An investigation into the rapidly induced chemical responses of *Myrica gale* to insect herbivory.

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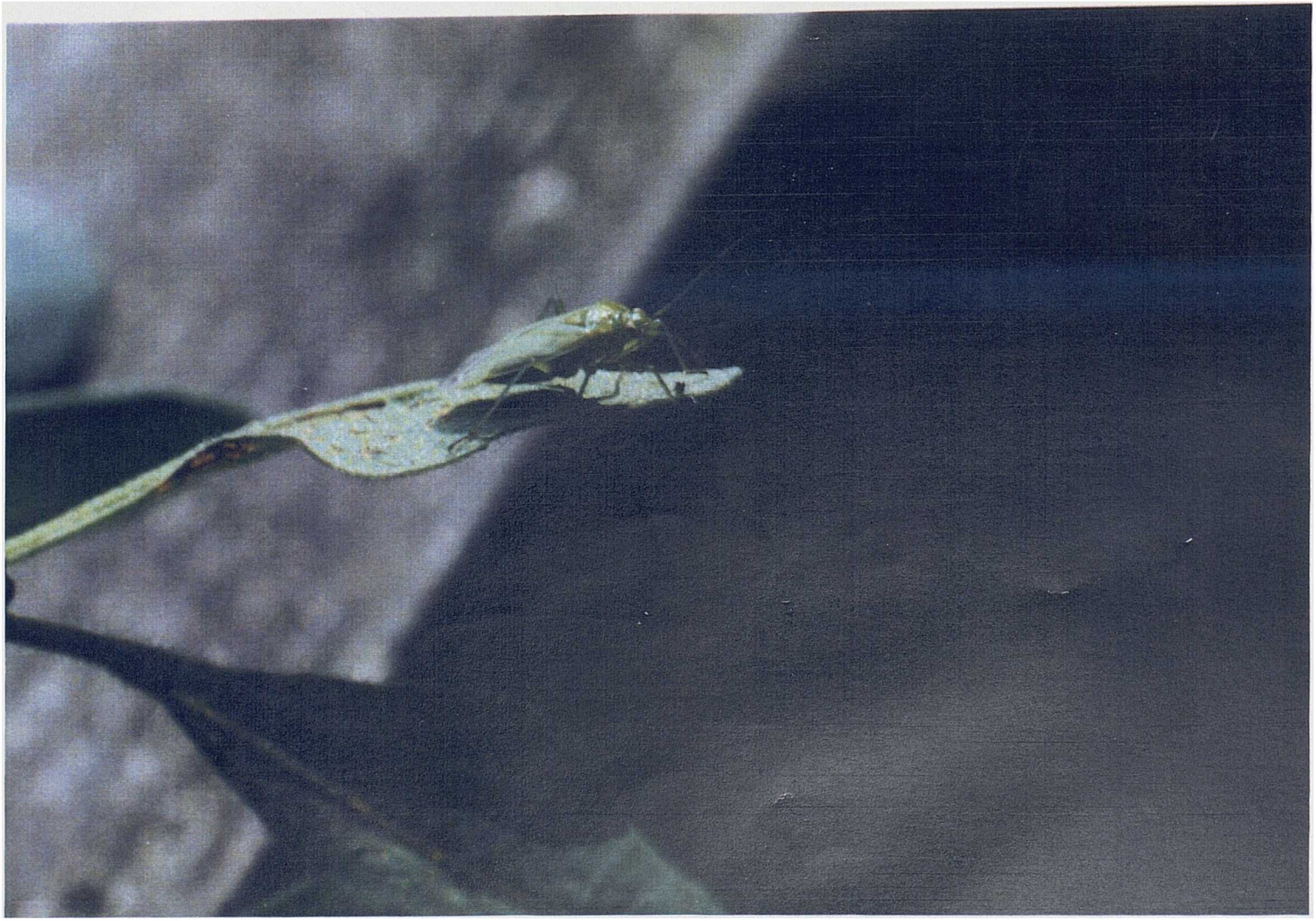


A capsid bug, herbivore of sweet gale, poses for a photo on a leaf exhibiting signs of ealier herbivory.

C O N T E N T S.

A B S T R A C T.

The effect of natural herbivory on the secondary metabolism of *Myrica gale* was investigated. In a field experiment herbivory was found to elicit changes in both the leaf phenolics and the density of volatile oil glands. *Lygocoris spinolai*, a capsid bug, accounted for most of the observed herbivory.

One set of plants (the controls) were kept free of insect herbivores while a second set sustained capsid bug herbivory. The terpenoid and phenolic profile of leaves from each set was obtained on a weekly basis using GLC and HPLC respectively. GC-MS was used to identify constituents of the volatile oil. UV, ^{1}H and ^{13}C NMR, EI-MS and FAB-MS were used to identify the phenolics.

Capsid bug damage induced a qualitative change in the phenolic content of the leaves. This induction revealed a new compound, kaempferol-3-(2,3-diacetoxy-4-*p*-coumaroyl)rhamnoside, which was isolated and identified. The leaves also proved to contain other flavonoids, the concentrations of which were not affected by herbivory. Artificial damage was observed to elicit a quantitative change in leaf phenolics, thus having a different effect than herbivore damage. Herbivore damage also induced quantitative changes in the volatile oil by eliciting gland production whilst having no effect on the composition of the oil.

The induced flavonoid and the volatile oil were tested for fungal growth inhibitory properties. Both were found to have marked antifungal activity at low concentrations when tested against fungal species isolated from the leaves of *M. gale* in the field.

The observed phenomena are discussed in terms of defenses against herbivores and pathogens. The relationship between the nitrogen economy of M. gale and leaf chemistry is also discussed.

Chapter 1. Introduction.

1.1 Objective

This work has two complementary objectives. The first is to investigate the effect that insect herbivory has on the production of leaf secondary metabolites in *Myrica gale* L. The second is to explore the possible roles, if any, that secondary metabolites play in the interactions of *M. gale* with its biotic environment.

1.2 Secondary metabolites and their possible roles.

Products of plant metabolism can be split into two broad categories: primary or secondary metabolites. In addition to primary metabolites, which are essential for plant life, plants produce a vast array of secondary metabolites. Primary metabolites tend to be common to large plant taxa whereas particular secondary metabolites are frequently restricted to families, genera and even individual species. They include terpenes, flavonoids, tannins, steroids, alkaloids, cyanogenic glycosides and amino acids (Conn 1981).

Although secondary metabolites may play an important part in sustaining a plant's success within a community under a particular set of environmental conditions they are not essential for growth in conditions where the plant is neither stressed by physical or biotic factors. The question arises: "What benefits do plants gain from their investment in secondary metabolites?"

Plant secondary metabolites often exhibit marked biological activity. Human perception of this phenomenon is well recorded from classical times. The basis of herbal medicine rests with this

phenomenon and the science of pharmacy has evolved largely from herbalism which has been practiced for thousands of years. However, without reference to a deity it is hard to explain the existence of plant secondary metabolites in terms of their efficacy as pharmaceutical agents. Perhaps, from a scientific point of view, a more acceptable basis for an explanation of secondary metabolites is in the theory of evolution.

From an evolutionary point of view secondary metabolites can be viewed as adaptations to environmental pressures on the plant. The selection is usually mediated by intraspecific competition whereby the reproductive success of individuals is a test of the benefits derived from particular characters. Selection for characters which do not improve the reproductive success of individuals within a species is unlikely. It follows that secondary metabolites must be of some benefit to plants or have been so at some point during the plant's evolution.

Secondary metabolites may have a variety of possible functions of benefit to a plant. Some secondary metabolites may be instrumental in protecting plant tissue from extremes of temperature and from harmful radiation. Others may act as storage molecules for excess assimilate or complex with soluble soil toxins rendering them less harmful (Rhoades 1985b). Perhaps the greatest scope for secondary metabolite activity, however, is in effecting the biotic environment. Within this area there exist possible roles in creating an environment unsuitable for other plant species competing for the same resources, providing attractants for mutualistic organisms and producing defences against herbivores and pathogens.

This work is primarily concerned with possible secondary

metabolite roles in defense although other roles will be examined. Plants form the major biomass on the earth and, as phototrophs, are at the start of all major food webs. Herbivores utilise living plant tissue and the vast proportion of herbivores are insects. However, of the twenty nine insect orders only nine have members able to exploit living plant tissue as a resource (Strong *et al.* 1984). In Britain the most casual observer cannot help but notice the mass of greenery during late spring, summer and early autumn in all but the most urban of environments. These plants cannot move to escape herbivores yet clearly they avoid herbivory to a large degree. To explain why such a relatively small number of insect taxa utilise plants as a food source Southwood (1973) contended that a major evolutionary barrier has to be negotiated before insects can exploit higher plants. Secondary metabolites can be seen, in this context, as a large part of this barrier.

In the past three decades the possibility that plants produce secondary metabolites to influence their biotic environment has generated considerable interest. It was Stahl (1888) who first suggested that secondary metabolites may have a defensive role in plants. Fraenkel (1959) provided evidence that secondary metabolites in plants can function as deterrents to herbivores. Since that time considerable attention has been focused on the subject. There are now many examples of secondary metabolites effecting herbivore success (Bryant *et al.* 1987, Cates *et al.* 1987, Harborne 1985, 1989, Rosenthal & Janzen 1979, Sutherland *et al.* 1980, Tahvanainen *et al.* 1985a).

1.3 Factors affecting evolution of secondary metabolites as defenses.

Empirical evidence that herbivores are not able to utilise the total resources available is apparent and obvious. Schultz (1983) suggested that primary productivity utilised by herbivores every year, excepting herbivore outbreaks when plants can be totally defoliated, is generally below 7%. To what extent plant investment in secondary metabolites regulates this figure is not clear although by investing in compounds with antifeedant or toxic properties a plant would deter generalist herbivores and reduce the success of specialist herbivores.

Any attempt, however, to explain the balance between herbivores and their hosts which relies solely on plant secondary metabolites affecting food quality runs into a problem when the generation length of a plant, such as a tree, is many times that of its herbivores (Schultz 1988a). The chemical defenses of a young tree would select for adapted herbivores rendering those defenses useless long before the tree reaches maturity. If plant chemical defenses were the sole regulators of herbivore population dynamics and could be readily overcome by adaptation mature trees would be rare. Fry (1989) found that the phytophagous mite (*Tetranychus urticae* Koch) can adapt to a new host, which initially causes low acceptance and high mite mortality, within 10 generations (approx. 27 weeks) and exhibit better growth on the new host than the control within 20 generations. Clearly plant chemistry is not the sole barrier to insect utilisation of plants and it is reasonable to assume that real ecosystems are somewhat more complex than this simple model.

The model does not take natural enemies of herbivores into

account although these are bound to reduce herbivore populations. Furthermore, plant chemistry may influence the susceptibility of herbivores to their natural enemies (Price *et al.* 1980, Schultz 1983). Even if natural enemies are not considered plants may be able to prevent rapid herbivore adaptation to their defenses by investing in complex mixtures of secondary metabolites. Whittaker and Feeny (1971) suggested that a cost should be associated with herbivore adaptations to plant chemical defenses so herbivores would be restricted in the number of defenses they could surmount. By evolving a unique set of defensive metabolites each plant species deters attack by most herbivores since they are unlikely to evolve counter adaptions to more than a few species (Rhoades 1985a). This, in turn, would lead to a degree of specialisation in the herbivores.

Two similar theories of defense strategy, now known as the plant apparency theory, were proposed in 1976 (Feeny 1976; Rhoades and Cates 1976). It was suggested that plants would evolve defence strategies which complemented their growth patterns. For example ephemeral species would invest in low concentrations of highly toxic defences which could only be metabolised by specialist feeders. Such plants, said to be unapparent, might expect to largely avoid predation by specialist feeders simply by virtue of their unpredictable distribution. Long living plants or species dominant in communities are apparent to herbivores and easy to locate. They would be expected to invest in high concentrations of complex mixtures of general antifeedants, such as phenolic resins and tannins which could act by precipitating protein in the gut, to which herbivores could not readily develop detoxifying systems.

Zucker (1983) questioned whether broad categories of secondary metabolites, specifically tannins, could be characterised as digestion inhibitors. He argued that tannins could have quite specific biological action and not necessarily be dosage dependant. Furthermore, he questioned whether tannins are necessarily an expensive form of defense. Haukioja *et al.* (1985b) found no relationship between leaf total phenolics and the ability of extracts to precipitate proteins. Recent research on the creosote bush (*Larrea tridentata* Coult.) has shown that although phenolic resins can reduce herbivory they do not act by reducing the digestibility of plant tissue but are likely to be toxic to herbivores (Meyer & Karasov 1989) and would, therefore, be less likely to act in the way that Rhoades and Cates (1976) had envisaged.

The results Coley (1983) obtained from a tropical rainforest did not agree with the plant apparency theory since the unapparent species, whilst investing in lower levels of simple phenolics and condensed tannins, did not escape herbivory and therefore tended to lose more tissue. She suggested that these species were able to sustain higher levels of herbivory since they tended to grow in higher quality habitats. She argued, therefore, that habitat quality is a major selective force in the evolution of defenses. This argument is closely allied to that of Bryant *et al.* (1983) who worked on Alaskan ecosystems and proposed that plants would adapt to low-resource environments by evolving strong constitutive defenses. Rundel (1982) and Mihaliak and Lincoln (1985) also predicted that plants with limited nitrogen would invest in high levels of defense. Coley *et al.* (1985) further argued that low habitat quality would

select for well defended slow growing plants. In conditions where optimal growth is slow it is to the advantage of the plant to maintain leaves for a longer period. It has been argued that the value of a leaf depends upon its input into the generation of future leaves (Harper 1989). In high quality habitats the value of a leaf is rapidly reduced with age as the plant is able to produce new leaves with comparative ease. In poor habitats plants cannot produce new leaves as easily so the value of leaves does not drop as rapidly and investment in defenses is advantageous.

The quality of habitat is not only likely to influence the degree of investment in secondary metabolites, it will also incline the plant towards particular types of compound. Perhaps the most important parameter dictating what types of allelochemicals a plant will invest in is the carbon/nutrient balance of soils on which the plant is adapted to grow (Waterman & Mole 1989). Plants have evolved secondary metabolites which incorporate nutrients which are not growth limiting in the environment to which the plant is adapted. Thus plants adapted to nutrient poor habitats will invest in secondary metabolites which do not contain soil nutrients, in particular nitrogen, since availability of such nutrients are, at best, unpredictable in such environments. On the other hand, growth of plants adapted to nutrient rich habitats is unlikely to be nutrient limited so nutrients are available for incorporation into secondary metabolites.

Another intriguing aspect of this hypothesis is that the pathway involved in secondary metabolite production often corresponds to the degree of plant evolutionary development. The shikimate pathway is more primitive and is typical of woody species

whereas the acetate and mevalonate pathways, common to many herbaceous species, are considered to be a more recent development (Bate-Smith 1972, Kubitzi & Gottlieb 1984).

1.4 Co-evolution of plants and herbivores - does the theory stand scrutiny.

Ehrlich and Raven (1964) argued that, in many cases, herbivores and their host plants may have co-evolved over a lengthy period of time resulting in close interactions and a high degree of specialisation. These ideas gained acceptance and many herbivore-host interactions have since been explained in terms of co-evolution. More recently, however, the general application of the theory has come into question (Janzen 1980, Fox 1981, Strong *et al.* 1984). Thompson (1982) argues that close reciprocal evolution (i.e. between a plant and a single herbivore or herbivore taxon) is unlikely except in certain restricted situations, but that in a wider sense co-evolution would be expected to occur between many groups of organisms (e.g. plants, pathogens, herbivores, pollinators).

More recently some authors (Jermy 1984, Bernays & Graham 1988) have questioned the evidence for plant-herbivore co-evolution and asserted that it was lacking. The crux of their argument is that, in general, herbivory has not been demonstrated to effect plant fitness and therefore workers should not assume that it is a selective force in the evolution of plants.

The effect of plant secondary metabolites on herbivores has also been scrutinised (Bernays & Graham 1988). Smiley (1985) questioned whether the host distribution of heliconid butterflies

could be explained in terms of host-plant (*Passiflora*) chemistry. He found that most host species within one subgenus (*Plectostema*) could support *Heliconius* species equally well, even though their distribution is far more restricted in nature, clearly reducing the importance of plant chemistry as an explanation of the distribution of the butterflies.

Strong *et al.* (1984) listed a number of studies where herbivory appears to severely restrict plant populations but they point out that these are *not* necessarily typical. They argue that in many, if not most, cases insect herbivores are too rare to effect plant fitness. Thompson (1988) argued that the observed ease with which insect herbivores change host (Strong *et al.* 1984) detracts from the importance of co-evolution.

However, Thompson (1988) makes the important point that it is differences between genotypes within a population that provide the basis for evolution. It follows from this that any pressure on the population, however small or intermitant, will select for the most successful genotype. Thus, herbivory does not have to devastate a population of plants, it simply has to tip the balance of plant fecundity in favour of a particular genotype for that genotype to become dominant within the population over a number of generations. Likewise, a secondary metabolite does not have to be toxic to a herbivore species to select for the herbivore genotype which can most efficiently metabolise it. In fact, Stipanovic (1983) has argued that an extremely toxic defense will rapidly select for herbivores able to overcome it whereas a less toxic substance will act by reducing herbivore damage without rapidly selecting for detoxifying adaptations. Some specialist herbivores are capable of

detoxifying secondary metabolites, for instance the bruchid beetle *Caryedes brasiliensis* can detoxify the normally toxic L-canavanine of the legume *Dioclea megacarpa* Rolf. using a similar catabolic system to that utilised by the plant (Rosenthal *et al.* 1978).

It seems likely that plants have evolved to produce some secondary metabolites in response to past herbivore pressure. Schultz (1988a) points out that there are many instances where these metabolites have a negative impact on plant enemies. Bryant *et al.* (1989) used the theory of co-evolution to make a series of predictions. They predicted that Alaskan and Siberian birch and willow would suffer greater herbivory than birch and willow in Finland and would, as a consequence, invest in a greater proportion of chemical defenses. This, in turn, they argued, would select for herbivores in Alaska and Siberia better able to utilise leaves high in secondary metabolites. These predictions were tested using snowshoe hares (*Lupus americanus*) from Alaska and mountain hares (*L. timidus*) from Finland and the results supported the hypothesis.

An inherent feature of evolutionary theory is that adaptations exhibited by a population in response to a pressure must result in positive feedback to that population. In the case of plant adaptations to herbivory difficulties arise when those benefits are couched in terms of influencing herbivore population dynamics. One reason for this is that reduced host plant quality has been shown, in at least one system, to benefit herbivores by reducing their susceptibility to pathogens (Schultz 1988a). Furthermore, studies in population modelling demonstrate that fluctuations in herbivore populations can be intrinsically chaotic (Schaffer & Kot 1985, Schaffer 1986). It would be naive, therefore, to assume that simple

feedback mechanisms involving population dynamics of herbivores select for chemical defenses in plants.

However, one of the most important ideas to come out of the co-evolution debate is that the evolution of plants and their herbivores cannot be taken out of the context of the environment where that evolution is taking place.

1.5 Induced defenses in plants

Levin (1971,1976) distinguished between constitutive and induced chemical defenses to herbivores. Constitutive defenses exist in the plant prior to any contact with herbivores whereas induced defenses are based on the accumulation or modification of existing host metabolites as a consequence of interaction between host and herbivore.

The existence of constitutive defenses had been accepted since the publication of Fraenkel's (1959) paper. More recently induced production of secondary metabolites, in response to herbivory, has been observed. The earliest reports of herbivore induced chemical changes are from conifers. Reid *et al.* (1967) observed that lodgepole pine (*Pinus contorta* Douglas) trees produce resins in response to bark beetle (*Dendroctonus ponderosae* Hopkins) attack. Berryman (1969) found that grandis fir (*Abies grandis* Douglas) responds to fir engraver beetles (*Scolytus ventralis* Lec.) by producing resins in the bark phloem parenchyma. These resins proved to be highly repellent to adult beetles and the success of the defensive response was proportional to its magnitude. The foliage of Scots pine (*Pinus sylvestris* L.) has been found to exhibit induced increases in polyphenolics in response to European sawfly

(*Neodiprion sertifer* Geoff.) damage (Theigles 1968). This response was not restricted to the damaged tissue.

Induced responses are not restricted to gymnosperms. Green and Ryan (1972) demonstrated that potatoes and tomatoes respond to wounding by the Colorado Potato Beetle with a dramatic increase (three fold or more) in endopeptidase inhibitor levels. It was suggested that such an increase in the tissue would decrease its palatability and possibly make it toxic.

Since the mid 1970's there has been a large volume of work devoted to investigating plant responses to herbivory. Much of that work has been focused on the effect of induced responses on herbivore population dynamics. Insects have frequently been used in such studies since, in addition to their importance as herbivores, they are manageable in experiments and usually have short lifecycles making studies on growth and reproductive success possible.

One direction in which research on induction has progressed has been through feeding trials using artificially damaged leaves. In general results of these experiments suggest that damage induces changes in leaf chemistry which reduce leaf palatability (Carroll & Hoffman 1980, Edwards & Wratten 1982, Wratten *et al.* 1984, Edwards *et al.* 1986, Raupp & Sakof 1989). In experiments where insect larval growth rate, insect mortality and larval development were gauged an array of results has emerged. In many cases insects fed on damaged tissue were less successful than controls (Rhoades 1983, Rottger & Klingauf 1976, Wallner & Walton 1979, Haukioja 1982, Valentine *et al.* 1983, Raupp & Denno 1984, Haukioja & Hanhimäki 1985, Rossiter *et al.* 1988, Hanhimäki 1989) although results occasionally indicated that damage may improve insect success

(Haukioja & Niemela 1979, Carroll & Hoffman 1980) and foliage palatability (Fowler & McGavin 1986, Carroll & Hoffman 1980).

Fowler and Lawton (1985) have cast doubt on the validity of the experimental designs which led to many of the early conclusions. In some cases they claim that statistics are flawed (Haukioja & Niemela 1979, Haukioja 1982, Wallner & Walton 1979), in others no statistics are given (Rottger & Klingauf 1976) and the effects measured are often so small as to be of doubtful biological significance (Haukioja & Niemela 1977, 1979, Wallner & Walton 1979). In many cases no differences were observed. Their conclusion was that the evidence they reviewed did not support a claim that damage induced changes can have anything but a negligible effect on insect herbivore population dynamics.

Not all early experiments on induction investigated the effect of artificial damage on palatability. Parker (1984) let some plants sustain natural damage whilst protecting others from damage and then assessed the plant palatability using a specialist grasshopper. Plants previously protected from damage proved to be more attractive to the grasshoppers. Karban and Karey (1984) reported that cotton seedlings subjected to five days of spider mite infestation at the cotyledon stage, and then left clear of mites for twelve days, proved to be a far poorer food source than previously uninfested controls. This work, along with that of Raupp and Denno (1984), was accepted by Fowler and Lawton (1985) to demonstrate large enough induced effects to be biologically significant.

Some evidence suggests that the effects of damage can be very localised. Haukioja and Hanhimaki (1985) found that artificial damage to birch resulted in a rapid reduction in quality of damaged

leaves (as a food for the geometrid moth *Epirrita autumnata* Bkh.) with leaf quality increasing with distance on the branch from the site of damage. Bergelson *et al.* (1986) found that casebearing moth larvae (*Coleophora serratella* Zeller) vacated their mines in birch leaves, following simple damage to adjacent tissue, but rarely left the leaf. This behaviour was evident within 24 hours of damage. Adult willow beetles (*Plagiodera versicolora* Laich.) also respond to damage within 24 hours by moving away from the leaves. Using computer generated images compiled from videos Croxford *et al.* (1989) have examined the effect of artificially damaging cotton and soya leaves on the feeding behavior of *Spodoptera littoralis* Boisd. Clear areas of avoidance are observed around sites of leaf damage and damaged leaves are frequently avoided altogether.

Avoidance of damaged tissue is restricted by herbivore mobility. *S. littoralis* and *P. versicolora* are relatively mobile whereas the mobility of *C. serratella*, which carries its leaf tissue case when moving, is far more restricted. Since the advantages of host selection behaviour mediated by locally induced plant responses are dependant upon the ability of the herbivores to move such behavior should be most pronounced in mobile insects. Harrison and Karban (1986b) have observed that, in choice experiments, adult spider mites (*T. urticae*) avoid damaged cotton plants without coming into physical contact with them, whereas this is not true of immature spider mites. This may be due to the fact that immature spider mites are far less mobile than adults so the advantages of avoidance are outweighed by the difficulties involved and likelihood of failure.

Plants may derive various benefits from stimulating localised

avoidance of damaged tissue by herbivores. It is possible that movement involves risks, particularly of predation (Croxford *et al.* 1989), although Bergelson and Lawton (1988) found no evidence to support this hypothesis. Movement also involves expenditure of energy and periods of time when feeding is not possible. Although this is unlikely to directly affect survivorship of herbivores it may delay maturation of larvae or reduce reproductive success.

By deterring feeding from damaged and adjacent tissue the plant limits the area of leaf likely to suffer herbivory and, in doing so, increases the competition amongst herbivores for resources (Hanhimäki 1989). This, in turn, reduces the total herbivore load on the plant and increases its chances of surviving the damage.

Plants may also benefit from deterring herbivores from sites of damaged since nitrogen mobilisation may accompany damage stress (White 1984). In addition to defending nitrogen resources the plant may also benefit by controlling herbivore reproduction. In some cases induced defenses have been demonstrated to effect the success of female herbivores more than males (Raupp & Denno 1984, Harrison & Karban 1986a). The protein requirement for reproduction is high so the availability of undefended mobilised nitrogen, in the absence of induced defenses, could actively promote reproductive success.

Recent work has demonstrated that induced changes triggered by one guild of insects can deter later guilds. Silkstone (1987) demonstrated that artificial damage early in the season reduced the palatability of birch leaves to *S. littoralis* later the same year. It has been demonstrated that plants sustaining natural early season damage are avoided by late season herbivores and that late season

herbivores feeding on plants damaged by earlier herbivory exhibit reduced fecundity and survivorship (Faeth 1986, Harrison & Karban 1986a). However, herbivory, especially when light, may occasionally improve the quality of leaves for late-season herbivores (Williams & Myers 1984). When the herbivore is not mobile previous damage can stimulate feeding to compensate for the reduction in the quality of the leaves as a food (Fowler & MacGarvin 1986). One study (Danell & Huss Danell 1985) demonstrated that moderate browsing of birch (*Betula* sp.) twigs by moose and simulated browsing both stimulated insect herbivory and further moose browsing. This was associated with increased levels of nitrogen in the twigs after browsing. Clearly, damage does not always reduce subsequent herbivory although this does seem to be true in general.

In addition to rapidly inducing short term defenses herbivory may influence the food quality of plant tissue in years subsequent to damage (Haukioja *et al.* 1985a, Haukioja & Neuvonen 1985, Neuvonen *et al.* 1987, Tuomi *et al.* 1988, Hanhimäki 1989). This implies a more fundamental response by the plant than simply producing defense metabolites in the vicinity of damage, especially when the long term induced responses are observed in deciduous species since the defenses exist in leaves temporally separated from damage. It is possible that long term induced responses reflect that plants sustaining herbivory suffer from nutrient depletion and, in accordance with the nutrient stress hypothesis, channel excess photosynthate into defenses. This implies a feedback loop whereby herbivory causes nitrogen depletion which, in turn leads to a carbon nutrient balance favouring secondary metabolite production rather than growth. The subsequent investment in secondary metabolites

leads to decreasing herbivory and an improvement in the carbon nutrient balance. Such a mechanism is not an adaptation to herbivory in itself but clearly it will select for secondary metabolites which reduce herbivory. However, insect frass applied at the base of trees also appears to cause long term induced defenses in birch (Haukioja *et al.* 1985a) even though it should improve the carbon nutrient balance of the trees. It is possible, therefore, that such defenses may, in some cases, be direct adaptations to herbivory.

The effect of herbivory appears to change over the season, the most marked effects being observed early in the season. Edwards and Wratten (1982) noted that the feeding rate of snails on damaged birch leaves was inhibited, relative to controls, in June but not in July. In a similar experiment, in which *S. littoralis* and *Orgyia antiqua* L. were test animals, Wratten *et al.* (1984) observed reduced feeding on damaged plants in April and June but not in August.

Hartley (1988) provided evidence that insect damage stimulates phenolic production in birch (*Betula* sp.) more than artificial damage. In a more detailed study Hartley and Firn (1989) found that both artificial damage and caterpillar (*Apocheima pilosaria*) damage to birch (*B. pendula*) caused an increase in the activity of phenylalanine ammonia lyase (PAL), an enzyme involved in the early stages of phenolic biosynthesis. The increase due to caterpillar damage was more than twice the increase due to artificial damage. Furthermore, changes due to artificial damage were restricted to the damaged leaves whereas insect damage resulted in additional PAL activity in adjacent intact leaves. The results of this work clearly give rise to questions about the use of artificial damage as

a model for herbivore damage.

The ability of plants to respond to herbivory is inversely proportional to the extent of the herbivory since the reserves utilised in induction can be exhausted (Raffa & Berryman 1983, Christiansen & Ericsson 1985, Christiansen *et al.* 1987). As a result failure to control herbivore populations at a low level can result in reduced defenses, rapid increases in herbivore density and be followed by plant death (Berryman *et al.* 1989). The picture that emerges, therefore, is that induced responses may have a major role in limiting, rather than eliminating, the populations of insects which are able to overcome the constitutive defenses of a particular plant. This strategy would benefit the plant more than an aggressive approach, aimed at reducing the populations of herbivores, since the latter would rapidly select for herbivores unaffected by the induced compounds. However, in some cases herbivores have been observed to overcome induced responses by simple density on the plant (Hodges *et al.* 1979, Berryman *et al.* 1989). Rhoades (1985a) points out that opportunistic herbivores, unable to minimise the effect of plant defenses, may take advantage of plants suffering stress. By overwhelming plants insects create severe conditions of stress seriously impairing the plants ability to respond to attack.

1.6 Plant pathogens and plant insect interactions.

Much of the work on plant-insect interactions has been motivated by an interest in insect population dynamics and host selection (Bernays & Graham 1988, Edwards & Wratten 1982 , Faeth 1986, Rossiter *et al.* 1988) and has concentrated purely on the

plant-herbivore interactions (Wallace & Mansell 1976, Denno & McClure 1983, Hedin 1983, Brattsten & Ahmad 1986). This approach runs the risk of over simplifying complex interactions between plants and their environment.

Plant pathogens exert pressures on plants which select for chemical defenses. Two groups of micro-organism, the fungi and the viruses, are responsible for the majority of infective plant diseases although bacteria also account for some diseases (Dickinson & Lucas 1977). Land plants appeared in the Silurean period (440 - 395m years B.P.) and some fossil plants from this age show clear signs of fungal infection (Kevan *et al.* 1975). Pathogens, therefore, have long exerted selection pressures on plants. The subject of plant chemical defenses induced by microbial innoculation is well researched (see Kuc 1976 and Brooks & Watson 1985). Such defenses are known as phytoalexins.

In situations where plants are under pressure from herbivores and pathogens, secondary metabolites in plants could be multifunctional (Whittacker & Feeny 1971, Harborne 1985). Herbivorous insect fossils are known from the lower Devonian period (395 - 370m yrs B.P.) and a variety of Devonian arthropods have been implicated in plant pathogen transmission (Kevan *et al.* 1975). Insects are known to act as vectors for plant diseases. Molnar (1965) found that mortality in *Abies lasiocarpa* (Hook) Nutt. was caused by a bark beetle-fungus association. One of the earliest reports of herbivore induced responses (Reid *et al.* 1967) involved a beetle-fungus association. Such responses effectively confine pathogen invasion thus resisting serious infection (Christiansen & Ericsson 1985), in addition to controlling the extent of herbivory.

Although herbivore-plant pathogen associations are rarely obligatory for fungal disease spread (Dickinson and Lucas 1977) insects can readily transport spores between host plants. Various authors (Kennedy 1951, van Emden et al 1969, Macias and Mink 1969, Hodgson 1981, Ajawi and Dewar 1982) have reported that aphids prefer, and are more successful, on diseased tissue. Clearly herbivores which actively seek out diseased plants are likely to act as disease vectors especially when disease is not widespread and herbivores are forced to utilise healthy tissue in addition to diseased tissue.

In the absence of vectors many diseases spread by airborne spores (Dickinson & Lucas 1977). These often require wounding of plant tissue to allow infection since the plant cuticle is the primary defense against pathogen invasion (Dickinson & Lucas 1977). Wounding associated with herbivory is likely to account for the most frequent source of infection by opportunist pathogens.

Costs associated with defense will select for plants producing secondary metabolites which confer protection against both herbivores and pathogens. It is not surprising, therefore, that there are examples of phytoalexins which are toxic to insects or inhibit larval growth as well as being toxic to pathogens (Sutherland *et al.* 1980, Smith 1982).

It would seem that there is a close link between herbivory and disease. One of the questions posed in the co-evolution argument is whether herbivory often constitutes a selective pressure to the host. Evidence of direct selective pressure from herbivores alone is lacking (Strong *et al.* 1984, Bernays & Graham 1988, Schultz 1988b). However, plants are unlikely to be able to distinguish between loss of fitness due to herbivory and loss of fitness due to

associated pathogen infection so it is probable that herbivores and pathogens in an association, however loose, exert a selective pressure on plants resulting in adaptations to either or both.

1.7 The chemical basis for induced responses

Although much of the work on induced responses concentrates on palatability bioassays, many attempts have been made to find a chemical basis for the observed phenomena. Such attempts have tended to focus on broad classes of secondary metabolites rather than specific compounds. Faeth (1986) found that condensed tannins were induced in oak (*Quercus emorgyi* Port. & Coult.) by leaf chewers and that this correlated with reduced survivorship of leaf miners. Bergelson *et al.* (1986) measured a rise in total phenolics within 24 hours of damage in damaged areas of birch leaves and a corresponding rise in undamaged areas of the same leaves within eight days. These changes in total phenolics corresponded very closely to movement of casebearing moths away from damaged areas and a reduction in growth, measurable within eight days of damage, of larvae on undamaged parts of damaged leaves. Rossiter *et al.* (1988) found that rises in red oak leaf phenolics correlated with insect damage and that both insect and artificial damage reduced the food quality of the leaves. In an investigation of long-term induced responses Tuomi *et al.* (1988) found that damage to birch leaves in June and August of one year led to increased leaf phenolics the following July, the largest response being observed in trees sustaining damage in June.

By correlating foliage phenolics with herbivore damage Haukioja *et al.* (1985b) found some evidence supporting the hypothesis that phenolics have a defensive role, although their results are far from

convincing in this respect. However, since the total phenolic content of leaf extracts did not correlate with the ability of these extracts to precipitate haemoglobin the authors question the importance of total phenolic measurements as indicators of leaf palatability. Hartley and Lawton (1987) found no correlation between reduced herbivory on damaged plants and either total phenolics or tannins. Bernays and Graham (1988) use this lack of clarity to argue that plant chemistry is unlikely to be important in herbivore dynamics.

Some of the confusion may be due to lack of specificity in chemical measurements. As discussed in Section 1.3 the plant apparency theory (Feeny 1976, Rhoades & Cates 1976) assumes that phenolics and tannins have general antifeedant activity. This assumption has now been questioned (Zucker 1983, Haukioja *et al.* 1985b, Mole & Waterman 1987, Meyer & Karasov 1989).

Thompson *et al.* (1989) observed increased grazing of fir twigs in thinned stands even though concentrations of secondary metabolites in the thinned stand twigs was higher. When Tahvanainen *et al.* (1985a) tried to explain differences between the palatability of a number of willow species (*Salix* spp) to mountain hares (*Lepus timidus*) they found negligible differences in willow total phenolics but large differences in specific phenolic glycosides and leucoanthocyanins. In a separate experiment Tahvanainen *et al.* (1985b) found that different willow phenolic glycosides could act as deterrents or feeding stimulants on leaf beetles. The success of sawflies on willow is also mediated by specific phenolic glycosides and the adult insects exhibit strong preferences for the willow species to which they are best adapted (Roininen & Tahvanainen

1989). Analogous results have been found for terpenes (Cates *et al.* 1987) and isomeric linear furanocoumarins (Berenbaum *et al.* 1989). Scriber *et al.* (1989) report that a single phenolic glycoside, tremulacin from quaking aspen (*Populus tremuloides* Michx.), exhibits differential toxicity to different subspecies and hybrids of the same species of herbivore (*Papilio glaucus* L.).

A better relationship between induced reduction in leaf quality and changes in leaf chemistry may become apparent as chemical analyses improve and more detailed feeding trials using natural herbivores are undertaken. Schultz (1988b) argued that too little is known about plant chemistry and that this is retarding research in the field.

