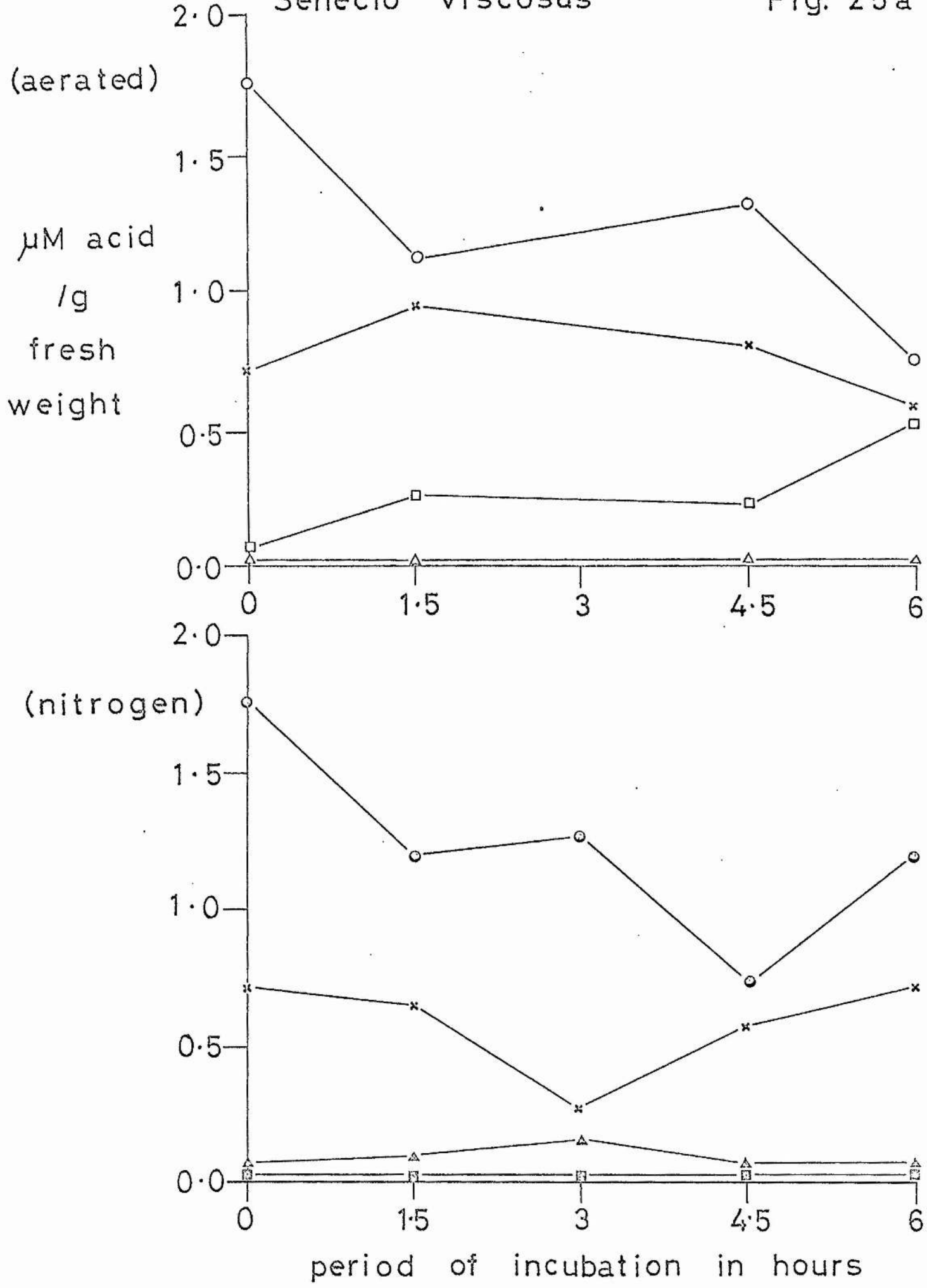
Fig. 25a. Senecio viscosus

25b. Ranunculus flammula

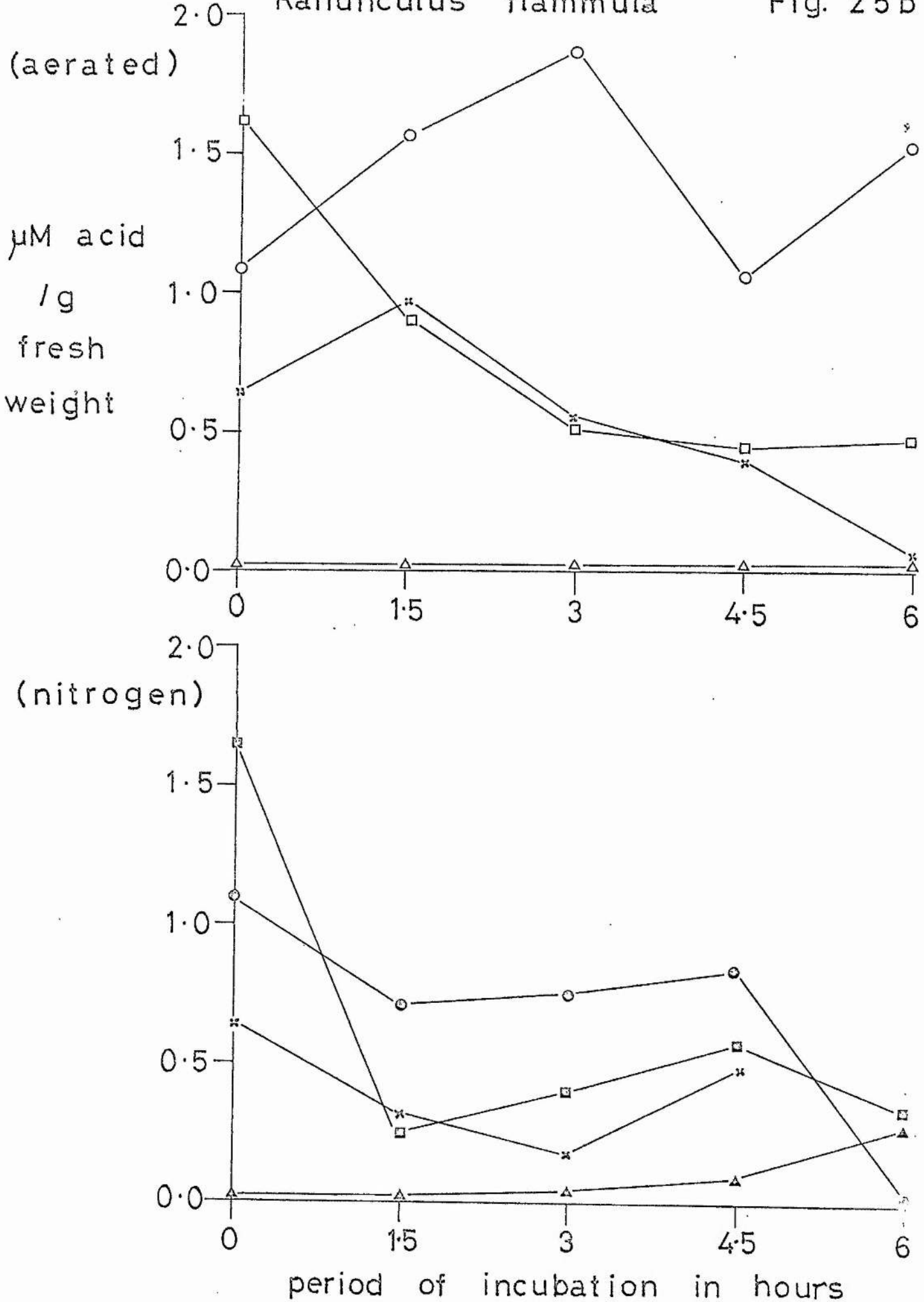
25c. S. aquaticus

Senecio viscosus

Fig. 25a



Ranunculus flammula Fig. 25b



Senecio aquaticus Fig. 25c

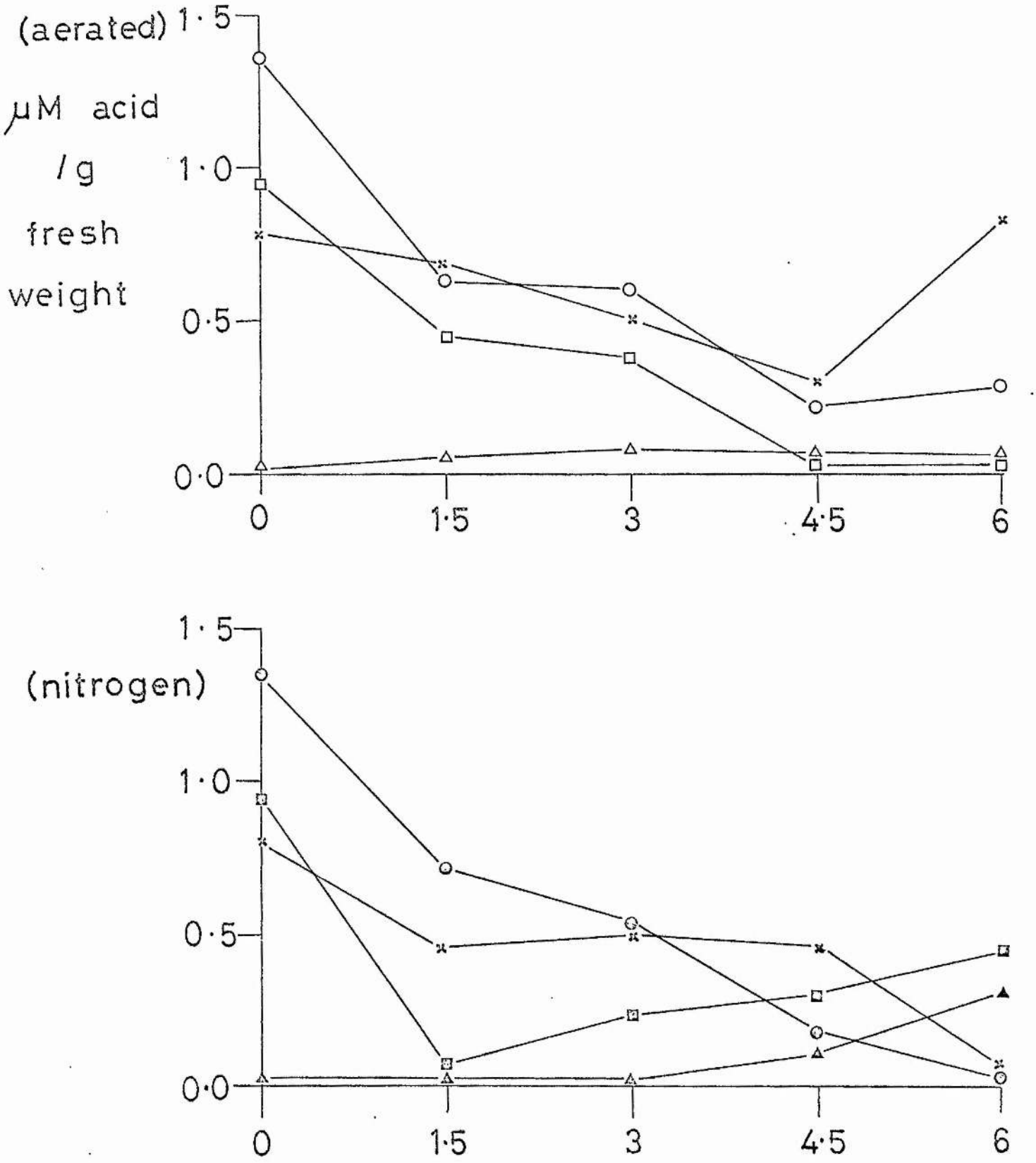
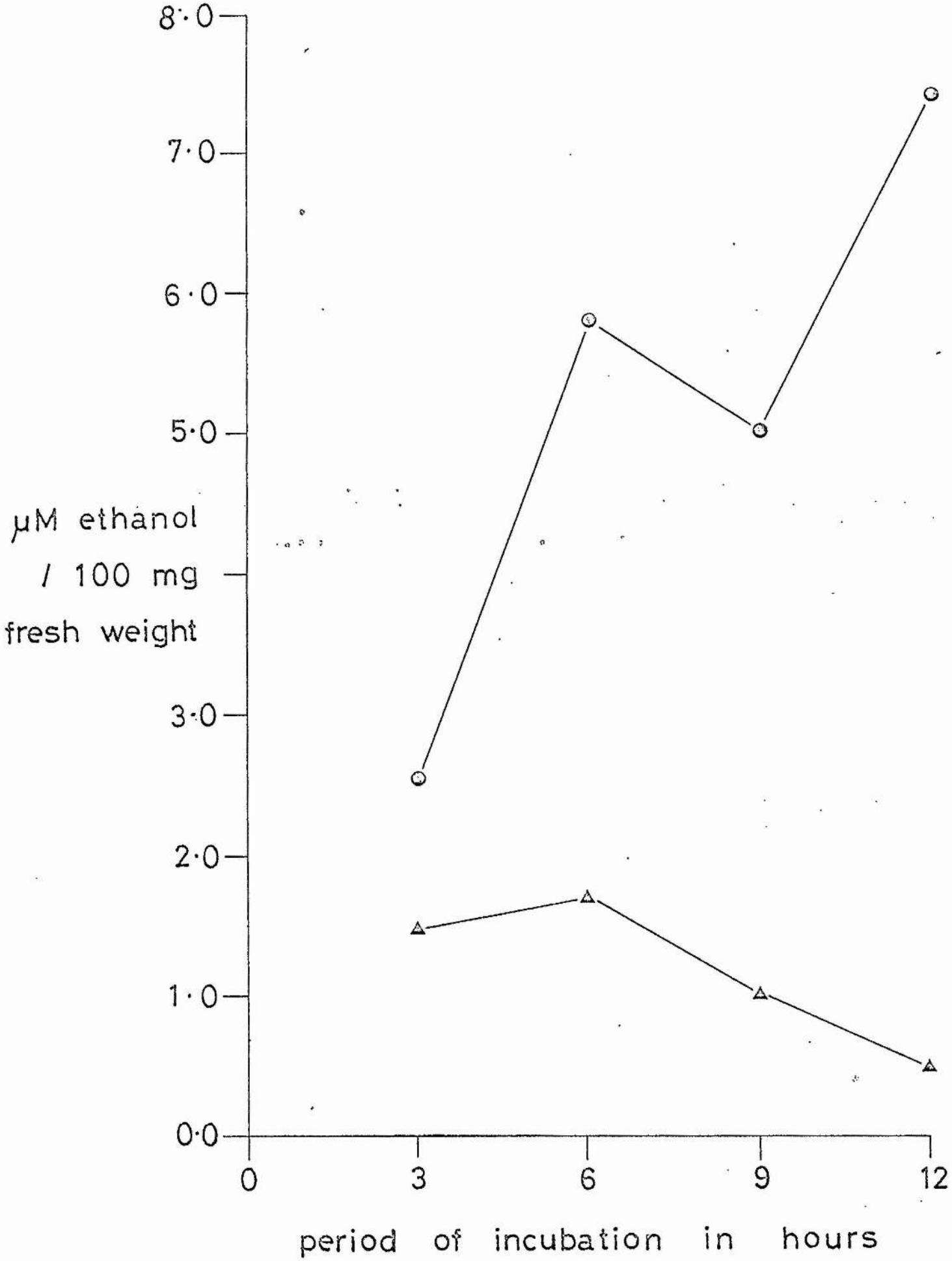