One study which investigated the effect of herbivory on specific compounds (Tallamy 1985) indicates that *Cucurbita pepo* L. responds to *Epilachna borealis* Fab. damage by increasing levels of cucurbitacins. These compounds are known to have anti-feedant properties and the insects were observed to display specialised feeding behavior to overcome the plant response.

Bryant *et al.* (1985) have shown that mature willow shoots respond to damage by reverting to juvenile shoots. Although both have similar levels of total phenolics the juvenile tissue is far more resistant to grazing due to far higher concentrations of specific phenolic glycosides and leucoanthocyanins (Tahvanainen *et al.* 1985b). Whether this morphological change, accompanied as it is by a chemical change, can be classed as an induced chemical response is a debatable point.

1.8 Priorities for this study.

The present study aims to investigate induced responses to herbivory in a long established ecosystem. Although artificial damage will be utilised, emphasis will be on changes in response to natural herbivory. Due to limitations of time no attempt will be made to investigate long-term induced responses. Plant chemistry will, however, be monitored throughout each growing season to account for the possibility that the magnitude of induced responses vary with time.

Myrica gale, an aromatic shrub common in Scottish wetlands, is used throughout the study since it grows in relatively undisturbed ecosystems, it has been the focus of various physiological and ecological studies (Chapter 2) and its chemistry is very interesting (Chapters 3 & 4). The emphasis in the chemical analyses will be on separation and identification of specific secondary metabolites. However, where appropriate, quantitative analyses will also be undertaken.

Bioassays will concentrate on micro-organisms isolated from the population of plants under investigation. In view of the limitations of expertise and equipment palatability tests involving natural herbivores will take a low priority.

Chapter 2. The study site and study plant.

2.1 The study site

2.1.1 Geography and Biology of Flanders Moss

Flanders Moss, thirty miles north of Glasgow, is an almost ideal site for this type of study (Fig 2.1). It is a raised peat bog of approximately 10 Km^2 (Ratcliffe 1964) and is a remnant of a much larger area of raised peat bog which began to accumulate on estuarine beach deposits 5500 years ago (Newey 1966). Core samples indicate deposition of Sphagnum/Calluna oligotrophic peat throughout its history.

Pollen in the core samples suggest that the community on the moss has been relatively stable in the past three to four thousand years (Newey 1966). Although *M. gale* is not represented in Newey's list *Corylus avellana* L. (hazel) pollen occurs at all depths in the peat. A more recent study (Edwards 1981) demonstrates that the pollen of *M. gale* and *C. avellana* are indistinguishable using light or phase contrast microscopy and can only be readily distinguished by scanning electron microscopy. Edwards concludes that occurance of *M. gale* pollen is largely underestimated in Scottish records. Since *C. avellana* is not a plant usually associated with peat bogs (Clapham, Tutin & Moore 1987) and is not presently found on Flanders Moss (Ratcliffe 1964, Bannister 1977), it seems likely that much, if not all, of the pollen Newey attributed to *C. avellana* came from *M. gale* and that a population of *M. gale* has existed on Flanders Moss for thousands of years.

The Flanders Moss peat is up to 10m deep, has very low pH (3.3-3.7 [Ratcliffe 1964]) and is ombrotrophic, deriving all new

Figure 2.1 A view across Flanders Moss displaying a swathe of reddish sweet gale, prior to budburst, in the foreground.

moisture and nutrients from rain (Bannister 1977). A feature of ombrotrophic bogs is high deficiencies of telluric minerals such as calcium (Schwintzer 1978). A Calluna-Eriophorum nodum is extant over much of the moss but this merges into a Molinea-Myrica nodum towards the edges and in flushes (Ratcliffe 1964). The moss is especially interesting to botanists due to the presence of Labrador Tea (*Ledum groenlandicum* Retz.) and Bog Laurel (*Kalmia polifolia* Wangenh.), both rare in the British Isles (Bannister 1977). It is also the most northern site where wild rosemary (*Andromeda polifolia* L.) is found.

The moss supports a population of blue hares (*Lepus timidus*) and a number of deer species. Adders (*Vipera berus*) are also common and frequently encountered on sunny mornings.

2.1.2 History of Flanders Moss

Flanders Moss is a remnant of a raised peat bog which extended over the Carse of Stirling, 24 km long and between 6 and 10 km wide. While the peat bog extended over the whole area it was a major obstacle to transport and this may have been a factor influencing the position of Stirling, which is adjacent to the narrowest part of the bog (now Causewayhead). The area was considered to be totally unproductive and its removal, in the eighteenth and nineteenth centuries, from a large area of the Carse of Stirling was seen as a great improvement to the area (Cadell 1913).

The removal of Blair Drummond Moss was initiated by Lord Kames, who took over the Blair Drummond estate in 1766, and was continued after his death, in 1782, by his heir (Cadell 1913). They achieved this remarkable feat by employing Highlanders, who had been evicted

from their homes during the Highland clearances. Each man was allotted 10 Scots acres of moss, given tools, timber for a house and 8 bushels of meal for sustenance. The men then had the lease rent free for seven years to establish a smallholding.

The peat was dug out and floated down channels, constructed at Lord Kames' expense, to the River Forth. As the cleared area expanded away from the river the channels required more sophisticated construction, to maintain a gradient, and in one case specialist engineers were employed to build a very large water wheel to feed the channel. However, when the operation was reaching its zenith fishermen in Edinburgh complained of the river being fouled so Lord Kames' tenants began drying the peat to sell.

Rather than construct a house a tenant would leave a thick walled shell of peat intact and roof it once it had dried. Although the conditions were extremely damp for some years the families living in these dwellings enjoyed better than average health, which Cadell (1913) ascribed to the antiseptic qualities of the peat.

This method of clearing the peat proved to be highly successful and, after initial reservations about the settlement of Highlanders associated with the Stewart cause, popular throughout the region. By the 1811 census a community of 150 families lived on 1440 imperial acres of cleared moss. Thus, whilst vastly improving his estate Lord Kames provided work for many destitute families.

During the removal of the moss at Blair Drummond the remains of a wooden causeway, believed to be Roman, was uncovered. The bones of a whale with neolithic harpoons nearby was also uncovered from the clay below the peat; possibly the earliest indication that the area had once been estuarine. A more detailed and extremely

entertaining, if occasionally over imaginative, account of the history of Flanders Moss is given by Cadell (1913).

2.2 The study plant

2.2.1 Its distribution

The species of plant selected for this study was *Myrica gale* L., a member of the Myricaceae, commonly known as bog myrtle or sweet gale in Britain. Sweet gale grows in wetlands in Western Europe from Spain to Scandinavia, across Northern Europe to North West Russia and in North America from Labarador to North Carolina and Alaska to Oregon. A subspecies or allied species is known from Eastern China. (Clapham *et al.* 1987). It is found from sea level to 600m (Clapham *et al.* 1987, Flatberg 1986). It is restricted to minerotrophic fens (Schwintzer 1985) and ombrotrophic bogs (Ratcliffe 1964, Bannister 1977).

2.2.2 Description.

M. gale (Fig 2.2) is a deciduous shrub which grows to 2.5m (Clapham *et al.* 1987). The leaves are simple, obovate and usually dentate at the apex. The leaves on mature stems range from 9mm x 3mm to 46mm x 13mm. It is a normally dioecious plant although individual plants can change sex on successive years and are occasionally monoecious, having male and female flowers on the same branch (Lloyd 1980). Catkins open two to three weeks prior to budbreak and pollenation is wind assisted (Clapham *et al.* 1987). Fertilization is delayed, occuring five weeks after pollenation (Hakanson 1955).

Budbreak is later in *M. gale* than in other deciduous plants in

Figure 2.2 Myrica gale L. growing in the Glasgow botanical gardens.

the community, typically occuring two weeks after flowering, and the first leaves reach maturity approximately six weeks later (Sprent *et al.* 1978, Schwintzer 1979). On Flanders Moss, in 1987, 1988 and 1989, flowering was observed between early and late April. Earliest budbreak was observed at the end of April and in the majority of plants budbreak occured in the first week of May. The timing of budbreak on Flanders Moss was similar to the findings of Schwintzer (1979) in North America but a month earlier than budbreak reported from the lower northern slopes of Schiehallion in the Tummel Valley, 60km north of Flanders Moss, in 1975 and 1976 (Sprent *et al.* 1978). On Flanders Moss mature fruit can be found in late July and August. End of season leaf senescence occurs between mid October and early November.

Once a plant is established it spreads vegetatively by rhizome extension and in favourable habitats can form dense clumps of branched stems (Bannister 1977, Schwintzer 1983). In less favourable habitats it exhibits sparse cover of short stems (up to 50cm) (Bannister 1977, personal observation). On Flanders Moss the stems rarely exceed 1.5m in height or 7mm in diameter.

Schwintzer (1983), working on a bog in North America, found no stems over seven years old and calculated that 94% of stems were less than five years old. The most rapid shoot growth was observed in the first three years. Juvenile stems commonly emerge, under the cover of mature stems, during June and July. The dimensions of leaves on these stems are typically twice those of leaves on mature stems.

New shoots were observed to develop from the primordia after budbreak, maturing into woody branches by the following year. For

the purposes of this thesis a current year's growth will be referred to as a shoot and the entire axial growth from a single root bud will be referred to as a stem.

2.2.3 The association between sweet gale and Frankia sp.

Sweet gale grows in association with a nitrogen fixing actimomycetous endophytic fungus, *Frankia* sp. (Schwintzer 1979). Root nodules, first described by Brunchorst (1886), are characteristic of this association. A fungus was observed in the nodules (Brunchorst 1886, Moller 1889) and named *Frankia* sp. A role in nitrogen fixation was proposed for them by Bottomley (1912) although he claimed that a bacterium was associated with sweet gale in the root nodules. Bond (1949) was the first to clearly demonstrate that the nodules were involved in nitrogen fixation but for many years attempts to isolate the causal organism were unsuccessful (Fletcher & Gardner 1974). The first successful isolation and reinoculation of *Frankia* sp. was in another member of the Myricaceae, *Comptonia peregrina* L. (Callaham *et al.* 1978). A number of strains of *Frankia* sp. have now been isolated from *M. gale* (Baker 1982, St Laurent & Lalonde 1987). It is possible that *Frankia* is not the only mycorrhizal fungus found in association with *M. gale*. Rose (1980) found a vesicular-arbuscular fungus associated with *M. gale*, which was tentatively identified as *Glomus tenuis* (Greenhall), in a sphagnum peat bog. However, much of the recent work on nitrogen fixation in *M. gale* has concentrated on its association with *Frankia* sp. (Wheeler 1984, Schwintzer 1985).

Biological nitrogen fixation has a high energy requirement (Haaker *et al.* 1980). The phosphate requirement is also large

(Gardner *et al.* 1984). Plant derived carbon, in the form of succinate or other dicarboxilic acids, is utilized in the endophyte metabolism. Energy from catabolism is converted to ATP, much of which is then used during nitrogen fixation (8 ATP per N reduced to NH_3). Further ATP (3.5 - 5.3 ATP/NH_3) is required for combined nitrogen assimilation into amino acids and amides (Wheeler 1984).

Root nodules generally live for three years although a small percentage (12%) survive for up to five years (Schwintzer *et al.* 1982). Young nodules exhibit maximum nitrogen fixing efficiency since only the young infected tissue has high nitrogenase activity but both old and young tissue has high metabolic activity. Thus, as the proportion of old to young tissue increases the nodules become less efficient (Schwintzer *et al.* 1982). However, when shoot growth is restricted by lack of nitrogen, photosynthate can be channelled into root nodule growth thus increasing the nodule capacity for nitrogen fixation (Tjepkema 1984).

It seems likely that stem lifespan (94%: 3yrs or less) is related to nodule lifespan (88%: 3yrs or less). This strongly suggests that, at least in ombrotrophic conditions, sweet gale is in an obligatory association with *Frankia* sp. It has also been calculated that nodule production accounts for over 40% of the plant's nitrogen requirement (Schwintzer 1983) which lends extra support to this idea. This, in turn, indicates that, in ombrotrophic conditions, sweet gale will be restricted to conditions which favour *Frankia* sp. growth.

2.2.4 Environmental conditions necessary for sweet gale success.

Grime (1977) produced a useful simple classification system for

plants based on three primary strategies: competitive, stress tolerant and ruderal. By this system sweet gale has many of the characteristics of competitive plants, for example a dense canopy of mesomorphic leaves, well defined peaks of leaf production and conspicuous persistent litter. However, sweet gale is restricted to nutrient stressed sites. Clearly its association with the nitrogen fixing *Frankia* sp. allows it to exhibit these characteristics in nutrient low soils. In practical terms this means that sweet gale, by virtue of its association with *Frankia* sp., can outcompete stress tolerant species which it overgrows.

Sweet gale does best in moderately wet sites. Extremely wet conditions do not favour growth and it is susceptible to drying (Schwintzer & Lancelle 1983). On lake shores it does best between the mean summer water level and high water level (Larsson 1976, pers. obs.). Sweet gale grows throughout Flanders Moss but shows most luxurient growth at the edges (Bannister 1977).

Bannister (1977) suggests that this distribution of sweet gale could reflect the degree of aeration in the water. Work on the affect of soil aeration on the distribution of *Molinia caerulea* L. (which is closely associated with sweet gale at the edge of Flanders Moss) lends support to this hypothesis. Armstrong & Boatman (1967) found that oxygen was not detected more than 6cm below the surface in peat bog sites where water was stagnant but could be detected to a depth of 16cm in flushes. Since Flanders Moss is raised above the surrounding farmland the moss edges, where sweet gale is most successful, slope down and this gradient will generate movement in the water immediately below the surface (Bache & MacAskill 1984) increasing the availability of oxygen to the roots of sweet gale.

Bond and MacConnell (1955) demonstrated the importance of oxygen in non-leguminous nitrogen fixation. As part of his studies on sweet gale Bond (1961) described the growth of negatively geotropic nodule hairs in response to a reduction in soil oxygen and these specialized roots have been shown to supply the nodules with oxygen (Silvester *et al.* 1988).

In addition to morphological adaptations the active tissue of sweet gale nodules has been found to contain both oxygenated and deoxygenated haemoglobin (Tjepkema 1983a) strongly suggesting the existence of an active oxygen transport system. Tjepkema (1978) demonstrated that soil oxygen alone is utilized in nitrogen fixation.

The effect of oxygen stress on sweet gale growth in the field was demonstrated by Schwintzer (1985) who observed that anaerobic conditions (site flooding) in spring and early summer retard nitrogen fixation by *Frankia* sp. associated with sweet gale and this resulted in reduced root and shoot growth.

It seems likely that the distribution of sweet gale in wetland habitats is dictated by the conditions essential for nitrogen fixation by *Frankia* sp. This would explain its preference for soils where the water around the roots is constantly changing. However, young nodule tissue exposed to excess oxygen exhibits a rapid reduction in nitrogenase activity (Tjepkema 1983b). It is not surprising, therefore, that sweet gale does not grow well in dry conditions.

Another edaphic factor which influences nitrogenase activity in nodules is soil temperature. Sprent *et al.* (1978) suggested that the timing of leaf burst is controlled by soil temperature and its

effect on nitrogen fixation. They calculated from field observations that a minimum temperature of 8°C was required before budburst. Schwintzer *et al.* (1982) showed that in laboratory conditions nitrogenase activity in sweet gale nodules was negligible below 5°C. The effect of this phenomenon on sweet gale was demonstrated in greenhouse conditions where a minimum temperature of 6°C was maintained and budburst occured in February. Work on the nodules of *Alnus rubra* Bong., induced by infection with *Frankia* sp., demonstrated that the efficiency of oxygen utilization in nitrogen fixation by nodules increases with temperature with optimal efficiency being achieved between 16°C and 28°C (Winship & Tjepkema 1985).

It appears that the distribution of sweet gale is determined by factors effecting the *Myrica/Frankia* association. Furthermore, the timings of sweet gale flowering, budburst, fertilization and fruit development are associated with edaphic conditions favourable for nitrogen fixation. It can be concluded that the availability of nitrogen governs the ecology and physiology of sweet gale to a large degree.

2.2.5 The nitrogen budget of sweet gale.

Schwintzer (1983) calculated that nitrogenase activity in the nodules can account for up to 43% of the plants annual nitrogen requirement. The remaining nitrogen is recycled from the litter. In sweet gale over 70% of the nitrogen is transported in the sap in the form of amides, serine making up more than 20% of the remainder (Wheeler 1984).

After winter dormancy nitrogenase activity becomes measurable

in nodules shortly after budbreak and peaks approximately six weeks later (Schwintzer 1979, Schwintzer *et al.* 1982, Sprent *et al.* 1978). The activity remains stable until late August and diminishes to nothing, after leaves have been shed, by late October. Good ambient conditions for nitrogenase activity persist into late September so the observed drop in activity during September probably reflects that the plant diverts assimilated carbohydrate away from root nodules and into reserves for the following spring (Wheeler 1984).

Leaf nitrogen, measured as a proportion of leaf biomass, is greatest at budbreak and decreases as the leaves mature (Fig 2.3). Total leaf and new stem nitrogen increases after budbreak (Fig 2.4) and peaks when leaf biomass peaks and nitrogenase activity is at its highest (Sprent *et al.* 1978). Nitrogen from stem and root tissue is translocated to leaf tissue early in the growing period, when nitrogenase activity in the nodules is minimal, and reserves are replenished in stems and roots after leaves reach maturity. At this time leaves cease to be a major nitrogen sink yet nitrogenase activity is at its peak so nitrogen availability increases. This also coincides with fruit reaching maturity.

In a comparison of leaf nitrogen (measured as % dry wt) in sweet gale and *Chamaedaphne calyculata* Moench., which were growing together, Schwintzer (1983) produced a higher figure for sweet gale (2.36%) than for *C. calyculata* (1.71%). Small (1972b) reported a similar proportion (2.5%) in the leaves of sweet gale growing in boggy woodlands. This was a higher percentage of nitrogen than he measured in any plant species from raised peat bogs (Small did not investigate *M. gale* from raised peat bogs). Sprent *et al.* (1978) have shown that leaf nitrogen drops throughout the growing season

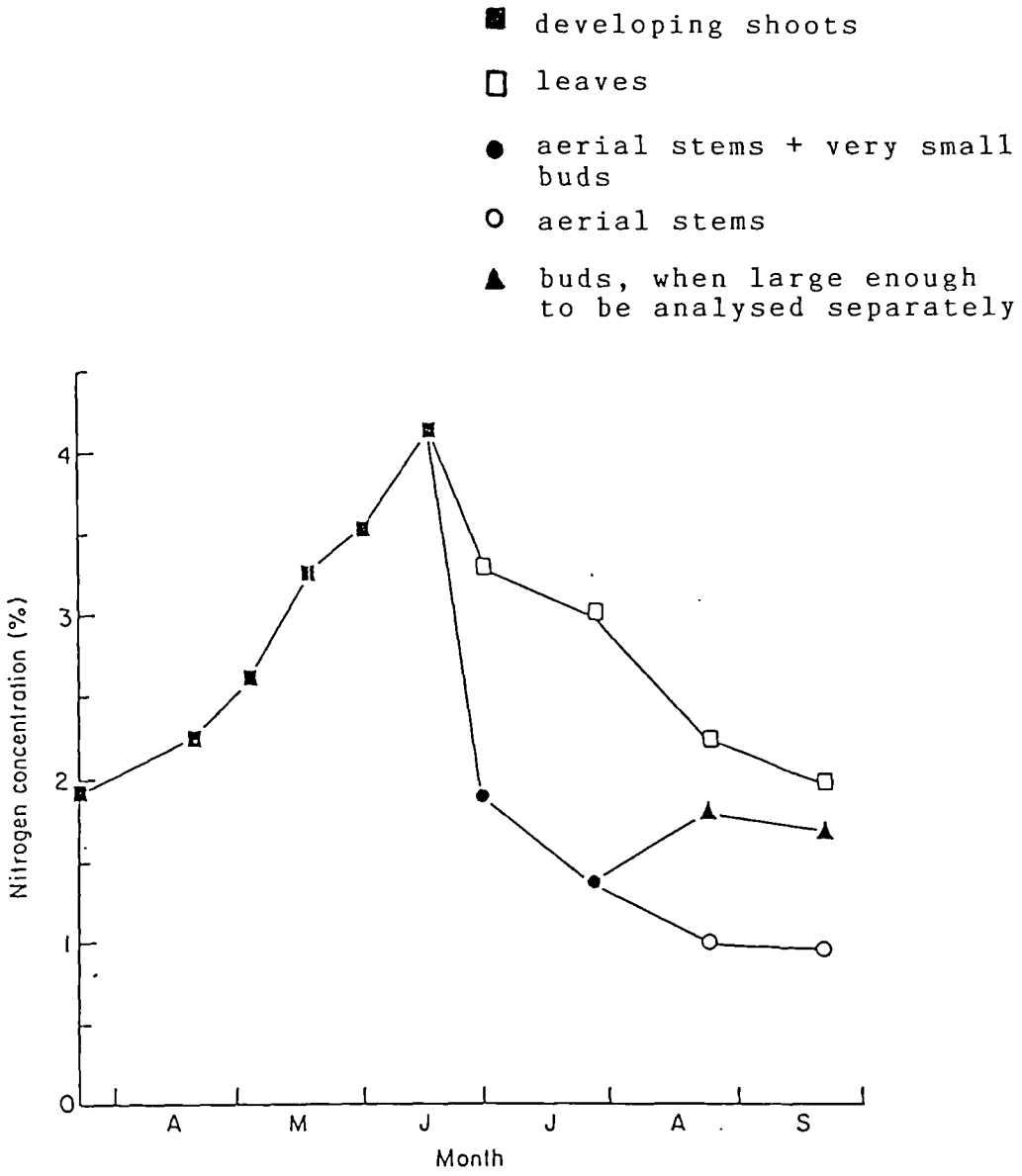


Figure 2.3 The concentration of nitrogen (% D.W.) in new shoot growth. (printed with permission: copyright Sprent et al. 1978)

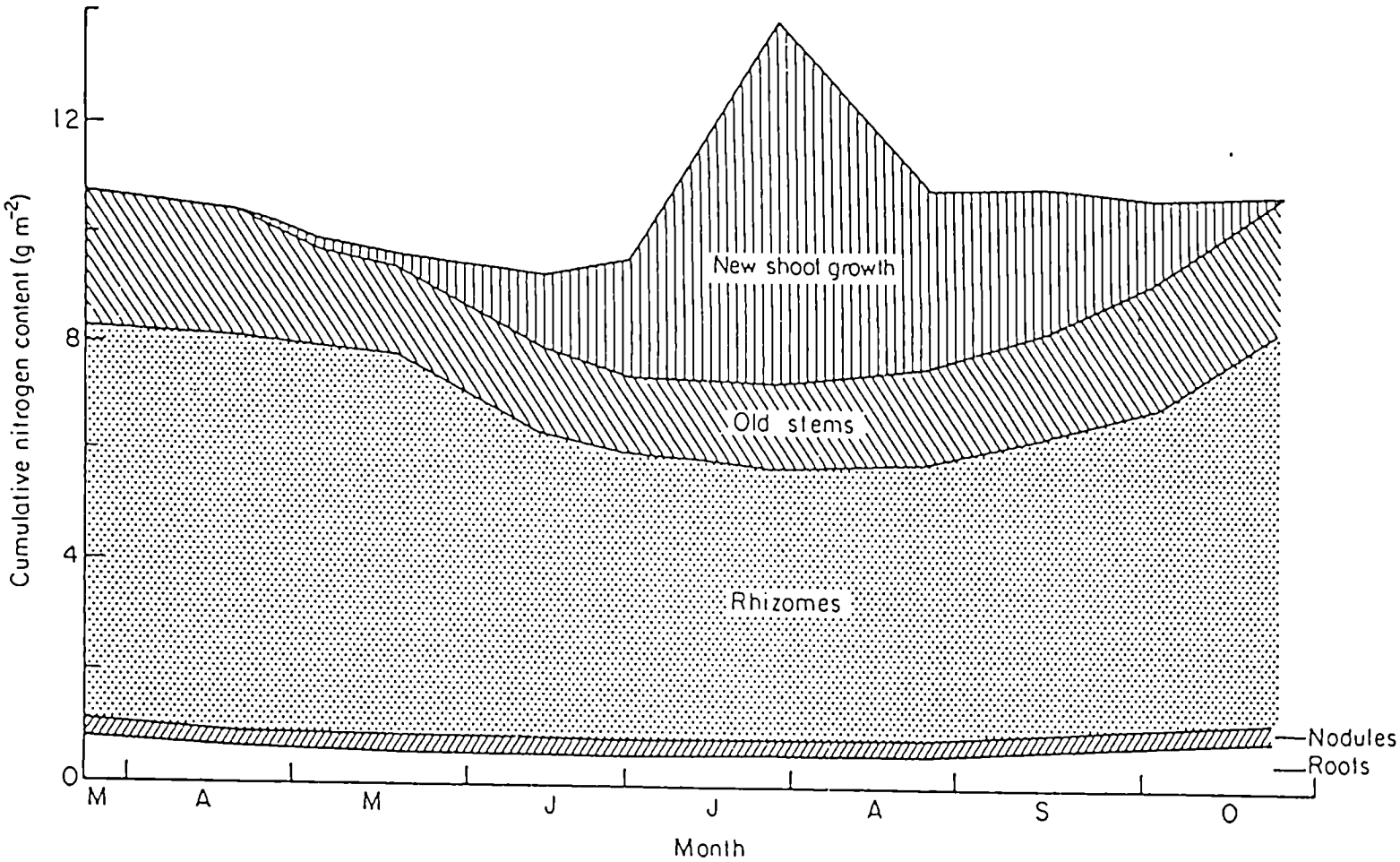


Figure 2.4 The allocation of nitrogen in sweet gale through the growing season. (printed with permission: copyright Sprent *et al.* 1978).

from over 3% just after budburst to around 2% towards the end of September (Fig.2.3). In terms of nitrogen, therefore, sweet gale provides the most nutritious tissue, for herbivores and pathogens, of any plant species on raised peat bogs.

Most bogland plants reabsorb much (up to 70%) of their leaf nitrogen before leaf loss (Small 1972a). It can be calculated from the figures of Sprent *et al.* (1978) that sweet gale reabsorbs no more than 42% of nitrogen present in leaves when they initially reach maturity. Sweet gale leaves are frequently richer in nitrogen when shed (1.73%) than the leaves of other bogland plants in mid summer, long before nitrogen has been reabsorbed (Small 1972b).

Clearly, the nitrogen budget of sweet gale differs from other plants growing under similar conditions. The loss of nitrogen to the litter makes sweet gale an ecologically important member of the community, a point originally made by Bond (1951, 1967) and emphasised by Sprent and Scott (1979). It is also of importance to sweet gale since more than 50% of the annual nitrogen requirement is not fixed and must, therefore, be absorbed from the soil. In ombrotrophic conditions almost all of this nitrogen must come from decomposing litter. However, since sweet gale is restricted to sites of moving soil water soluble nitrogen made available by rapid litter decomposition could easily be leached. Furthermore, high combined nitrogen in the soil inhibits elemental nitrogen fixation (Stewart & Bond 1961).

It follows that slow litter decomposition would favour sweet gale growth. None the less, sweet gale litter has been shown to have a near optimal carbon to nitrogen ratio for decomposition by microorganisms (Allard & Moreau 1986). It is very interesting,

therefore, that sweet gale litter has been shown to decompose less rapidly than associated species and this results in significantly less leaching (Richard & Moreau 1982, Richard *et al.* 1982). Richard *et al.* (1982) suggest that leaf cuticle strength may effect colonization of litter by saprophytic fungi. However, the existence of antifungal secondary metabolites in the leaves would also influence this aspect of the nitrogen budget of sweet gale.

2.2.6 The herbivores of sweet gale.

A complete list of herbivores reported from sweet gale in Scotland is given in Table 2.1. No systematic attempt was made to collect and identify all the herbivores of sweet gale on Flanders Moss. However, herbivores of interest were collected and, when possible, identified.

Two capsid bugs were collected, *Lygocoris spinolai* (Meyer-Dur) common from early to mid summer, and *Lygus rugulipenis* (Poppius) found in September. *L. spinolai* is common on sweet gale (Southwood & Leston 1959). *L. rugulipenis*, however, has not been recorded on sweet gale before. It is polyphagous and there are generally two generations every year. During the second, autumn, generation in September the species is numerous and very mobile (Southwood & Leston 1959). Although it appeared to be associated with the sweet gale on Flanders Moss (Hancock pers. comm.) it is unlikely to be a major herbivore. Both species of capsid bugs were restricted to the area of most luxuriant sweet gale growth along the upper outer edge of the moss.

In the laboratory *L. spinolai* (Fig 2.5) was observed to feed by inserting its proboscis through the leaf upper epidermis and

Table 2.1 The phytophagous insects which feed on sweet gale in Scotland. (compiled from the Phytophagous Insects Data Bank (NERC), with additional data provided by B.G. Hancock, K. Bland and M. Young).

Species	Order	Family	No of hosts	Frequency on M. gale
Lygocoris spinolia	Hemiptera	Miridae	6	common
Lygus rugulipennis			many	rare
Plesiocorisrugicollis			5	common
Aphrophora alni		Cercopidae	10	?
A. alpina			Specific 1	?
Phileanus spumarius			24	?
Myzocallis myricae		Callaphididae	Specific 1	?
Phenacoccus aceris		Pseudococcidae	9	?
Ypsolopha parenthesella	Lepidoptera	Yponomeutidae	5	occasional
Coleophera viminitella		Coleophoridae	5	common
C. seratella			4	occasional
C. pyrrhulipennella			?	very rare
Teleiodes paripunctella		Gelechiidae	5	common
Acleris aspersana		Tortricidae	?	common
A. caledoniana			7	common
A. lipsiana			5	common
A. maccana			2	common
A. notana			3	common
A. rufana			4	common
Aphelia viburnana			3	common
Archips rosana			?	common
Argyrotaenia pulchellana			4	common
Choristoneura hebenstreitella			7	rare
Clepsis senecionana			5	common

Table 2.1 (cont).

Species	Order	Family	No of hosts	Frequency on M. gale
Epinotia caprana	Lepidoptera	Tortricidae	2	common
Hedya atropunctana			3	common
Orthotaenia undulana			4	occasional
Pammene luedersiana			Specific 1	very rare
Philedonides lunana			7	common
Spilonota ocellana			many	occasional
Macrothylacia rubi		Lasiocampidae	many	occasional
Cleora cinctaria		Geometridae	7	occasional
Ematurga atomaria			11	occasional
Lycia lapponaria			4	common
Rheumaptera hastata			5	common
Selenia dentaria			13	occasional
Semiothisa brunneata			2	rare
Diacrisia sannio		Arctiidae	10	occasional
Acronicta euphorbiae ssp. myricae		Noctuidae	15	common
A. menyanthidis			9	common
A. rumicis			many	occasional
Blepharita adusta			10	occasional
Eurois occulta			13	occasional
Lacanobia contigua			13	occasional
Orthosia gracilis			5	common
Papestra biren			16	occasional
Polia hepatica			10	occasional
Agrilus viridis	Coleoptera	Elateridae	16	?
Otiorhynchus morio		Curculionidae	13	?
Rhynchaenus iota			4	locally common

removing all the tissue, bounded by the smallest visible lattice of veins, between the abaxial epidermis and adaxial epidermis, creating hollows in either surface. The remaining layers of epidermis rapidly died leaving small brown spots on the leaves. Usually the spots were regularly distributed. If, however, feeding had been concentrated in one part of the leaf the whole section of the leaf would die.

The other order of herbivores causing major damage to sweet gale on Flanders Moss was the Lepidoptera. The majority of lepidoteran larvae found grazing on sweet gale leaves were members of the Tortricidae although *Teleoides paripunctella* (Thun.) (Gelechiidae) and *Rheumoptera hastata* (Prout)(Geometridae) were collected. None of the tortricids were identified as all the pupae collected for hatching proved to contain parasitoids.

The feeding habit of the majority of moth larvae on sweet gale is to spin or roll leaves to form tents around the apical bud of a shoot (M. Young pers. comm.) and chew leaves inside, although on Flanders Moss the immature apical leaves were rarely eaten. The tents became common during July and could be found until late September.

Such tents were abundant on sweet gale in the interior of the moss but were very infrequent (on < 9% of plants) at the edge of the moss where capsid bugs were abundant. The sweet gale population could, thus, be split into two sub groups according to position on the moss and this was reflected in the type and density of herbivore.

Capsid bug damage became apparent before moth larvae damage so the capsid bug distribution was unlikely to be influenced by the

Figure 2.5 Lygocoris spinolia on sweet gale leaves.

An immature (3rd or 4th instar) capsid bug.

A mature capsid bug drinking with immature capsid bug on the left.

distribution of moth larvae. It is possible that the leaf quality of sweet gale at the edges of the moss, where the gradient ensured water movement, is better and this favours *L. spinolai* growth. In addition to growing on a gradient, much of the edge sweet gale is also less exposed to wind, mainly by virtue of its dense luxurient growth. This would afford the capsid bugs, which were always observed to feed on the upper surface of the leaves, some protection against desiccation, a major problem for insects (Southwood 1973).

The distribution of the moth larvae may have been influenced by the distribution of capsid bugs. It has been demonstrated that late season herbivores avoid leaves damaged early in the growing season, either by early season herbivores (Faeth 1986), or artificially (Silkstone 1987). Faeth (1986) also demonstrated that the survivorship of late season herbivores was reduced when larvae were given previously damaged leaves and Harrison and Karban (1986a) found that spring feeding by *Platyprepia virginalis* Bdv. on *Lupinus arboreus* Sims (bush lupine) reduces the growth and fecundity of summer feeding *Orgyia vetusta* Bdv. females on the same plants. On Flanders Moss moth larvae may be avoiding sweet gale previously damaged by *L. spinolai* and, thus, be restricted to the more exposed plants in the interior of the moss where they escape desiccation by feeding in tents. This phenomenon of late season herbivores avoiding plants which have sustained early season herbivore damage may be mediated by plant secondary metabolites and, in sweet gale, is a possible indication of induced chemical responses to capsid bug feeding.

One lepidopteran, *Coleophera viminetella* Zeller, was found throughout the sweet gale population. The larvae of this species

are leaf miners. Although it was found on the same plants as *L. spinolai* it was restricted to otherwise undamaged leaves. *C. viminetella* was not common and the extent of its damage to leaves was insubstantial. *Coleophera pyrrhulipennella* Zeller was also found mining the leaves of one plant. This is the first recording of this species from sweet gale.

Very little has been published on vertebrate utilization of sweet gale. However, there is good evidence that fallow deer (*Dama dama*) in the New Forest (U.K.) avoid sweet gale even when it is widespread in the community (Jackson 1977). In Sweden the mountain hare (*Lepus timidus*) occasionally utilizes sweet gale although this is the least utilized species in its diet (Lindlof *et al.* 1974). On Flanders Moss there was some evidence of vertebrate browsing on sweet gale stems in February 1987 during a very cold spell. No vertebrate damage was observed at any other time.

2.2.7 Physiological effects of herbivory.

The study plants for the field experiment were selected from the edge of the moss where the main herbivore was *L. spinolai*. In 1987, 1988 and 1989 *L. spinolai* damage became apparent in late May and was widespread by the end of the first week in June. A survey of *L. spinolai* damage was made on August 18, 1987. More than 95% of stems which were allowed to sustain natural damage showed signs of capsid bug feeding. It was estimated that a mean of 53.2% of leaves on each stem had sustained damage.

Sweet gale exhibited a clear physiological response to herbivory. It was observed that 74% of plants which had sustained damage on 50% or more of the leaves were producing new shoots

whereas this figure was 9% for plants with less than 50% of the leaves damaged. Once new shoots were produced damaged leaves were rapidly shed.

In 1987 the new leaves, produced in response to damage, sustained fairly high levels of capsid bug damage. In 1988 sweet gale was again observed to produce new shoots to compensate for herbivory. This flush coincided with a week of high winds and very few fresh signs of damage were subsequently observed.

2.2.8 The pathogens of sweet gale.

The fungi which have been isolated from sweet gale in Britain are listed in Table 2.2. Only two of these species, *Ramularia destructiva* Phil. & Plow. and *Septoria myricae* Trail are primary pathogens in Scotland (Foister 1930, Grove 1935, Ellis & Ellis 1985).

On Flanders Moss very few plants were observed to exhibit the symptoms of disease. Occasionally small (< 10mm diam.) brown spots were observed on a few leaves of otherwise healthy plants. The lower side of these spots appeared to have a fine covering of white hyphae. The spots were very similar to the symptoms of *R. destructiva* (*Ovularia destructiva* [Phil. & Plow] Massee) described from Scotland by Foister (1930).

For most of the season the brown spots were only observed on leaves which had not sustained herbivory. Immediately prior to leaf drop, however, a large number of herbivore damaged leaves developed similar symptoms. At this time many leaves also developed other signs of fungal infection (black spots and discolouration) but infection did not appear to persist into the following year.