Fig. 26. ROOT TISSUE ETHANOL CONTENT UPON INCUBATION UNDER ANAEROBIC CONDITIONS AFTER GROWTH FOR 1 MONTH IN FLOODED CULTURE

Key: ● Senecio squalidus

▲ Senecio aquaticus

Fig. 26


8·0
7·0
6·0
5·0
µM ethanol
/ 100 mg
fresh weight
3·0
2·0
1·0
0·0
0
3
6
9
12
period of incubation in hours

DISCUSSION

INTRODUCTION

Periods of waterlogging, whether they be short-term or prolonged, greatly reduce the availability of oxygen to the roots of plants. The development of aerenchymatous tissue in the roots (Conway 1937, McPherson 1939, Sifton 1945), although commonly associated with many wet-land species, does not fully explain why certain species tolerate flooding and others do not (see Introduction p. 10-15). Metabolic adaptations, which need operate only during the period of reduced oxygen supply concomitant with waterlogging, have been suggested as a probable means of effecting flood tolerance, especially over a range of structurally different species.

Since 1957, when Mazelis and Vennesland first suggested that malic acid should be regarded as the natural end-product of anaerobic carbohydrate breakdown in plants, there have been several reports of various organic acids being produced during oxygen deficiency. Thus in addition to the malic acid postulated by Mazelis and Vennesland (1957), there has also been evidence of succinic acid accumulation during anaerobiosis in peas (Wager 1961), under high carbon dioxide concentrations in castor oil bean mitochondria (Bendall, Ranson & Walker 1960), and in apples suffering

from carbon dioxide poisoning (Hulme 1956). These succinate accumulations were usually associated with adversely affected tissue metabolism, whereas malate accumulations have been found during partial anoxia in undamaged tissues (Henshaw, Coult & Boulter 1962). The conflicting reports on which is the major acid to accumulate in plants upon anoxia have had one thing in common - little or no bearing on the ecology of the species examined. It is therefore of greater relevance to note that in the present work, the organic acid changes observed during experimental flooding have been related to a group of flood tolerant plants taken from naturally wet sites, and a group of flood intolerant plants taken from dry sites. Thus the uniform organic acid changes in the helophyte group can be shown to have a direct bearing upon the success of these species in waterlogged areas, and the lack of such a flood response in the non-helophytes to attribute to their lack of flooding survival properties. There is also some agreement in the literature when the various acid accumulations upon anoxia are re-examined in the light of the ecology of the species concerned.

MALIC ACID CHANGES

The most striking organic acid change during the four-day period of experimental flooding was the rise in malic acid in

all the helophytes and the drop in all the non-helophytes (Fig. 3). This finding has some agreement in the literature, for the production of malic acid in the helophyte Iris pseudacorus in the presence of CO_2 and other respiratory inhibitors has been reported by Bown, Boulter and Coult (1968), and malate decrease under anaerobic conditions has been found in buckwheat seedlings (Effer & Ranson 1967) and in maturing seeds of Pisum sativum (Wager 1961). When investigated under natural conditions during the spring period as the dune slack habitat is reverting from a flooded winter state to a non-flooded summer one, a malate decrease was demonstrated in Filipendula ulmaria, Erica tetralix and Glyceria maxima (Fig. 16). The experimental finding of malic acid accumulation in helophytes during periods of flooding therefore has some field confirmation. Other work has indicated a seasonal variation in the malate and shikimate content of Iris rhizome under natural conditions (Table 6).

Table 6. Seasonal variation in malic and shikimic acid content of Iris rhizome, in mg / 100 g fresh weight. (From Henshaw, Coult and Boulter 1962)

Date	malic acid	shikimic acid
17th February	163	739
8th March	225	878
1st April	137	610
10th June	67	545

However, when aquatic, rather than marsh, plants were investigated, the malic acid content of the root and rhizome tissues of Iris pseudacorus did not relate particularly to periods of flooding in the field (Fig. 15). It may be that the organic acid metabolism of I. pseudacorus, together with that of the other rhizomatous species studied, Nuphar lutea, is of greatest importance in carbohydrate storage, and that under the conditions of an aquatic environment malic acid assumes a greater importance as a carbon energy store. It is possible, however, that although the root and rhizome tissues of N. lutea are permanently submerged by water, the seasonal fluctuations in concentration of dissolved oxygen in the loch water could affect the degree of anaerobiosis in these tissues. It is tempting to suggest that the low malate levels in the root and rhizome tissue between December and March (Fig. 14), when the oxygen concentration of the water would be at its highest, and the malate peak in June, when the concentration of dissolved oxygen would be lower, indicate such an effect. Further tissue sampling, with concurrent measurements of the oxygen concentration of the water, might confirm this influence on the malate levels in these submerged roots and rhizomes.

There were considerable changes in malic acid levels upon incubation of root material under aerated and completely anaerobic conditions (Fig. 21). In aerated culture the malate content of Senecio viscosus and S. aquaticus fell sharply whereas that of

Ranunculus flammula rose slightly. Similar reductions in malate content during constant aeration have been observed in the roots of pumpkin, tomato and willow, Salix cinerea (Dubinina 1961). The same author suggested that this drop in malic acid indicated an increased utilisation of organic acids in the respiratory cycle, providing for protein synthesis and good root growth. A rise in malate accompanying aerated culture has been demonstrated in maturing seeds of Pisum sativum upon their return to air after a 24-hour period of anaerobiosis (Wager 1961).

During anaerobic incubation the malic acid content of all three species fell. This malate decrease has similarly been found in buckwheat seedlings under anoxia (Effer & Ranson 1967) and in anaerobically treated pea seeds (Wager 1961); although in the roots of pumpkin, tomato and Salix cinerea, an oxygen deficiency was accompanied by a rise in malic acid content (Dubinina 1961). These latter findings were not, however, from tissues kept in completely anaerobic conditions.

Malic acid has often been described in the literature as being of common occurrence in various plant tissues, and has not always been particularly linked with changes induced by partial anoxia. Thus an early review (Bennet-Clarke 1933) lists 'malic acid plants', such as members of the genera Ranunculus, Berberis, Rhamnus and Fraxinus, as containing chiefly malic acid. Plants

with the Crassulacean type of acid metabolism, in which malic acid is formed from carbohydrate in the dark and reconverted in the light, also show a high malate content (Ranson & Thomas 1960). Malic acid constitutes 30-40% of the total organic acid content of the cotton plant (Ergle & Eaton 1949), is predominant in the roots of Vicia faba (Schramm & Piatkowska 1961), in the fruits of lemon (Sinclair & Eny 1945) and in the leaves of all 27 species of Leguminosae examined by Bentley (1952). Table 7 shows the leaf malate levels recorded by other investigators.

Table 7. Malic and citric acid content of leaf tissues.

Plant	Malate	Citrate		
Pea	56.1	86.0	m equiv. / 100 g dry weight	(Pierce &
Spinach	6.5	3.7	" "	Appleman
Beet	21.2	20.6	" "	1943)
Wheat	1.7	0.6	µ mole / g fresh weight	(MacLennan, Beevers & Harley 1963)
Rhubarb	3.5	-	% dry weight	(Pucher, Clarke & Vickery 1937a)

Root tissues of maize, carrot and beet contain large amounts of malic acid relative to the other organic acids (Table 8), and malate has been recorded in the rhizomes of rhubarb (Pucher, Clarke & Vickery 1937a) and in those of Iris pseudacorus (Henshaw, Coult & Boulter 1962).

Table 8. Malic and citric acid content of root tissues. (From MacLennan, Beevers & Harley 1963)

Plant	Malate	Citrate			
Maize (1st 1 cm)	7.5	1.5	µ mole / g	fresh weight	
Carrot	15.9	1.2	"	"	
Beet	1.1	1.8	"	"	
I. pseudacorus	0.11	0.03	"	"	(own data)
N. lutea	0.18	-	"	"	(own data)

CITRIC ACID CHANGES

The fluctuations in citric acid content of the roots of flooded helophytes and non-helophytes do not fit into any distinguishable pattern (Fig. 7). It could have been expected that changes in citrate would have been similar to those of malate, for previous workers have found changes in the two acids to be generally linked (Dubinina 1961, Wager 1961, Bourne & Ranson 1965). If that were to be the case, then the fall in citrate upon flooding in Senecio aquaticus and Carex lasiocarpa is atypical, and the rise in citrate shown by the other helophytes is the more expected behaviour. The changes in citrate levels accompanying flooding in the non-helophytes are so different from one species to another that it is impossible to draw any conclusions. While some non-helophyte

species show an initial citrate decrease, as also found in buckwheat seedlings in anoxia (Effer & Ranson 1967), so others exhibit an initial citrate increase.