Table 2.2 Fungal species previously reported from sweet gale.

Species	Substrate	Symptoms (if pathogen)	Reference
Anthostomella myricae	branch	v. little damage	c
Ciboria acerina	male catkins (on the ground)		a
Cronartium comptoniae	leaves (in U.S.)	rust	e
Cryptodiaporthe aubertii	dead branches		a
Cytospora myricae-gales	dead twigs		d
Dasyscyphus sulohurellus	dead branches		a
Diplodia sp.	twigs (in U.S.)	blight	e
Fusicoccum myricae	dead leaves		d
Gymnosporangium ellissii	leaves (in U.S.)	rust	e
Hyalotricha corticola	dead twigs		a
Pestalotiopsis oxyanthi	dead branches		a
Phoma incommoda	dead leaves		d
Ramularia destructiva	leaves	brown spots	a,b & e
Septoria myricae	leaves	green spots	d & e

Key to references: a: Ellis & Ellis (1985), b: Foister (1930)
c: Grove (1933), d: Grove (1935), e: Pirone (1978)

2.2.9 Uses of sweet gale.

Sweet gale extracts have been shown to inhibit viruses, fungi and bacteria (Chantrill *et al.* 1952, Negueruela *et al.* 1982) and in some cases bioactive compounds have been isolated (Malterud & Faergi 1982).

It is not surprising, therefore, that sweet gale is known for its pharmaceutical properties. It has been used as a treatment for burns and other skin complaints and has been utilized as an emmenagogue and an abortifacient. It has also been used as a treatment for dysentery (Malterud & Faergi 1988).

Sweet gale is most commonly associated with ancient brewing practices. Malterud and Faergi (1982) suggest that the presence of compounds in sweet gale similar in structure to compounds isolated from hops, which provide beer with its characteristic bitter taste and act as conservatives, may explain its popularity as an alternative to hops. The leaves of sweet gale were widely used throughout the middle ages in Europe (although not in Britain, the Netherlands or Belgium) to flavour beer (Behre 1983, Malterud 1982a, Malterud & Faegri 1982, 1988). Earliest records of sweet gale in human settlements come from excavations of a Southern Holland ice age community and a two thousand year old Finnish community but no connection between sweet gale and brewing was established at these sites (Behre 1983). In the twelfth century Saint Hildegard extolled the health giving properties of beer flavoured with sweet gale and in 1152 an order of Danish monks moved their monastery to a site close to a sweet gale community for brewing purposes. The Danish trade in sweet gale was large enough by 1284 to warrant taxation. As late as the eighteenth century Tabernaemontanus (1731)

recommended the use of sweet gale in beer as a preservative, for its good taste and its beer strengthening properties.[1] However, by the late middle ages its use had fallen away since it proved to present a health risk. Too much sweet gale beer was found to cause blindness and even death so, in 1723, a complete ban on the use of sweet gale was established in the North of Germany. That ban was later adopted by many countries and remains in force.

Sweet gale is still used in parts of Scandinavia to flavour liquors (Malterud & Faergi 1982). It has also been recently used to drive out vermin and to repel moths and mosquitoes (Behre 1983, Malterud & Faergi 1988)

[1]Behre refers, in part, to notes by Tabernaemontanus on Gallapfel (Fr: noix de gal) which Behre clearly considers to be the fruit of sweet gale. The identity of this fruit is questionable, however, as the name Tabernaemontus uses for sweet gale (galen) is not mentioned in these notes and the accompanying drawing of the plant looks unlike sweet gale but has some resemblance to oak. Tabernaemontanus does make direct reference to galen in a section on brewing.

Chapter 3. Extraction, separation and identification of phenolics.

3.1 Introduction.

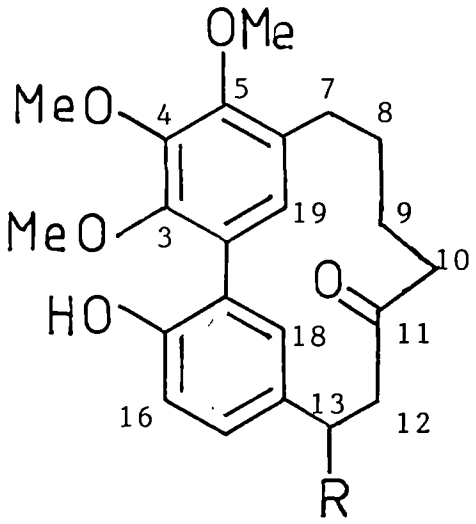
A variety of classes of phenolic compounds have been isolated from members of the Myricaceae. The phenolics of sweet gale have been extensively studied and the plant has been found to produce a number of interesting diarylheptanoids, chalcones, dihydrochalcones and flavonol glycosides. This information is summarised in Figure 3.1.

3.2 Methods.

3.2.1 Extraction of leaf extract for analytical HPLC.

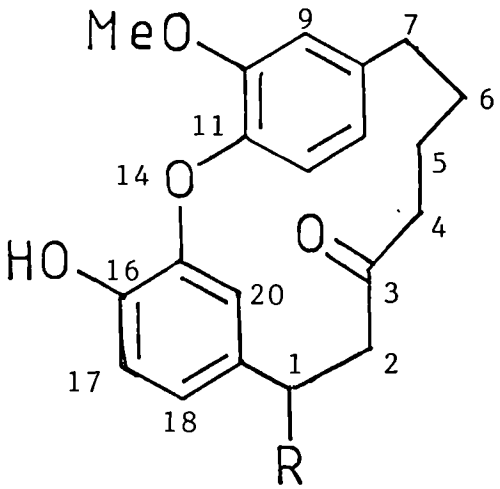
Fresh leaves were ground with acid washed sand and extracted in a Soxlet apparatus with methanol for 2 h. Water was added to create 20% aqueous methanol and the leaf was extracted for another 2 h. The extract was dried under vacuum at 60°C and the residue refluxed with petroleum ether/ethyl acetate (99:1) for 1 h. to extract the non-polar components. The non-polar fraction was decanted and filtered through an 0.45 m nylon 66 membrane filter. The filtrate was taken to dryness and the residue redissolved in 3.5ml petroleum ether/ethyl acetate (90:10) and stored in a freezer. The extract residue which was not soluble in petroleum ether/ethyl acetate was redissolved in 3.5 ml methanol/water (70:30) and filtered through the same membrane filter that had been used for the non-polar extract.

Caffeic acid was added to a 2 ml aliquot of each extract as an internal standard at a concentration of 2.5×10^{-4} M. The extracts were stored at 4°C until analysed by HPLC.



R = OH: Porson
Anthonsen et al. (1975)

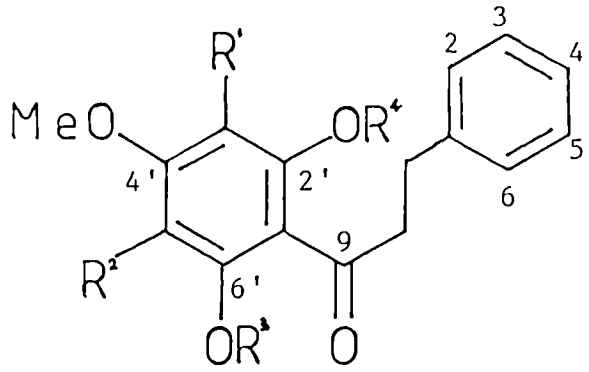
R = H: Myricanone
Malterud (1981)



R = H: Galeon
Malterud et al. (1976)

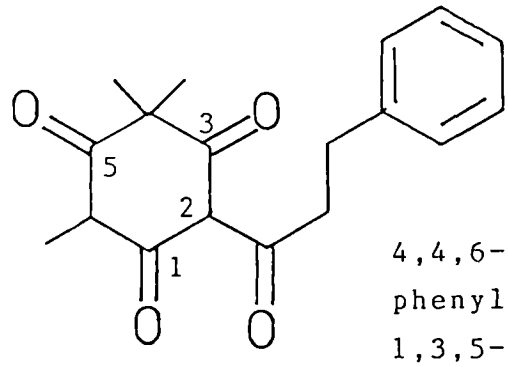
R = OH: Hydroxygaleon
Malterud et al. (1976)

Figure 3.1 Phenolic compounds isolated from Myrica gale.
I. Cyclophanes.

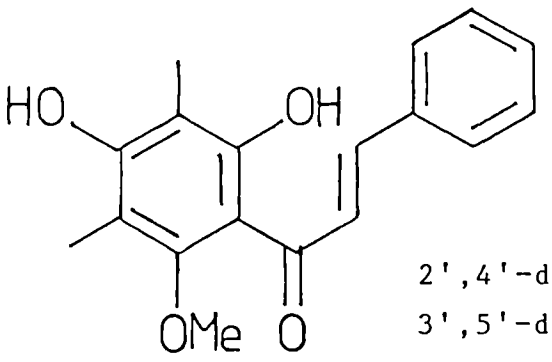


R' = R² = Me, R³ = R⁴ = H: 2',6'-dihydroxy-4'-methoxy-3',5'-methyldihydrochalcone
Anthonsen et al. (1971)

R' = H, R² = Me, R³ = H, R⁴ = Me:
2'-hydroxy-4',6'-dimethoxy-3'-methyldihydrochalcone
Malterud et al. (1977)

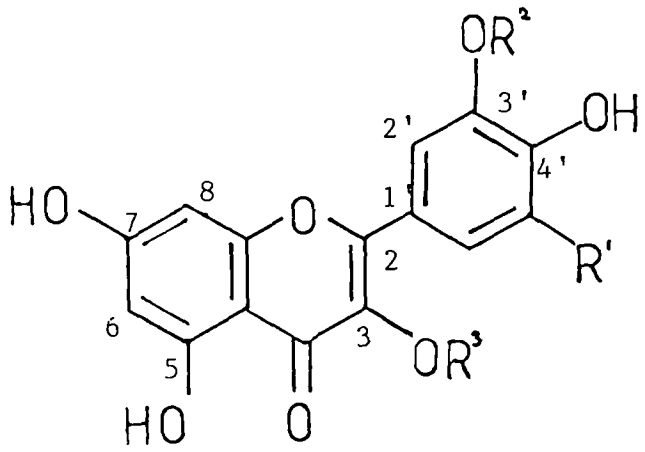


4,4,6-trimethyl-2-(phenylpropionyl)cyclohexane-1,3,5-trione
Anthonsen et al. (1971)



2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone
Malterud et al. (1977)

Figure 3.1 Phenolic compounds isolated from Myrica gale. II. Chalcones and dihydrochalcones.



R' = H, R^2 = H, R^3 = glucosyl: quercetin-3-glucoside*

R' = H, R^2 = H, R^3 = galactosyl: quercetin-3-galactoside*

R' = H, R^2 = H, R^3 = H: quercetin*

R' = OH, R^2 = H, R^3 = galactosyl: myricetin-3-galactoside*

R' = OH, R^2 = R^3 = galactosyl: myricetin-3,3'-digalactoside*

*Bodalski & Rhadkowska-Bodalska (1969)

Figure 3.1 Phenolic compounds isolated from Myrica gale. III. Benzo-γ-pyran derivatives.

3.2.2 Separation of phenolics by analytical HPLC.

Instrumentation: Gilson 303 HPLC ternary gradient system controlled by Gilson 'HPLC Manager' run on an Apple IIe microcomputer. Shimadzu SPD-6AV UV spectrophotometric detector.

An aliquot of the polar extracts from the Soxlet extractions (20 µl / run) was separated by reverse phase HPLC with a Techsphere octadecylsilicate (ODS) S5 (5µ) column (25 x 0.46 cm). A ternary gradient system was developed from the system of Hahn *et al.* (1983). The solvents were 40 mM formic acid (A), 8% BuOH in MeOH (C) and 40% A in C (B): 60 min. run, 0-25 min. 95 % A, 5 % B changing 50 % A, 50 % B; 25-40 min., B increasing to 100 %; 40-50 min., C increasing to 100 %; 50-57 min., 100 % C; 57-59 min., changing to 95% A, 5 % B; 59-60 min., 95 % A, 5 % B. The flow conditions were as follows: 0-52 min., 1 ml min^{-1}; 52-56 min., increasing to 3 ml min^{-1}; 56-60 min., decreasing to 1 ml min^{-1}. Compounds were detected at 280nm. The data was transferred to the integrator and stored on disc.

A new column (Techsphere ODS S5) was used in year 2 which required altered conditions as follows: solvents A,B and C as above, 0-35 min. 95 % A, 5 % B changing to 50 % A and B; 35-45 min., B increasing to 100 %; 45-53 min., C increasing to 100 %; 53-58 min., 100 % C; 58-59 min., changing to 95 % A, 5 % B; 59-60 min. 95 % A, 5 % B. The phenolic profiles differed in respect of the retention times of peaks, most notably peak 44 which changed from 44.3 min (year 1) to 52.3 min (year 2). However, it can be seen from Fig 3.2 that the phenolic profiles were comparable to those of the previous year. The phenolic analyses are discussed in more depth in Chapter 5.

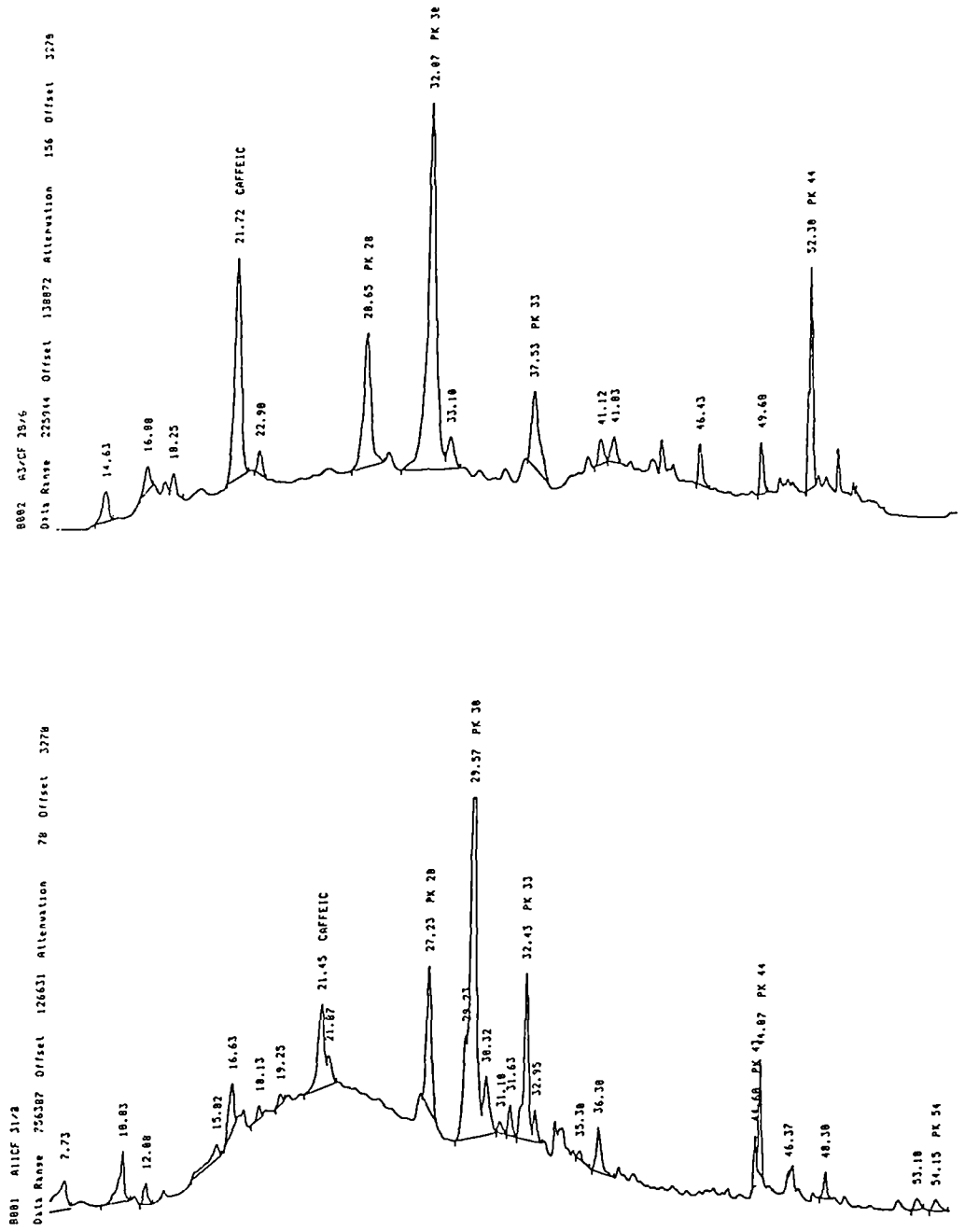


Figure 3.2 HPLC chromatographs of sweet gale leaf methanol extracts from 1987 (lower) and 1988 (upper).

3.2.3 Isolation of phenolic compounds.

A large Soxlet (500 ml) was used to extract 50 g dried leaf with 20 % aqueous methanol. The extract was taken to dryness under vacuum at 60°C. The residue was refluxed in 50 ml petrol/ethyl acetate (99:1) and the non-polar extract was discarded. The residue was dissolved in 50 ml 30 % aqueous methanol. Two fractions of the extract were prepared by semi-preparative HPLC (2 ml / run) with a Spherisorb-ODS2 5 column (25 mm x 0.9 mm) using a binary system (3 ml min^{-1}) as follows: solvent A (40 mM formic acid), solvent B (8 % nBuOH in MeOH): 35 min. run, 0-30 min., 5 % B in A increasing to 100 % B; 30-33 min., 100 % B; 33-35 min., 100 % B reducing to 5 % B in A. Fraction 1, containing peaks 28, 30 and 33, was collected between 15 and 22 min. Fraction 2 containing peaks 43, 44 and 54 was collected between 26 and 33 min.

Fraction 1 was taken to dryness under vacuum at 60°C and redissolved in 20 ml 70 % aqueous methanol. It was further separated (2 ml per run) into 3 sub-fractions using the following system (3 ml min^{-1}): solvent A (25 % B in water), solvent B (8 % nBuOH in MeOH): 25 min. run, 0-20 min., 100 % A reducing to 50 % A; 20-23 min., 50 % A; 23 to 25 min., 50 % A increasing to 100 % A. Fraction 1a was collected between 10 and 12 min. and contained approximately 45 % peak 28 (by area). Fraction 1b was collected between 17 and 18 min. and consisted of approximately 80 % peak 30. Fraction 1c was collected between 19 and 21 min. and consisted of approximately 60 % peak 33. Each sub-fraction was concentrated under vacuum and further purified on the same system.

In fraction 2 peak 44 and peak 43 eluted from the semi-preparative column together. Despite numerous purification

steps it proved extremely difficult to isolate almost pure (approx. 95 %) peak 44 from the fraction. Attempts to isolate peak 43 (a minor peak) from fraction 2 proved unsuccessful. To overcome this two differing preparative techniques were used in tandem.

Dried leaf (75 g) was blended in 750ml 30 % aqueous methanol. The mash was filtered (Whatman N° 1 paper) and the filtrate was taken to dryness under vacuum at 60°C. The extract was redissolved in 50ml 60% aqueous methanol. The extract was separated into fractions on TLC grade acetylated polyamide column (15 cm x 5 cm) with 50 ml aliquots of solvent. The concentration of methanol in each aliquot was 5% greater than the previous, starting at 50 % aqueous methanol and ending with 100% methanol. The solvent was eluted under vacuum.

The fraction which contained peak 44 (fraction 8) was further separated on a 0.9 cm x 25 cm 5 m ODS2 column using a binary system (3 ml min^{-1}) as follows: solvent A (60 % solvent B in water), solvent B (8 % n BuOH in MeOH): 30 min. run, 0-10 min. 90 % A; 10-20 min. A reducing to 60 %; 20-25 min., A reducing to 0 %; 25-29 min., A increasing to 90 %; 29-30 min., 90 % A. Peak 44 was eluted between 16.5 and 17.5 min. Peak 54 was also present in fraction 8 and was collected between 21 and 22 min. The fraction containing peak 43 (fraction 9) was separated on the same system and was collected between 16.5 and 17.0 min.

3.2.4 Structure elucidation of phenolics.

Instrumentation: Nuclear magnetic resonance (NMR) spectrometry: Bruker WM 250 (250.13 MHz for ^{1}H) and Bruker AC300 modified for inverse acquisition (300.13 MHz for ^{1}H and 75.49 MHz for ^{13}C).

Mass spectrometry: electron impact (70ev): AEI MS 902 double focusing spectrometer; fast atom bombardment: VG ZAB-E MS in the positive ion mode. Ultra violet (UV) spectrophotometry: Unicam SP.800.

Once purified compounds were subjected to ^{1}H NMR spectrometry (250 MHz) with decoupling experiments, mass spectrometry (electron impact) and UV spectrophotometry with addition of sodium hydroxide, sodium acetate, boric acid and aluminium chloride using the methods described by Harborne (1984). These techniques proved adequate for structure elucidation on peaks 30, 43 and 54.

Peak 33 proved to be a flavonoid glycoside and was not amenable to electron impact MS. FAB-MS (Schulten 1989) provides a soft ionization method for phenolic glycosides and was used in this case with 3-nitrobenzyl alcohol as matrix.

Peak 44 proved to be a complex molecule requiring more sophisticated NMR experiments, specifically two dimensional long range heteronuclear correlations (^{1}H-^{13}C) (Derome 1989). FAB-MS was used to aid structure elucidation.

3.3 Results and Discussion.

3.3.1 Peak 30.

Extraction of 50 g of fresh leaves rendered 14 mg compound in the form of a yellow amorphous solid. UV data were as follows: λ_{max} nm : 258, 304sh, 364; (+ NaOH) 275sh, 320sh, 380sh; (+ NaOAc) 272, 340sh, 388; (+ NaOAc & H_3BO_3) 260, 305sh, 393; (+ $AlCl_3$) 270, 310sh, 350sh, 425.

^{1}H NMR (250 MHz, CD_3OD) gave the following data (Fig 3.3): δ7.37 (2H, *s*, H-2', H-5'), 6.39 (1H, *d*, *J* = 2 Hz, H-8), 6.19 (1H, *d*,

J = 2 Hz, H-6), 5.18 (1H, *d*, *J* = 6.8 Hz, H-1"), 3.83 (2H, *m*), 3.60 (4H, *m*).

High resolution EI-MS (Fig 3.4) gave a molecular ion of 318.0374 which has the solution $C_{15}H_{10}O_8$ (theoretical mass: 318.0375, error = -0.4 ppm).

The singlet at δ 7.37 indicates two equivalent protons on an aromatic ring which is substituted at all other positions. The pair of signals at δ 6.39 and 6.19 which exhibit the same *J* value indicate two meta coupled unequivalent protons. The doublet at δ 6.19 is typical of a galactosyl or glucosyl anameric proton and the presence of a sugar is confirmed by a series of overlapping signals integrating for six protons at δ 3.83 and 3.60.

The UV data indicate a flavonone and correspond to myricetin (Mabry *et al.* 1970). A glycoside of myricetin is, therefore, indicated and myricetin-3-galactoside has previously been isolated from sweet gale (Bodalska and Rzadkowska-Bodalski 1969). Although individual couplings cannot be determined in the multiplet between δ 4.0 and 3.5 there appear to be doublets with small coupling constants consistent with a galactosyl. The UV of this compound exhibited a hypsochromic shift of 10 nm with respect to myricetin which is consistent with a glycosyl at the 3 position (Harborne 1984). The bathochromic shifts associated with addition of aluminium chloride, sodium acetate and boric acid demonstrate that the remaining hydroxyls are unsubstituted. This UV data broadly agrees with that of Bodalski and Rzadkowska-Bodalska (1969) for myricetin-3-galactoside although they observed no hypsochromic shift in band III compared to the aglycone. The mass spectrum confirms the presence of the aglycone with major fragments at *m/z* 153.0170

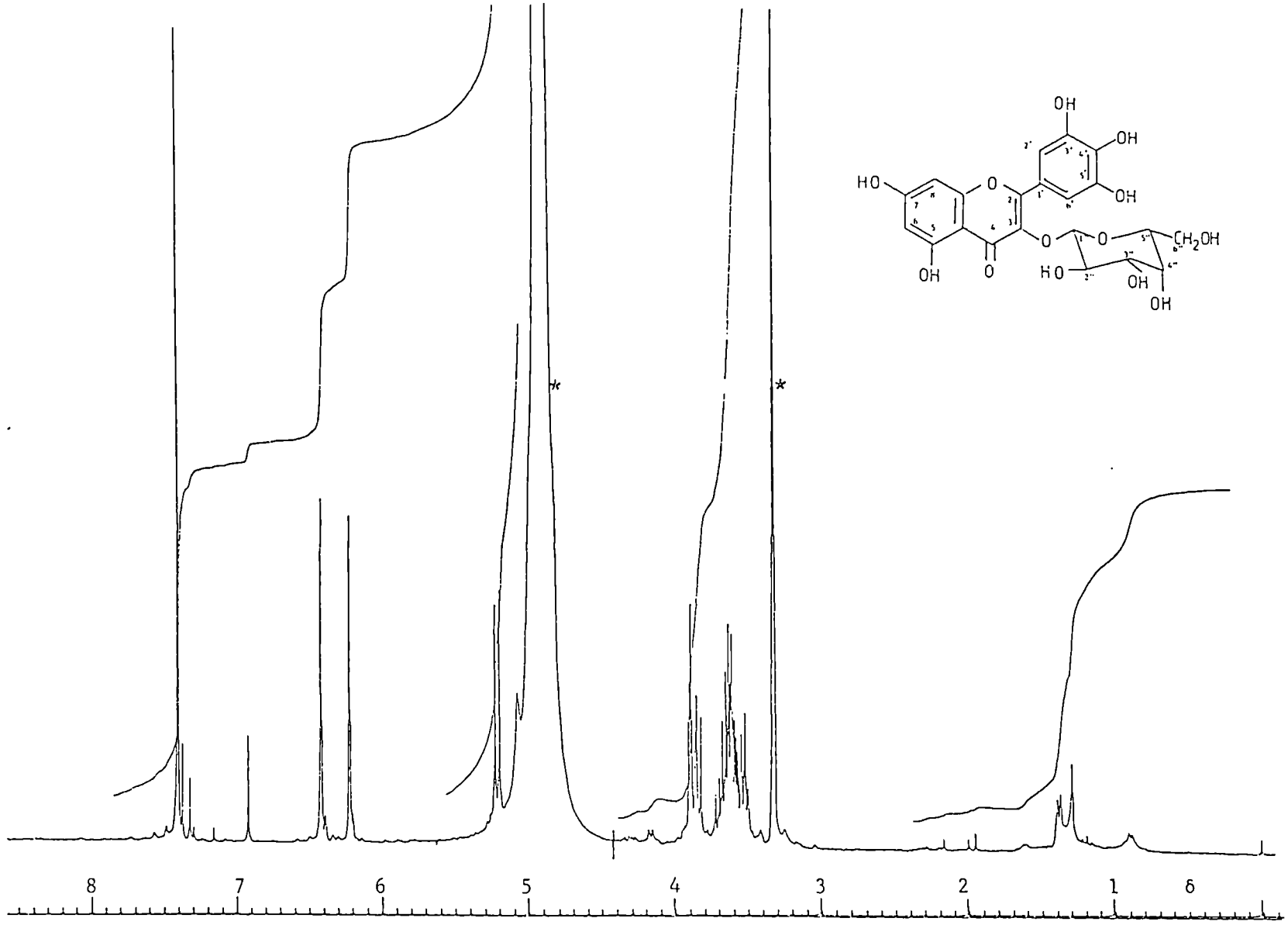


Figure 3.3 The 250 MHz ^{1}H NMR of peak 30 (asterisks denote solvent signals).

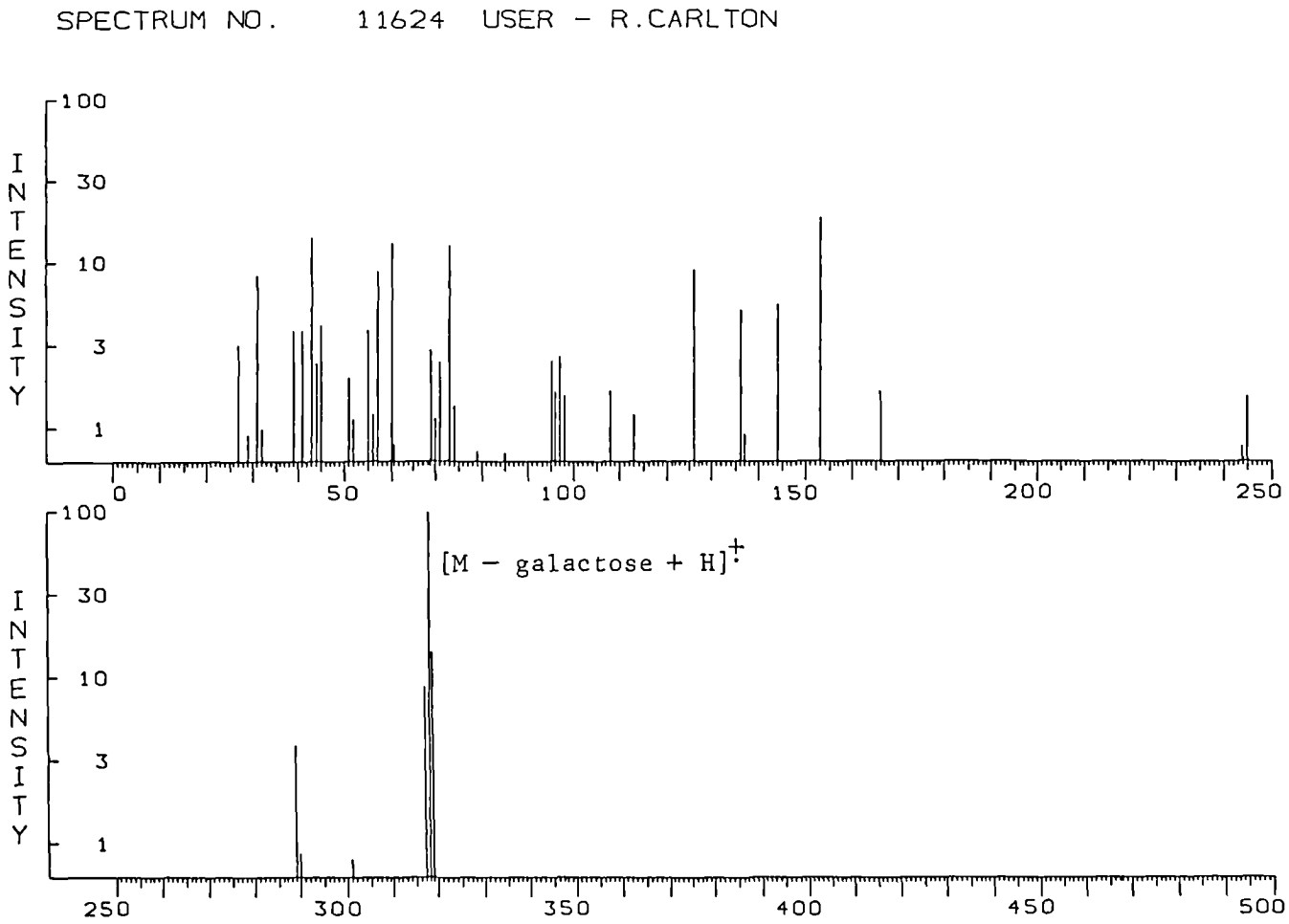
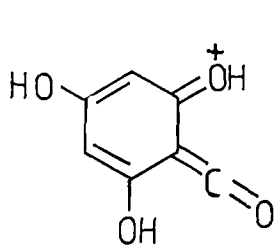
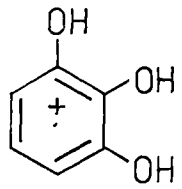


Figure 3.4 The EI-MS spectrum of peak 30.

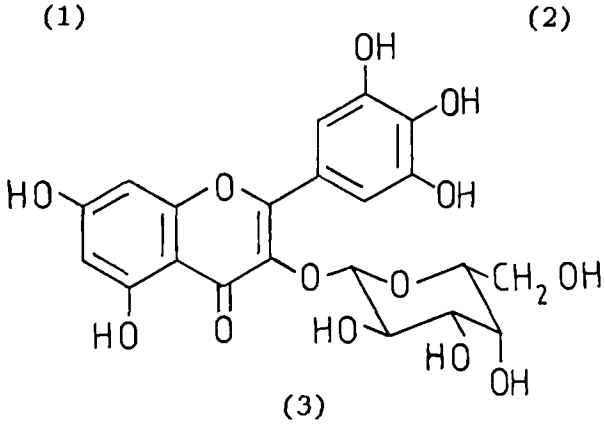
which solves for $C_7H_5O_4$ (1) and 126.0317 which solves for $C_6H_6O_3$ (2) Peak 30 proved to be myricetin-3-galactoside (3).



(1)



(2)



(3)

3.3.2 Peak 33.

Extraction of 50 g fresh leaf rendered 5 mg amorphous yellow solid with UV λ_{max} nm: 259, 267sh, 299, 366; (+ NaOH) 280, 340sh, 400sh; (+ NaOAc) 269, 300sh, 330sh, 384; (+ NaOAc & H_3BO_3) 268, 300sh, 394; (+ $AlCl_3$) 274, 302sh and 429. ^1H NMR (250 MHz, CD_3OD) gave the following data : δ 7.83 (1H, *d*, *J* = 2.2 Hz, H-2'), 7.58 (1H, *dd*, *J* = 2.2, 8.5 Hz, H-5'), 6.85 (1H, *d*, *J* = 8.5 Hz, H-4'), 6.40 (1H, *d*, *J* = 2.0 Hz, H-8), 6.20 (1H, *d*, *J* = 2.0 Hz, H-6), 5.16 (1H, *d*, *J* = 7.9 Hz, H-1"), 3.81 (2H, *m*), 3.60 (4H, *m*).

FAB-MS (Fig 3.5) gave a pseudo molecular ion (M + Na^+) of 487 which solves for $C_{21}H_{20}O_{12}$.

Like peak 30 the NMR of this compound exhibits signals at δ 6.40, 6.20, 5.16, 3.83 and 3.60 suggesting a benzopyran-4-one

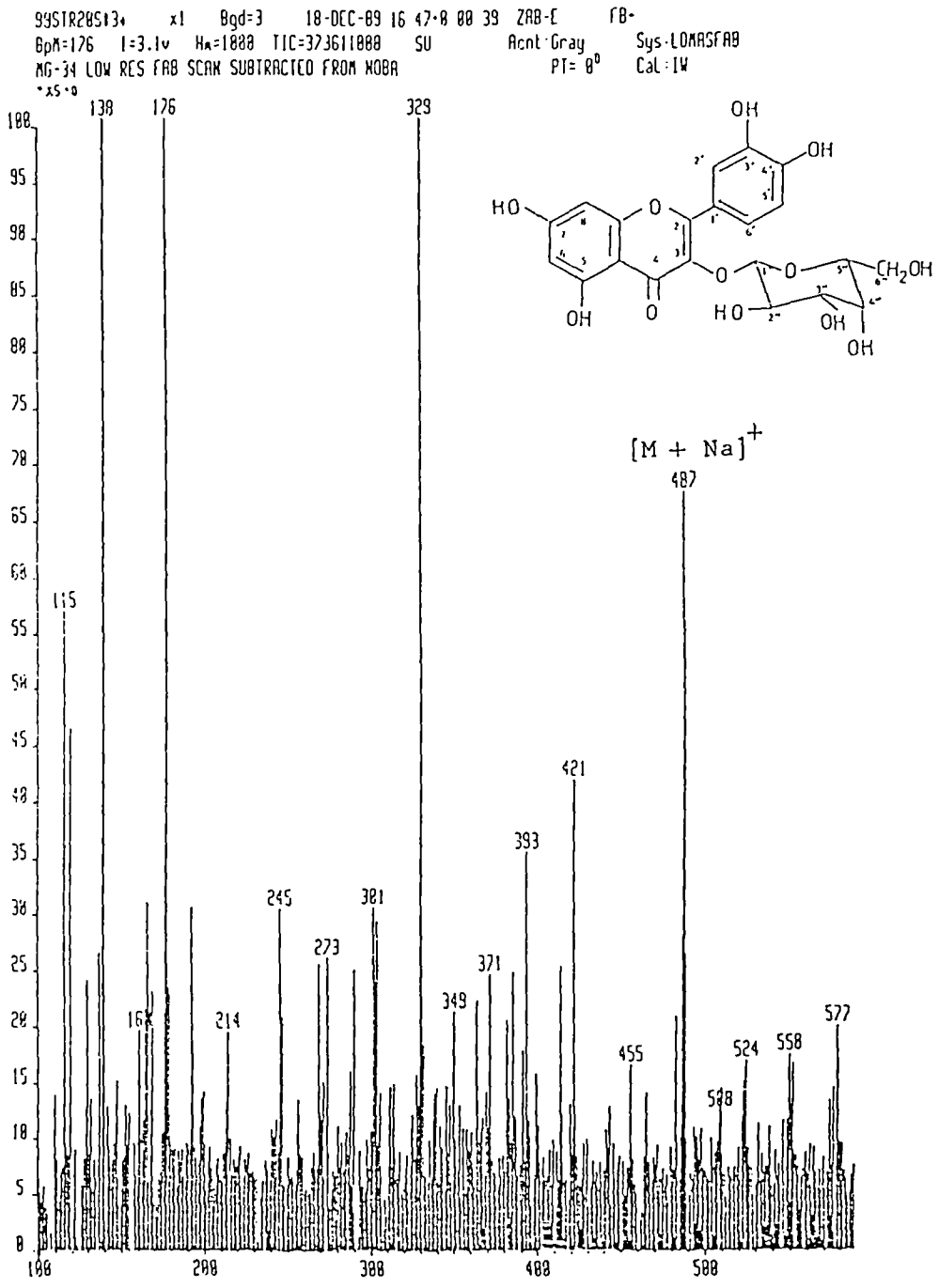
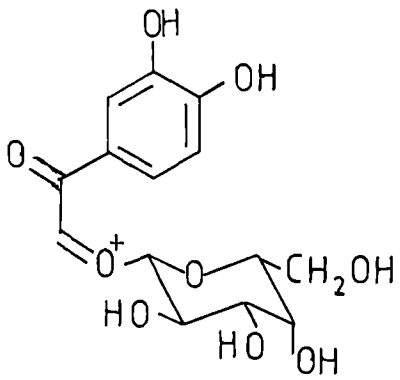


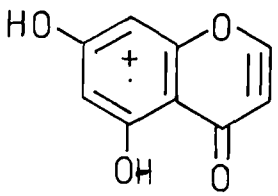
Figure 3.5 FAB-MS spectrum of peak 33.

oxygenated at the 5 and 7 positions and with a galactoside moiety at C-3. The signals at δ 7.83, 7.58 and 6.85 indicate three protons on a phenyl substituted at the 3 and 4 position.