Monthly samples of Iris pseudacorus root and rhizome tissue contained citric acid in varying amounts. Although the levels were less than those of malic and shikimic acid in this field material, citrate was the only other organic acid to be present in detectable quantities. The citric acid content of the rhizome was generally greater than that of the root, and showed well-marked summer low and winter high levels. Such an annual fluctuation could be indicative of a link between malic and citric acid, for malic acid accumulation has already been observed in the naturally flooded roots and rhizomes of some helophyte species.

Under aerated incubation citric acid shows a drop in level similar to that of malic acid, which, it has already been suggested, could indicate an increased utilisation of organic acids in the respiratory cycle. The two helophytes exhibit this citrate drop, whereas in the non-helophyte Senecio viscosus there is a small citrate rise during the 6 hours of aerated incubation.

Citric acid changes in the helophytes during incubation in nitrogen were also similar to those of malate. Both species showed considerable decrease over the 6 hour period, and this simultan-

eous decrease in citric and malic acid content is probably further evidence that the tricarboxylic acid cycle in these species is in fact retarded by completely anaerobic conditions.

A certain degree of similarity between citrate and malate levels in the experimental material was to be expected, since citric acid is very commonly found in plant tissues and may occur in quantities at least equal to, if not greater than, malic acid. Citric acid comprises 30-40% of the acidity in the roots of the grapevine, although comprising less than 2% of that in all other parts (Kliewer 1966). In Vicia faba citrate is the only organic acid in the flowers and is the major one in the dry and soaked seeds (Schramm & Piatkowska 1961).

Fleshy fruits may be as rich in citric acid as they are in malic acid (Bennet-Clarke 1933), and in the fruits of Citrus spp. concentrations of citrate as great as ten times that of malate have been recorded (Sinclair & Ramsey 1944, Sinclair, Bartholomew & Ramsey 1945). The citric acid content of apple fruit pulp, however, is much lower than that of malic acid (Hulme & Wooltorton 1958).

Citric acid was as widespread and as abundant as malic acid in the leaves of the Leguminosae species investigated by Bentley (1952), although in general smaller concentrations have been ob-

served by other workers (Table 7). Similarly, the citrate content of root tissues was found to be lower than that of malate in maize and carrot, and only slightly higher in beet (Table 8).

LACTIC ACID CHANGES

The four-day period of flood treatment was accompanied by a rise, albeit a small one, in the lactic acid levels of all the non-helophytes (Fig. 6). Similar treatment to the helophytes resulted in slight decreases in lactate levels, with the exception of Juncus effusus. If, as Effer and Ranson (1967) have suggested, the activity of the tricarboxylic acid cycle is retarded during anoxia, then the accumulation of lactate in the non-helophytes could be indicative of this effect. There might also be concurrent accumulation of pyruvate and ethanol. In the helophytes, however, the absence of any lactate rise would indicate that in those particular flood tolerant species the tricarboxylic acid cycle was not being retarded during the period of flooding. There is evidence of a large accumulation of lactic acid in pea seeds during a 24-hour period of anaerobiosis (Wager 1961).

The lactate changes in the root tissue incubated under nitrogen in some respects support the above findings. During the first three hours there was a rapid rise in the lactic acid con-

tent of the non-helophyte Senecio viscosus (Fig. 22), but in the helophytes only a very small rise in Ranunculus flammula and no change at all in S. aquaticus. During a further three hours anaerobiosis, however, the lactate in the helophytes rose more sharply. It is probable that the helophyte species, although able to tolerate the near anaerobic conditions which are set up during the four-day flooding period in sand culture, cannot tolerate the completely anaerobic conditions during incubation under nitrogen. In this respect it is important to note that completely anaerobic conditions would never arise in the field, as there would always be some oxygen diffusion down the shoots to the waterlogged roots. Thus the helophyte root tissues in nitrogen are being subjected to oxygen deficiencies greater than those normally encountered and to which they have some tolerance, and their metabolic behaviour in these conditions can be likened to that of a non-helophyte. Consequently the tricarboxylic acid cycle may be retarded, with the accompanying rise in lactic acid as shown between 3 and 6 hours incubation. In detached rhododendron leaves a rise in lactic acid of a very similar magnitude and time course to the above experiments has been demonstrated during storage in nitrogen (Table 9).

If the above hypothesis is correct, that helophytes are tolerant to partial, but not complete, anoxia, then it would explain why there were no differences in the malate changes between helo-

phyte and non-helophyte tissue during incubation under nitrogen. In these conditions, the normally operating metabolic adaptations of the helophytes to reduced oxygen supply can no longer continue, and their behaviour in essence becomes that of a non-helophyte. Since lack of oxygen retards the tricarboxylic acid cycle in non-helophytes, with a consequent drop in malate level, then a similar fall in malate is inevitable in the helophytes whenever the conditions become completely anaerobic. Fig. 22b shows that there was a decrease in malate of this nature when root tissues of Senecio aquaticus and Ranunculus flammula were incubated under nitrogen.

Table 9. Organic acid contents (μ mole / g fresh weight) of detached rhododendron leaves stored under nitrogen in darkness. (From Bourne & Ranson 1965)

Time	lactic acid	succinic acid	malic acid	citric acid
Start	0.04	0.24	2.49	0.82
1.5 hours	0.09	0.64	1.99	1.28
3 "	0.09	0.74	2.14	1.02
6 "	0.25	0.78	2.35	1.22
12 "	0.28	1.01	2.05	0.86

While the changes in lactate levels in flooded and incubated root material are therefore of importance, the relatively small

magnitude of these levels suggests that lactate does not figure predominantly in the organic acid metabolism. Evidence from other investigations shows that lactic acid is not as widespread in plant tissues as it is, for example, in animal tissues following anaerobic respiration. The normally accepted end-product of anaerobic respiration in plants is ethanol. However, lactic acid has been recorded and measured in potato discs during a period of anaerobiosis, before the build-up of ethanol (Barker & el Saifi 1953) and was formed during anaerobic respiration in the rhizome of Equisetum limosum (Barber 1957). In germinating peas the level of lactic acid was found to fluctuate as ethanol was formed and metabolised (Cossins 1964). The presence of lactic acid in grapevines has also been shown (Kliewer 1966). In general, though, there are no accumulations of lactic acid in plant tissues, and whenever it is formed it tends to be as a result of anaerobic conditions in species very adversely affected by anoxia.

SUCCINIC ACID CHANGES

With the exception of Juncus effusus there were no rises in the succinic acid contents of the roots of helophytes after four days flooding. In all the flooded non-helophytes, however, there were steady, if slight, rises in succinate throughout the flood

period (Fig. 8). Previous workers have usually found evidence of succinate increases in tissues subjected to various levels of oxygen deficiency, and the general lack of any such increase in the flooded helophytes could indicate that these species are avoiding whatever metabolic change is causing the succinate accumulation in other species. Succinic acid has thus been found to accumulate in carrot root and oat coleoptile at 20% CO_2 in air, but not in air itself (Table 10), and very similar results were obtained using the pulp of apples (Table 11).

Table 10. Occurrence of succinic acid in plant tissues after storage in gas mixtures. + indicates high (relative) concentration, - indicates low concentration. (From Ranson 1953)

Plant organ/ storage time	air	2-10% CO_2 in air	20-90% CO_2 in air	Pure N_2; CO_2 or 1% O_2 in N_2
Young carrot root (3 days)	-	-	+	+
Older carrot root (3 days)	-	-	+	+
Oat coleoptile	-	-	+	+

An explanation of this succinate accumulation in anoxia has been suggested from acid changes in Ricinus mitochondrial preparations. Whereas in air (0.05% CO_2) there is rapid conversion of citrate into succinate, fumarate and malate, in CO_2 concent-

rations above 20% the conversion to fumarate and malate is reduced and succinate accumulated (Ranson, Walker & Clarke 1957). This accumulation of succinic acid was shown to result from the inhibition of succinic dehydrogenase at that 20% CO_2 level (Bendall, Ranson & Walker 1958). More recent work has further shown that in the absence of oxygen it is the flavin component of succinic dehydrogenase which cannot be regenerated, so that the oxidation of succinic acid itself becomes progressively retarded and succinate accumulates (Effer & Ranson 1967).

Table 11. The quinic, succinic and malic acid content of pulp of apples stored at 37°C under various CO_2 concentrations, expressed as mg / 100 g fresh weight. (From Hulme 1956).

Treatment	Days	Quinic	Succinic	Malic	Condition of tissue
Air	31	84.0	-	1,222	no damage
10% CO_2	31	87.5	6.4	1,229	no damage
20% CO_2	11	89.5	21.0	1,225	CO_2 damage

During the incubation of root tissues under completely anaerobic conditions there were succinate decreases in the two helophytes, Ranunculus flammula and Senecio aquaticus, and a slight overall rise in the non-helophyte S. viscosus. Storage of detached rhododendron leaves under similar conditions of complete anaero-

biosis was also accompanied by succinate accumulation (Table 9).

The succinate changes which have been followed in the experimental material have not been of large magnitude, relative to the changes in, say, malate. This is not unexpected, since although other workers have found succinic acid in several plant tissues, the quantities have generally been small. In Phaseolus coccineus succinic acid occurs in the stem tissues, but in concentrations considerably lower than either malic or citric acid (Bentley 1952). Succinate has been detected at low levels (0.2 μ mole / g fresh weight) in maize root and wheat leaf, but not at all in carrot root or beet root (MacLennan, Beevers & Harley 1963). In Vicia faba it was detected in all tissues except the flowers (Schramm & Piatkowska 1961), and one of the few examples of succinate accumulation was found to accompany carbon dioxide poisoning in apples (Hulme 1956).

SHIKIMIC ACID CHANGES

Shikimic acid was not detected in either helophytes or non-helophytes upon experimental flooding, nor upon incubation of the root tissues in aerated and anaerobic conditions. However, when shikimic acid was found, in the roots and rhizomes of Iris pseudacorus and Nuphar lutea, growing under natural conditions,

it was usually the major acid present in those tissues. The levels of shikimate altered during the year, and these changes have already been shown in Figs. 18 and 19.

The shikimic acid content of the rhizome of I. pseudacorus, which was always greater than that of the root, showed a steady decrease from March to June. An identical change in levels was found in this tissue by Henshaw, Coult and Boulter (1962) (see Table 6), who also found related changes in quinic acid during the same period (see p. 66 for role of quinic acid as shikimic acid precursor). The shikimic acid content of N. lutea rhizome, which fluctuated within the range of the root shikimate content, followed a similar downward course from February to May. The levels of shikimic acid in the roots of I. pseudacorus, although always less than 0.6 μ mole / g fresh weight, also showed a decrease from March to May.

The high levels of shikimic acid in the submerged tissues of these large rhizomatous species, and the predominance of this acid over all others, suggests that the shikimate plays an important role in an organic acid metabolism which is peculiar to plants such as Nuphar and Iris. Shikimic acid could function as a carbohydrate reserve in these tissues, with the high levels of the acid being accounted for by the large amounts of rhizome storage tissues which adapt these plants to their predominantly wet environ-

ment. The utilisation of this carbohydrate reserve would be reflected in the shikimate fluctuations observed *in situ* throughout the year.