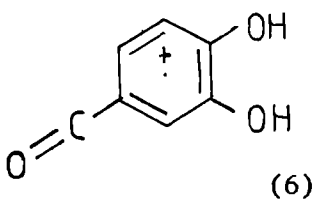
The UV data are very similar to quercetin-3-*O*-galactoside data given by Bodalski and Bodalska (1969) and Mabry *et al.* (1970). When compared with quercetin the UV of peak 33 exhibits a hypsochromic shift in band III characteristic of a substituted 3 position (Harborne 1984). UV data after addition of reagents confirm that the other hydroxyls (at 5, 7, 3' & 4') are free. The mass spectrum confirms the presence of a glycoside of quercetin with major fractions at *m/z* 329 which solves for $C_{14}H_{17}O_9$ (4), *m/z* 176 which solves for $C_9H_4O_4$ (5) and *m/z* 138 which solves for $C_7H_6O_3$ (6). Peak 33 proved to be quercetin-3-galactoside (7).



(4)



(5)



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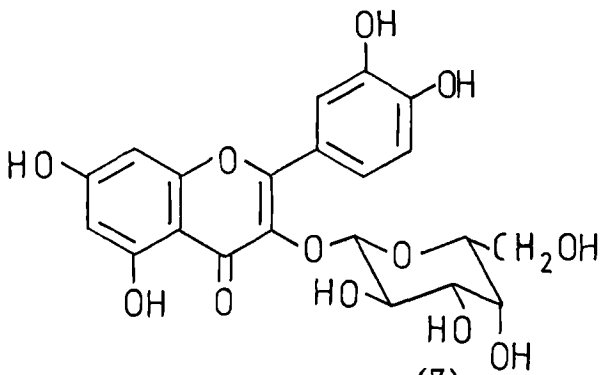


(7)

3.3.3 Peak 43.

Extraction of 150 g fresh leaf rendered 4 mg of amorphous yellow solid with UV λ_{max} nm: 286, 326sh; (+ NaOH) 250, 342 (+ NaOAc) 255, 285, 342; (+ NaOAc & H_3BO_3) 255, 285, 342; (+ $AlCl_3$) 285.

^1H NMR (250 MHz, CD_3OD) gave the following data (Fig 3.6): δ 7.60-7.30 (5H, *m*), 5.43 (1H, *dd*, *J* = 3.3, 12.6 Hz, H-2), 3.72 (3H, *s*, 5-MeO), 2.96 (1H, *dd*, *J* = 12.6, 16.8 Hz, H-3), 2.76 (1H, *dd*, *J* = 3.3, 16.8 Hz, H-3), 2.08 (6H, *s*, 6-Me, 8-Me).

High resolution EI-MS gave a molecular ion of 298.1197 (Fig 3.7) which solves for $C_{18}H_{18}O_4$ (theoretical mass: 298.1206, error = -2.8 ppm).

The signals at δ 7.50, 7.41 and 7.35 correspond to an unsubstituted phenyl attached to an electronegative group. Three signals at δ 5.43, 2.96 and 2.76 indicate an ABX system. The presence of a signal for 6H at 2.08 correspond to a pair of equivalent methyls on a second phenyl ring leaving a signal for 3H at δ 3.72 which concurs with the presence of a methoxyl on the same phenyl. The mass spectrum gives the major fraction at *m/z* 194.0566 which solves for $C_{10}H_{10}O_4$ (8).

This data is compatible with 5-hydroxy-7-methoxy-6,8-dimethylflavanone which has previously been reported from *Unona lawii* (Joshi & Gawad 1974) and 7-hydroxy-5-methoxy-6,8-methylflavanone previously isolated from *Eugenia javanica* Lam. (Mitscher *et al* 1973). Only one maximum was seen in the UV spectrum which is consistent with the methoxyl at C-5 (Malterud 1982b). No shift was observed on or within 60 min of addition of aluminium chloride, confirming the position of the methoxyl at C-5 where it

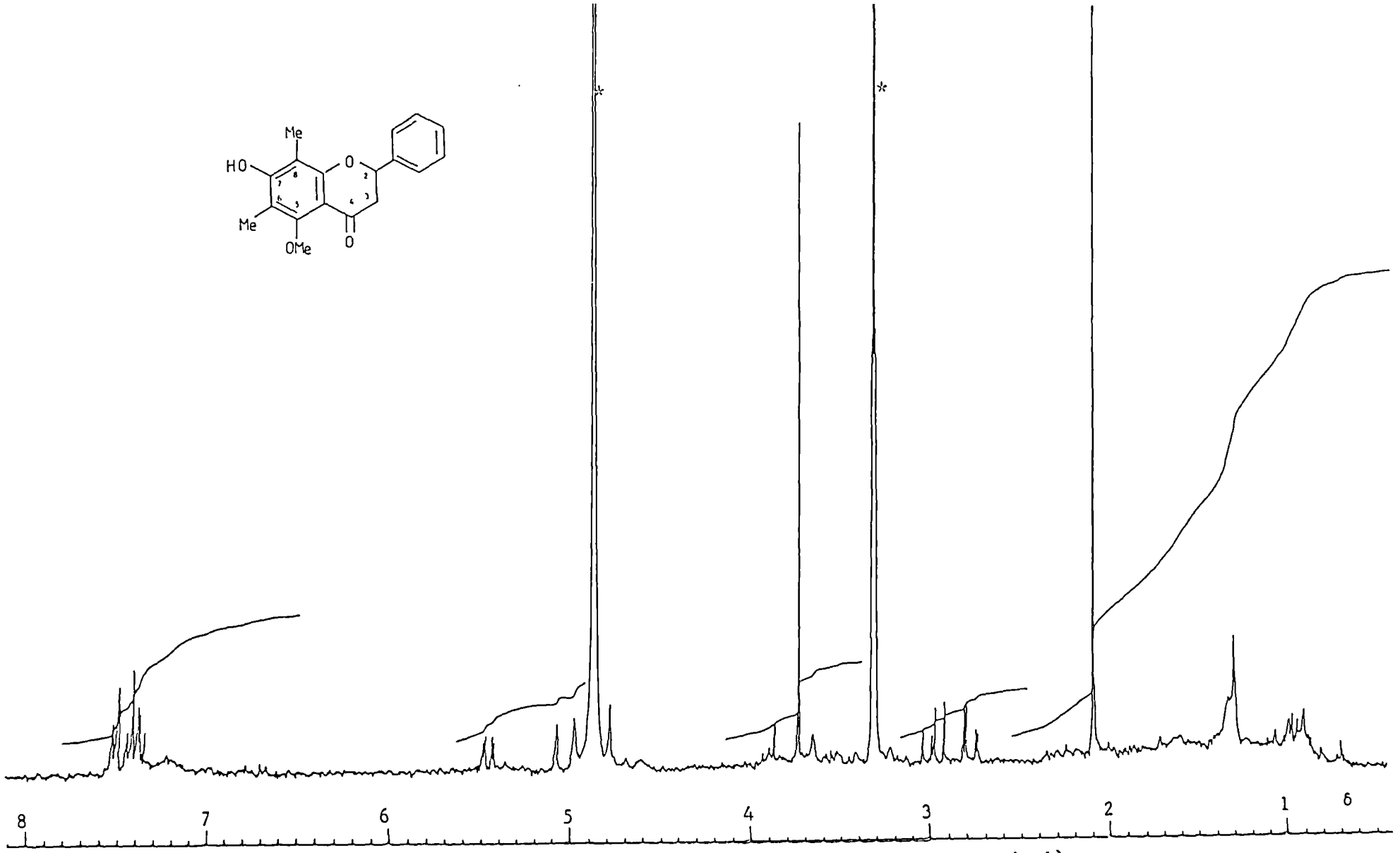


Figure 3.6 The 250MHz ^{1}H NMR of pk 43 (solvent peaks are asterisked).

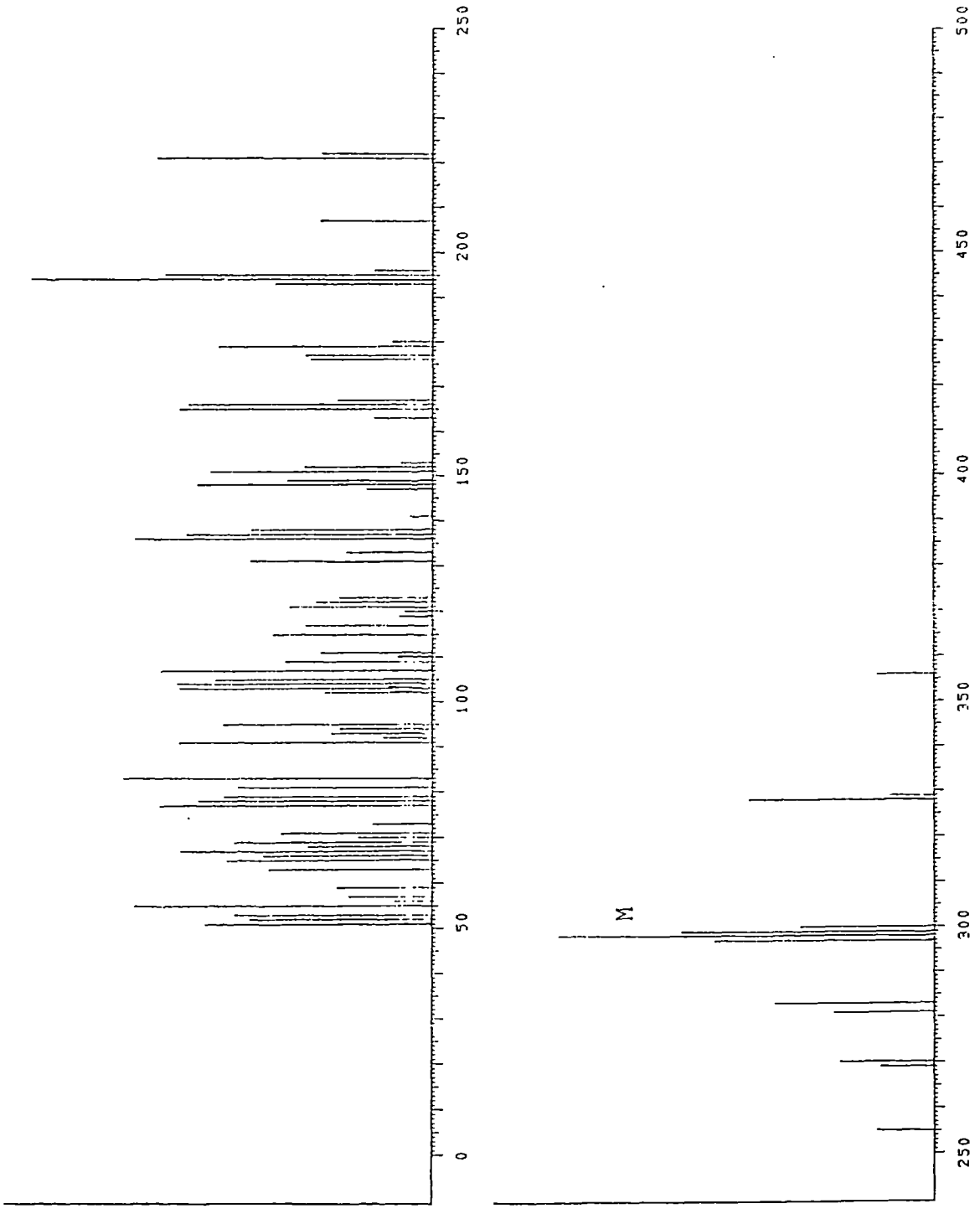
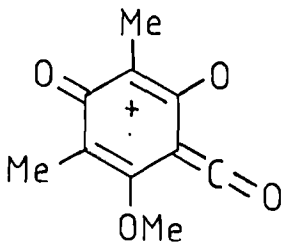


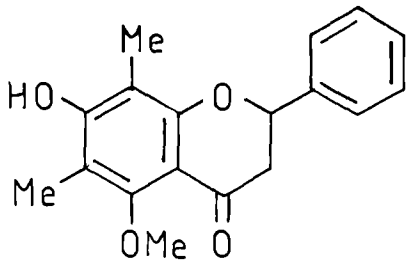
Figure 3.7 The EI-MS of peak 43.

inhibits chelation.

Peak 43 proved to be 7-hydroxy-5-methoxy-6,8-methylflavanone (9) which had not previously been isolated from sweet gale .



(8)



(9)

3.3.4 Peak 54.

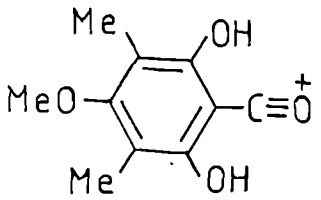
Extraction of 75 g fresh leaf rendered 68 mg yellow amorphous solid. UV λ_{max} nm: 282, 345; (+ NaOH) 238sh, 295; (+$AlCl_3$) 287 sh, 307. ^1H NMR (250 MHz, $CDCl_3$) gave the following data (Fig 3.8): δ 9.43 (2H, *br s*, 2'-OH, 6'-OH), 7.26 (5H, *m*, H-2, H-3, H-4, H-5, H-6), 3.71 (3H, *s*, 4'-MeO), 3.45 (2H, *t*, J = 7.8 Hz, 2H-8), 3.06 (2H, *t*, J = 7.8 Hz, 2H-7), 2.11 (6H, *s*, 3-Me, 5-Me).

High resolution EI-MS gave a molecular ion of 300.1357 which solves for $C_{18}H_{20}O_4$ (theoretical mass: 300.1362, error = -1.4 ppm).

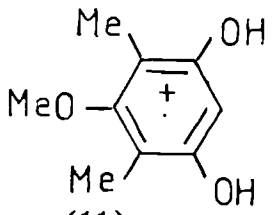
The multiplet at δ 7.26 which integrates for five protons indicates an unsubstituted phenyl, in which all of the protons are almost equivalent. The singlet at δ 3.71 indicates the presence of a methoxyl group and the singlet for six protons at δ 2.11 indicates two equivalent methyls on an aromatic ring. The signals at δ 3.45 and 3.06, both for two protons, have identical J values which suggests that they were coupled to one another. Both signals are deshielded indicating the close proximity of electron withdrawing

groups to each carbon. The presence of two benzene rings has been indicated and either would account for the signal at 3.06 ppm. However, the presence of a more electronegative group, such as a ketone, is indicated by the double doublet at δ 3.45. The final signal at δ 9.43 points to two equivalent hydroxyls on an aromatic ring.

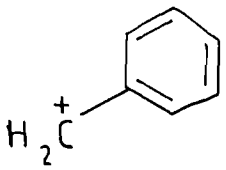
Altogether the NMR indicates two aromatic rings, one unsubstituted and one substituted (2 OH, 2 Me, 1 MeO) linked by a propyl chain containing a ketone. This data corresponds to 2',6' dihydroxy-4'-methoxy-3',5'-dimethyl-dihydrochalcone (13) previously isolated from sweet gale (Anthonsen *et al.* 1971). The mass spectrum confirms this, with the anticipated molecular ion, a base peak at *m/z* 195 which solves for $C_{10}H_{11}O_4$ (10) and major fragments at *m/z* 168 which solves for $C_9H_{12}O_3$ (11) and *m/z* 91 which solves as C_7H_7 (12). The UV data are similar to those given for this compound by Uyar *et al.* (1978). Addition of aluminium chloride caused a gradual bathochromic shift which was complete within 25 min. in agreement with the results of Malterud (1982b).



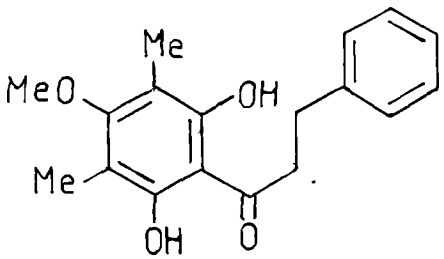
(10)



(11)



(12)



(13)

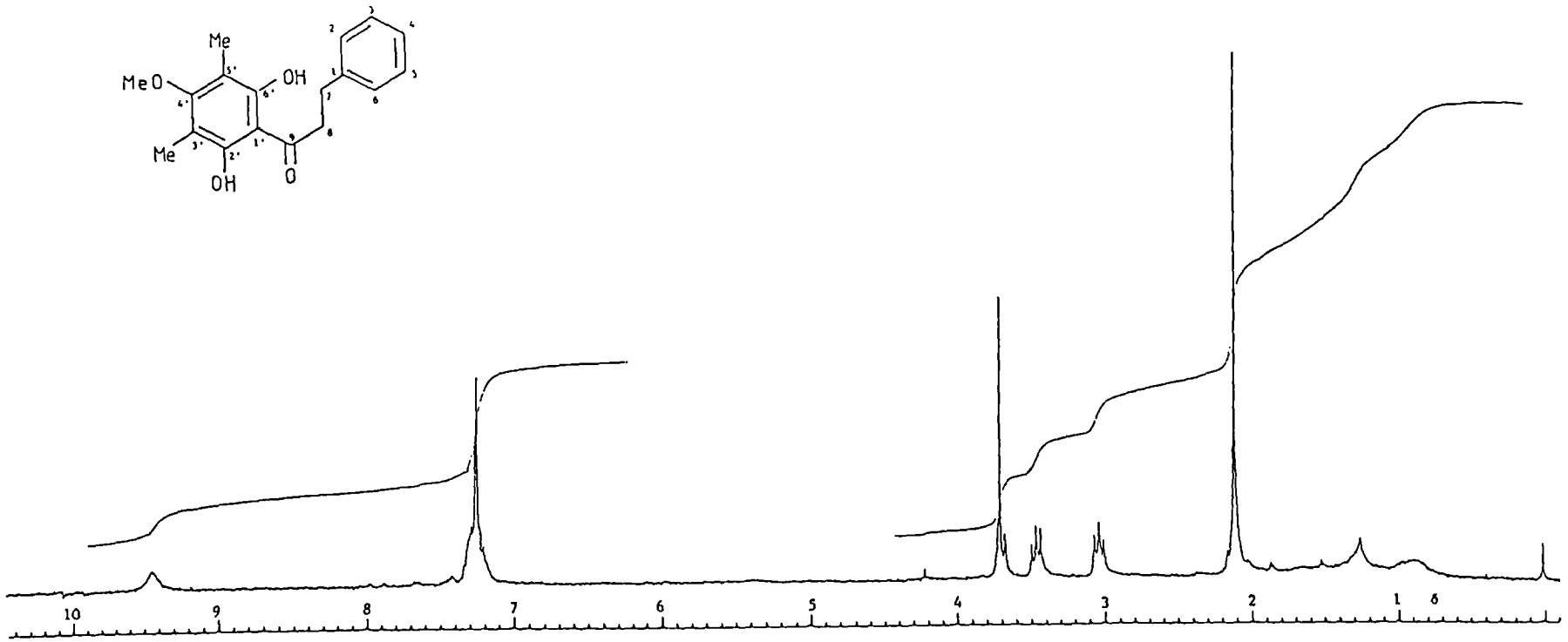


Figure 3.8 The 250 MHz 'H NMR of pk 54.

3.3.5 Peak 44.

Extraction of 150 g fresh leaf rendered 13 mg amorphous yellow solid. UV data were as follows λ_{max} nm: 263, 297sh, 312; (+ NaOH) 270, 365; (+ NaOAc) 270, 297, 310; (+ $AlCl_3$) 278, 302, 325, 398.

^1H NMR (300 MHz, CD_3OD) gave the following data (Fig 3.9): δ 7.78 (2H, *d*, *J* = 8.8 Hz, H-2', H-6'), 7.51 (1H, *d*, *J* = 16.0 Hz, H-3"'), 7.46 (2H, *d*, *J* = 8.7 Hz, H-5"', H-9"'), 7.01 (2H, *d*, *J* = 8.8 Hz, H-3', H-5'), 6.82 (2H, *d*, *J* = 8.7 Hz, H-6"', H-8"'), 6.38 (1H, *d*, *J* = 2.1 Hz, H-8), 6.23 (1H, *d*, *J* = 2.1 Hz, H-6), 6.19 (1H, *d*, *J* = 15.9, H-2"'), 5.72 (1H, *d*, *J* = 1.6 Hz, H-1"), 5.61 (1H, *dd*, *J* = 1.6, 3.3 Hz, H-2"), 5.36 (1H, *dd*, *J* = 3.3, 9.0 Hz, H-3"), 5.00 (1H, *t*, *J* = 9.0 Hz, H-4"), 3.37 (1H, *dq*, *J* = 7.3, 9.0 Hz, H-5"), 2.12 (3H, *s*, 2"-Ac), 1.93 (3H, *s*, 3"-Ac), 0.88 (3H, *d*, *J* = 7.3 Hz, 6"-Me). ^{13}C NMR (75.4 MHz, CD_3OD) gave the following data (Fig 3.10): ppm *s* at 178.9 (C-4), 171.6 (C-3"-Ac), 171.3 (C-2"-Ac), 167.8 (C-1"'), 165.4 (C-7), 161.3 (C-5, C-4'), 161.0 (C-7"'), 159.0 (C-2), 158.2 (C-9), 134.3 (C-3), 126.8 (C-4"'), 122.1 (C-1'), 106.0 (C-10); *d* at 147.4 (C-3"'), 131.7 (C-2', C-6'), 131.3 (C-5"', C-9"'), 116.8, 116.7 (C-3', C-5', C-6"', C-8"'), 114.0 (C-2"'), 100.1 (C-6), 98.7 (C-1"), 95.0 (C-8), 71.2 (C-5"), 70.4, 70.3 (C-2", C-3"), 69.6 (C-4"; *q* at 21.1 (2 x Ac), 17.7 (C-6"').

FAB-MS gave a pseudo molecular ion ($M + Na^+$) of 685 (Fig 3.11) which solves for $C_{34}H_{30}O_{14}$.

The ^1H NMR of peak 44 exhibits similar signals to peaks 30 and 33 at δ 6.38 and 6.23 for meta split protons. Signals for two sets of coupled protons at δ 7.78 (2H) with 7.01 (2H) and 7.46 (2H) with 6.82 (2H) indicated two *para* substituted phenyl groups, the more deshielded pair of signals being compatible with a flavonone B ring.

A pair of signals at δ 7.51 and 6.19 indicate deshielded *trans*-coupled protons compatible with a *para*-coumaroyl substituent.

An anomeric proton typical of a rhamnosyl is present at δ 5.72 and the presence of this hexose is also indicated by the methyl doublet at δ 0.88 coupled to a proton (H-5") at 3.37. However, three of the remaining protons (H-2", 3" & 4") are deshielded indicating substitution of the three hydroxyls. The *para*-coumaroyl is a probable substituent as are the pair of acetates denoted by signals at δ 2.12 and 1.93.

The FAB-MS spectrum concurs with the hypothesis that the molecule is a diacetoxy and *p*-coumaroyl substituted rhamnoside of kaempferol, the major fragment at *m/z* 377 solving for the diacetoxy-1-desoxyrhamnosyl-*p*-coumarate (14).

Two structural questions still needed to be answered. The probable position of the rhamnoside is at the 3 position on the flavonoid. However, this needed confirmation. Secondly, the data discussed above gives no indication of the substitution pattern on the rhamnoside. These two questions were answered using two dimensional $^{13}C/^{1}H$ NMR in a heteronuclear multiple bond correlation (HMBC) experiment (Summers *et al.* 1986) which allows connectivities of protons and carbon molecules across two and three bonds. This often allows the linkage between quaternary carbons and the nearest protons to be identified.

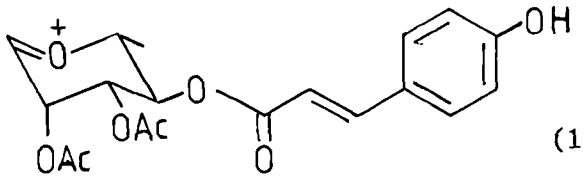
The experiment was run with a 90 ms delay (Fig 3.12). This demonstrated a linkage between the rhamnoside 1 proton (δ 5.70) through the oxygen to the quaternary C-3 carbon (134.32 ppm). This confirms the position of the rhamnoside at the 3 position.

The substitution pattern of the rhamnoside was elucidated in

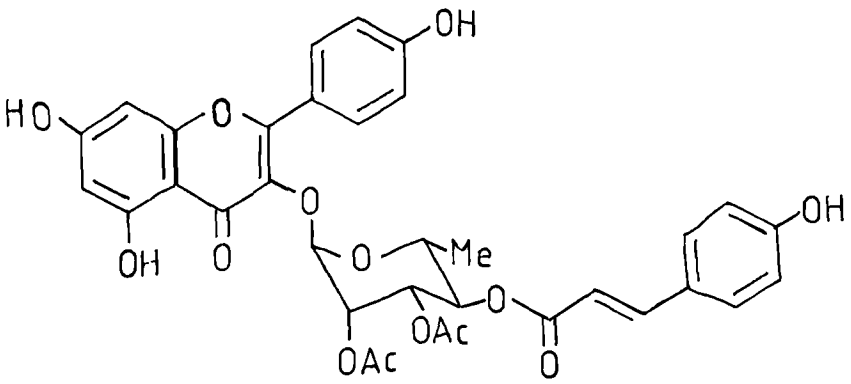
the same experiment. In the first instance the quaternary carbon of the *p*-coumarate carbonyl (167.79 ppm) was identified by its connectivity with the trans coupled protons (H-2"',3"'). The connectivity between this carbon and the proton triplet of the rhamnoside 4 position (δ 5.00) is quite clear.

The position of the acetates at the 2 and 3 position are verified with their carbonyl carbons (171.27 & 171.59 ppm) exhibiting connectivities with the rhamnoside 2 proton (δ 5.60) and 3 proton (δ 5.37) respectively. Thus, peak 44 proved to be kaempferol-3-(2,3-diacetoxy-4-*p*-coumaroyl)-rhamnoside (15) (Carlton *et al.* 1990).

It is interesting that the UV data do not exhibit absorbance bands typical of a kaempferol-3-glycoside (Mabry *et al.* 1970). It is probable that this is caused by the large (diacetoxy-*p*-coumaroyl) rhamnoside moiety forcing the flavonol B ring out of plane thus disrupting the extended conjugation of the kaempferol system.



(14)



(15)

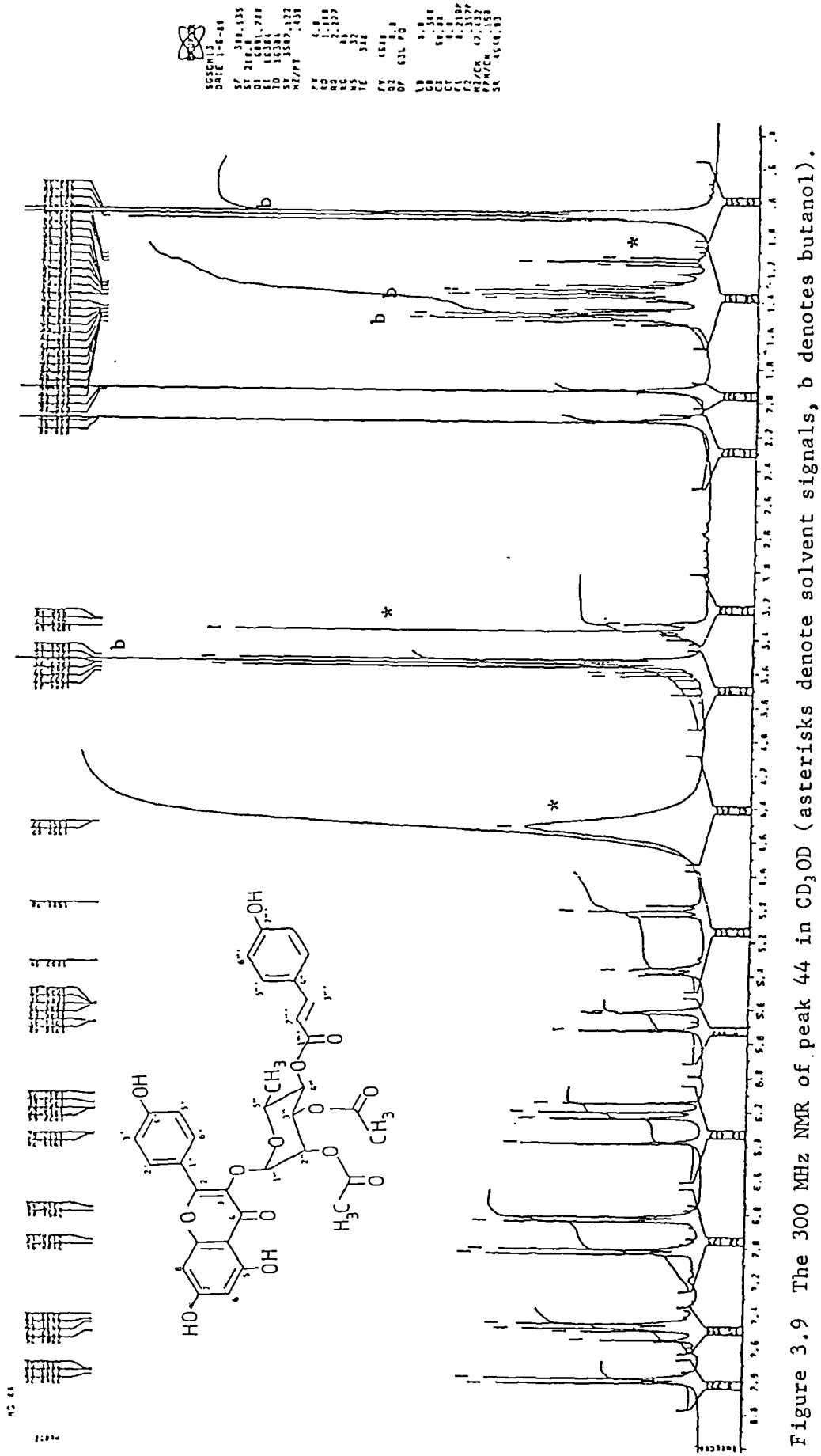


Figure 3.9 The 300 MHz NMR of peak 44 in CD_3OD (asterisks denote solvent signals, b denotes butanol).

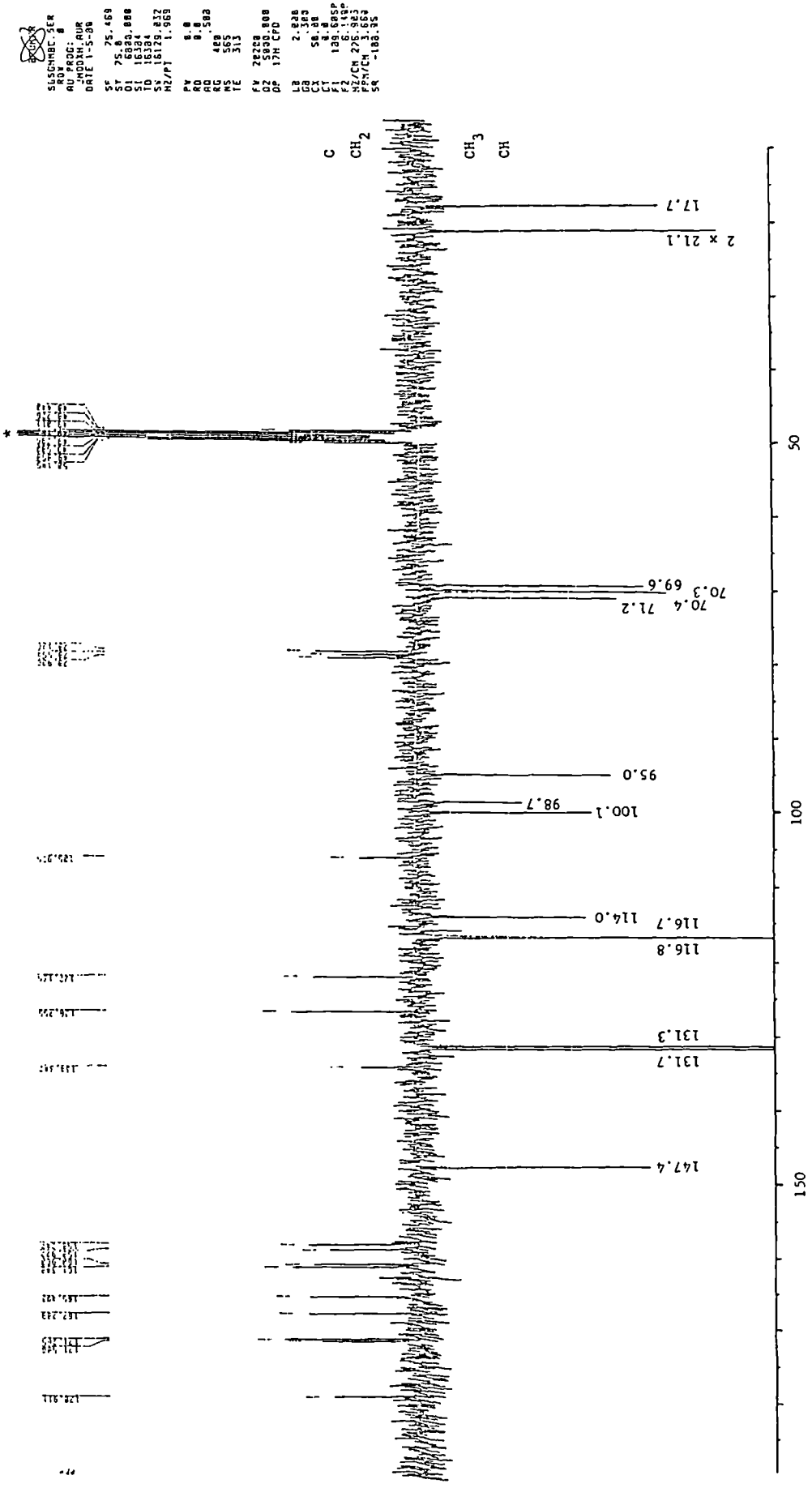


Figure 3.10 The ^{13}C NMR of peak 44.

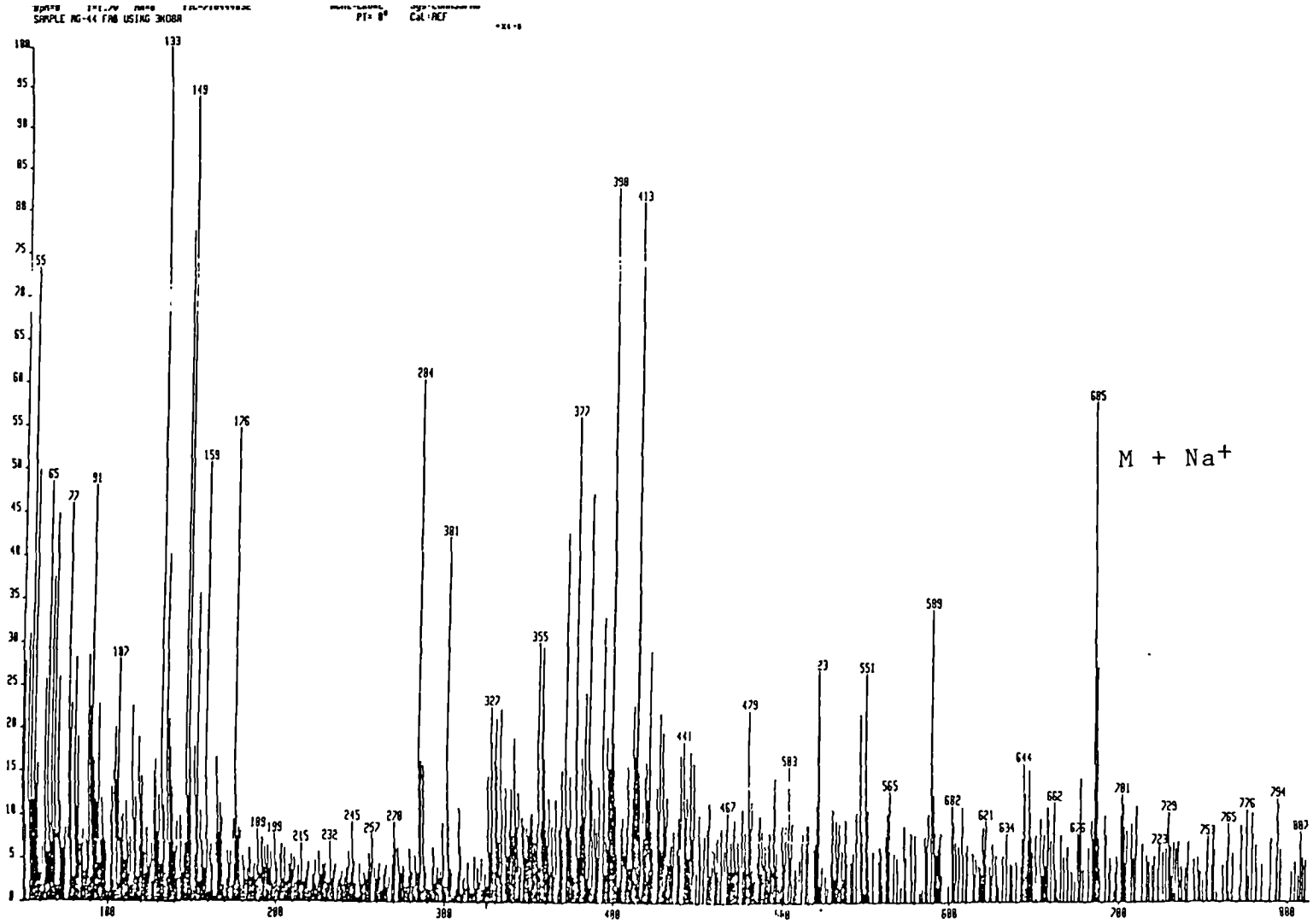
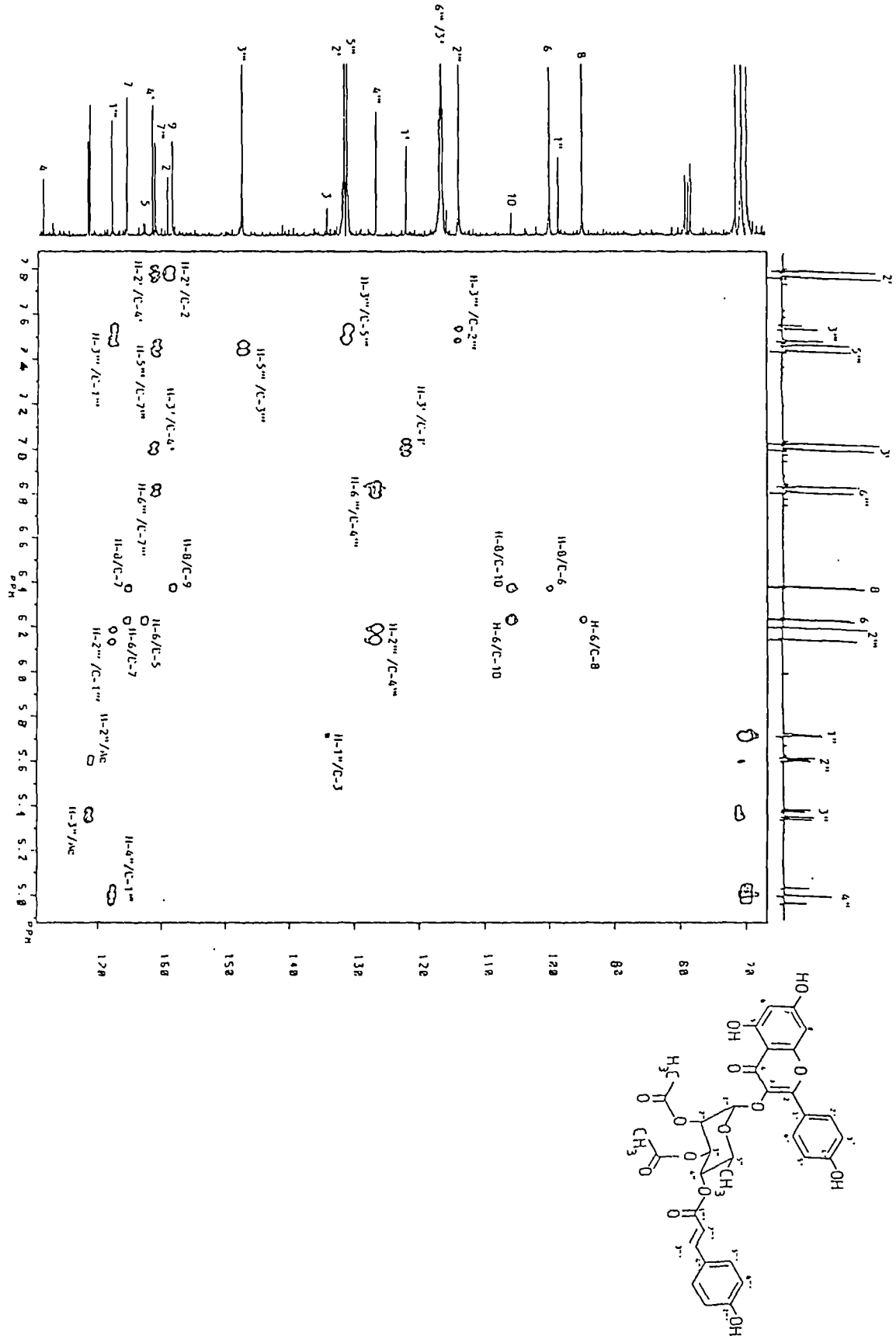


Figure 3.11 The FAB-MS spectrum of peak 44.

Figure 3.12 The HMBC spectrum of pk 44.



Chapter 4. The volatile oil of Myrica gale.

4.1 Introduction.

Monoterpenes are ubiquitous in higher plants (Loomis & Croteau 1980) but sweet gale is relatively unusual among temperate plants because it produces glands, situated on the surfaces of leaves (Fig 4.1), fruit and catkins, which contain a complex mixture of terpenes which form a distinctive volatile oil. The volatile oil of sweet gale has long attracted the attention of chemists and biologists (Chevalier 1909, Enklar 1911, Perrot 1911, Schoofs 1921) and more recently has been analysed on a number of occasions (von Schantz & Kapetanidis 1970, Halim & Collins 1973, Tattje & Bos 1974, Lawrence & Weaver 1974, Negueruela, Alonso & Rico 1982). The results of these later studies are summarised in Table 4.1. It can be seen that the volatile oil is a complex mixture made up of relatively few major and numerous minor terpenes. Halim and Collins (1973), in a comparison of members of the Myricaceae in North America, found that the volatile oil of sweet gale markedly differed from the oils of its relatives and used this as an argument for the reclassification of the species in a genus of its own. However, close scrutiny of Table 4.1 reveals that the oil of separate populations of sweet gale differ considerably. A high proportion of limonene is the only common major constituent and many terpenes were present in only one population. Clearly the production of a volatile oil stored in glands is a fixed characteristic whereas the composition of the oil is variable within the species. It is not clear from the reports of analyses summarised in Table 4.1 whether variation exists within the populations sampled.

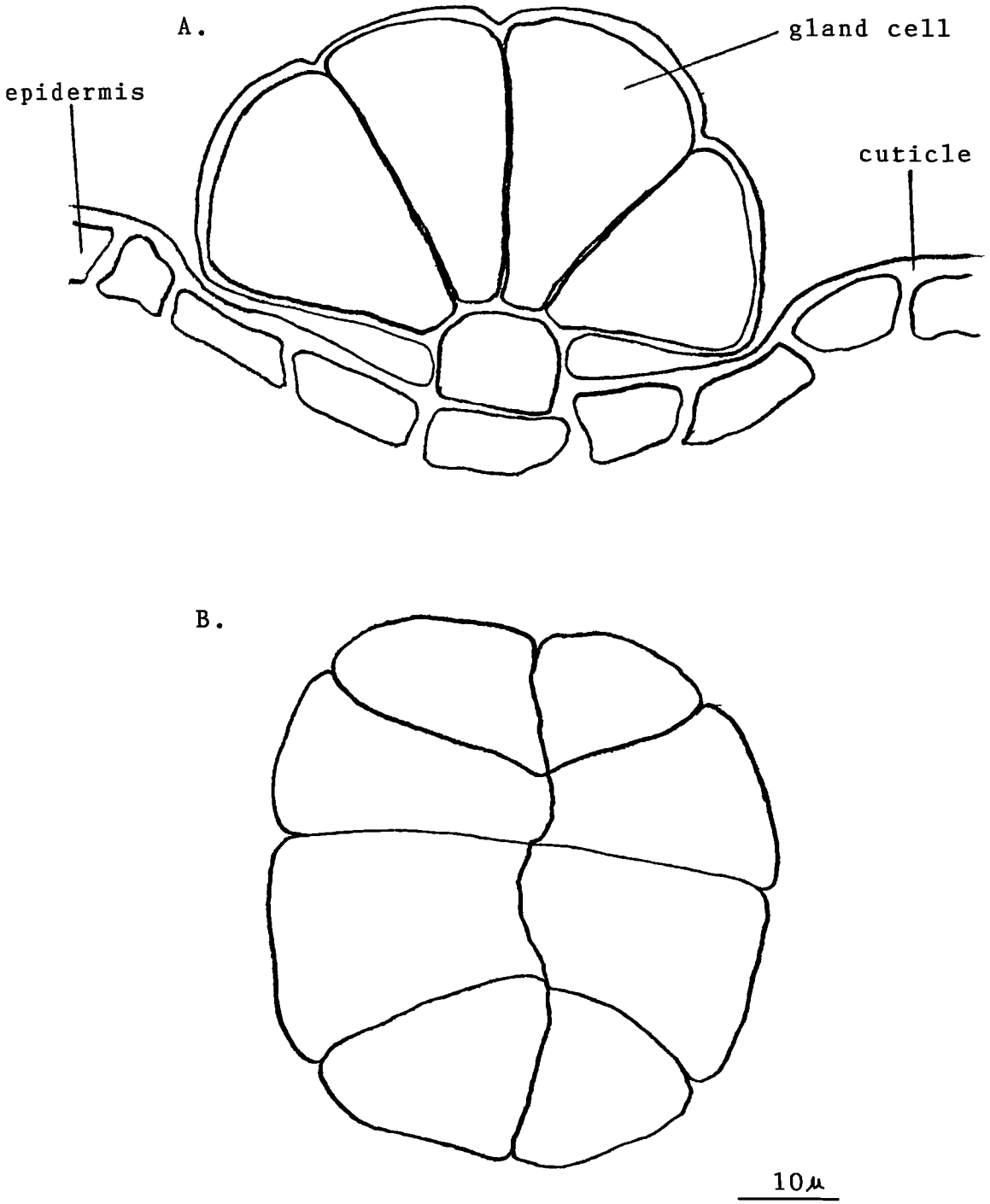


Figure 4.1 A cross section (A) and a top view (B) of a multicellular leaf gland from Myrica gale.

Table 4.1 Comparison of the composition of sweetgale volatile from different sources.

	Percentage of total peak area (if data available).				
	I	II	III	IV	V
alpha-thujene	0.50	n.d.	n.d.	n.d.	0.12
alpha-pinene	3.00	17.80	4.70	p.	41.38
camphene	<0.10	0.70	0.10	p.	2.33
beta-pinene	0.10	1.70	0.30	p.	1.09
amyl acetate	<0.10	n.d.	n.d.	n.d.	n.d.
myrcene	16.20		29.10	p.	2.84
alpha-phellandrene	5.10		n.d.	p.	3.60
alpha-terpinene	0.10	1.00	5.60	p.	3.25
limonene	10.80	10.00	14.60	p.	8.35
1,8-cineol	2.80	7.10	0.10	p.	13.62
beta-phellandrene	n.d.	3.20	n.d.	p.	n.d.
trans-ocimene	1.20	1.10	4.40	p.	n.d.
cis-ocimene	0.90	n.d.	3.60	p.	n.d.
gamma-terpinene	0.50	0.60	n.d.	p.	1.82
para-cymene	4.70	4.70	5.50	p.	n.d.
terpinolene	0.20	0.80	0.20	p.	0.99
cis-3-hexenol	n.d	n.d	0.20	p.	n.d.
methyl heptenone	0.10	n.d.	n.d.	n.d.	n.d.
alpha-para-dimethylstyrene	<0.10	n.d.	n.d.	n.d.	n.d.
alpha-cubinene	<0.10	n.d.	n.d.	n.d.	n.d.
octyl acetate	<0.10	n.d.	n.d.	n.d.	n.d.
copaene	0.50	2.80	n.d.	n.d.	0.70?
linalool	0.70	0.40	1.30	p.	0.43
borneol	n.d.	n.d.	n.d.	n.d.	0.44
benzaldehyde	<0.10	n.d.	n.d.	n.d.	n.d.
guaia-3,7-diene	n.d.	0.70	n.d.	n.d.	0.72?
trans-alpha-bergamotene	1.30	n.d.	n.d.	n.d.	n.d.
bornyl acetate	n.d.	0.20	n.d.	p.	0.56
terpinen-4-ol	0.90	1.00	0.30	p.	2.20
caryophyllene	<0.10	2.60	5.50	p.	0.34
elemene	n.d.		n.d.		0.52?
gamma-elemene		0.20		p.	
guaia-6,9-diene	<0.10	n.d.	n.d.	n.d.	n.d.
selina-4,11-diene	6.00	n.d.	n.d.	n.d.	n.d.
carvotanacetone	0.20	n.d.	n.d.	n.d.	n.d.
alpha-terpineol	2.10	2.10	0.40	p.	2.20
alpha-terpenyl-acetate	0.20	7.00	n.d.	p.	0.59
humulene	n.d.	n.d.		p.	n.d.
alpha-humulene			3.40		
longifolene	n.d.	0.20	n.d.	n.d.	n.d.
eremophilene	n.d.	n.d.	0.90	n.d.	n.d.
alpha & beta-selinene	0.10	n.d.	0.90	p.	n.d.
delta-selinene	<0.10	n.d.	n.d.	n.d.	n.d.
trans-beta-farnesene	1.00	n.d.	n.d.	n.d.	n.d.
aromadendrene	n.d.		n.d.	n.d.	0.72?
allo-aromadendrene		0.40			

Table 4.1 (cont).

	I	II	III	IV	V
piperitone	0.10	n.d.	n.d.	n.d.	n.d.
beta-bisabolene	3.00	n.d.	n.d.	n.d.	n.d.
carvone	0.10	n.d.	n.d.	n.d.	n.d.
citronellal	n.d.	n.d.	n.d.	n.d.	0.72?
citronellol	n.d.	n.d.	0.50	p.	n.d.
alpha-muurolene	0.20	0.10	n.d.	n.d.	n.d.
gamma-muurolene	n.d.	0.30	n.d.	n.d.	n.d.
epsilon-muurolene	n.d.	0.20	n.d.	n.d.	n.d.
geranyl acetate	0.30	n.d.	n.d.	n.d.	n.d.
alpha-farnesene	0.10	n.d.	n.d.	n.d.	n.d.
epsilon-cadinene	n.d.	n.d.	n.d.	n.d.	0.72?
delta or gamma-cadinene	2.50	12.90	0.30	n.d.	5.53?
nerol	n.d.	n.d.	0.10	p.	t.?
ar. curcumene	0.20	n.d.	n.d.	n.d.	n.d.
cubenene	0.30	n.d.	n.d.	n.d.	0.70?
geraniol	n.d.	n.d.	0.10	n.d.	n.d.
calamenene	0.30	2.40	0.10	n.d.	5.53?
benzyl isovalerate	<0.10	n.d.	n.d.	n.d.	n.d.
calacorene	n.d.	0.30	n.d.	n.d.	n.d.
caryophyllene oxide	1.80	n.d.	n.d.	n.d.	0.76
nerolidol				p.	0.69?
trans-nerolidol	1.00	5.90	0.80		
humelene epoxide II	0.80	n.d.	n.d.	n.d.	n.d.
eudesmol	n.d.	n.d.	3.60	n.d.	0.25?
cadinol	n.d.		n.d.	n.d.	3.27?
delta-cadinol		0.20			
farsenol	n.d.	n.d.	0.10	n.d.	n.d.
alpha-bisabolol	5.00	n.d.	n.d.	n.d.	n.d.
selin-11-en-4-ol	14.60	n.d.	n.d.	n.d.	n.d.
valencene	n.d.	n.d.	n.d.	p.	n.d.
beta-elemenone	n.d.	n.d.	n.d.	p.	n.d.
germacrone	n.d.	n.d.	n.d.	p.	n.d.
juniper camphor	n.d.	n.d.	n.d.	p.	n.d.

p. = present; t. = trace;
n.d. = not detected;
? = questionable identification.

Key to sources:

I = Lawrence & Weaver (1974),
II = von Schantz & Kapetanidis (1971),
III = Halim & Collins (1973),
IV = Tattje & Bos (1974),
V = Negueruela, Alonso & Rico (1982).

All reports cited above gave analyses of steam distilled oil. Bicchi *et al.* (1985) demonstrated that steam distillation can lead to significant changes in the composition of volatile oils. It follows that some of the listed results are questionable. In addition to problems of thermal decomposition steam distillation is also a relatively time consuming method which requires fairly large samples. The necessity for large samples often dictates that the leaves of more than one plant are distilled and this masks differences between plants. The damage incurred by collecting enough leaves from one plant for distillation could induce changes masking induction from herbivory. These factors are problematic in a study such as the present one which aims to sample a large number of plants on a regular basis. A cold extraction technique, developed to avoid these problems, is reported below.

Terpenes in sweet gale are not restricted to the volatile oil. Rashid (unpublished) has isolated a number of triterpenes from the root bark although only two of these are present in the leaves. No attempt was made in this work to investigate leaf triterpenes.

4.2 Materials and methods.

4.2.1 Extraction procedures.

Initially volatile oil was steam distilled, from 100 g batches of leaves taken from a large number of plants, according to the method described in the British Pharmacopoeia (1988). This method was repeated whenever a large volume of oil was needed.

In the first new method developed nitrogen was passed at a low flow rate (approx 1 cm^3 min^{-1}) through a 5 cm^3 round bottomed flask, heated to 50°C, containing 0.35g of fresh leaf (Fig 4.2). The

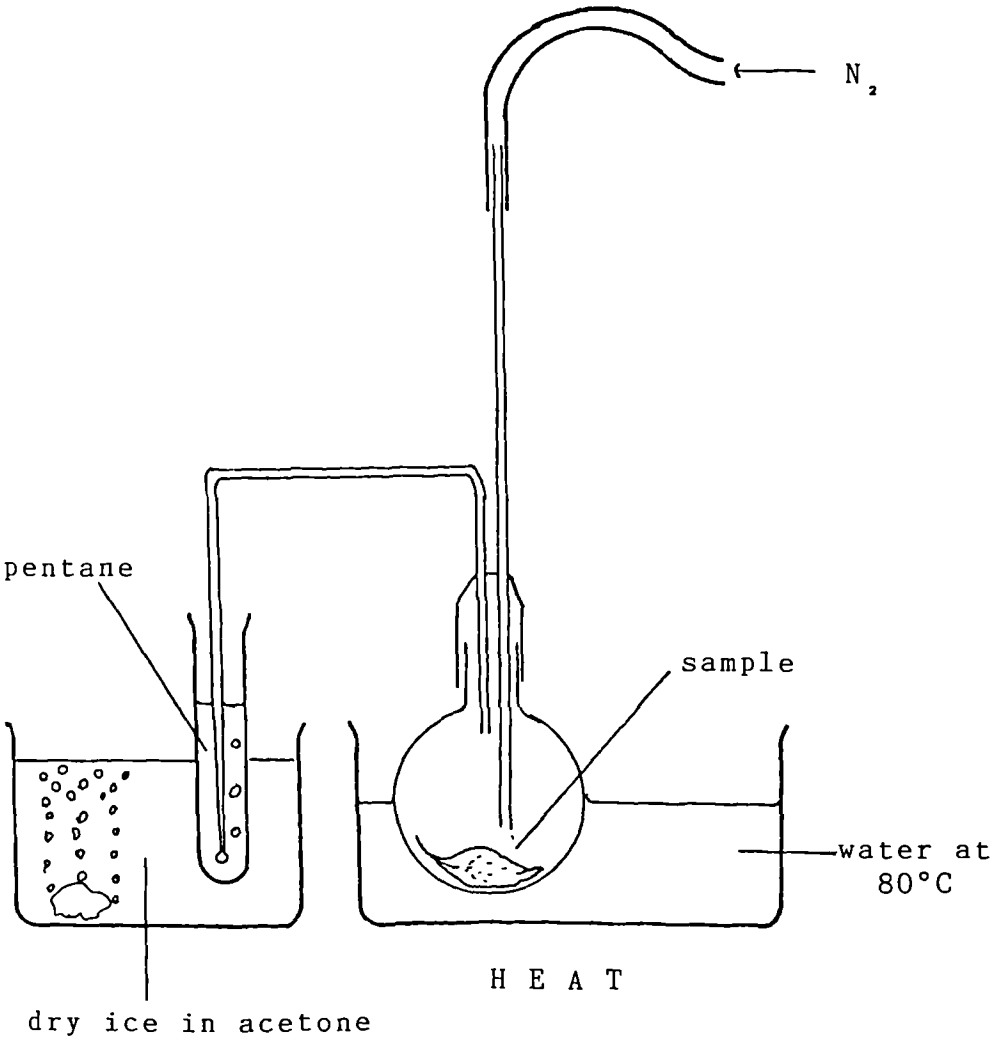


Figure 4.2 Apparatus for extraction of volatile oils from small leaf samples.

nitrogen passed out through a fine capillary tube into pentane cooled to -70°C in order to condense the oil vapour. After 1 h pentane was removed from the apparatus and evaporated under nitrogen to concentrate the sample. The method was tested using a single fruit of *Zanthoxylum americanum* and this provided ample oil for analysis on GLC. Unfortunately, when fresh leaves of sweet gale were placed in the heated chamber they gave off water vapour in addition to the oil and this water frequently froze in the fine capillary tube in the cold trap. Once five pieces of apparatus were running in tandem this proved to be a very awkward problem.

A second method was attempted which involved piercing the larger glands with a micropipette and extracting the oil directly. Micropipettes were produced by drawing out heated capillary tubes. This was initially done by clamping the top of the tube vertically, weighting the bottom and flaming the middle. This method provided micropipettes of similar dimensions but was fairly laborious. It was found that, with practice, micropipettes could successfully be produced manually by holding the tube horizontally above a flame and pulling the ends apart.

Once produced the micropipette was attached to a vacuum and clamped above a microscope platform at an angle of approximately 30 degrees from horizontal. A leaf was fixed to a slide and placed on the platform. Using low magnification the tip of the micropipette was lowered almost to the surface of the leaf and the leaf manoeuvred so the tip ruptured a gland. This was repeated ten times before the micropipette was removed and the oil was washed out with pentane.

This method proved to be very difficult to repeat frequently

since the leaf surfaces were rarely flat and prolonged exposure to the microscope light caused the glands to heat and burst. In addition, it proved very difficult to avoid contamination of the sample from the vacuum apparatus.

A third method was developed which involved mechanically rupturing the oil glands and collecting the liberated oil in pentane. Leaves (3.5 g) were taken from fresh or frozen shoots. The leaves were compressed on a sheet of glass with a hand held wallpaper seam roller to rupture the glands and then washed in pentane. The glass and roller were washed in the pentane after each sample. The pentane was driven off under nitrogen, the mixture being cooled to approximately 0°C during this process. The residue from each sample was made up to 0.35 ml with pentane and stored in a gas tight vial in the freezer until analysed by GLC.

4.2.2 Separation procedure.

Instrumentation: Carlo Erba HRGC 5300 with MFC 500 console using flame ionisation detection. Trivector Trio integrator. Column: Analytical SE30 (25m x 0.32mm). Mobile phase: nitrogen.

A GLC method was developed for steam distilled oil and the cold pressed extracts. Samples were injected directly on to the column at 80°C. The oven was kept at 80°C for ten minutes then heated at 45°C min^{-1} to 100°C. This temperature was maintained for 15 min and then increased to 150°C at 45°C min^{-1}. After 10 min the temperature was ramped at 10°C min^{-1} to 280°C and this temperature was maintained for 25 min.

A relatively low injection port temperature was selected to avoid initial thermal degradation of extracts. The lengthy isocratic stage at 100°C was required for the separation of sesquiterpenes although these were not detected until the following isocratic stage. The final isocratic stage was added for the fresh pressed leaf samples to allow leaf cuticle waxes, extracted by the pentane wash, to be eluted from the column.

The volatile oils of ten plants, extracted in 1987 before herbivory was evident, were investigated in detail to assess variation within the Flanders Moss sweet gale population. To assess changes during the summer a comparison was made between oils extracted from five plants in early and late summer.

4.2.3 Identification of peaks in the chromatograph.

Instrumentation: Hewlett Packard 5988A Mass Spectrometer. Hewlett Packard 5890 GLC. Column: Analytical BP1 (12m x 0.32mm). Mobile phase: helium.

The constituents of both steam distilled oils and cold pressed oils were identified. Monoterpenes were identified by a combination of GC-MS and comparing peak retention times with those of available standards. Sesquiterpenes were identified by GC-MS. For GC-MS analysis the oils were injected on column, the injection port being maintained at 250°C, and the column temperature was ramped from 50°C to 250°C at 5° min^{-1}. Identification by GC-MS involved a comparison of the peak spectrum with spectra stored in the computer's library. Similarity is presented as a percentage and the five library spectra demonstrating the greatest similarity to

the peak spectrum are listed by compound name.

Some difficulties were encountered whilst using the GC-MS. The signals of ions resulting from the disintegration of minor oil peaks were frequently distorted by background noise. In these cases the similarity coefficients were frequently low (< 70%). Occasionally more than one solution of equal likelihood was given. These problems were largely overcome by running a number of different samples on separate occasions and comparing the results. Pentane was driven off cold pressed oil samples to concentrate them, thereby improving the mass spectra of minor peaks. Use of standards was especially useful when multiple solutions to monoterpene peak spectra were given.

A number of the peaks gave no solution. In these cases the molecular ion was often apparent so a probable formula could be ascribed to the compound. When no likely molecular ion was apparent the peak was designated as unknown.

4.3 Results.

Steam distillation provided relatively large samples of oil. Distillation of 100 g leaves typically liberated 0.8 ml oil.

The cold pressed method of extracting oils proved to be very successful. Fig. 4.3 gives a complete chromatograph of one such extract. The terpene constituents are listed in Table 4.2. No terpenes were detected after 47 minutes, most of the further peaks either proving to be long chain hydrocarbons and their alcohols or not exhibiting a mass spectrum recognised by the computer. The major component of all these extracts was hexatriacontane, a leaf cuticle wax. The presence of this compound set a limit on the

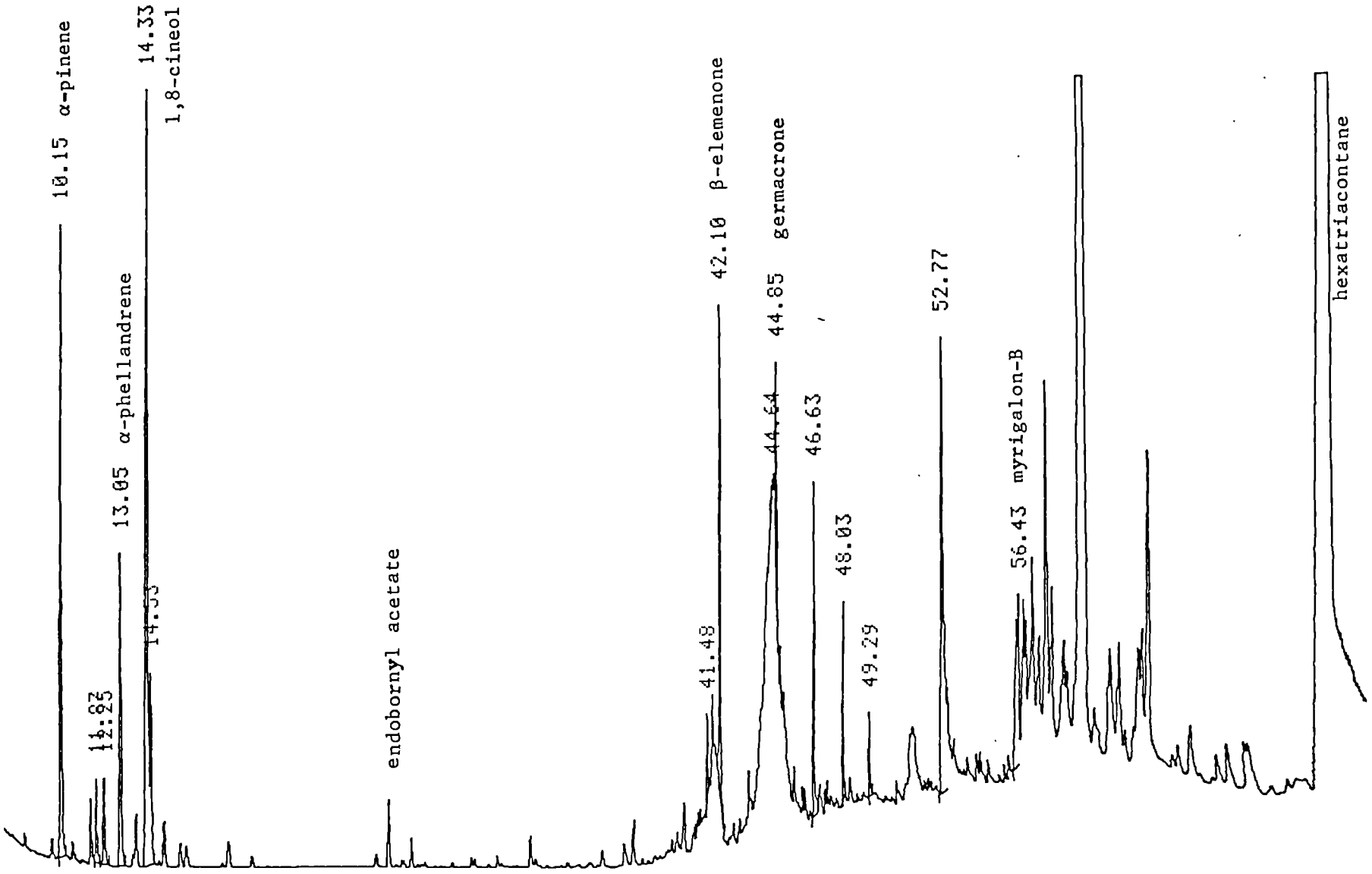


Figure 4.3 A GLC chromatograph of sweet gale volatile oil extracted by cold pressing.

Table 4.2 The composition of leaf gland oil extracted by cold pressing and steam distillation. The similarity indeses refer to GC-MS comparisons with library spectra. Percentage areas were calculated from cold extractions of 10 separate plants.

Compound	Peak	Formula	Cold pressed oil: percent similarity	Steam dist. oil: percent similarity	Mean % area	Max % area	Min % area
alpha-thujene	1	C10H16	98.00	95.00	0.273	0.54	0.03
alpha-pinene	2	C10H16	96.00	96.00	12.337	19.08	5.82
camphene	3	C10H16	97.00	97.00	0.354	0.56	0.13
cyclofenchane	4	C10H16	75.00	n.d.	0.009	0.06	0.00
sabinene	5	C10H16	76.00	n.d.	0.506	0.80	0.19
beta-pinene	6	C10H16	97.00	96.00	1.750	3.01	0.84
beta-myrcene	7	C10H16	87.00	88.00	1.357	2.21	0.84
alpha-phellandrene	8	C10H16	94.00	83.00	3.241	4.44	2.25
delta-4-carene	9	C10H16	94.00	78.00	0.148	0.15	0.00
para-cymene	10	C10H14	93.00	96.00	1.144	2.09	0.71
1,8-cineol	11	C1 H180	97.00	95.00	10.626	13.33	7.93
limonene	12	C10H16	95.00	97.00	1.470	3.04	0.00
3,7-dimethyl,1,3,6-octatriene	13	C10H16	89.00	n.d.	0.338	0.91	0.01
trans-ocimene	14	C10H16	89.00	n.d.	0.212	0.42	0.00
1,2-dichloro-4-methyl-benzene	15	C7H6C112	95.00	n.d.	0.001	0.01	0.00
octanoic acid,methyl ester	16	C9H1802	61.00	n.d.	0.056	0.35	0.00
alpha terpinolene	17	C10H16	94.00	90.00	0.072	0.25	0.00
nonanal	18		n.d.	95.00			
1-isopropenyl-?-methyl benzene	19	C10H12	n.d.	87.00			
gamma-terpinene	20	C1 H16	96.00	n.d.	0.205	0.44	0.00
alpha-terpinene	21	C10H16	93. 0	n.d.	0.033	0.08	0.00
linalool	22	C10H180	34.00	93.00	0.401	0.59	0.23
D-frenchyl alcohol	23	C10H180	93. 0	n.d.	0.045	0.11	0.00
butanoic acid, 3-methyl, 3-methyl, 3-methybutyl ester	24	C10H2002	n.d.	94.00			
rose oxide	25	C10H180	n.d.	70.00			
alpha-campholenealdehyde	26	C10H160	84.00	n.d.	2.450	0.40	0.15
C10H180	27		n.d.	ni			
rose oxide	28	C10H180	n.d.	68.00			
trans-pinocarveol	29	C10H160	n.d.	88.00			
trans verbenol	30	C10H180	84.00	n.d.	0.022	0.06	0.00
C10H180	31		n.d.	ni			
C10H180	32		n.d.	ni			
C12H24	33		n.d.	ni			
endo-borneol	34	C10H180	74.00	71.00	0.043	0.28	0.00
undecane	35	C11H24	63.00	n.d.	0.010	0.09	0.00
1-terpinen-4-ol	36	C10H180	97.00	89.00	0.084	0.78	0.00
alpha terpineol	37	C10H180	93.00	81.00	0.118	0.54	0.00
dodecane	38	C12H26	64.00	n.d.	0.007	0.07	0.00
2-butyl octan-1- ol	39	C12H260	70.00	n.d.	0.015	0.10	0.00
beta-citranellol	40	C10H200	86.00	n.d.	0.403	0.83	0.03
bornyl formate	41	C11H1802	78.00	n.d.	0.005	0.04	0.00
endobornyl acetate	42	C12H2002	95.00	98.00	0.049	0.22	0.00
tridecane	43	C13H28	67.00	n.d.	0.040	0.19	0.00
1-p-menthen-8-ylacetate	44	C12H2002	83.00	89.00	0.264	0.43	0.12

Table 4.2 (cont).