Under experimentally induced anaerobic conditions shikimic acid did not accumulate in *Iris* rhizome (Boulter, Coult & Henshaw 1963), so that if shikimate is being produced anaerobically it must be utilised in further biosynthesis. The bulk of experimental evidence shows that shikimic acid is an aromatic precursor, being formed as an intermediate between the carbohydrates formed by photosynthesis and aromatic ring compounds (Brown & Neish 1955, Eberhardt & Schubert 1956, Hasegawa, Higuchi & Ishikawa 1960, Isherwood 1963). The aromatic compounds are utilised in the formation of a wide variety of secondary growth substances, including lignin (Davies 1959, Neish 1960). It has also been shown that in roses quinic acid can be converted to shikimic acid (Weinstein, Porter & Laurencot 1959), thus possibly explaining the related changes in quinic and shikimic acids found in *Iris* rhizome by Henshaw, Coult and Boulter (1962).

The exact role of shikimic acid in plant organic acid metabolism has therefore yet to be clarified. Although at one time shikimate was thought to be a rarely occurring plant acid, it has recently been identified in a large range of plant tissues (Hattori, Yoshida & Hasegawa 1954, Yoshida & Hasegawa 1957) and is now more

widely recognised. It has also been detected in grasses (Richardson & Hulme 1955), in the Gymnospermae and some primitive families of the Angiospermae, and in some mosses and ferns where often considerable quantities are present (Kinzel & Walland 1966).

CHANGES IN OTHER ORGANIC ACIDS

The paper chromatograms of root extracts from experimentally flooded material, and of root and rhizome extracts taken from field material, did not detect any organic acids other than those already measured by specific enzymic analysis. The enzymic analysis of some extracts for oxaloacetic acid also had negative results, which agrees with other evidence that oxaloacetate does not accumulate in plant tissues (Davies, Giovanelli & Rees 1964). It was therefore concluded that malate, citrate, lactate, succinate and shikimate were the only important accumulating acids in the metabolism of the tissues studied, although several other cyclic acids, such as aconitic, isocitric and fumaric, and extra-cyclic acids, such as oxalic, tartaric and malonic, are to be found in plant tissues (Ranson 1963). Most of these acids occur in small quantities, although there are examples where one or other may accumulate. For instance, tartaric acid is prevalent to the same extent as malic acid in the grapevine (Kliewer 1966), oxalate is the major acid in Begonia seedlings (Crombie 1954) and formate

accumulates in the stinging nettle, Urtica dioica (Davies 1959).

OPERATION OF THE TRICARBOXYLIC ACID CYCLE

Theo rganic acid changes upon experimental and natural flooding and aerobic and anaerobic incubation which have been described above require explanation in terms of the operation of possible involved metabolic pathways. Citric, succinic and malic acids are all members of the tricarboxylic acid (TCA) cycle, and the changes in levels of these acids may be examined in relation to the operation of this acid.

Evidence for the operation of an active Krebs or TCA cycle in plants, which was at first restricted to the particular tissues studied, such as pea seedlings (Davies 1953), potato tubers (Barker & Mapson 1955), green shelled peas (Turner & Quartley 1956, Quartley & Turner 1957) and root tissues of the swede, Brassica napus (Truelove 1962), has now become sufficient for the wide acceptance that the TCA cycle is functional in higher plants (Davies 1959, Ranson 1963, Davies, Giovanelli & Rees 1964).

It is possible for the lack of oxygen which accompanies flooding to retard the TCA cycle, through gradual inhibition of the oxidative steps involving nicotinamide-adenosine dinucleotide (NAD),

or nicotinamide-adenosine dinucleotide phosphate (NADP) co-enzymes. While these oxidative steps may continue for a short period in anoxia by coupling with reductive reactions in the cells (Effer & Ranson 1967), nevertheless continued lack of oxygen will gradually increase their inhibition. It is probable, though, that if there is no oxidative regeneration of the flavin component of succinic dehydrogenase during anoxia, that this flavo-protein co-enzyme will be more rapidly and completely inhibited than NAD or NADP. This latter effect will result in a build-up of succinate (1), a block in the TCA cycle at that point, and a probable decrease in fumarate and malate levels while these two acids are metabolised further (2), (3). In the flooded non-helophytes the

(1) $COOH.CH_2.CH_2.COOH \xrightarrow[\text{SDH}]{\text{oxidised flavoprotein} \rightarrow \text{reduced flavoprotein}} COOH.CH.CH.COOH + H_2$

succinate — fumarate

(SDH - succinic dehydrogenase)

(2) $COOH.CH.CH.COOH + H_2O \xleftrightarrow[\text{fumarase}]{} COOH.CHOH.CH_2.COOH$

fumarate — malate

(3) $COOH.CHOH.CH_2.COOH \xleftrightarrow[\text{MDH}]{\text{NAD} \rightarrow \text{NADH}_2} COOH.C{=}O.CH_2.COOH$

malate — oxaloacetate

(MDH - malic dehydrogenase)

acid changes fitted this hypothesis, for while there was an immediate increase in succinate upon flooding, there was an equally rapid decrease in malate.

Experimental flooding, and the lack of oxygen that accompanies it, did not have the same effect on the helophytes. There was generally no succinate increase in these species, and rather than a decrease in malic acid content there was a rapid and pronounced rise in malate. If lack of oxygen was having a retarding effect on the reactions of the TCA cycle, it would presumably be manifested first in a succinate build-up following succinic dehydrogenase inhibition, as in the non-helophytes.

Malate could accumulate at the expense of another acid already in the cells, or at the expense of a reserve food whose catabolism gave rise to one of the TCA cycle intermediates (Ranson 1963). Thus it is conceivable that rapid protein catabolism could yield quantities of -ketoglutarate sufficient for transformation within the TCA cycle and subsequent accumulation as malic acid (4), (5).

(4) $COOH.CH_2.CH_2.CO.COOH \xrightarrow[KDH]{NAD \quad NADH_2 \quad CO_2} COOH.CH_2.CH_2.CO.CoA$

-ketoglutarate — succinyl CoA

(KDH - ketoglutarate dehydrogenase)

(5) $COOH.CH_2.CH_2.CO.CoA \xrightarrow{ADP+P \quad ATP} COOH.CH_2.CH_2.COOH$

succinyl CoA — succinate

Thereafter: succinate ⟶ fumarate ⟶ malate

The loss of TCA cycle intermediates during anaerobiosis may also be through leakage of the acids from the roots as a result of cell membrane injury (Hiatt & Lowe 1967). On the other hand, Grineva (1961) concluded that excretion of these intermediates was an active process.

METABOLIC PATHWAYS INVOLVING MALIC ACID

Malic acid levels can be affected by reactions involving carbon dioxide fixation in plant tissues. There are three mechanisms of carbon dioxide fixation in plants. The 'malic enzyme' catalyses the decarboxylation of oxaloacetate at acid pH (6).

(6) $COOH.CO.CH_2.COOH \xrightleftharpoons{Mn^{++}} COOH.CO.CH_3 + CO_2$

oxaloacetate — pyruvate

Oxaloacetate and malate are interconvertible in the presence of malic dehydrogenase (3), so that the net reaction catalysed

by the 'malic enzyme' is: (7) (Vennesland & Conn 1952).

(7) $CH_3.CO.COOH + CO_2 + NADPH_2 \xrightleftharpoons{Mn^{++}} COOH.CH_2.CHOH.COOH + NADP$

pyruvate — malate

The above reaction is fully reversible, and the extent of CO_2 fixation is determined by the ratio NADP / $NADPH_2$.

Oxaloacetate can also be formed by carbon dioxide fixation to phospho-enolpyruvate (PEP), catalysed by pepcarboxylase (8).

(8) $CH_2.CO.PO_3.H_2COOH + H_2O + CO_2 \xrightleftharpoons{Mg^{++}} COOH.CH_2.CO.COOH + H_3PO_4$

PEP — oxaloacetate

The equilibrium of the above reaction is strongly in favour of carboxylation.

Fixation of carbon dioxide to PEP can also be catalysed by pepcarboxykinase (9).

(9) $CH_2.CO.PO_3.H_2.COOH + ADP + CO_2 \xrightleftharpoons{Mg^{++}} COOH.CH_2.CO.COOH + ATP$

PEP — oxaloacetate

In the pepcarboxykinase reaction, photosynthetic utilisation of carbon dioxide would lead to malate disappearance and synthesis

of carbohydrate from organic acids. On the other hand, increase in carbon dioxide would lead to malate formation.

The widespread occurrence of the 'malic enzyme' , pepcarboxykinase and pepcarboxylase in plant tissues (Davies, Giovanelli & Rees 1964), and the readily available interconversion of oxaloacetate and malate by malic dehydrogenase, suggest that carbon dioxide fixation could have a considerable influence on malate levels in plant tissues. The metabolic switch which leads to malate accumulation in helophytes upon experimental flooding may involve some of the reactions outlined above. For instance, the increased carbon dioxide levels which accompany oxygen reductions in waterlogged soils could lead to oxaloacetate (and hence malate) formation by the pepcarboxykinase reaction described above (9). Upon the return to non-flooded, aerated conditions, the photosynthetic utilisation of carbon dioxide would shift the equilibrium of the pepcarboxykinase reaction, and the accumulated malate would be metabolised to carbohydrate. Upon experimental flooding in the non-helophytes there is no switch to malate accumulation, but increases in the activities of alcohol and malic dehydrogenases (Crawford & McManmon 1968). It appears that the acetaldehyde produced from the anaerobic breakdown of pyruvate (10) is being metabolised to ethanol (11). There is evidence that flood intolerant species are stimulated to greater alcohol dehydrogenase activity by acetaldehyde, but that this effect is not so marked

in flood tolerant species (Crawford & McManmon 1968, Crawford 1969).

(10) $CH_3.CO.COOH \xrightarrow{\text{carboxylase}} CH_3.CHO + CO_2$

pyruvate — acetaldehyde

(11) $CH_3.CHO + NADH_2 \xrightarrow{ADH} CH_3.CH_2OH + NAD$

acetaldehyde — ethanol

(ADH - alcohol dehydrogenase)

It is therefore suggested that, whereas intolerant species cannot adapt to periods of experimental flooding and produce toxic quantities of ethanol, flood tolerant species can switch their metabolism to accomodate an accumulation of non-toxic malic acid for the duration of the anaerobic period.

METABOLIC PATHWAYS INVOLVING SUCCINIC ACID

The rise in succinic acid in experimentally flooded non-helophytes has already been discussed in terms of the inhibition of the flavin component of succinic dehydrogenase by an oxygen deficiency, and has been reported by Crawford and Tyler (1969). It is therefore probable that the aerobic respiratory steps of the TCA cycle are retarded upon flooding in non-helophytes, and that evidence of this effect is a build-up of succinate and a decrease

of malate, which is further in the cycle than succinate. This blockage of the TCA cycle was found in all the experimentally flooded non-helophytes, and represents one of the adverse effects of flooding in these species.

METABOLIC PATHWAYS INVOLVING LACTIC ACID

Although lactic acid is not generally found in higher plants, it may be detected in the tissues during periods of anaerobiosis (Kenefick 1962). With blockage of the oxidative steps of the TCA cycle upon anoxia, pyruvate may go straight to lactic acid formation (12).

(12) $CH_3.CO.COOH + NADH_2 \xrightarrow{LDH} CH_2.CHOH.COOH + NAD$

pyruvate — lactate

(LDH - lactic dehydrogenase)

In the present work there was evidence of the anaerobic formation of lactic acid in experimentally flooded non-helophytes and in root tissues under nitrogen incubation. The quantities of lactate so formed were generally small, and other experimental evidence (McManmon 1969) reports only small activities of lactic dehydrogenase in flooded root tissues.

PATHWAYS INVOLVING ETHANOL

The anaerobic utilisation of pyruvate may go via acetaldehyde (10) to ethanol (11) (Ranson 1953). Thomas (1925) found ethanol and acetaldehyde accumulating in apple cells in the absence of oxygen, and tomato fruits under anaerobiosis also developed increased quantities of acetaldehyde and ethanol (Gustafson 1934). There are therefore several instances of ethanol formation in plant tissues during periods of anaerobic respiration, and in the present work there was a rapid increase in ethanol content in the excised roots of Senecio squalidus incubated under nitrogen (Fig. 26). Similarly, ethanol was formed in barley roots in nitrogen (Nance 1949) and Crawford (1967b) demonstrated ethanol production under nitrogen in several species which had been found intolerant of experimental flooding. The ethanol production in flood tolerant plants during periods of oxygen scarcity was much less (Crawford 1967b) and therefore sets them apart from the flood intolerant species in their avoidance of this type of anaerobic respiration.

The disadvantage of anaerobic respiration lies not only in the production and accumulation of possibly toxic products such as ethanol, but also in its inefficient utilisation of carbohydrates. Anaerobic respiration requires a greater rate of carbohydrate breakdown than does aerobic respiration for the liberation

of the same amount of energy (Grable 1966, Larkum & Loughman 1969). This phenomenon, known as the Pasteur effect, was first demonstrated in yeast cells, but has also been found to operate in higher plants such as maize (Neal & Girton 1955). The avoidance of the Pasteur effect in flood tolerant plants is therefore to their advantage during periods of flooding, when flood intolerant plants are suffering from a reduced energy yield and are competitively less vigorous.

There is some evidence for the conversion or further metabolism of the products of anaerobic respiration, for the ethanol which accumulates when pea seeds imbibe water prior to germination may be subsequently metabolised by the germinating seedling (Cossins & Turner 1963). The conversion of ethanol-1-C^{14} was followed in several tissues, including carrot discs, pea cotyledons, castor bean endosperm, potato tubers and corn coleoptiles, and the results suggested its conversion to acetyl co-enzyme A (Cossins & Beevers 1963). This acetyl co-enzyme A could then be further metabolised by the established pathways. In addition, plants grown under continuous and prolonged lack of oxygen supply to the roots do not exhibit the high ethanol content found in normally grown plants subjected to short term severe oxygen dificiencies (Aubertin, Rickman & Letey 1966). This latter finding means that not only is the ethanol which forms initially upon oxygen deficiency metabolised further, but that thereafter some other metabolic route

other than to ethanol production must be operating.

Even allowing for the possible utilisation of fermentation products in further metabolism, the reduced energy yield of anaerobic respiration compared with aerobic respiration and the accumulation of toxic products such as ethanol impart definite disadvantages to those tissues in which it occurs. While the switch to anaerobic respiration may be to overcome merely intermittent periods of anoxia, nevertheless those plants which can evade anaerobic respiration altogether are at an advantage, and there is evidence that beneficial adaptations of this nature operate with regard to flood tolerance. Under anaerobic conditions the roots of species intolerant to flooding showed marked increases in carbon dioxide and ethanol production, and an increase in alcohol dehydrogenase activity (Crawford 1966, Crawford 1967b, Crawford & McManmon 1968). Under similar conditions there were none of these responses in the flood tolerant species, and it is suggested that it is this avoidance of anaerobic respiration which helps their survival during periods of flooding.

INFLUENCING FACTORS OTHER THAN ANAEROBIOSIS

While considering the effect of flooding-induced anaerobiosis on the organic acid component of root tissue metabolism, it is

also important to consider any other factors which might be affecting the acid levels. One such factor is the cation-anion balance in the root tissues.

The absorption of cations and anions by plant roots does not necessarily occur at equal rates from even a single salt solution, and preferential absorption of ions from the external environment is not an uncommon phenomenon (Jacobson & Ordin 1954). Since the total charge of the ions inside plant cells must be electrically equal to zero, there must be mechanisms available for maintaining cation-anion equivalence following unequal ion absorption. Notwithstanding exchange for previously absorbed ions, the maintainance of ion balance may depend upon metabolic changes within the root, and there is evidence that the most important means of ion compensation lies within alterations to the malic acid fraction of the organic acids of the roots (Jacobson & Ordin 1954). Excess cation uptake, relative to anion uptake, has been shown to result in an increase in TCA cycle acids (Johnson, Jackson & Adams 1963), and when excess anion uptake occurs there is likewise a decrease in the acids. Changes in cell sap pH, which were proportional to organic acid changes in the roots, were induced by unbalanced ion uptake in excised barley roots within 15 minutes (Hiatt 1967).

The acid changes which result from excess cation or anion

uptake probably occur within cell vacuoles (Torii & Laties 1966). Certainly, the normal functioning of the TCA cycle cannot account for the changes, as malic acid is a member of the cycle, and some pool system has to be visualised to accommodate the malate increases and decreases which have been observed. It is also unlikely that organic acid production upon excess cation uptake occurs through the oxidative deamination of amino acids, for in excised barley roots there was no correlation between the formation of organic acids and that of ammonia or amides, as would have been necessary to support this theory (Ulrich 1941).

It could be argued that, since excess cation uptake can lead to increased malic acid content in the roots, the observed rise in malate level in the roots of flooded helophytes may be a reflection of increased cation uptake following the provision of Hoagland's solution. However, it must be pointed out that any increase in the rate of cation uptake in turn suggests that the rate of respiration in the root tissues has increased. Furthermore, this increase in tissue respiration upon flooding in the helophytes can be compared with the situation in the flooded non-helophytes, where there has been no increase in malic acid, hence no increased cation uptake and hence no increase in the rate of respiration. In other words, the validity of the theory that flood tolerance in roots can be related to aspects of the organic acid metabolism is not affected by any acid change brought about

by unbalanced ion absorption.

A further influencing factor on the organic acid levels has been mentioned earlier, namely that of a role in carbohydrate storage. The high levels of shikimic acid in the large, fleshy rhizomes of Iris pseudacorus and Nuphar lutea suggest that changes in levels of this acid, and probably of malate also, are more related to the utilisation of carbohydrate reserves than to pathways concerned with flood tolerance.

Organic acid fluctuations in plant tissues may be also attributed to 'compartmentation' of these acids into 'pools' within the cells. The presence of such pools, where large amounts of individual acids are physically remote from the respiratory centres, has been demonstrated in root tissues in maize, carrot and beet (Table 12).

Table 12. Amounts of individual acids estimated to be in turnover pools (μ mole / g fresh weight). (From MacLennan, Beevers & Harley 1963).

Acid	maize root (1st cm)	carrot root	beet root	wheat leaf
Citric	0.35	0.60	0.86	0.09
Succinic	0.18	-	-	0.15
Malic	1.95	0.80	0.76	0.39

In the present work the large changes which were found in several of the acids indicate that they could not be storage products isolated from active cell metabolism, and the formation of new acids must have been in the cytoplasm and not isolated from enzymes.

METABOLIC ADAPTATIONS WITH REGARD TO ECOLOGICAL AMPLITUDE

Plant species which are metabolically adapted to withstand periods of adverse conditions, such as flooding, are at an advantage over those in whom there is no such adaptation. The former type of plant may survive and even flourish in a habitat where a temporary, short-term period of flooding excludes those non-adapted, non-tolerant species. The distribution of species in and around such sites illustrates their ecological amplitude in respect of flooding. For example, within the genus Erica, experimental flooding retards the growth and may even kill E. cinerea, but does not affect E. tetralix, and in the field those sites supporting E. tetralix tend to be waterlogged, yet those supporting E. cinerea much drier (Bannister 1964a, 1964b). In addition to interspecific differences there may also be intraspecific ones, for in Senecio vulgaris the flooding tolerance of a garden race was found to be greater than that of a sand-dune race (Crawford 1966). This intraspecific difference in tolerance to flooding.

which in the previous example was ascribed to the ability or otherwise to avoid periods of glycolysis, indicates some of the range and potential of metabolic adaptations.

One of the advantages of metabolic over morphological adaptations is that the former operate only when conditions make it necessary, and at other times and under normal conditions the metabolism of adapted and unadapted plants would be indistinguishable. Under times of stress, such as flooding, the species which can adapt its metabolism does so for as long as the conditions persist, then reverts to its normal metabolism, leaving the non-adapted plant checked in its growth and perhaps even killed through its inability to avoid the ill-effects of the particular stress. It is easy to visualise that even a temporary check on growth will put the non-adapted plant at a permanent disadvantage, especially if, say, the causal effect was a period of flooding in spring when growth is normally most vigorous. It can thus be seen how the adapted plants can survive and flourish in areas from which the non-adapted ones are excluded, even though the particular metabolic adaptation is in operation for only a small part of the time. Morphological adaptations, while enabling a plant to survive in areas from which it would otherwise be excluded, do not have the plasticity in operation of metabolic adaptations. Morphological adaptations cannot be switched on and off in response to changes in conditions and are therefore more suited to a par-

sistent environmental stress rather than a temporary one.

Metabolic adaptations to adverse conditions other than flooding have been observed, and have shown some similar points of interest. For example, populations of Agrostis tenuis capable of growing in lead contaminated soil could be taken from those growing naturally in a lead mine, whereas populations from normal pastures could not tolerate the experimental levels (Bradshaw 1960). Similarly, populations of Agrostis tenuis and A. stolonifera from sites contaminated by heavy metals were tested for tolerance to copper, nickel, zinc and lead, and each population showed a marked degree of resistance to poisoning by the metals known to be present in the collection sites (Jowett 1958). Interspecific differences have also been described, as in the increasing tolerance of aluminium shown by Agrostis stolonifera, A. tenuis, A. canina and A. setacea respectively (Clarkson 1966). Furthermore, intra-specific differences in resistance to aluminium toxicity have been shown by ryegrass varieties (Vose & Randall 1962). Apart from these results in higher plants, there is also the resistance to greatly increased copper levels in the soil as shown by the 'copper mosses' Dryptodon atratus and Mielichhoferia elongata (Martensson & Berggren 1954). A different type of metabolic adaptation has been reported in Polygonum bistortoides, where the alpine plants have higher respiration rates than the coastal ones in order to adapt to the thermal regime of the environment (Mooney

1963). Thus not only are metabolic adaptations more likely to operate only when the conditions necessitate, but they can include tolerance to a wide range of adverse effects against which there is no morphological adaptation.

The widening range of metabolic adaptations that have been reported confirm the existence of physiologically different populations, or 'edaphic ecotypes' (Bradshaw & Snaydon 1959, Snaydon & Bradshaw 1961). Where there are these differential responses to environmental factors within species themselves, then it follows that those populations possessing the ability to respond to some particular stress will be ecologically favoured, and have a greater amplitude than those populations without the ability. Temporary flooding of the habitat is an example of such an environmental stress, and the metabolic adaptations possessed by some plants to withstand this adverse condition are factors in their ability to invade these waterlogged sites and extend their ecological range.

CONCLUSIONS

The work described in this thesis has attempted to study the organic acid metabolism of the roots of higher plants in relation to their flooding tolerance. Micro-estimations of the various

organic acids have indicated that there are fluctuations in their levels which can, in some part, be attributed to periods of flood-induced anaerobiosis in the roots. The organic acid metabolism of flood tolerant species differs upon flooding from that of non-tolerant species, and it is suggested that these changes are part of the metabolic adaptations possessed by the former group which enable them to compete successfully in habitats prone to water-logging.

The acid levels in the root tissues tend to follow the general pattern for all plant tissues, with malic > citric > succinic > lactic. The relation of the acid metabolism to flooding tolerance lies in the succinate increase in flooded non-helophytes, which suggests an inhibition and block of the TCA cycle, and the malate increase in flooded helophytes, which suggests continued respiratory activity with the temporary provision for malic acid accumulation. These acid changes were rapidly induced by the flooding treatment, and in the helophytes this is important not only from the viewpoint of a survival mechanism upon the onset of flooding, but as a means of quick and reliable prediction of a plant's flood tolerance.