Compound	Peak	Formula	Cold pressed oil: % similarity	Steam dist. oil: % similarity	Mean % area	Max % area	Min % area
citronellyl acetate	45	C12H2002	88.00	94.00	0.079	0.29	0.02
geranyl acetate	46	C12H2002	94.00	94.00	0.078	0.30	0.00
C12H220	47		ni	n.d.	0.057	0.13	0.00
butanoic acid, 3-methyl, phenylmethyl ester	48	C12H1602	n.d.	91.00			
alpha-ylangene	49	C15H24	98.00	n.d.	0.067	0.36	0.00
alpha-cubenene	50	C15H24	98.00	95.00	0.050	0.12	0.01
beta-bourbonene	51	C15H24	67.00	n.d.	0.049	0.26	0.00
beta-elemene	52	C15H24	59.00	80.00	0.096	0.34	0.02
C15H24	53		ni	ni	0.005	0.03	0.00
C15H24	54		n.d.	ni			
trans-caryophyllene	55	C15H24	99.00	99.00	0.187	0.51	0.00
4,6-dimethyldodecane	56	C14H30	82.00	n.d.	0.073	0.19	0.00
C15H24	57		ni	n.d.	0.090	0.51	0.00
gamma-elemene	58	C15H24	99.00	98.00	0.450	0.97	0.07
C15H24	59		ni	ni	0.192	0.33	0.07
C15H240	60		n.d.	ni			
C15H240	61		n.d.	ni			
aristolen	62	C15H24	71.00	n.d.	0.251	0.74	0.00
delta-cadinene	63	C1'H24	86.00	95.00	1.108	2.68	0.47
1,2,3,4,4a,7-hexahydro-1,6-dimethyl-?-(i-methylethyl)-napthalene	64	C15H24	n.d.	79.00			
calarene	65	C15H24	84.00	n.d.	1.282	2.58	0.05
C15H24	66		ni	n.d.	0.052	0.09	0.01
eremophilene	67	C15H24	67.00	70.00	0.042	0.08	0.02
selina-4,11-diene	68	C15H24	59.00	n.d.	0.059	0.09	0.02
valencene	69	C15H24	75.00	53.00	0.124	0.52	0.02
C15H24	70		ni	n.d.	0.126	0.33	0.00
alpha-humulene	71	C15H24	57.00	n.d.	0.180	0.43	0.04
beta-farnasene	72	C15H24	95.00	n.d.	0.362	1.10	0.04
beta-selinene	73	C15H24	89.00	87.00	0.658	1.59	0.01
alpha-selinene	74	C15H24	84.00	89.00	0.483	1.41	0.01
delta-selinene	75	C15H24	62.00	n.d.	0.423	1.84	0.00
beta-guaiene	76	C15H24	73.00	n.d.	1.003	2.37	0.30
gamma-selinene	77	C15H24	79.00	74.00	1.000	2.07	0.14
C15H240	78		ni	ni	0.957	1.79	0.09
C15H240	79		ni	ni	2.701	6.65	0.03
nerolidol	80	C15H260	87.00	89.00	3.913	6.67	1.41
C15H240	81		ni	ni	4.374	9.27	0.00
beta elemenone	82	C15H220	94.00	99.00	14.271	25.35	0.03
C15H26	83		ni	n.d.	0.172	0.36	0.09
C15H26	84		ni	n.d.	0.258	0.67	0.15
C15H24	85		ni	ni	0.236	0.54	0.01
C15H24	86		ni	ni	0.351	0.96	0.10
C15H240	87		ni	n.d.	0.333	0.58	0.17
C15H240	88		ni	n.d.	0.338	0.45	0.18
unknown	89			n.d.	0.220	0.29	0.19
unknown	90			n.d.	0.228	0.38	0.14

Table 4.2 (cont).

Compound	Peak	Formula	Cold pressed oil: % similarity	Steam dist. oil: % similarity	Mean % area	Max % area	Min % area
unknown	91			n.d.	0.548	0.70	0.43
unknown	92			n.d.	0.406	0.76	0.22
C15H24	93		mi	mi	0.530	1.80	0.26
germacrone	94	C15H220	99.00	97.00	11.567	30.76	0.92
juniper camphor	95	C15H260	59.00	n.d.	0.469	1.51	0.00
C15H24	96		mi	n.d.	1.061	2.06	0.00
C15H240	97		mi	n.d.	0.538	3.02	0.00
benzoic acid,phenyl methyl ester	98	C14H1202	86.00	97.00	2.323	5.54	0.41
C15H240	99		n.d.	mi			
C15H24	100		mi	n.d.	0.607	1.13	0.15
C15H24	101		mi	n.d.	0.246	0.42	0.06
C15H24	102		mi	n.d.	0.197	0.45	0.00
		Total number of peaks	86	57			

n.d. = not detected; mi = formula ascertained from molecular ion.

concentration of the extracts since removal of too much pentane resulted in solidification of the extract.

With the exception of peaks 79 and 81 all major terpenes were identified by the computer with a high similarity coefficient (Table 4.2). Figure 4.4 is a typical example of a comparison of peak and library spectra. Most of the minor constituents were similarly identified although the similarity coefficients were often reduced. In these cases manual comparison of spectra generally confirmed identification (Fig. 4.5). However, isomers and other compounds of similar structure often fragment in an almost identical fashion so identification of some peaks may be suspect.

Use of the spectra to ascertain the molecular ion, and thus to assign a formula to unidentified peaks, may give rise to errors since many more complex terpenes are labile in which case the molecular ion may not appear. In these cases the apparent molecular ion is the first fragment with some stability under the conditions within the mass spectrometer. Peaks 96 and 100 - 102, all apparently hydrocarbon sesquiterpenes, may fall into this category since they are detected among oxygenated sesquiterpenes.

As a major peak germacrone exhibited a characteristic large front slope which obscured a number of minor peaks. As a result good mass spectra were not obtained and these have been listed as unknown peaks. The fronting of this peak was almost certainly due to thermal degradation of germacrone to ß-elemenone (Fig. 4.6) (Reichardt *et al.* 1989).

Wollenweber (1984) isolated a series of flavonoid aglycones in the leaf glands of *Comptonia peregrina* (L.) Coult. and *Myrica pensylvanica* Loisel including myrigalon B (peak 54: 2'6'-dihydroxy-

```
 1. ALPHA-PINENE, (-)-                                    136 C10H16
 2. ALPHA-PINENE, (-)-                                    136 C10H16
 3. ALPHA-PINENE, (-)-                                    136 C10H16
 4. Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-            136 C10H16
 5. d-Limonene                                            136 C10H16

 Sample file: >CB436     Spectrum #:        64
Search speed: 1       Tilting option: N       No. of ion ranges searched:   50

        Prob.      CAS #   CON #    POOT      K    DK  #FLG TILT   %    CON  C_I  P_IV
 1.      86*      80568   15565   "BIGDB    81    25     1    0   71      8   59    77
 2.      86*      80568   15359   "BIGDB    80    24     1    0   85      8   59    77
 3.      86*      80568   15563   "BIGDB    73    31     1    0   92      8   59    72
 4.      84*    4889832   15350   "BIGDB    69    24     1    0  100     10   55    67
 5.      84*    5989275   15517   "BIGDB    64    34     1    0   84     10   55    63

:AL
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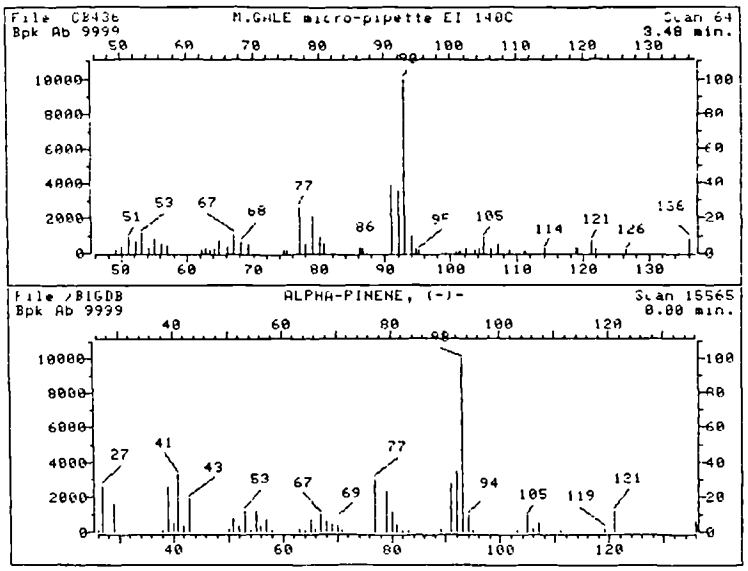

Figure 4.4 A GC-MS comparison of a peak spectrum with a library spectrum of α-pinene.

```
 1. Linalool                                          154 C10H18O
 2. Linalool                                          154 C10H18O
 3. Linalool                                          154 C10H18O
 4. Cycloheptane, metho›y-                            128 C8H16O
 5. Pentane, 3-chloro-                                106 C5H11Cl

 ample file:  CB422     Spectrum #:       710
Search speed: 1       Tilting option: N       No. of ion ranges searched:   53

      Prob.      AS #   CON #   ROOT     F   DF  #FLG TILT   %   CON  C_I R_IV

 1.     34      7R706    7697   BIGDB   53   41    2    0   47   34   12   17
 2.     31      78 06    7695   BIGDB   44   44    2    0   49   34   12   14
 3.     26       8706    7396   BIGDB   39   48    2    0   48   36   10   12
 4.     25   42604046    7333   BIGDB   39   43    2    0   62   50    7   13
 5.     15*     6 6.06    66J0  "BIGDB   25   78    3    0  109   57    3   13

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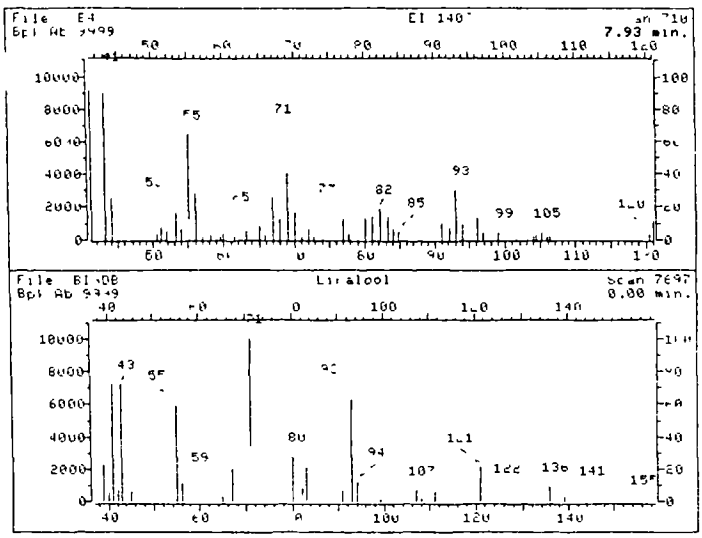
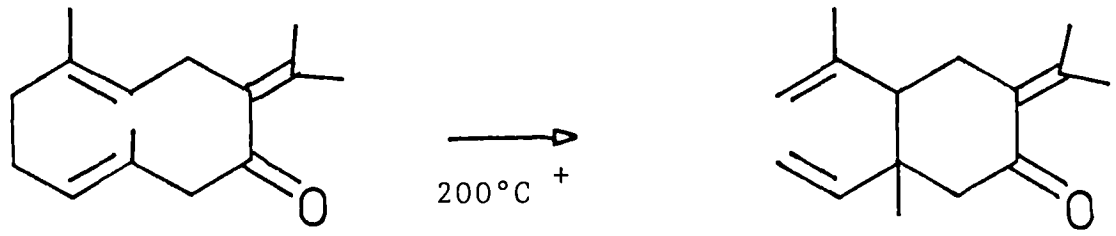


Figure 4.5 A GC-MS comparison which gives a poor similarity coefficient yet the identity of the peak can be confirmed by manual comparison.



germacrone beta-elemenone

Figure 4.6 Thermally induced rearrangement of germacrone to beta-elemenone.

4'-methothy-3',5'-dimethyl-dihydrochalcone). Since this compound had been isolated from sweet gale leaves in this study (Chapter 3.3.4) a sample was run through the GC-MS and the spectrum was manually compared with unidentified spectra from the cold pressed oil. One peak (rrt = 56.43 min) exhibited a spectrum which was almost identical to the peak 54 spectrum (Fig 4.7) indicating the presence of this compound in the glands.

A second unidentified spectrum, corresponding to a major oil gland peak (rrt = 52.77 min), exhibited the same molecular ion suggesting that the compound was probably an isomer of peak 54. This spectrum (Fig 4.8) appears to be similar to the previously reported mass spectrum for 4,4,6-trimethyl-2-(ß-phenylpropionyl) cyclohexane-1,3,5-trione (Anthonsen *et al.* 1971) with major fragments at *m/z* 168 and *m/z* 230, although the base peak (*m/z* 91) differed from the earlier report in which the molecular ion (*m/z* 300) furnished the base peak. This difference may result from the mass spectrum being run under different conditions.

It can be seen from Table 4.2 that in early summer the cold pressed oil of sweet gale consisted of a possible 86 terpenes. Only 57 compounds were separated in the steam distilled oil, of which 41 were also present in the cold pressed oil. The steam distilled oil contained none of the long chain alkanes or flavonoids. The cold pressed oil exhibited a more complex sesquiterpene profile than the steam distilled oil whereas the oxygenated monoterpene profile of the steam distilled oil was more complex. The major peaks were generally apparent in both types of extract although ß-elemenone was never a major constituent of steam distilled oil.

The cold pressed extracts were largely similar except in

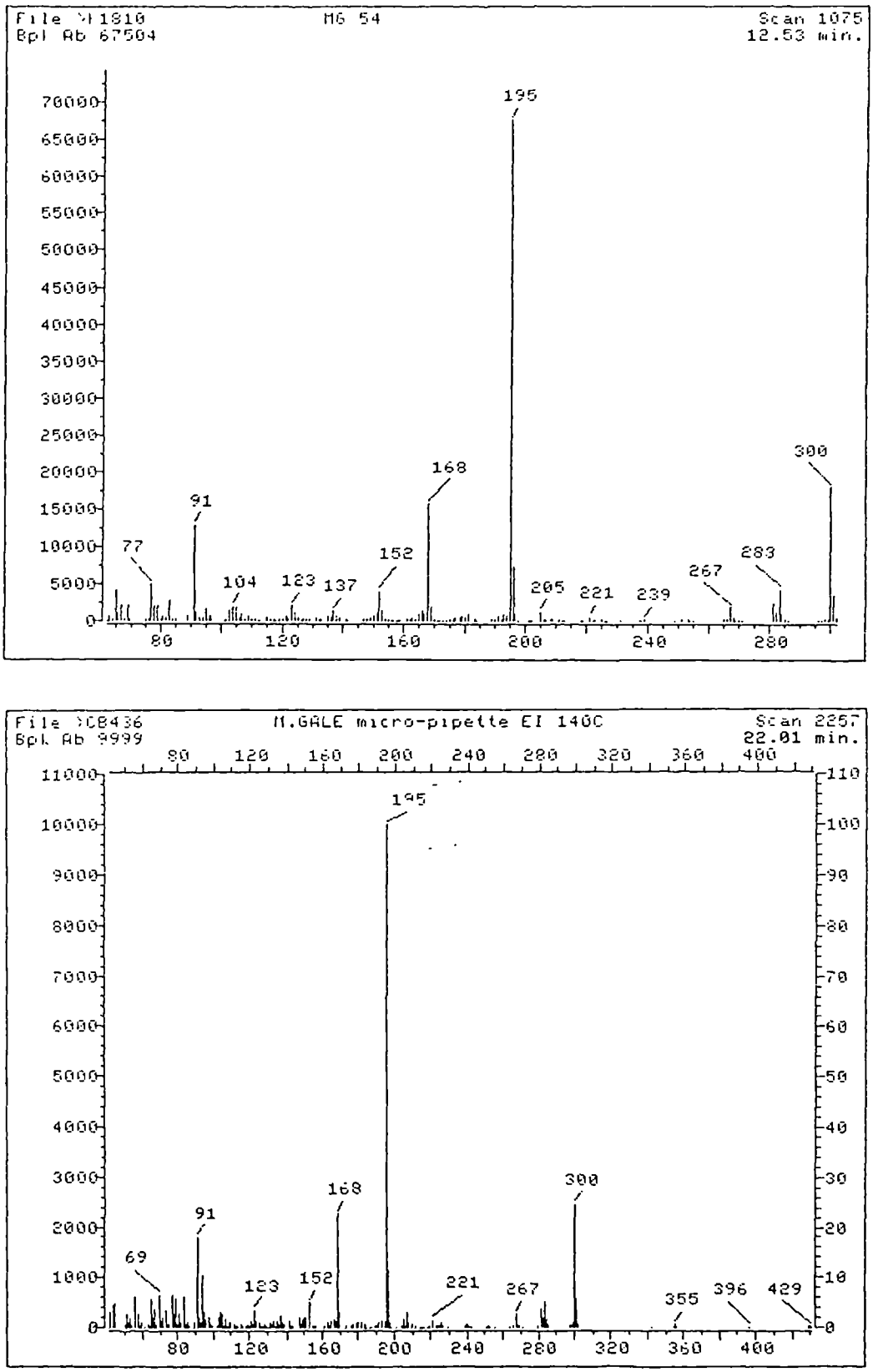
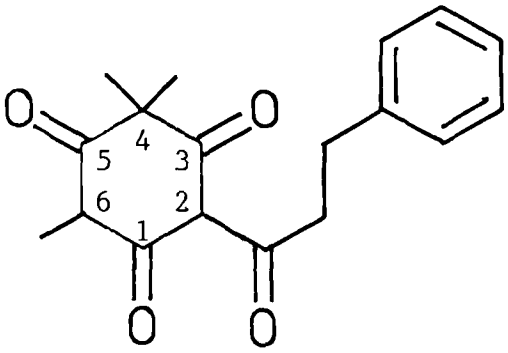


Figure 4.7 The GC-MS spectra of peak 54 (top) and a leaf gland extract peak (rrt = 55.8 min) (bottom). Comparison indicates that both are from the same compound.



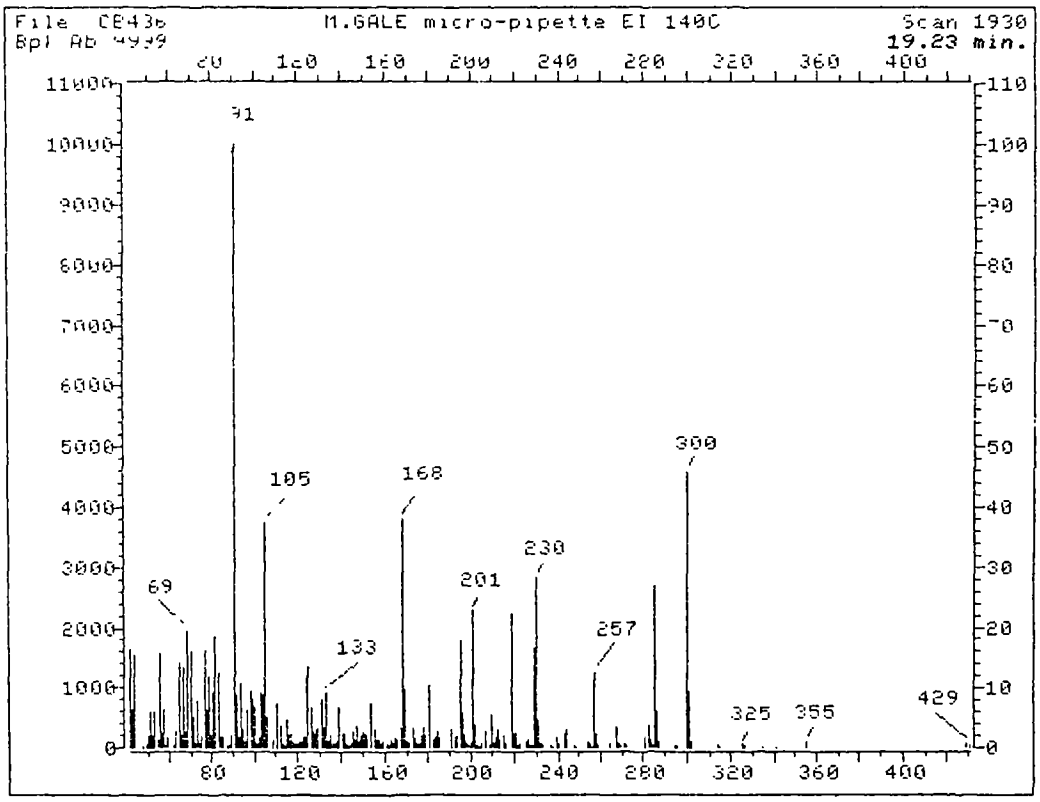


Figure 4.8 The GC-MS spectrum of an isomer of peak 54 with its probable structure (above).

respect of the major sesquiterpene peak. It can be seen from the percentage areas given in Table 4.2 that germacrone and ß-elemenone peaks accounted for a maximum of 30.65% and 25.35% of total peak area respectively. These, however, did not occur in the same extract and when ß-elemenone was the major sesquiterpene germacrone was present as a comparatively minor constituent. However, when germacrone was the major sesquiterpene the area under the ß-elemenone peak was frequently also large (Fig. 4.9). In only one extract was the ß-elemenone peak less than 7% of the total peak area. The monoterpene profiles of the extracts were broadly similar. Limonene was the only substantial monoterpene which did not always appear to be present. Limonene and 1,8-cineol have very similar retention times and it is possible that occasionally the column failed to separate them. However, all plants were extracted on more than one occasion and the presence or absence of a limonene peak was a consistent feature of oil from the same plant (Fig. 4.9).

The volatile oils of all plants were observed to change during the summer (Table 4.3). The most noticeable difference was the large reduction in the area of the ß-elemenone peak. In those plants where germacrone was a major constituent a smaller reduction in the germacrone peak area was also observed (Fig 4.9). The relative areas of α-pinene and 1,8-cineol both increased in late summer but this probably reflected no more than their rise as a proportion of the whole oil due to the reduction in the sesquiterpenes. Minor differences were apparent such as a reduction in α-phellandrene and a corresponding increase in *p*-cymene. In late summer significant increases in the areas of both 1-terpinen-4-ol and α-terpineol (t-test: $p < 0.001$) were also observed.

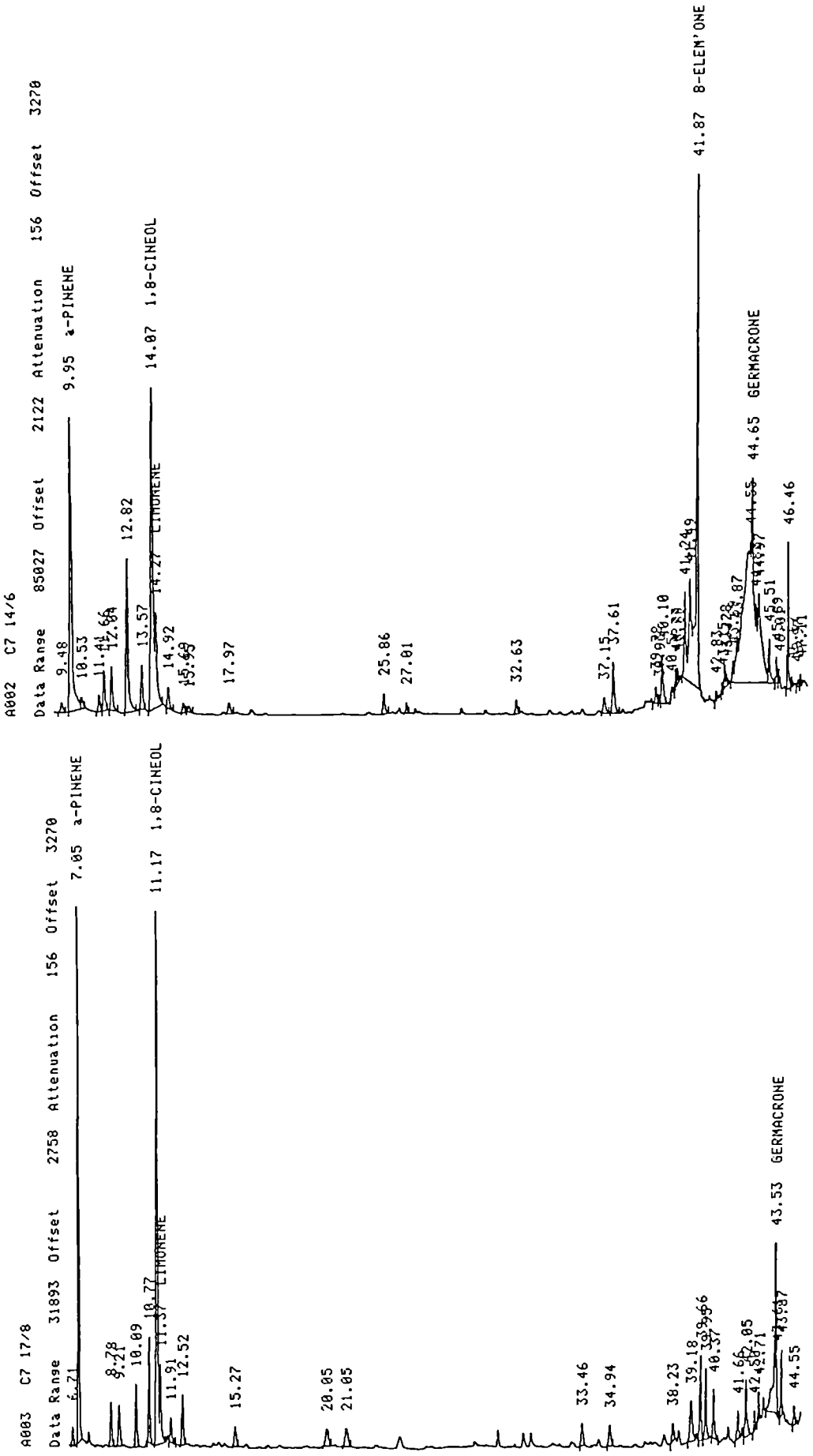


Figure 4.9 A comparison of the volatile oil of a sweet gale plant in early and mid summer.

Table 4.3 A comparison of the mean areas of selected peaks of volatile oils from five plants sampled on 14 June 1987 and 17 August 1987.

	peak	mean % area 14/6/87	mean % area 17/8/87	% change
alpha pinene	2	12.434	19.994	60.8
beta-myrcene	7	1.410	2.674	89.6
alpha-phellandrene	8	3.292	1.352	-58.9
para-cymene	10	1.338	3.768	181.6
1,8-cineol	11	12.098	20.566	70.0
3,7-dimethyl, 1,3,6-octatriene	13	0.610	0.860	41.0
trans ocimene	14	0.334	1.626	386.8
1-terpinen-4-ol	36	0.098	1.056	977.6
alpha-terpineol	37	0.216	1.304	503.7
nerolidol	80	3.202	1.762	-45.0
beta-elemenone	82	13.532	2.412	-82.2
germacrone	94	10.174	4.592	-59.9

4.4 Discussion.

4.4.1 The benefits of cold pressing extraction of leaf glands.

These results support the decision to develop a cold extraction method for the volatile oils. Not only does steam distillation fail to extract compounds such as flavonoids from the leaf glands, it also alters the composition of the terpenes in many respects. Both of these drawbacks were highlighted in this study. The differences between the cold pressed oil terpenes and the steam distilled oil, notably the absence of certain sesquiterpenes and the presence of oxygenated monoterpenes in the steam distilled oil, support the contention that some constituents undergo thermal decomposition during distillation.

One feature of the steam distilled oil was the absence of ß-elemenone. This, however, may not indicate thermal degradation but have been due to the fact that most leaf collections for steam distillation were made between late summer and mid autumn when the volatile oil of all plants contained little ß-elemenone.

4.4.2 Variation within and between populations of sweet gale.

A comparison of previous studies (Table 4.1) demonstrates that sweet gale volatile oil differs between populations. A comparison of Tables 4.1 and 4.2 lends support to this conclusion since the population on Flanders Moss is different again from other populations. This study also demonstrates that it is important to distinguish between individual plants since differences occur within the population.

When plants within the population differ, in respect of their oils, the differences are maintained throughout the year. This

indicates that the composition of the oil is under close genetic control. The fact that the oil composition is a variable characteristic, and that large differences occur between populations, may indicate that the genes controlling this characteristic are susceptible to relatively rapid changes, possibly resulting from local pressures.

The presence of ß-elemenone and/or germacrone in the volatile oil is interesting. These were major components of the oil in Scotland but were only detected in one other population (Lawrence & Weaver 1974), and then as very minor components. There appears to be a link between these two sesquiterpenes, and the ability of germacrone to degrade to ß-elemenone by a retro Deils-Alder rearrangement (Reichardt *et al.* 1989) lends some support to this supposition. In early summer the presence of a mixture of both sesquiterpenes in some plants and only ß-elemenone in others suggests that all plants produce ß-elemenone but some plants are able to convert it to germacrone whereas others lack that ability. If this hypothesis is correct then the conversion of ß-elemenone to germacrone may confer some advantage to the plant and represent a recent adaptation.

It would appear that ß-elemenone production diminishes later in the summer since little of this compound was found in the late summer oil. The continuing presence of germacrone later in the summer may be due to its lower volatility. However, plants are able to recycle terpenes (Loomis & Croteau 1980) but if plants within the Flanders Moss population have only recently acquired the ability to produce germacrone they may yet lack the ability to rapidly metabolise it.

The differences between the oil composition in early and late summer are intriguing. It is extremely difficult to explain them in terms of passive activity (such as loss by evaporation). Uncontrolled oxidation or reduction may account for some of the observations, such as the relative changes in *p*-cymene and α-phellandrene, but this does not explain the loss of sesquiterpenes. If some of the changes are under active control it suggests that the function of the oil differs between early and late summer. It is possible that the sesquiterpenes provide a store for excess carbohydrate and energy. Production of isopentenyl pyruvophosphate, the five carbon building block of terpenoids, from mevalonic acid requires 3ATP (Spurgeon & Porter 1980). However, it is difficult to envisage a use for this stored carbon and energy at a time when above-ground growth has virtually stopped yet photosynthesis is peaking due to large leaf areas and high temperatures. If, however, the sesquiterpenes have a defensive role then the importance of defense possibly diminishes later in the summer. This would be in accord with the reduction in leaf nitrogen (Fig 2.3) after leaf burst and observed ability of sweet gale to shed old damaged leaves and produce new ones in mid and late summer.

Clearly the composition of sweet gale leaf gland volatile oil is a variable characteristic within the population and, furthermore, individual plants appear to be able to vary the composition with time.

Chapter 5. The effect of herbivory on the leaf chemistry of sweet gale.

5.1 Introduction.

The object of the field study was to investigate the effect of insect herbivory on the secondary chemistry of sweet gale in its natural environment. To achieve this an experiment was planned in which one set of plants would be kept free of insects whilst a second set, from the same population, would be allowed to sustain natural insect damage.

Since sweet gale plants comprise of stems connected by roots and frequently no clear boundaries exist between plants it is often not possible to distinguish one plant from another. To circumvent the problem of identifying entire plants before bud-burst in year 1 individual stems were selected for the experimental groups. The stems were selected as a source of leaves, for collection on a regular basis, for chemical analyses. By the beginning of year 2 a number of clearly delineated plants had been identified and these were used in subsequent studies.

The experiment was designed to facilitate simple parametric statistical manipulation of the data. A fundamental aspect of this design was the method employed to keep one set of stems free of insects. Two possibilities were considered. The first involved the application of insecticide on a regular basis and the second required physical barriers, such as fine mesh bags, to be used to keep insects from these stems. Both methods had possible drawbacks but the former was chosen for a variety of reasons.

From a purely practical point of view there were difficulties

involved in physically isolating the stems from insect herbivores and effectively maintaining that isolation. These difficulties included the possible presence of insect eggs on the stems, such as those of *Lygocoris spinolai* which lays its eggs in bark fissures on sweet gale stems (Southwood & Leston 1959), which would have to be eradicated before the plant was isolated. Maintenance of barriers on such an exposed site could also be problematic.

Any practical method of physically isolating a plant within a community from insects would also involve affecting the immediate environment by cutting down airflow, increasing the relative humidity and possibly effecting the temperature around the plant. Furthermore, the simple method of using fine mesh to bag the plants would cut the levels of incident light reaching the plant. Any one of these factors could have an influence on plant metabolism leading to artifacts in the experimental results. The combined effect of suppressed herbivory and such artifacts could be extremely complex and the results of subsequent analyses would be open to misinterpretation.

The use of insecticide presented none of the practical difficulties associated with physical barriers. The possibility existed that it would have an effect on plant metabolism but this was amenable to investigation in greenhouse experiments. However, care had to be taken in the choice of insecticide as Flanders Moss is a site of special scientific interest. The insecticide had to be active at low concentrations, be non-persistent and leave no harmful residues. Cypermethrin, a chlorinated pyrethroid, was selected. It is both more active and more stable than the natural pyrethrin yet it has a short half life in the soil. Furthermore, it has to be

ingested in very high doses to be toxic to mammals (LD_{50} for rabbits > 24g kg^{-1}: Worthing 1983).

Production of data amenable to statistical manipulation was a major consideration in the design of the experiment. By demonstrating statistical errors Fowler and Lawton (1985) were able to question the validity of conclusions in many of the early studies of induction. To fulfil the requirements for statistical manipulation a random selection of stems for the experiment was included in the design. Before this could be carried out the number of replicates for each experimental group had to be adopted. Perhaps the most common error in statistical analyses is a type II error (Sokal & Rohlf 1981) in which data from distinct populations do not prove to be significantly different (i.e. the null hypothesis is not rejected). A common cause of this type of error is the use of too few replicates. However, in the absence of data on the normal distribution of the parameter under investigation it is not possible to calculate the number of replicates needed in the experiment. In this case it was not possible to search for information on the variability of induced secondary metabolite concentrations before the experiment investigating this phenomenon had taken place. The decision on replicate number, therefore, was made on the basis of the maximum number of replicates which could be practically analysed within a week.

It was possible that damage of any sort would trigger a response and that sampling could itself induce changes and thus obscure any effect of herbivory. To reduce this danger it was determined that very small quantities of tissue should be collected with a minimum of wounding from each stem. To achieve a further

reduction in the danger a multiple of the replicate number of stems were incorporated in each group. This catered for the creation of subsets allowing the rotation of sampling among the subsets (i.e. a different subset to be sampled each week) resulting in extended periods between samples being collected from any particular stem.

5.2 Methods.

5.2.1 Field Experiment Year 1 (1987): Herbivore damage.

Sixty sweet gale stems were selected at random in an area along the Northern edge of Flanders Moss. These were divided into three groups. These groups were denoted by the letters A, B and C. Groups A and B were subdivided into five subsets of five stems each. Group C comprised of the remaining ten stems divided into two subsets.

The stems in group A were allowed to sustain natural herbivory. Group B provided the controls. These stems were sprayed on a weekly basis with cypermethrin for the duration of the experiment. Plants in group B sustained no damage except that incurred by sampling. Group C provided stems for artificial damage. They were sprayed every week with cypermethrin to keep them free of insect herbivores until damaged. Damage incorporated approximately 50% of leaves being cut diagonally to the midrib two to four times.

Approximately one gram of leaves were collected per stem. Groups A and B were sampled on a weekly basis from May to June and every two weeks from July until mid September. Each sample consisted of two shoots taken cleanly from different branches on the stem. The subsets were rotated so the minimum period between consecutive sampling on a particular stem was 5 weeks (Table 5.1).

Table 5.1 The feild experiment sampling reg'me in year 1 (1987).

Plant set	4 May	11 May	18 May	25 May	1 Jun	8 Jun	15 Jun	22 Jun	6 Jul	20 Jul	3 Aug	17 Aug	31 Aug
A1-A5	x					x					x		
C1-C5	x					x					x		
A6-A10		x					x					x	
C6-C10		x					x					x	
A11-A15			x					x					x
C11-C15			x					x					x
A16-A20				x					x				
C16-C20				x					x				
A21-A25					x					x			
C21-C25					x					x			

x denotes when plants were sa pled

5.2.2 The field experiment Year 1: Artificial damage.

On June 8 five stems from group C were damaged. Samples were collected three times: immediately prior to being damaged, one week after and two weeks after damage. On July 6 the method was repeated using the second subset. Four collections of samples were made from this subset, one prior to damage and three over the following weeks (Table 5.1). As the group C stems were being artificially damaged the effect of sampling the same stem every week was not considered to be a problem.

5.2.3 Collection considerations.

In order to avoid rapid chemical changes in the collected leaves due to sampling itself care was taken not to damage leaves on the shoots during collection. The shoots were immediately placed in a chilled container.

On return to the laboratory the material either immediately underwent extraction or was deep frozen awaiting later extraction. Leaves were subjected to volatile oil extraction first and then the same leaves were used for the extraction of phenolics.

5.2.4 Field Experiment Year 2 (1988).

Ten clearly delineated plants were selected and divided into two groups. Cypermethrin was applied to one group, the controls, on a weekly basis and the other group was left to sustain natural herbivore damage.

Weekly sampling was carried out in the same way as year 1 from early May 1988 until early August 1988. No plants were artificially damaged. Phenolic extracts were prepared from the leaves. A single

collection of immature leaves just burst from intermediate primordia was made in early August and extracted in methanol as above.

5.2.4 The greenhouse experiment.

In March 1987 one hundred sweet gale stems with roots were collected from a ride in the Forestry Commission woods by Loch Lomond (grid ref 695238). These were a planted in large pots (35cm tall x 30cm diameter) and the pots placed in beds in Glasgow Botanic Gardens. The pots were buried, with the rims just submerged, at 1 m intervals.