The latter point can be expanded to cover the problem of flood tolerance in cultivated plants, for as long as spring flooding continues to cause loss and damage to crops then any means

of increasing the plants' resistance to flood injury is worthy of investigation. There are several reports on the adverse effects of spring flooding on different clovers, and indications that some of these varieties possess greater tolerance to flooding than others and are therefore more suited to flood-prone areas (McKenzie 1951, Bendixen & Peterson 1962, Hoveland & Webster 1965). The present work suggests that if strains of a particular species are flood tolerant, they can be detected on the basis of their organic acid metabolism upon experimental flooding. A programme of plant breeding in which selection was for the ability to adapt metabolically and withstand flooding could then produce the seed material required for use, in preference to that of susceptible strains, on ground prone to periods of waterlogging. The segregation of physiological characteristics is as feasible as that of morphological ones (Hiesey & Milner 1965). Selection for resistance to aluminium has already been reported (Vose & Randall 1962), and Marshall and Millington (1967) have successfully crossed strains of clover for the production of increased flood tolerance in the progeny.

SUMMARY

1. The organic acid metabolism of the roots of wet-land species (helophytes) and that of dry-land species (non-helophytes) has been examined in relation to their tolerance to periods of experimental flooding. Growth differences between helophytes and non-helophytes were apparent only after an 18-day flood period, yet within four days of flooding differences could be observed in the levels of certain organic acids. Flooding in helophytes increased the level of root malic acid, and decreased that of succinic and lactic acid, whereas the reverse was found in non-helophytes.

2. There was evidence that under natural flooded conditions the root tissues of some wet-land species contain larger amounts of malic acid than when the flood water has receded and ground aeration improves. Shikimic acid has been detected in aquatic macrophytes and the fluctuating levels of shikimate in Iris pseudacorus and Nuphar lutea are discussed.

3. The organic acid changes related to flood tolerance operate only under partial anoxia, and under the strictly anaerobic conditions imposed by incubation of root tissue under nitrogen, there was a general reduction in all acid levels except lactate.

4. It is suggested that non-helophytes cannot tolerate flooding through an inability to continue TCA cycle respiration during periods of reduced oxygen supply, and through the poisoning effects of ethanol accumulation. Helophyte species appear to be metabolically adapted to overcome periods of flood-induced anoxia, and tissue respiration continues with the provision for malic acid accumulation and no build-up of ethanol.

5. A tolerance of flooding, involving adaptations of the organic acid metabolism of the roots, has been demonstrated in helophyte species. This metabolic adaptation is rapidly induced, thus offering immediate protection upon flooding anoxia, and its importance in determining the ecological amplitude of a species and its possible role in future production of flood tolerant strains, are discussed.

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APPENDIX

APPENDIX CONTENTS

HOAGLAND CULTURE SOLUTION

From Thomas, Ranson and Richardson (1956).

Reagents: A. Solution of essential macronutrients.

	ml in 1 litre of nutrient solution
M/1 KH_2PO_4	1
M/1 KNO_3	5
M/1 $Ca(NO_3)_2$	5
M/1 $MgSO_4$	2

B. Solution of essential micronutrients (less iron).

	g dissolved in 1 litre of water
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$CuSO_4.5H_2O$	0.08
$H_2MoO_4.H_2O$	0.09

Method: To each litre of solution A, 1 ml of solution B is added, so giving ppm. of the micronutrients as stated in brackets: boron (0.5), manganese (0.5), zinc (0.05), copper (0.02) and molybdenum (0.05). Iron should be added in the form of 0.05% ferric tartrate solution, at the rate of 1 ml per litre. The reaction of the solution should be adjusted to pH 6.0 by adding 5% KOH or 0.1N H_2SO_4.

ENZYMIC DETERMINATION OF MALIC ACID

From Bergmeyer (1963).

Reagents: Hydrazine-glycine buffer (0.4 M hydrazine; 1 M glycine; pH 9.5). Dissolve 7.5 g glycine, 5.2 g hydrazine sulphate and 0.2 g EDTA-$Na_2H_2.2H_2O$ in distilled water and dilute to 100 ml. This stock solution is stable and small portions can be adjusted to pH 9.5, using 2N NaOH, as required.

Malic dehydrogenase (MDH) (Commercial preparation).

Nicotinamide-adenine dinucleotide (NAD) (Commercial preparation).

Method:

$$\text{Malate} + \text{NAD} \underset{}{\overset{\text{MDH}}{\rightleftharpoons}} \text{oxaloacetate} + \text{NADH}$$

The amount of NAD reduced to NADH is determined from the change in extinction ($\Delta E_{1\ cm}$) at the end of the reaction, measured on the spectrophotometer at 334 nm. The $\Delta E_{1\ cm}$ for 1 μ mole of malate is 2.0. Test reactions are carried out as shown:

Cuvette	I	II	III
Glycine buffer	1.9	1.9	1.9
Extract	0.5	0.25	0.0
H_2O	0.5	0.75	1.0
NAD	0.03	0.03	0.03
MDH	0.03	0.03	0.03
Total volume in ml	2.96	2.96	2.96

ENZYMIC DETERMINATION OF LACTIC ACID

From Bergmeyer (1963).

Reagents: Hydrazine-glycine buffer (0.4 M hydrazine; 1 M glycine; pH 9.5). Dissolve 7.5 g glycine, 5.2 g hydrazine sulphate and 0.2 g EDTA-$Na_2H_2 \cdot 2H_2O$ in distilled water and dilute to 100 ml. This stock solution is stable and can be adjusted to pH 9.5, using 2N NaOH, as required.

Lactic dehydrogenase (LDH) (Commercial preparation).

Nicotinamide-adenine dinucleotide (NAD) (Commercial preparation).

Method:

$$\text{Lactate} + \text{NAD} \underset{}{\overset{\text{LDH}}{\rightleftharpoons}} \text{pyruvate} + \text{NADH}$$

The amount of NAD reduced to NADH is determined from the change in extinction ($\Delta E_{1\ cm}$) at the end of the reaction, measured on the spectrophotometer at 334 nm. The $\Delta E_{1\ cm}$ for 1 µ mole lactate is 2.0. Test reactions are carried out as shown below:

Cuvette	I	II	III
Glycine buffer	1.9	1.9	1.9
Extract	0.5	0.25	0.0
H_2O	0.5	0.75	1.0
NAD	0.03	0.03	0.03
LDH	0.03	0.03	0.03
Total volume in ml	2.96	2.96	2.96

ENZYMIC DETERMINATION OF OXALOACETIC ACID

From Bergmeyer (1963).

Reagents: Triethanolamine buffer (0.4 M; pH 7.6). Dissolve 18.6 g triethanolamine hydrochloride in about 200 ml distilled water, add 3.7 g EDTA-$Na_2H_2.2H_2O$ and about 18 ml 2N NaOH to adjust the pH to 7.6. Dilute to 250 ml with distilled water.

Malic dehydrogenase (MDH) (Commercial preparation).

Reduced nicotinamide-adenine dinucleotide (NADH) (Commercial preparation).

Method:

oxaloacetate + NADH $\rightleftharpoons^{MDH}$ malate + NAD

The amount of NADH oxidised to NAD is determined from the change in extinction ($\Delta E_{1\ cm}$) at the end of the reaction, measured on the spectrophotometer at 334 nm. The $\Delta E_{1\ cm}$ for 1 μ mole oxaloacetate is 2.0. Test reactions are carried out as shown below:

Cuvette	I	II	III
Triethanolamine buffer	1.9	1.9	1.9
Extract	0.5	0.25	0.0
H_2O	0.5	0.75	1.0
NADH	0.03	0.03	0.03
MDH	0.03	0.03	0.03
Total volume in ml	2.96	2.96	2.96

PREPARATION OF ACONITASE ENZYME

From Bergmeyer (1963).

Reagents: 90% acetone.

Citrate buffer. 0.004 M citric acid; add 0.1 M NaOH to adjust the pH to 5.8.

Method: The ventricular muscle of a freshly killed pig's heart was homogenised with three times its own volume of chilled citrate buffer. The homogenate was left to stand at $4^{\circ}C$ for 20 minutes, then centrifuged at 9,000 r.p.m. at $0^{\circ}C$ for 20 minutes. The supernatant was decanted, then chilled 90% acetone was added to make the concentration of acetone up to 35%. After standing at $4^{\circ}C$ for 20 minutes the preparation was centrfuged at 9,000 r.p.m. at $-5^{\circ}C$ for 20 minutes, decanted and chilled 90% acetone added to the supernatant to make the concentration of acetone up to 45%. After standing at $4^{\circ}C$ for a further 20 minutes, centrifuging at 9,000 r.p.m. at $-10^{\circ}C$ for 20 minutes and decanting, the precipitate was dissolved in chilled distilled water to give an aqueous solution of aconitase. The activity of the enzyme preparation, which is retained for almost two weeks at $4^{\circ}C$, was checked with a known citrate solution before use on test solutions.

ENZYMIC DETERMINATION OF CITRIC ACID

From Bergmeyer (1963).

Reagents: Tris buffer (0.1 M; pH 7.4). Disslove 6.047 g tris-hydroxymethyl-aminomethane in 100 ml distilled water, add 0.186 g of EDTA-$Na_2H_2.2H_2O$, adjust to pH 7.4 with approximately 22 ml 2N HCl and dilute to 500 ml with distilled water.

Manganous sulphate (0.02 M). Dissolve 67.6 mg $MnSO_4.H_2O$ or 74.8 g $MnSO_4.2H_2O$ in distilled water, and make up to 20 ml.

Potassium-sodium tartrate (0.3 M; pH 7.4). Dissolve 8.467 g $KNaC_4H_4O_6.4H_2O$ in 50 ml distilled water, adjust to pH 7.4 with 1N KOH and dilute to 100 ml with distilled water.

Aconitase (see p.v.)

Isocitric dehydrogenase (IDH) (Commercial preparation).

Nicotinamide-adenine dinucleotide phosphate (NADP) (Commercial preparation).

Method:

$$\text{citrate} + \text{NADP} \underset{\text{IDH } (Mn^{2+})}{\overset{\text{aconitase}(Fe^{2+})}{\rightleftharpoons}} \alpha\text{-oxoglutarate} + CO_2 + \text{NADPH} + H$$

The amount of NADP reduced to NADPH is determined from the change in extinction ($\Delta E_{1\ cm}$) at the end of the reaction, measured on the spectrophotometer at 334 nm. The $\Delta E_{1\ cm}$ for 1 μ mole of citrate is 2.0. Test reactions are carried out as shown

below:

Cuvette	I	II	III
Tris buffer	0.8	0.8	0.8
Manganous sulphate	0.1	0.1	0.1
Tartrate solution	0.1	0.1	0.1
Aconitase	0.1	0.1	0.1
Extract	0.5	0.25	0.0
H_2O	1.3	1.55	1.8
IDH	0.03	0.03	0.03
NADP	0.1	0.1	0.1
Total volume in ml	3.03	3.03	3.03

PREPARATION OF SUCCINIC DEHYDROGENASE ENZYME

From Rodgers (1961).

Reagents: Phosphate buffer (0.01 M; pH 7.4 and 0.06 M; pH 7.0).

Made up with KH_2PO_4 and Na_2PO_4.

Method: 50-100 g of freshly killed pig's heart ventricular muscle was minced and washed four times with 10 volumes of ice-cold phosphate buffer (0.01 M; pH 7.4). The suspension was filtered through muslin under reduced pressure after each wash. This washed tissue could be stored for several weeks if deep frozen. For use, the preparation was resuspended in phosphate buffer (0.06 M; pH 7.0), homogenised for $2\frac{1}{2}$ minutes in a Waring Blendor and filtered through muslin. The homogenate remained active for only a few hours, even when kept at $4^{\circ}C$, and should therefore be used immediately.

ENZYMIC DETERMINATION OF SUCCINIC ACID

From Rodgers (1961).

Reagents: Phosphate buffer (0.06 M; pH 7.0). Made up with KH_2PO_4 and Na_2HPO_4.

Standard succinic acid solution, 0.1 mM. Succinic acid is dissolved in phosphate buffer (0.06 M; pH 7.0).

Succinic dehydrogenase (see p. viii.)

Cyanide reagent. 2.72 g of KH_2PO_4, 1 g of crystalline bovine plasma albumin, and 60 mg EDTA are dissolved in 900 ml distilled water, the pH adjusted to 7.0 with KOH, 780 mg KCN added and the mixture diluted to 1 litre.

2:6 dichlorophenol-indophenol indicator solution. Stock solution (0.15%) is diluted 1:10 with phosphate buffer (0.06 M; pH 7.0) immediately before use.

Method: The method is based on the reduction of 2:6 dichlorophenol-indophenol by succinic acid in the presence of succinic dehydrogenase. The measurement of the extinction change of the dye is measured on the spectrophotometer at 600 nm. Although the calibration curve showing the relation between the amount of succinic acid added and the resulting change in extinction ($\Delta E_{1\ cm}$) at the end of the reaction is always linear, the slope varies from one enzyme preparation to another. Test reactions are therefore carried out as shown below, with the inclusion of a set of succinate

standards. A typical standard curve is shown in Fig. 27.

The activity of the succinic dehydrogenase is rapidly lost after preparation, and enzyme homogenates taking longer than 20 minutes to complete the oxidation of 0.1 μ mole of succinic acid should be discarded. It is convenient to set up the reaction tubes in sets of 20, including the calibration tubes, and measure the extinction values serially at one minute intervals.

Tube no.	1	2	3	4	5	6	7
cyanide reagent	1.0	1.0	1.0	1.0	1.0	1.0	1.0
dye	-	1.0	1.0	1.0	1.0	1.0	1.0
buffer pH 7.0	3.0	2.0	1.5	1.0	-	1.0	1.0
succinate soln.	-	-	0.5	1.0	2.0	-	-
sample	-	-	-	-	-	1.0	0.5
SDH	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Total vol. in ml	6.0	6.0	6.0	6.0	6.0	6.0	6.0

After 20 minutes the extinction of the contents of tube no. 2 (the blank reaction) is measured in a 1 cm cuvette against the contents of tube no. 1. The extinction values of the remaining tubes are also measured against the contents of tube no. 1, and the values corrected for the extinction of the blank.

MICROCOLORIMETRIC DETERMINATION OF SHIKIMIC ACID

From Yoshida and Hasegawa (1957).

Reagents: Periodate reagent. Dissolve 160.5 mg sodium periodate in 25 ml freshly prepared acetate buffer.

Acetate buffer (pH 4.7). Mix equal volumes of 1N acetic acid and 1N sodium acetate.

Ethylene glycol solution. Dissolve 1 ml ethylene glycol in 100 ml distilled water.

Aniline solution. Saturate distilled water with freshly distilled aniline at 25°C and take the aqueous layer. This solution should be freshly prepared before use.

Absolute alcohol.

Method: 1 ml periodate reagent was added to a test tube containing 1 ml neutral solution of shikimic acid (40-200 µg / ml). The tube was placed in a water bath at 30°C for 15 minutes, then 1 ml ethylene glycol solution added and the tube maintained in the above conditions for 5 minutes. After removing from the water bath, 1 ml aniline solution was added to the tube, and it left standing at room temperature for 5 minutes. 5 ml of absolute alcohol were added, with thorough mixing, and a clear red solution was formed. The optical density of the solution was determined at 510 nm. The absorption maximum of the pigment is sharp and the

optical densities are proportional to the quantities of shikimic acid between 40 and 200 µg / ml (good results have been obtained down to 10 µg / ml). Shikimic acid standards were run with each set of determinations, since fluctuations in the optical densities occurred between those carried out under even slightly different conditions. A typical standard curve is shown in Fig. 28.

PAPER CHROMATOGRAPHY OF ORGANIC ACIDS

Paper: Whatman no. 1.

Solvent: From Higgins and von Brand (1966).

Propanol / eucalyptol / formic acid / water in the ratio: 50 / 50 / 20 / 5.

Indicator reagent: From Carles, Schneider and Lacoste (1958).

Aniline-glucose. Dissolve 2 g glucose and 2 ml aniline in 200 ml distilled water. To this solution add 20 ml 95% ethanol and 60 ml butanol.

Method: The chromatography cabinet was equilibrated with the solvent for at least 24 hours. An ascending run of approximately 30 cm took 20-22 hours at room temperature, after which the solvent was dried off by placing the paper in an oven at 115°C for 1 hour. The dried paper was sprayed with aniline-glucose reagent, then returned to the oven at 115°C for 5 minutes. The development of brown spots indicated where organic acids were present. A copy of a typical chromatogram of known organic acids is shown in Fig. 29.

Using the technique described above the following Rf values and lower limits of demonstrability were obtained:

acid	Rf value	lower limit of demonstability		
		20 µg	10 µg	5 µg
succinic	0.71	+	+	-
lactic	0.69	+	+	-
malic	0.45	+	+	+
citric	0.37	+	+	+
shikimic	0.23	+	+	+

In the chromatogram of an acid mixture containing 5 µg of each acid, the succinate and lactate spots were indistinguishable.

ENZYMIC DETERMINATION OF ETHANOL

From Bergmeyer (1963).

Reagents: Semicarbazide-glycine buffer. Dissolve 20 g $Na_4P_2O_7 \cdot 10H_2O$, 5 g recrystallised semicarbazide hydrochloride and 1 g glycine in distilled water. Add 20 ml 2N NaOH and dilute to 600 ml. Adjust pH to approximately 8.8.

Alcohol dehydrogenase (ADH) (Commercial preparation).

Nicotinamide-adenine dinucleotide (NAD) (Commercial preparation).

Method:

ethanol + NAD $\rightleftharpoons^{ADH}$ acetaldehyde + NADH

The amount of NAD reduced to NADH is determined from the change in extinction ($\Delta E_{1\ cm}$) at the end of the reaction, measured on the spectrophotometer at 334 nm. The $\Delta E_{1\ cm}$ for 1 μ mole of ethanol is 2.0. Test reactions are carried out as shown below:

Cuvette	I	II	III
Buffer	1.9	1.9	1.9
Extract	0.5	0.25	0.0
H_2O	0.5	0.75	1.0
NAD	0.03	0.03	0.03
ADH	0.03	0.03	0.03
Total volume in ml	2.96	2.96	2.96

Fig. 27. SUCCINIC ACID STANDARD CURVE

Wavelength: 600 nm.

Fig. 27

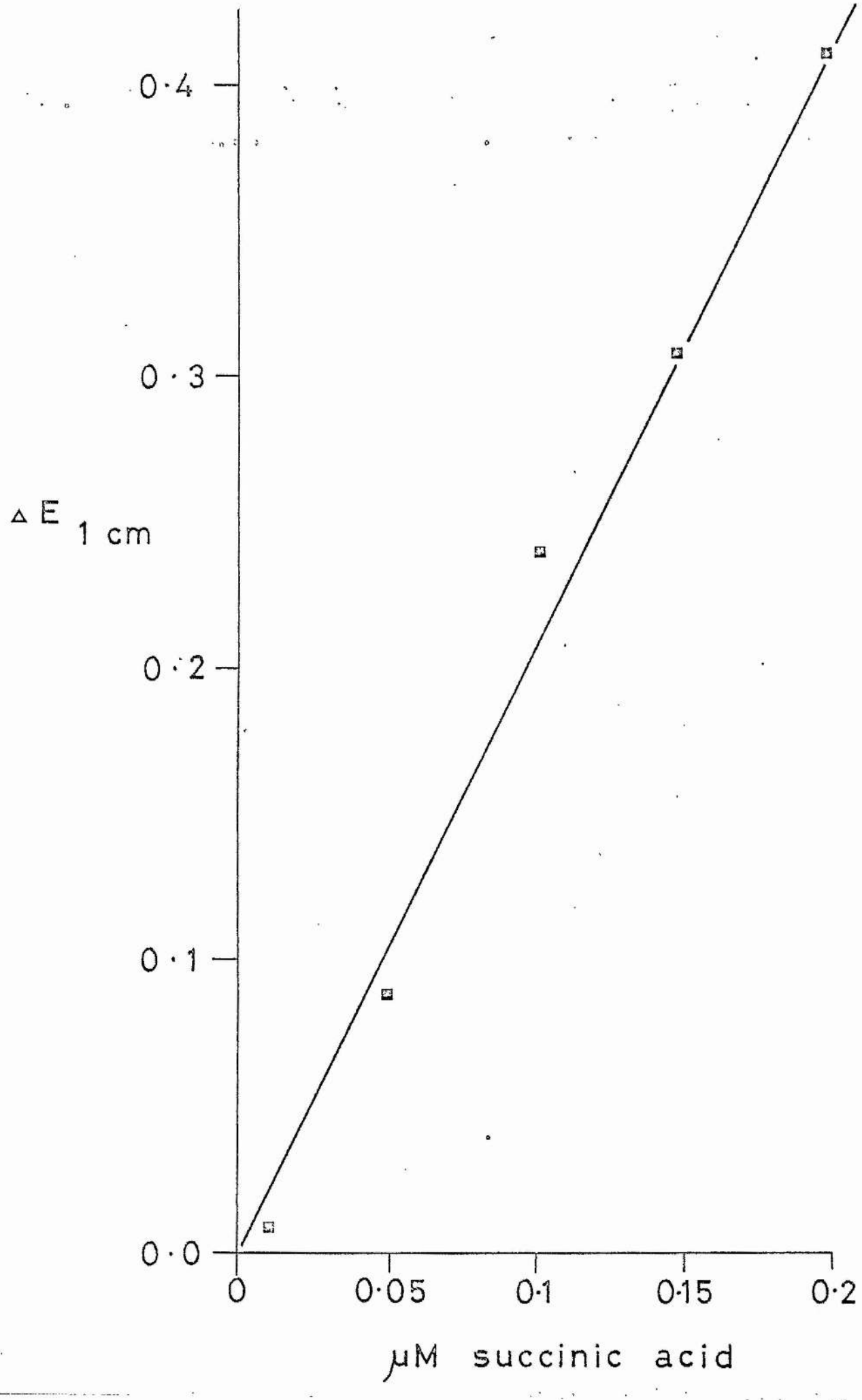

Fig. 28. SHIKIMIC ACID STANDARD CURVE.

Wavelength: 510 nm.