Thirty plants were dug up, in their pots, on 15 April 1989. These were removed to the roof of the Royal College Building. Ten of the most healthy looking plants were taken into the greenhouse and each plant was placed in a wooden frame (80cm x 40cm x 40cm). To exclude insects the sides and base of each frame was covered with a fine mesh nylon net and an acetate hinged lid was fixed over the top (Fig 5.1)

All ten plants were initially sprayed once with Malathion (PBI) to kill any insects which had overwintered on the plants. The group was divided in two and five plants were sprayed weekly with cypermethrin whilst five plants were left untreated. Fresh leaves from each plant were collected and phenolics were extracted on six dates between 15 May and 27 June.

5.3 Data analysis.

5.3.1 Analysis of the volatile oil data.

The methods used to extract and separate the leaf volatile oils are described in Chapter 4, sections 4.2.1 & 4.2.2. The GLC

Figure 5.1 The greenhouse experiment.

chromatograms were stored on disc. The stored data was retrieved once a series of samples had been separated and comparisons were made between the experimental groups. Visual inspection of chromatographs revealed possible differences between groups. When tentative differences were observed the peaks in question were integrated and the areas were calculated as a proportion of the area under the 1,8-cineol peak. The 1,8-cineol peak was chosen for this function as it was both large and appeared to vary very little between samples. An internal standard was not used in the volatile oil analysis because the yield of oil is a very variable characteristic of sweet gale. The concentration of internal standard relative to the constituents of the oil would be dependant on the yield. Thus large variations would be expected which would bear no relation to the relative concentrations of constituents within the oil.

5.3.2 Analysis of phenolics data.

Extraction and separation of the leaf phenolics was achieved using the method described in Chapter 3, sections 3.2.1 and 3.2.2. In year 1 the chromatograms were inspected visually to initially discern possible differences between groups. The areas of peaks which appeared to differ between groups were integrated as was the area of caffeic acid (internal standard). The area of each peak was divided by the area of the corresponding internal standard peak to account for variation between runs. Standard curves of the standardised peak area against concentration for two of the compounds were plotted to check. The data from each peak was statistically analysed for each sampling date and the group means

were plotted against time. In addition to peaks which appeared visually to exhibit differences between the groups the analysis was carried out on one other major peak to check whether the any effect of herbivory was general to all phenolics or specific to a few compounds.

In year 2 compounds which the year 1 analysis indicated were linked to herbivory were further investigated. In addition a further peak, which appeared as a shoulder of one peak apparently linked to herbivory, was included in the analyses. The results from the single collection of mid-season immature leaves were compared with results from early season immature leaves.

In year 2 fresh leaves from each sample throughout the season was weighed, dried at 60°C. for 24 h and reweighed. The proportion of water was calculated. These results were plotted against time.

5.4 Results.

5.4.1 Year 1: The effect of herbivory.

The first sign of insect damage was on one plant on 25 May. By the following week approximately 60% of plants exhibited some degree of herbivore damage and this had increased to almost 100% by 23 June.

The standard curves (Figs 5.2 & 5.3) demonstrate that a good linear relationship exists between compound peak area/caffeic peak area and compound concentration. Groups A and B were observed to differ in respect of peaks 28, 30 and 44. The concentration of peak 28 (Fig 5.4) in the leaves of group A was significantly higher than in the leaves of group B on 25 May and significantly lower on 2 June. The magnitude of the these differences were not great and on

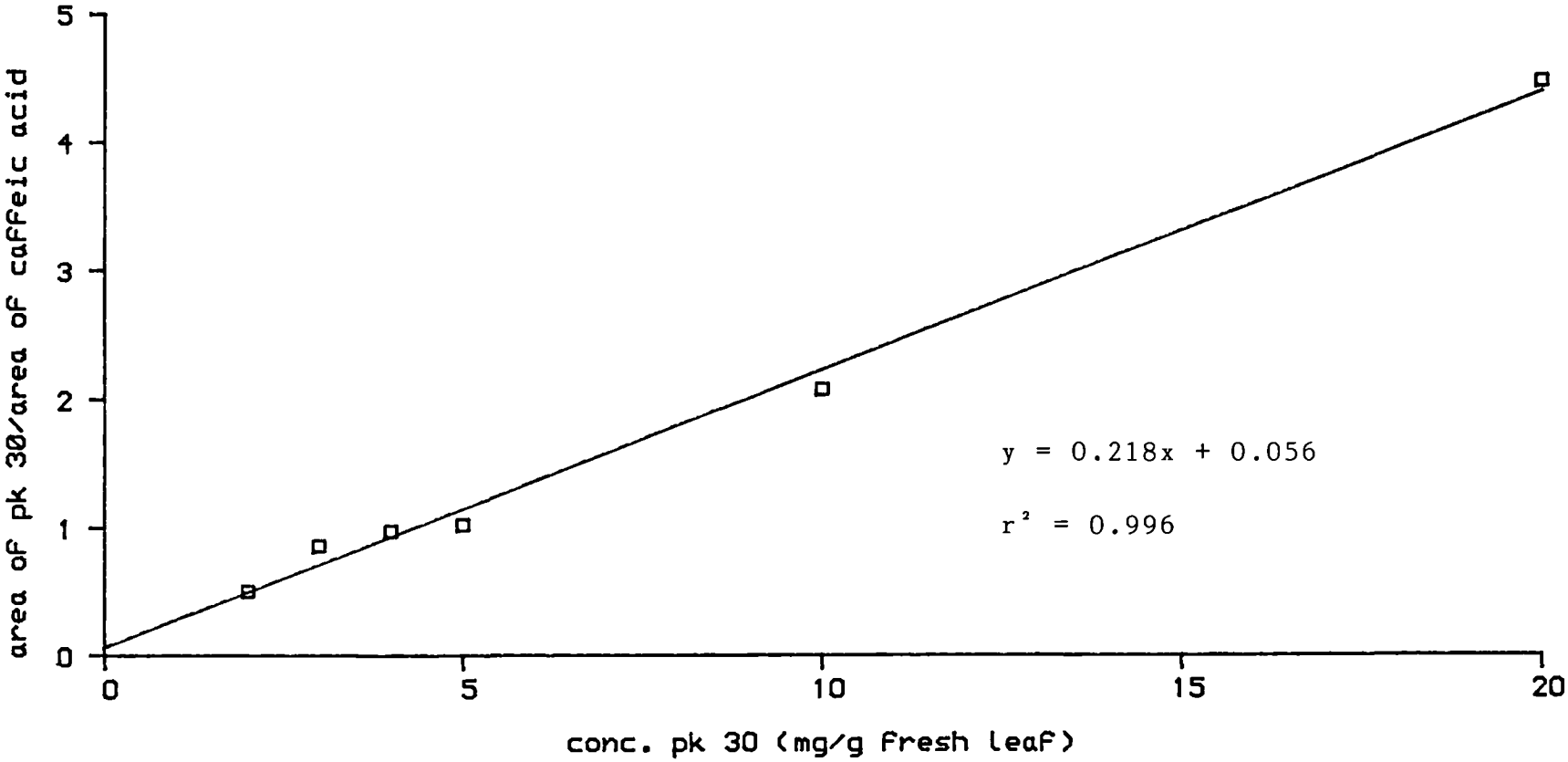


Fig. 5.2 The relationship between the concentration of pk 30 and the area under pk 30 divided by the area under the caffeic acid peak.

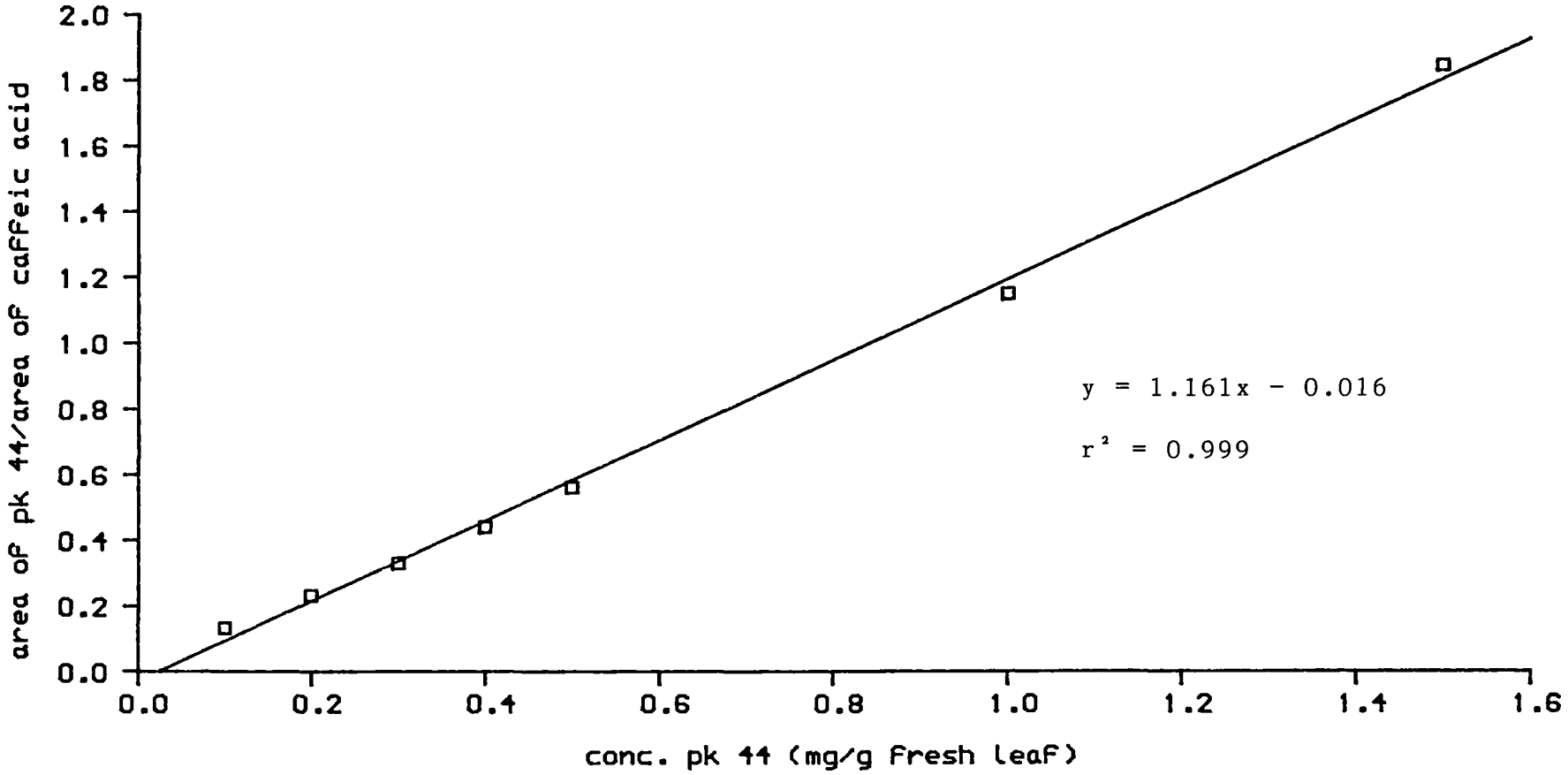


Fig. 5.3 The relationship between the concentration of pk 44 and the area under pk 44 divided by the area under the caffeic acid peak.

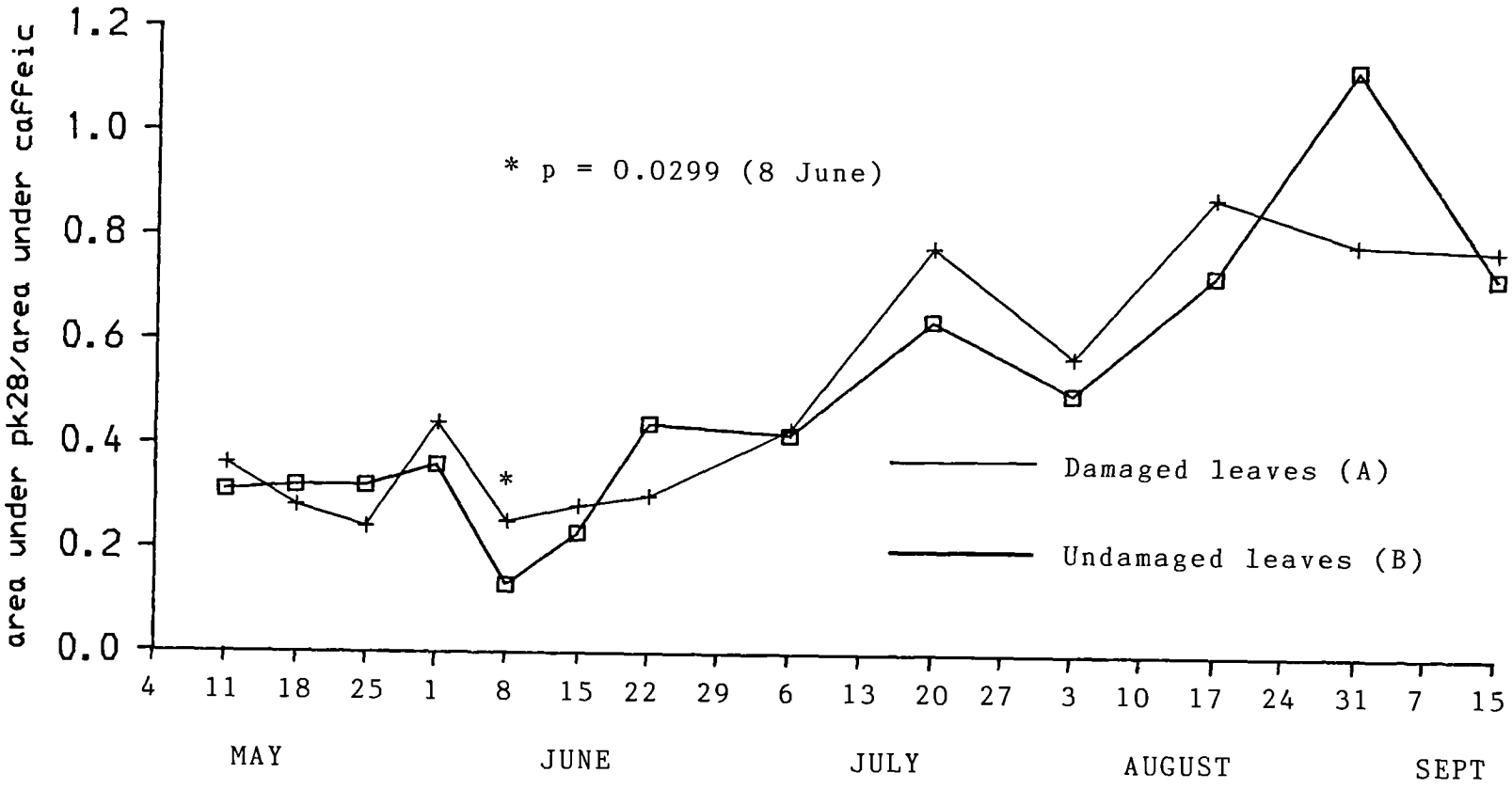


Fig. 5.4 A comparison of the leaf concentrations of peak 28 between groups A and B during the summer of 1987. (Confidence limits have been ommitted for the sake of clarity).

other weeks the means differed by a similar magnitude (i.e. 2 June, 23 June, 20 July, 3 August and 18 August) although these were not significant at the 5% level. The fluctuations continued throughout the season. In both groups an upward trend in concentration was observed throughout the period.

The mean concentrations of peak 30 (Fig 5.5) were comparatively low until 25 May and then increased rapidly to approximately ten times their initial concentration. This rise was most rapid in group B resulting in a significantly greater concentration in this group on 8 June. The concentration of peak 30 in group A had risen to a similar level by 23 June although it remained marginally lower than that of group B until late August.

The mean concentration of peak 44 (Fig 5.6) was initially low in both groups and remained stable for three weeks. On 2 June group A exhibited a rise in peak 44 resulting in a significant difference on this day. The upward trend continued until early August and was mirrored by a rise in group B after 14 June. In group A the mean $\log_{10}$ concentration of peak 44 was significantly greater than that of group B on 2, 14 and 23 June. The groups were similar in respect of peak 44 after 13 July.

The plants did not appear to differ in respect of the other major peak in the chromatograph, peak 33 (Fig 5.7). The groups exhibited no significant differences in respect of peak 33 during the season. With the exception of an early fluctuation the concentration of this compound remained steady throughout the season.

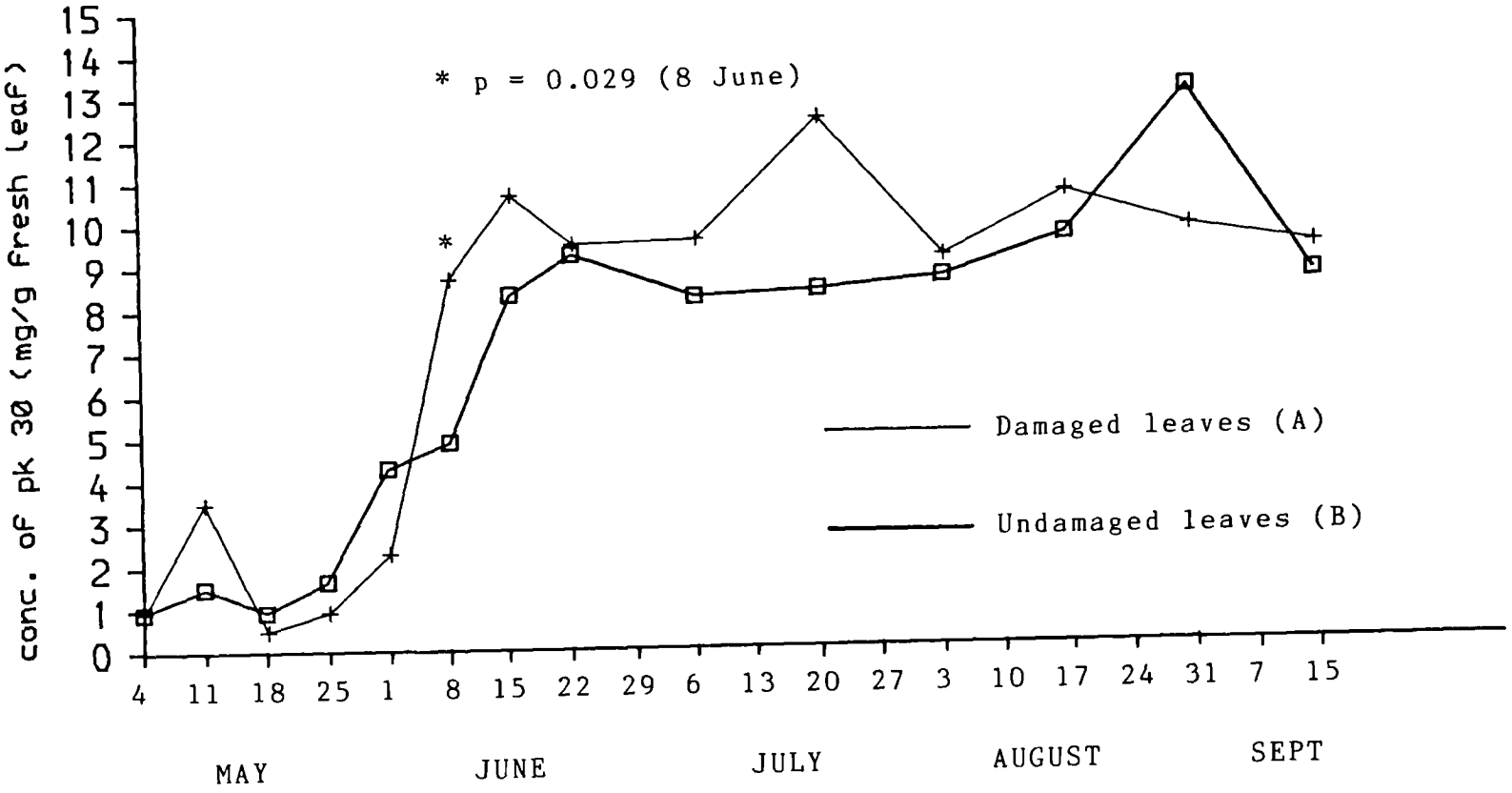


Fig. 5.5 A comparison of the leaf concentrations of pk 30 between groups A and B during the summer of 1987 (confidence limits have been ommitted for the sake of clarity).

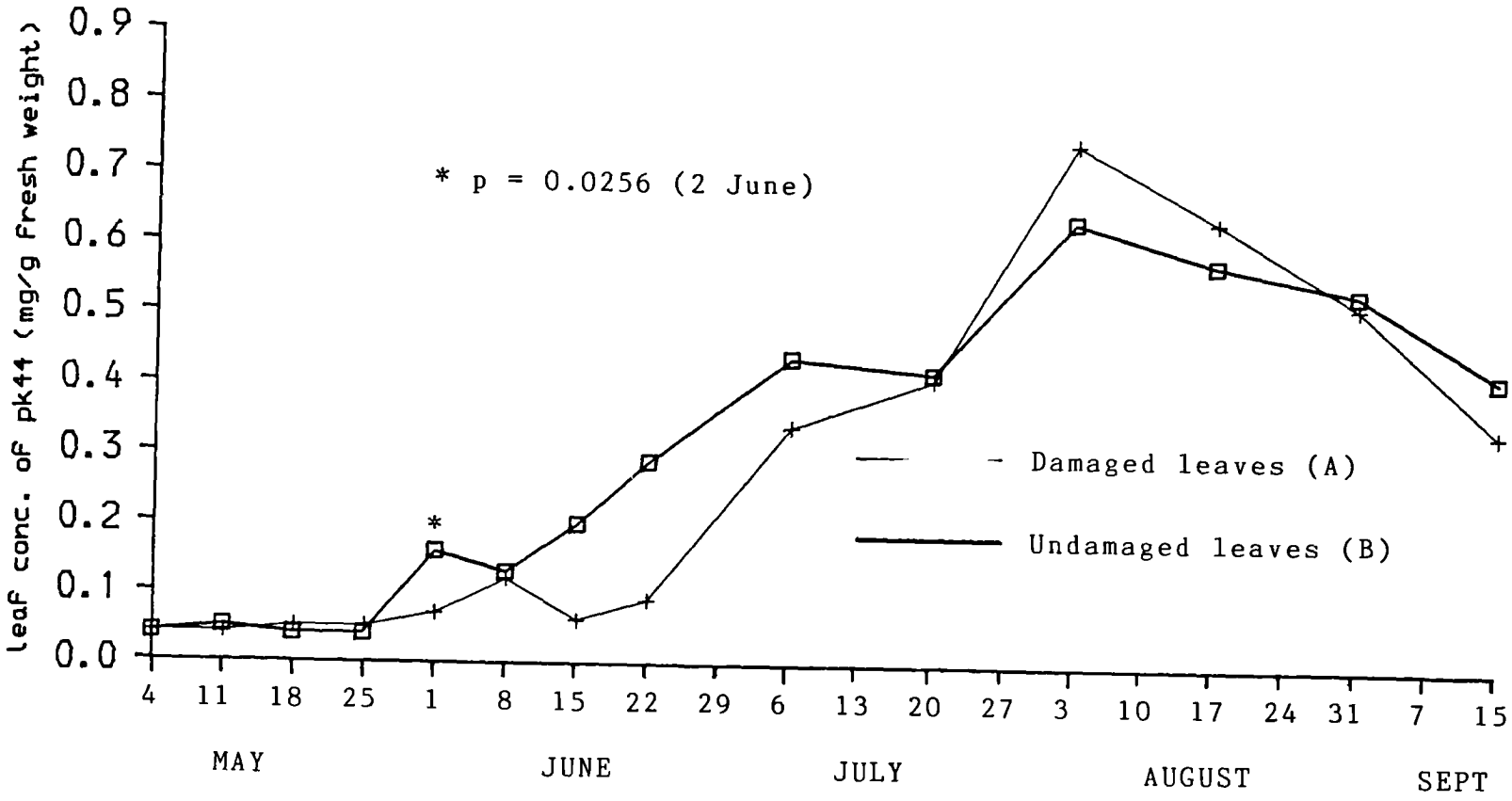


Fig. 5.6 A comparison of the leaf concentrations of peak 44 between groups A and B during the summer of 1987. (Confidence limits have been ommitted for the sake of clarity).

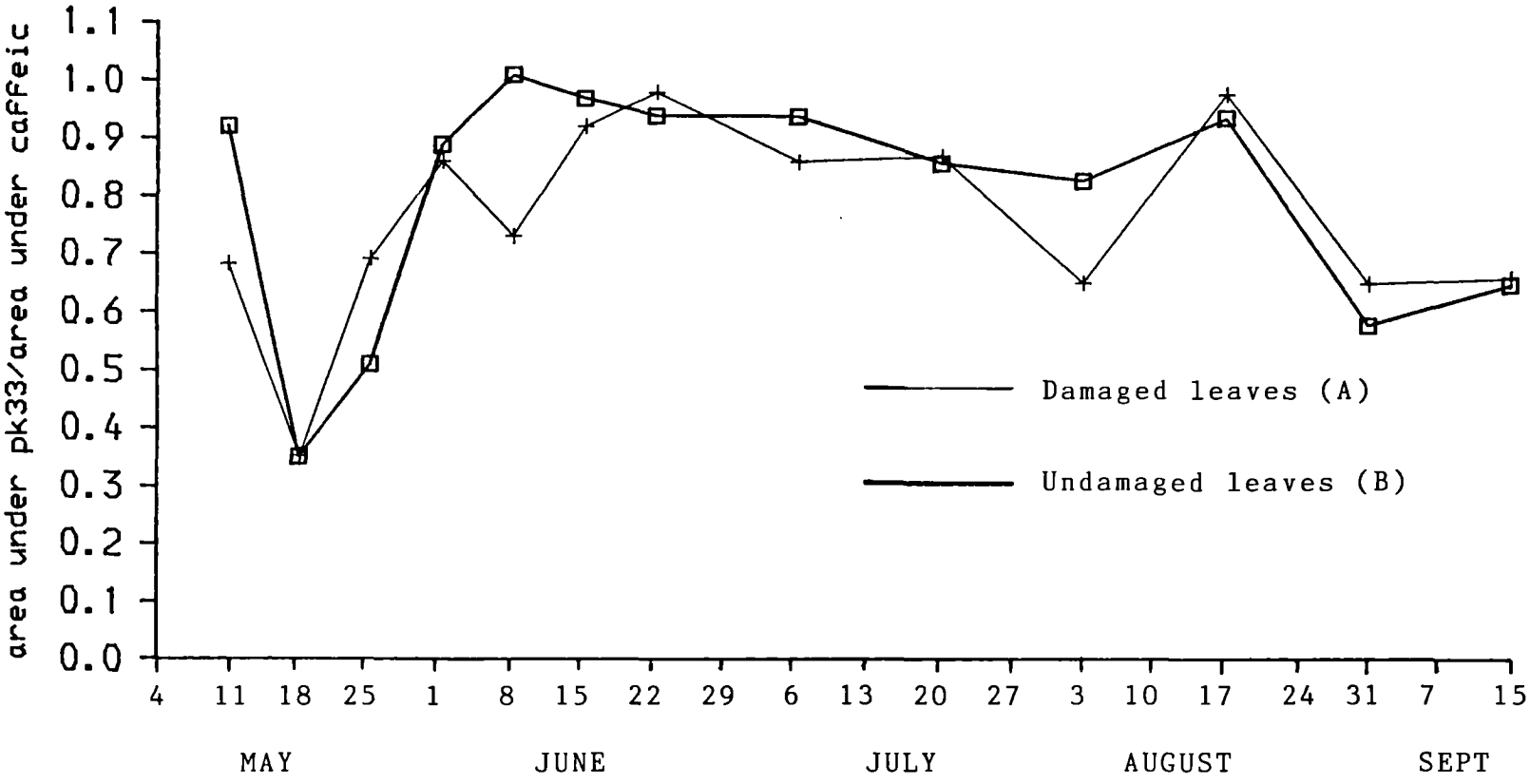


Fig. 5.7 A comparison of the leaf concentrations of peak 33 between groups A and B during the summer of 1987. (Confidence have been ommitted for the sake of clarity).

5.4.2 Year 1: The effect of artificial damage.

The analysis of artificially damaged plants was kept separate to the group A and B analyses. The measurements of peaks 28, 30, 33 and 44 on consecutive weeks after damage were subjected to analysis of variance.

In the first week following artificial damage on June 8 the concentrations of peaks 27, 30 and 33 increased whereas the concentration of peak 44 decreased (Fig 5.8). During the second week the concentrations of peak 27, 30 and peak 33 continued to increase and the peak 44 concentration exhibited a marked rise. Overall the plants exhibited a significant increase in the concentration of peak 44 ($p < 0.05$). The increases measured in peaks 27, 30 and 33 were not statistically significant (Table 5.2).

In the week following damage on July 6 the concentrations of all compounds under investigation increased (Fig 5.8). Peaks 27, 30 and 33 exhibited similar relative increases between 35% and 40 % (Table 5.3). Peak 44 exhibited the greatest relative increase, approximately three time as great as the corresponding increases in the other peaks. In all cases the first week's increase was followed by a decrease during the second and third week after damage.

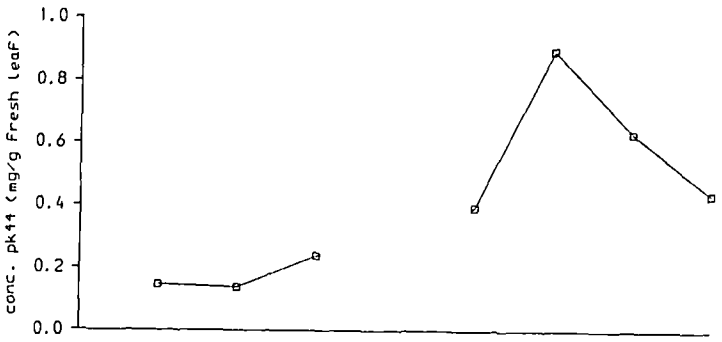
5.4.3 Year 2: The effect of herbivory.

Leafburst occurred during the first week in May and the first collection was made on May 7. Herbivore damage was first observed on June 6 and was extensive on all five plants on June 13.

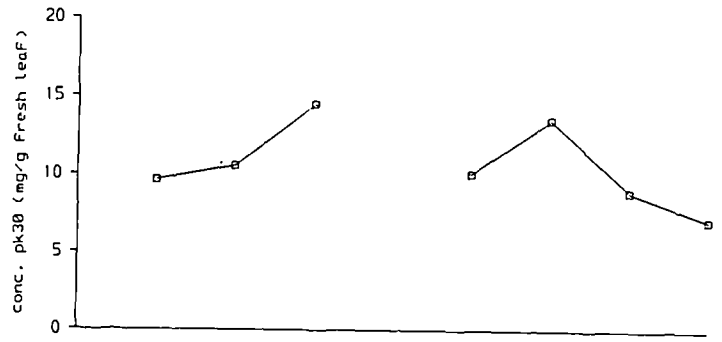
Peaks 30 and 44 were selected for analysis in year 2. In addition, peak 43, the shoulder of peak 44, was also included as it

Figure 5.8 The effect of artificial damage.

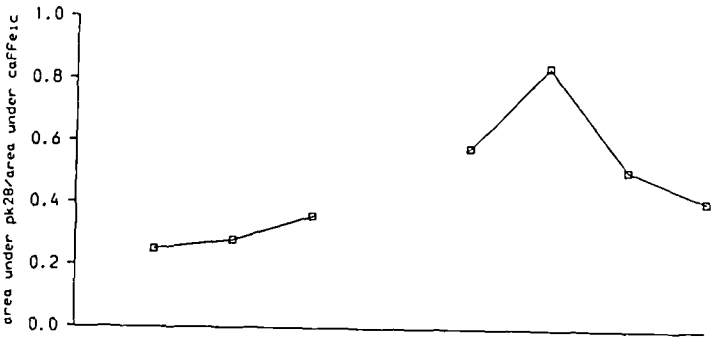
a. Peak 44



b. Peak 30



c. Peak 28



d. Peak 33

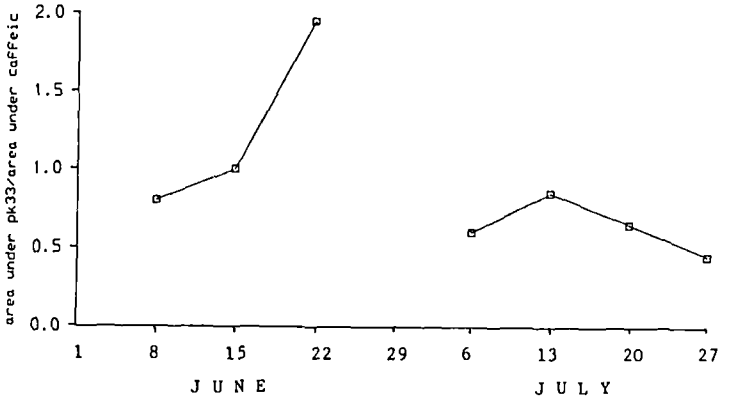


Table 5.2 The percentage changes in the leaf concentration of peaks 28, 30, 33 and 44 resulting from artificial damage on 8 June 1987 and 6 July 1987.

Period	Peak 28	Peak 30	Peak 33	Peak 44
8/6-15/6	16.3	14.4	20.0	-17.8
15/6-22/6	26.4	33.0	40.2	117.0
8/6-23/6	47.0	52.1	68.2	78.4
F statistic	0.76	1.08	1.53	5.19*
6/7-13/7	38.4	35.1	39.8	110.4
13/7-20/7	-35.8	-33.6	-20.3	-26.2
20/7-27/7	-19.2	-19.9	-31.7	-30.6
6/7-27/7	-28.3	-26.8	-23.9	7.7
F statistic	1.45	2.27	2.12	2.17

*: $p < 0.05$

appeared to follow a similar pattern to peak 44. The concentration of peak 44 was initially very low in both groups (Fig 5.8). The concentrations in both groups remained similar for four weeks, both rising gradually. Between 6 June and 13 June the mean concentration of peak 44 in group A began to rise sharply in contrast to little change in group B. The rise in group A continued until 28 June and a corresponding rise was observed in group B which started after 13 June. The two groups exhibited similar mean concentrations of peak 44 on 5 July. By this time a thirty fold increase had been observed and the rise continued in both groups throughout the season.

The mean concentration of peak 44 in group A was significantly greater than that of group B on 13 June ($p = 0.0096$) and 20 June ($p < 0.0001$). The rise in mean concentration in group B mirrored the group A rise almost exactly but was delayed by one week. Thus the year 2 results for peak 44 followed a similar pattern to the corresponding results of year 1.

The immature leaves collected in August exhibited a lower concentration of peak 44 than mature leaves at that time (Fig 5.8). However, the concentration of peak 44 was considerably greater in these leaves than in comparable leaves collected early in May and the difference was significant (t-test: $p = 0.013$).