Fig. 28

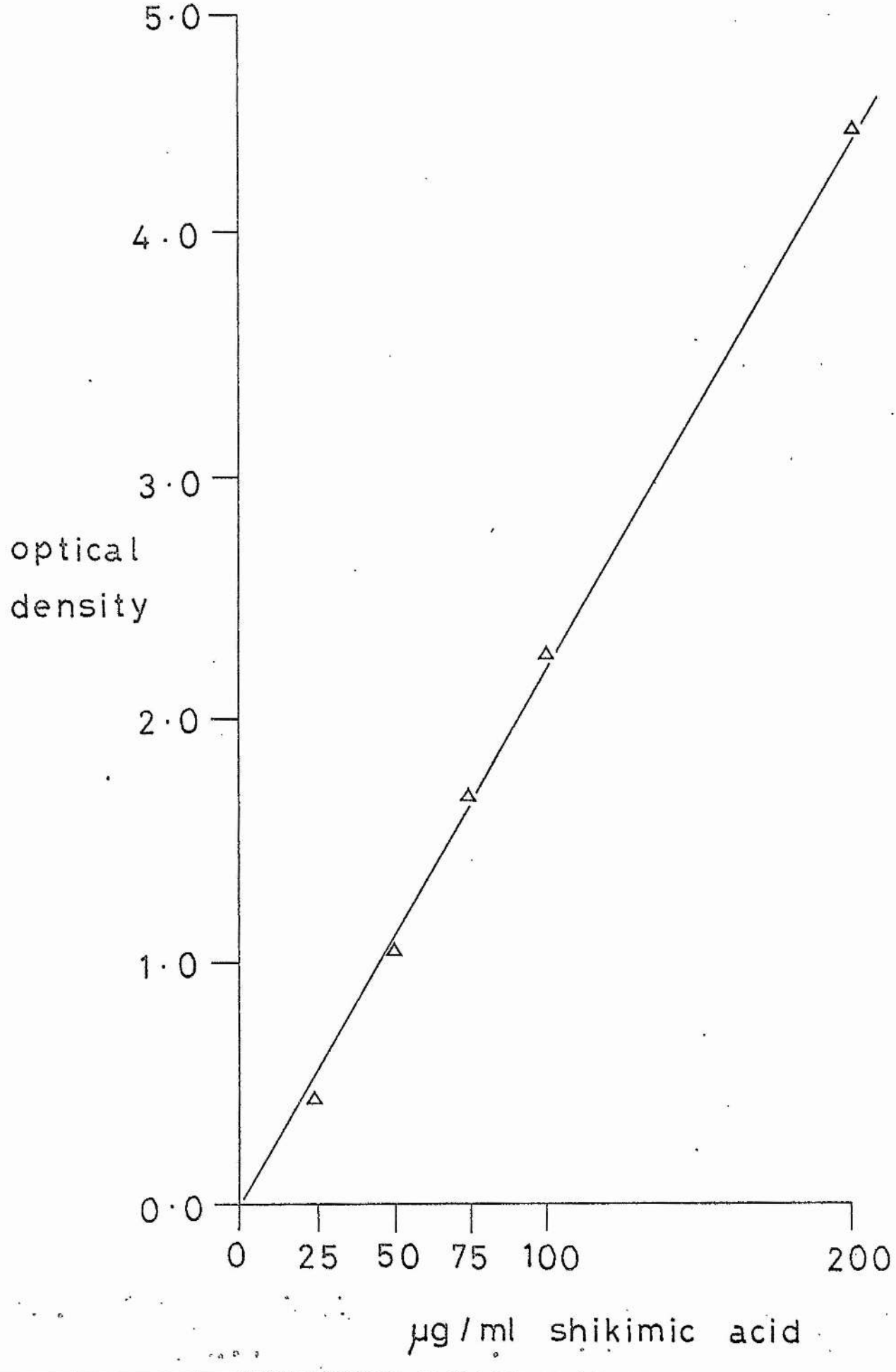
5·0
4·0
3·0
2·0
1·0
0·0
optical
density
0
25
50
75
100
200
μg / ml shikimic acid

Fig. 29. COPY OF PAPER CHROMATOGRAM OF ORGANIC ACIDS

Paper: Whatman no. 1.

Solvent: Propanol / eucalyptol / formic acid / water.

50 / 50 / 20 / 5.

Indicator: aniline-glucose.

Key: su - succinic acid

l - lactic acid

m - malic acid

c - citric acid

sh - shikimic acid

x - mixture containing 10 µg of each of the above organic acids.

Fig. 29

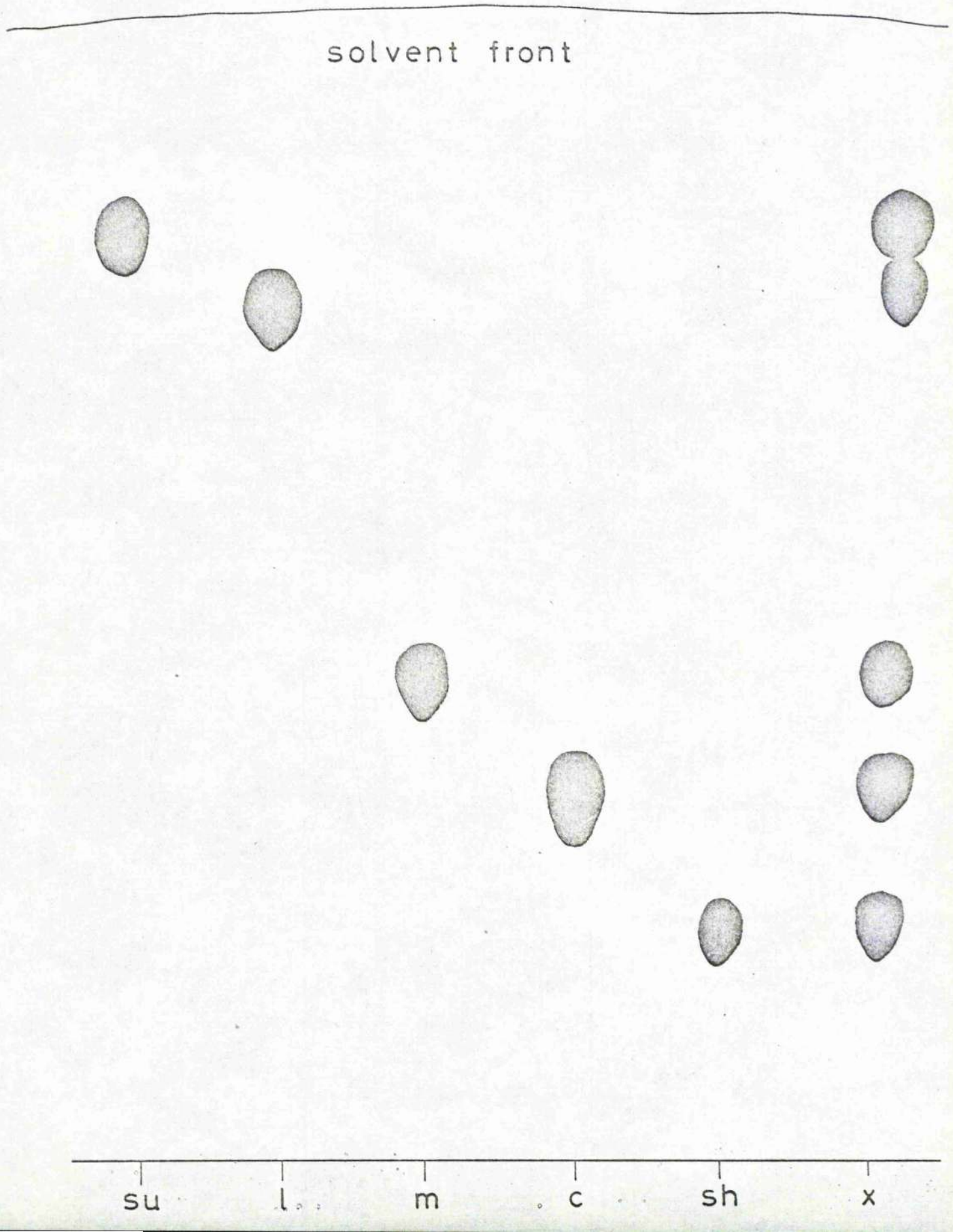

Plate I. Loch of Lowes; 11th June 1968. Base-poor marsh developed at the loch margin. Note the predominance of *Myrica gale*, with new and old shoots of *Phragmites communis*; ground layer contains *Carex lasiocarpa* and *Menyanthes trifoliata*.

PLATE I

Plate II. Loch Clunie; 11th June 1968. Marsh vegetation developed at the loch margin. Note tall, blade-like leaves of Iris pseudacorus in the foreground, and dense growth of Filipendula ulmaria elsewhere excluding other species.

PLATE II

Plate IIIA Sand dunes at Tentsmuir, Fife; June 1969. Note the open nature of the dry, sandy substrate, the ridge pattern of the dune formation, and the predominance of grasses such as Agropyron junciforme and Elymus arenarius.

Plate IIIB Dune-slack at Tentsmuir, Fife; June 1969. Note the closed plant community developed on the damp substrate, with Filipendula ulmaria in the foreground and an area of Glyceria maxima in the middle distance (marked ☆).

PLATE III A

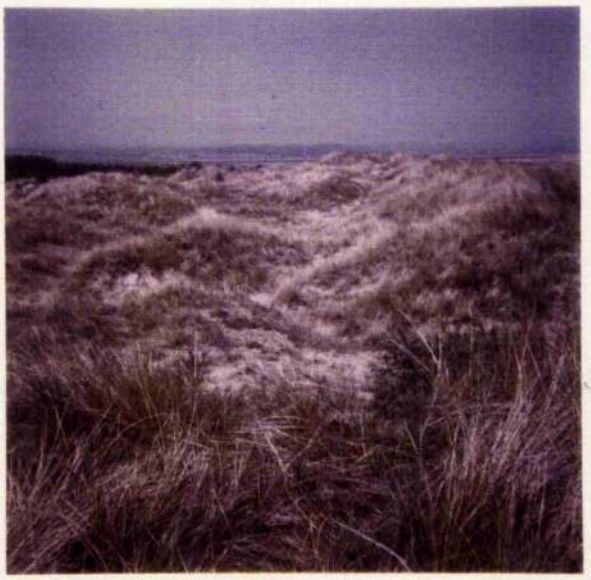

PLATE III B

Plate IVA Culture buckets showing growth of Ranunculus flammula in high and low water table conditions. Note the central perforated metal tube for visual check on the level of the water table.

Plate IVB Culture bucket of Ranunculus flammula in high water table conditions. Note the level of nutrient solution just above the sand surface.

PLATE IV A

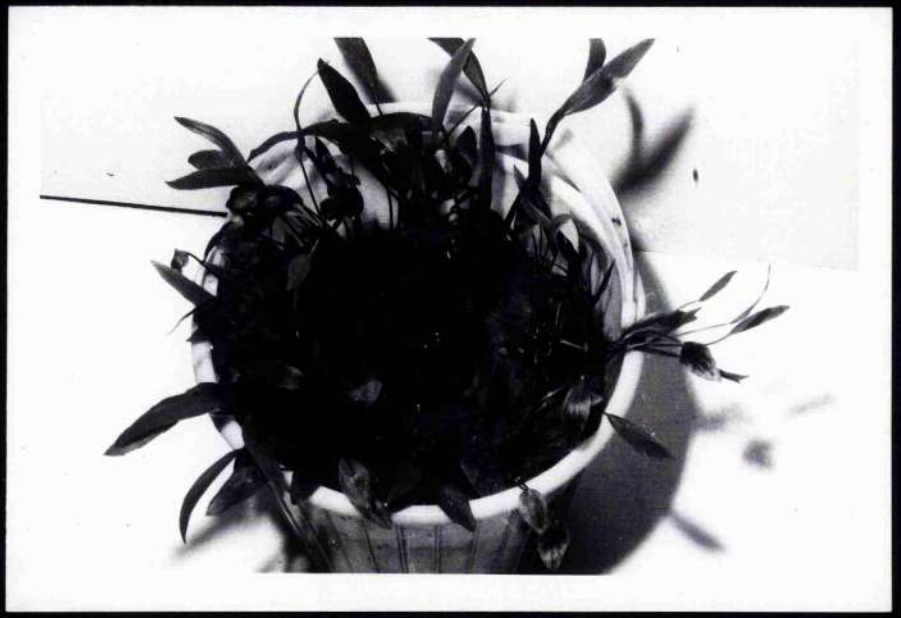

PLATE IV B

Plate VA Loch Clunie; 6th March 1968. Note the extensive flooding beyond the loch margin (belt of trees) into the foreground marsh area.

Plate VB Loch Clunie; 14th May 1968. Note the drop in water table from Plate VA, no flooding in the marsh area, and growth of Iris pseudacorus and Filipendula ulmaria in the foreground.

PLATE V A

PLATE V B