Peak 43 (Fig 5.10) exhibited a similar pattern to peak 44, with group B lagging a week behind group A. However, the rise in the mean concentration of peak 43 in group A was first observed two weeks earlier than the rise in peak 44 and the group means converged a week earlier. At no time was the difference between the groups significant at the 5% level although it was very close on 13 June ($p = 0.055$). On this date the $\log_{10}$ data gave a significant

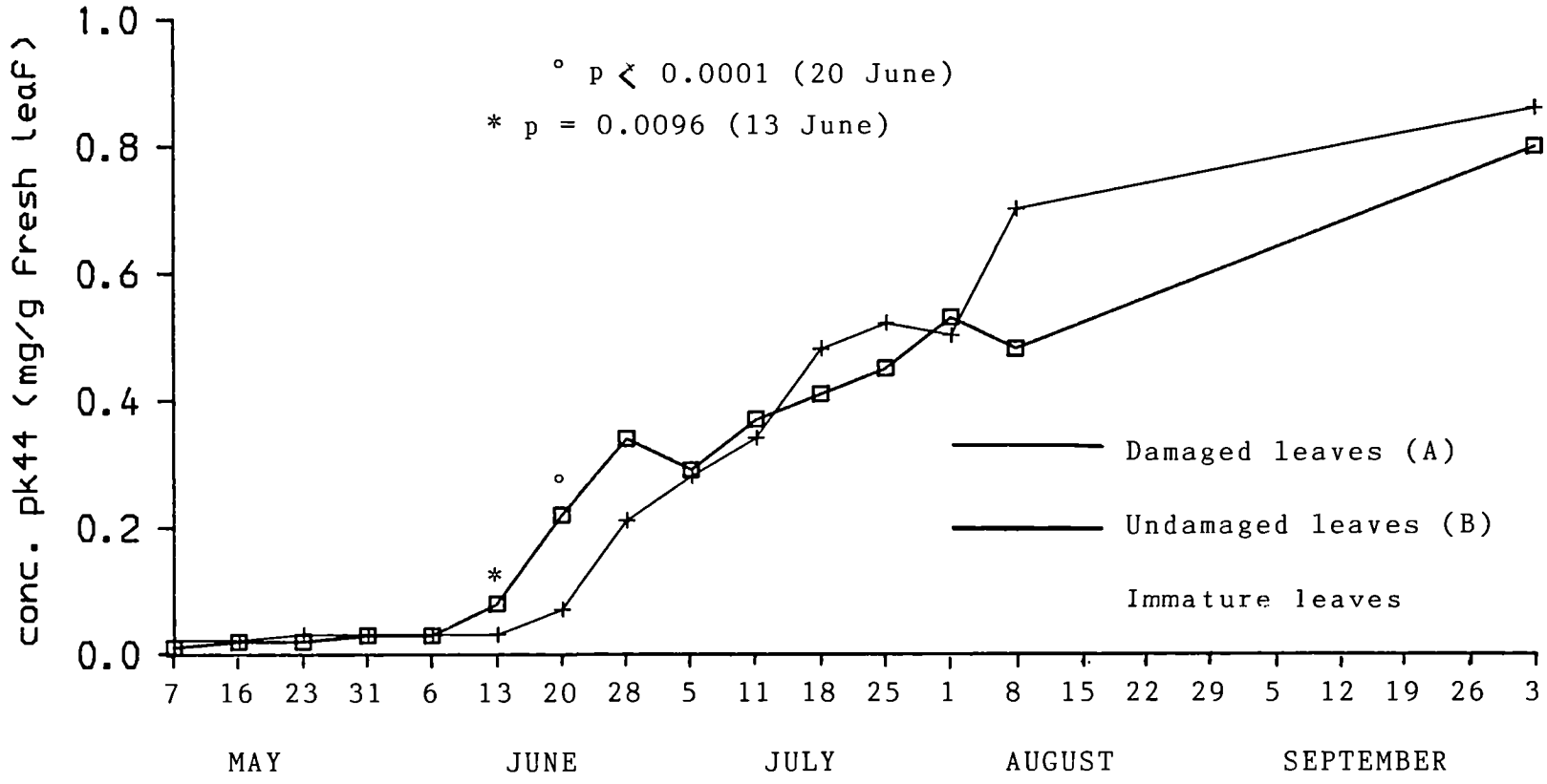


Fig. 5.9 A comparison of the leaf concentrations of peak 44 between groups A and B during the summer of 1988. (Confidence limits have been ommitted for the sake of clarity).

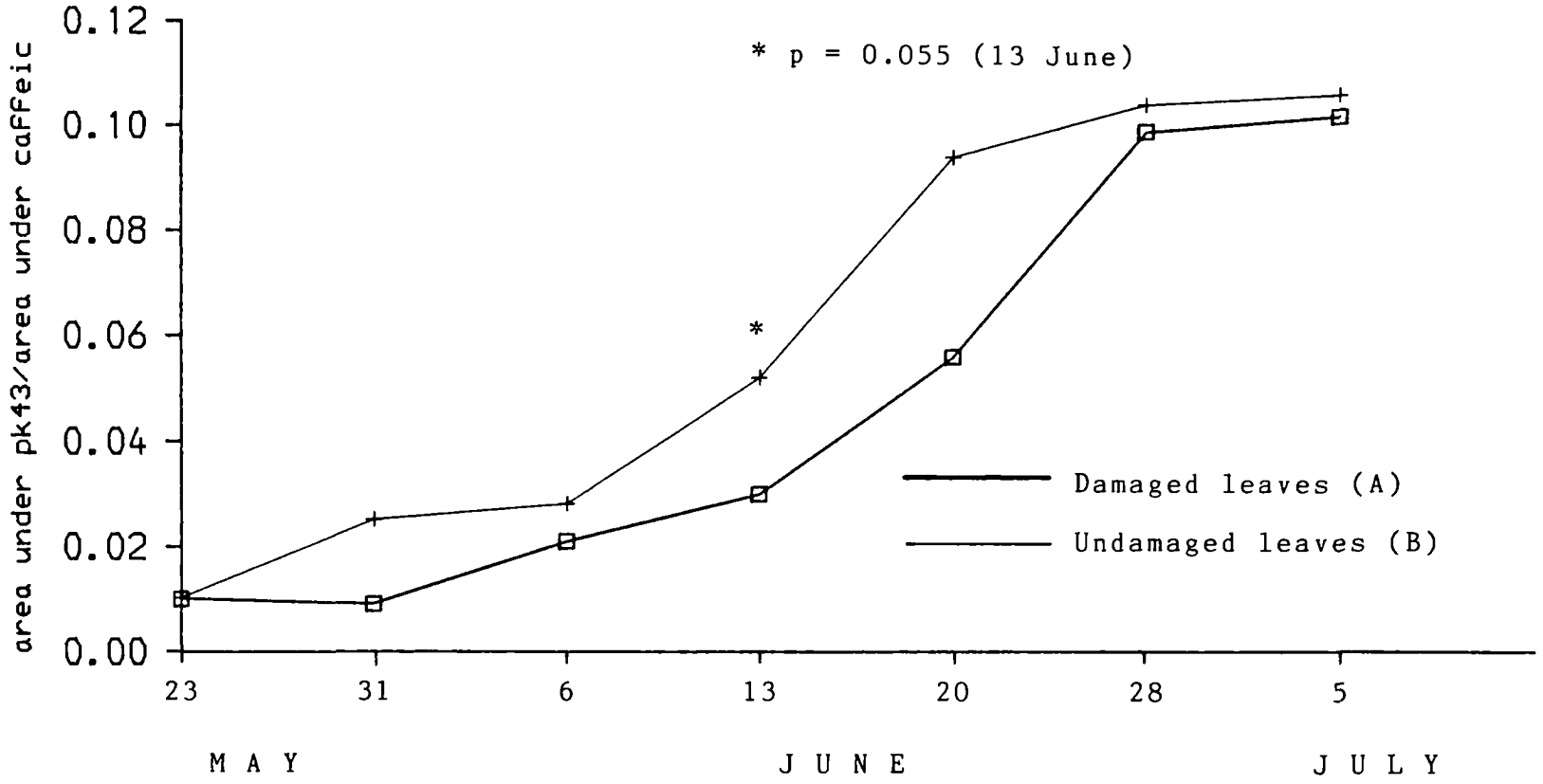


Fig. 5.10 A comparison of the leaf concentrations of pk43 between groups A and B during the early summer of 1988. (Confidence limits have been ommitted for the sake of clarity).

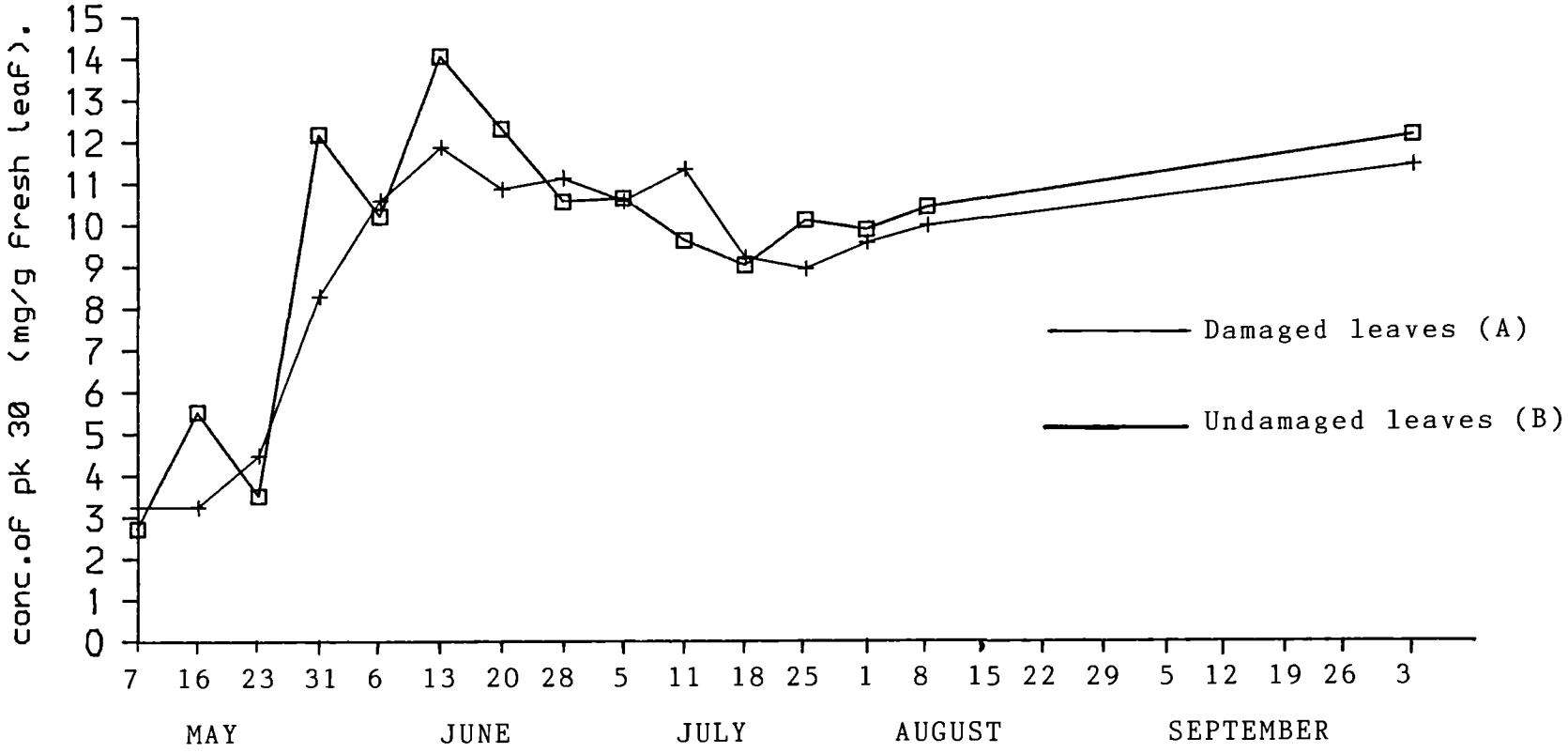


Fig. 5.11 A comparison of the leaf concentrations of pk 30 between groups A and B during the summer of 1988 (confidence limits have been ommitted for the sake of clarity).

difference ($p = 0.033$). A tenfold order of increase was observed during the period.

In 1988 the mean concentrations of peak 30 in the groups did not closely follow the pattern of 1987 (Fig 5.11). The concentrations did not differ significantly between the groups on any week. Where the means did show large differences (16 May, 31 May & 13 June) group A had the higher mean concentration. The concentrations of both groups were initially considerably higher than the previous year. In both groups these concentrations rose in late May to slightly higher levels than observed in 1987 before falling gradually.

The leaves of both groups increased in percentage dry weight from 27% in early May to approximately 40% in early August (Fig 5.12). Although the groups differed significantly on 23 May no pattern became apparent until after 13 June when group A exhibited a marginally greater mean dry weight (never greater than 3 percentage units) for five consecutive weeks. The dry weight of group A leaves levelled off at 41% on 11 July. In group B the dry weight exceeded group A on 25 July (at 43%) before dropping back to 39% on 8 August.

5.4.4 The greenhouse experiment

The sprayed and unsprayed plants all showed a rise in peak 44 concentration. The increase was evident for both groups on 31 May and continued throughout the sampling period (Fig 5.13). In both groups the increase was large (80 fold in the unsprayed plants and 120 fold in the sprayed plants). The mean concentration of the sprayed group was higher than that of the unsprayed group at all times but at no time did the groups differ significantly.

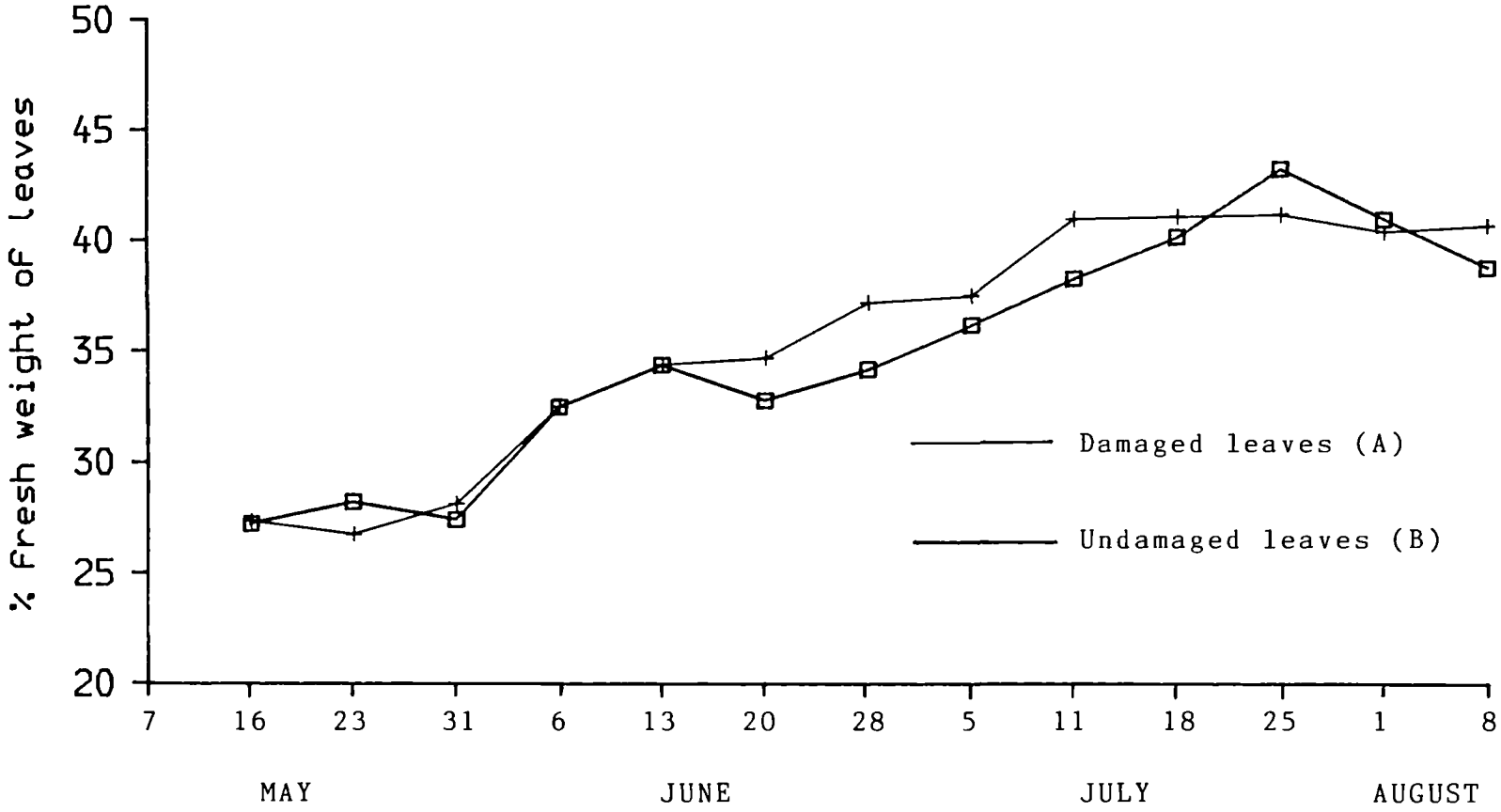


Fig. 5.12 A comparison of the dry weight of leaves from groups A and B, expressed as a percentage of fresh leaf weight.

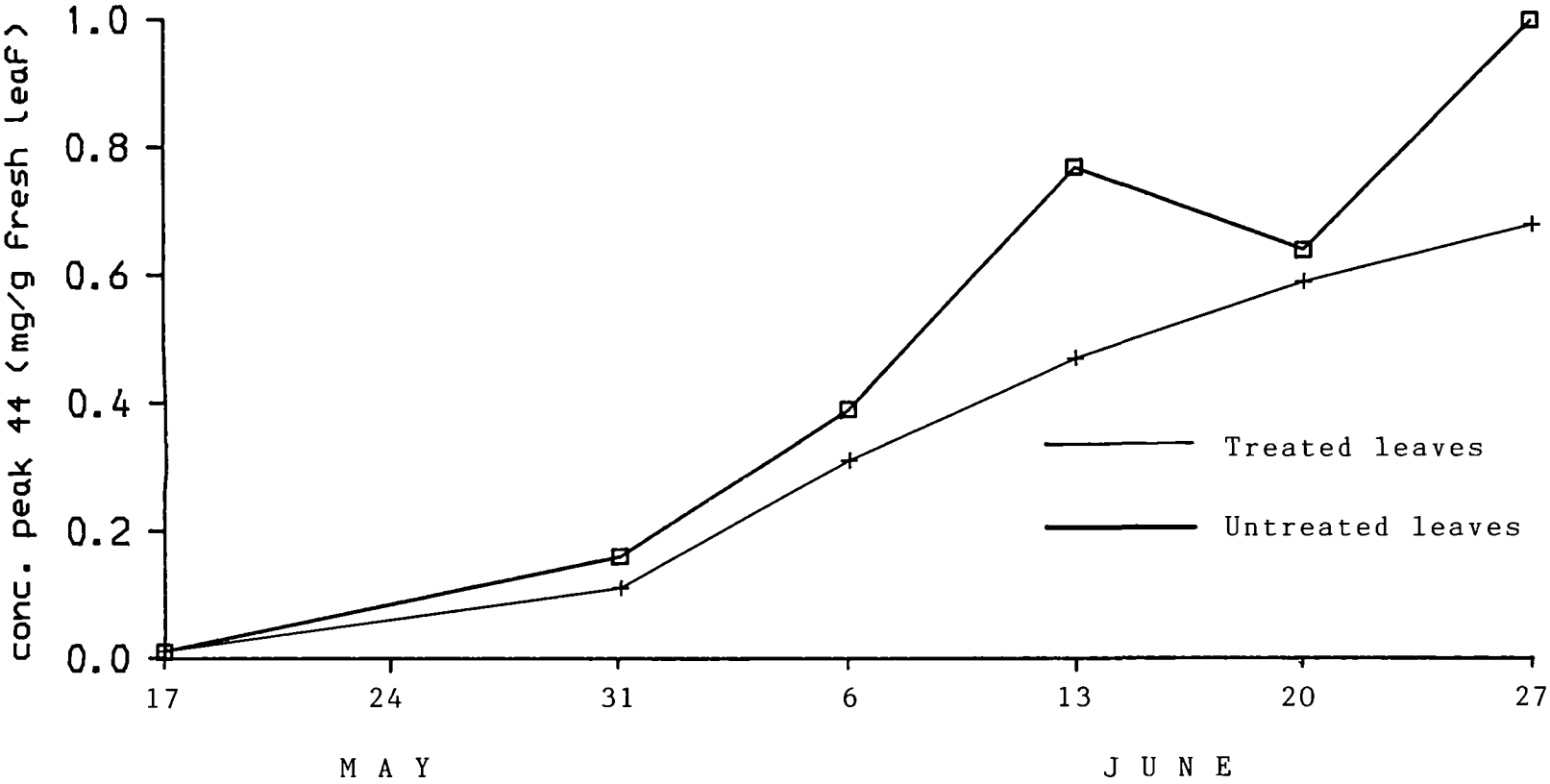


Fig. 5.13 A comparison of the leaf concentrations of peak 44 between plants treated and untreated with cypermethrin. (Confidence limits have been ommitted for the sake of clarity).

5.4.5 The effect of damage on the volatile oil.

The composition of the essential oil did not differ greatly either between plants or during the season. There were no obvious differences between the experimental groups. However, there did appear to be a greater density of oil glands on the surface of leaves which had sustained herbivore damage. No attempt was made at this time to quantify this phenomenon.

5.5 Discussion.

5.5.1 The phenolics.

The results of the phenolic analyses in year 1 provided some evidence that sweet gale exhibits chemical changes induced by herbivory. The most interesting results concerned peaks 30 and 44. These compounds both exhibited a large increase following the first signs of herbivory.

The concentration of peak 44 was greater in the leaves of the damaged (group A) plants for four weeks following herbivory, a significant difference occuring on the first week. The converse was true of peak 30, the leaves of damaged plants exhibiting less of an increase than the controls. Thus, it appeared that herbivory stimulated peak 44 production in group A but inhibited peak 30 production. The difference between groups A and B in respect of peak 44 was sustained for longer than the difference in respect of peak 30. It is important to note that the statistical analyses in year 1 only provided an indication of the differences since the peaks under investigation were not selected *a priori*. However, the difference between groups A and B in respect of peak 44 was observed consecutively in four separate sets of plants (Fig 5.6) strongly

indicating that the observed difference was real.

It was not obvious why the differences between groups A and B did not persist. In the year 1 experiment single stems were sprayed and it was probable that the sprayed stems were connected with untreated stems through the roots. There was a possibility, therefore, that treated and untreated stems were communicating and that the treated stems were demonstrating a delayed response to damage sustained by neighbouring, untreated, stems of the same plant. In view of this possibility clearly delineated plants were selected for the year 2 experiment so that all the stems of the control plants could be identified and sprayed.

Although there were significant differences between groups A and B in respect of peak 28 these differences did not appear to follow any pattern and they could not be related to the experiment. The fact that the groups were significantly different probably results from both sets of data having distributions far smaller than that of the population as a whole ($s << \sigma$) on those occasions (i.e. a type I error). This is not uncommon where an *a priori* decision is not made to investigate specific characteristics, but rather that the characteristics are selected in retrospect, since any two groups selected at random are likely to differ in respect of some characteristics. It seems unlikely that any biological significance should be attributed to these results.

Peak 33 did not differ greatly between the groups clearly demonstrating that, unlike artificial damage, herbivory did not have an effect on all compounds in the aqueous/methanol fraction. The fact that some phenolics are effected by herbivory yet others are not also lends support to the argument that crude chemical analyses,

such as measurement of total phenolics, at best cannot give an accurate picture of plant responses and at worst may be misleading.

The result from the artificially damaged plants lent some support to the possibility that an induced response was being observed in respect of peak 44 concentrations. Artificial damage on June 8 did not trigger an immediate positive change in peak 44 concentrations. In fact, the mean peak 44 concentration in group C plants fell between June 8 and June 14, in a similar fashion to group B (the undamaged plants). The rapid increase in the peak 44 concentration occured in the second week after damage. This was a week after the corresponding rise in group A (plants sustaining herbivory) and a week before the corresponding rise in group B.

Not only was the response to artificial damage delayed in June, immediately after herbivory became apparent, it was also not as great as the response to herbivory over the same period. This, perhaps, is not surprising since the mechanism mediating an induced response might be expected to be an adaptation to pressures associated with herbivory in which case herbivory would be expected to elicit the maximum response.

It is surprising that the greatest response, that of peak 44, was delayed by one week at a time when group A leaves were already exhibiting a rise in peak 44. One month later, on July 6, similar damage elicited an immediate response. These results indicate that artificial damage triggered a response in sweet gale but that, unlike the response to herbivory, it was suppressed early in the growing season.

By July 6 peak 44 concentrations had risen considerably in groups A, B and C and there were no indications of suppression of

responses to artificial damage. Artificial damage led to an increase in the concentration of all compounds measured whereas herbivory appeared to only be associated with an increase in peak 44. On July 6 the relative increase of peaks 28, 30 and 33 were very similar (35% - 40%) and were possibly a result of leaf dehydration caused by the severe wounding. Peak 44 was distinguished by increasing to a far greater extent although this was not statistically significant. The ensuing drop in the concentrations of the four compounds also distinguished artificial damage from herbivory.

The artificial damage experiments aided the identification of peak 44 as a possible induced compound. However, all compounds investigated exhibited a similar pattern of change in response to artificial damage whereas this was not true of herbivore damage. Cutting the leaves was clearly not a good model for capsid bug herbivory. The rate of artificial damage (50% of leaves severely damaged in less than 30 minutes) was far greater than that of herbivory yet in June, when plants exhibited the greatest response to herbivory, the effect was smaller. For these reasons no further work was carried out on the effects of artificial damage.

At the end of year 1 further investigation was required to clarify the peak 30 and peak 44 results so the year 2 field experiment was planned. The design was altered in the hope of generating more clear cut data. In essence, however, the experiment remained the same.

The main consideration in the year 2 experiment was to select plants which were clearly delineated so all the stems in plants selected for spraying could be kept entirely free of insects. A

second benefit of using entire plants derived from having a large number of stems from which each sample could be selected. This allowed collection of samples from the same plant each week, with minimal effect to the plant, giving more continuity in the results.

In year 2 the pattern for peak 44 concentrations in the two groups was similar to the same period in year 1. The initial rise in group A was observed within a week of the first signs of herbivory. The corresponding rise in group B was observed to start one week later and the difference between groups A and B was highly significant on the two weeks following group A's initial rise. This result indicates that sweet gale does respond to herbivory.

An alternative hypothesis which could account for the results is that the cypermethrin application to group B plants accounted for the difference between the groups. If enhanced production of peak 44 is associated with maturation of leaves it was possible that cypermethrin caused the observed differences between the groups by delaying leaf maturation. The possibility also existed that cypermethrin directly effected peak 44 production although it was difficult to see why it would act only on one plant product and only delay its production by one week. The results from the artificially damaged plants, which had been sprayed with cypermethrin yet exhibited an immediate increase in peak 44 once they had sustained damage on July 6, had indicated that cypermethrin was having no effect on leaf chemistry. However, this damage was applied once the leaves were mature. The greenhouse experiment was specifically designed to check whether cypermethrin had any effect on leaf chemistry. It clearly demonstrated that the cypermethrin did not delay the production of peak 44. Both sprayed and unsprayed plants

exhibited a large increase in peak 44 concentrations early in the season. It would seem, therefore, that enhanced peak 44 production is triggered by herbivory rather than cypermethrin application.

Given that peak 44 is induced it would be of interest to know the mechanism of induction. Perhaps the most widely studied biosynthetic pathway associated with induced defenses is that which gives rise to phenolics. Infection with fungal mycelium stimulates mRNA activity associated with phenylalanine ammonia lyase (PAL), 4-coumarate coenzyme A ligase (CCL), chalcone synthase (CHS) activity (Grisebach 1985) and hydroxyproline-rich glycoprotein (HRPG) (Showalter et al 1985). Lawton and Lamb (1987) showed that infection, fungal elicitors and wounding induced transcription of genes for PAL, CHS and HRGP.

The mechanism regulating induction of peak 44 is likely, however, be more precise than the broad stimulation of enzymes involved in fundamental phenolic biosynthesis since the total leaf phenolics of sweet gale are not affected by herbivory. The specific enzymes which catalize peak 44 production would require substrates, these being intermediate products of the phenolic metabolic pathway. It follows that some stimulation of this pathway would necessary for peak 44 production. It is possible that herbivory provides this stimulation. However, synthesis of products by enzymes is often regulated by feedback inhibition (Umbarger 1956, Stadman 1970) whereby high concentrations of the product inhibit further product synthesis. Thus, the first enzyme of a biosynthetic pathway can be inhibited by the final product. The biosynthetic pathway leading to the production of phenolics is likely to be regulated by the concentration of products at the site of synthesis. Since Peak 44

production utilises the same pathway and substrates as other phenolics, which are produced irrespective of herbivory, its production would act as a sink for intermediate products. As a result the production of peak 44 from these intermediate products could stimulate the entire biosynthetic pathway. Further more, this pathway is likely to be in continual employment as plant phenolic compounds exist in a dynamic equilibrium, constantly undergoing synthesis and metabolism (Barz *et al.* 1985). It is probable, therefore, that no further genetic stimulation of the basic phenolic pathway is necessary during induction of peak 44 and the eliciting factor works directly on the gene or operon exclusively responsible for this compound.

The remaining question was whether or not the peak 44 increase in plants protected from herbivory, shortly after the induced response was exhibited by plants sustaining herbivory, was triggered by some other unknown factor or whether sweet gale begins to invest in peak 44 by mid June under any set of conditions. In the field experiment the group B rise cannot be ascribed to communication along the roots between damaged and undamaged plants since the plants in group B were isolated in space from other plants. However, it could be argued that the plants in group B were responding to some airborne signal. This hypothesis is similar to those of Baldwin and Schultz (1983) and Rhoades (1983) who suggested that induced chemical changes in the leaves of artificially damaged trees were being mirrored in the leaves of neighbouring undamaged trees due to inter-tree communication.

The results of the greenhouse experiment appear to discount the possibility of any inter plant communication triggering the enhanced

production of peak 44. These plants were separated from the nearest community of sweet gale, at Glasgow Botanic Gardens, by more than a kilometre yet they exhibited increase in peak 44 before herbivore damage was evident on the Botanic Gardens plants.

However, in studies of plant responses to fungal invasion it has also been shown that synthesis of enzymes, involved in phytoalexin production, is not only induced at the site of infection but also in uninfected cells (Showalter *et al.* 1985, Lawton & Lamb 1987). It has long been known that ethylene enhances the activity of PAL (Chalutz 1973) and recently this stress hormone has been linked directly with increases in mRNA for PAL, CCL and CHS (Ecker & Davis 1987). Ecker and Davis (1987) argue that since ethylene is associated with plant pathogen interactions it would be an ideal messenger to initiate induced responses in uninfected tissue.

The evidence for ethylene being able to induce phenolic production is very strong. It may not appear unreasonable, therefore, to associate ethylene with the induction of peak 44. If high levels of ethylene elicit the production of peak 44, its production in undamaged plants could be triggered by ethylene from damaged plants. In the greenhouse experiment the plants were in a confined space where ethylene levels would easily rise. Thus, if ethylene does trigger peak 44 production, the argument that the results of the greenhouse experiment refute the 'talking plants' hypothesis in respect of sweet gale is seriously weakened.

However, ethylene triggered induction of the phenolic biosynthetic pathway would be expected to lead to an increase in levels of all products of the pathway. When the plants sustained herbivore damage no quantitative change in phenolics was observed.

Induction of peak 44 is primarily a qualitative change. It is probable, therefore, that ethylene is not the specific trigger for induced peak 44 production.

The group B peak 44 rise could have been an induced response to challenges by pathogens. There was little evidence of this in terms of signs of disease (one group B plant exhibited leafspot on a few leaves) but the apparent lack of pathogens could be interpreted as a measure of the defenses' success.

The design and situation (rooftop) of the greenhouse provided a good, if not rigorous, test of whether the peak 44 increase in plants not sustaining herbivory could be ascribed to pathogen challenge. All plants in the greenhouse experiment exhibited an increase in peak 44 in the absence of herbivory. Total exclusion of fungal spores is a practical impossibility in a greenhouse. However, it was extremely improbable that the peak 44 increase observed in the greenhouse plants was caused by challenges to each plant by pathogens since the plants were protected from airborne spores by the glass of the greenhouse. In a still airspace, such as the interior of a greenhouse, spores descend by gravity. Had any spores entered the greenhouse the acetate lids of the plant frames would have acted as a further barrier between the spores and the plants.

The hypothesis which fits the data best is that the increase in levels of peak 44 in sweet gale, like that of peak 30, occurs regardless of herbivory but that herbivore pressure triggers an earlier increase. Embodied within any explain this phenomenon in terms of evolutionary theory is an implicit assumption is that some cost (not necessarily energetic) is associated with peak 44

production, but that high concentrations of peak 44 confer some advantage to the plant. A defensive role for peak 44 fits this hypothesis well. In such a system the cost and advantage would be balanced and in this case the cost prohibits enhanced peak 44 production until a certain period into the growing season, possibly corresponding with the leaves reaching maturity. To plants which suffer herbivory before this date the cost relating to possible further herbivory or associated pathogen attack outweighs the peak 44 production cost so early production of the defensive agent, as observed in year 1 and year 2, is triggered.

One such possible cost is that high concentrations of peak 44 are detrimental to early leaf development. The low concentrations observed in leaves in the month following budbreak, when determinate shoot leaves are developing, lends support to this idea. However, the concentration of peak 44 in leaves just bursting from indeterminate primordia, once determinate shoots were mature, exhibited much higher levels of peak 44. Leaves which burst late in the season import almost all assimilates from older leaves (Thrower 1962, Harper 1989) so it follows that the plants were actively importing peak 44 to the indeterminate young leaves. This is not in accord with peak 44 activity detrimental to leaf development.

A second possibility is that nitrogen is limiting at budbreak. Flanders Moss is a nutrient poor environment. Sweet gale gets a proportion of its nitrogen from its association with the actinomycete *Frankia* sp. The efficiency of this association is greatly reduced when the roots plus Frankia nodules are starved of oxygen, at times of when the surface water is high for instance. In Scotland the winters preceding year 1 (1987) and year 2 (1988) were

wet and the moss did not begin to dry until early summer. Nitrogen quite possibly limited on growth in these years. It was observed in 1988 that the rise in peak 44 concentration in group B coincided with the majority of determinate shoot leaves achieving their maximum size. Once early growth ceases nitrogen can be diverted from primary metabolic pathways to secondary pathways. In practical terms this means the nitrogen would be available for the production of enzymes to mediate the production of chemical defences. The defense compounds themselves do not contain nitrogen. The greenhouse plants were grown in soil which was not nutrient limiting. It is interesting to note that the increase in peak 44 concentration in these plants occured almost three weeks earlier than undamaged plants in the field on previous years and that the rise was considerably greater. However, it is important that these comparisons are not given too much weight as the plants came from different communities, were grown under different conditions and the analyses were undertaken on a different year.

A similar pattern to that of peak 44 was observed for peak 43 although the response to herbivory was far smaller. However, since peak 43 appeared as a shoulder of peak 44 it is difficult to assess whether it was riding up on the front of peak 44 and thus appearing to increase in area, or whether its area was actually increasing.

Peak 30 does not appear to respond in any direct way to herbivory. The significant difference recorded in year 1 was on one week only and this was not repeated in year 2. It seems likely, therefore, that statistic significance of the year one difference was misleading and that it did not represent a biologically important difference in the context of the experiment. In both

plants sustaining and plants escaping herbivory levels of peak 30 are enhanced at the time of the first signs of herbivory. Clearly this is not in direct response to herbivory. Peak 30 could be a constitutive defense. If this is case it is possible that the population has evolved a pattern of production to complement patterns of herbivory. Production would be enhanced at the time when general herbivores become a threat regardless of their actual presence.

5.5.2 The volatile oil

The results suggest that herbivory does not affect the composition of the volatile oil. However, the observation that herbivory appeared to trigger an increase in the density of oil glands suggested that production of the oil as a whole could be induced and this could be part of the species' defenses. This possibility required further investigation. This subsequent work is described in chapter 6.

5.5.3 Summary

There seems to be no doubt in the light of this experiment that sweet gale produces peak 44 and, possibly, peak 43 in response to herbivory earlier than would happen in its absence. This raises various questions about its function in the plant. The fact that these compounds are induced by herbivory suggests that they may have a role to play in deterring herbivores. A second possibility is that they have a role to play in resistance to pathogens. The two are not mutually exclusive and a defense which was active against both pathogens and herbivores would greatly benefit the plant.

Chapter 6. The effect of herbivory on sweet gale leaf gland density.

6.1 Introduction.

Myrica gale is characterised by its investment in volatile oil which is stored in glands on the leaf surfaces. Both leaf trichome glands and, larger, multicellular glands are utilized for this storage.

As demonstrated earlier (Chapter 5) herbivory did not appear to affect the relative concentration of any particular component of the oil. However, during the field experiment of 1987 it was observed that the leaves of plants which had sustained insect damage appeared to have a higher density of the larger multicellular glands. This observation raised the possibility that herbivory induces changes in total volatile oil production.

Volatile oils are known to have various modes of biological action. The success of insect herbivores is known to be adversely affected by monoterpenes (Annila & Hiltunen 1977, Cates & Redac 1986, Cates *et al.* 1987, Mihalaik *et al.* 1987) and sesquiterpenes (Langenheim 1980, Stipanovic *et al.* 1986). Terpenes, in particular oxygenated terpenes, have been demonstrated to deter grazing by ungulates (Scholl *et al.* 1977). Mammalian herbivores avoid sweet gale (Lindlof *et al.* 1974, Jackson 1977, Sprent *et al.* 1978, personal observation). The reason for this behaviour may well be smell or taste (the oil is extremely bitter to human taste). The odour of terpenoids in gymnosperms have been shown to deter feeding by red deer (Elliot & London 1987). Flanders Moss supports a population of red deer and it is probable that they avoid sweet gale

because of its volatile oil. The basis for this avoidance possibly lies in the ability of some volatile oils to inhibit herbivore gut microbial activity. Terpenes have been demonstrated to reduce ungulate digestion (Oh *et al.* 1968, 1969, Nagy & Tengerdy 1968) by depressing gut microbial activity.

The antimicrobial activity of volatile oils is not restricted to inhibiting herbivore digestion. There are numerous reports of antimicrobial activity of volatile oils in vitro (Maruzzella 1962, 1963, Maruzzella *et al.* 1963, Morris *et al.* 1978, Deans & Richie 1987, McDowall *et al.* 1988) and this activity has been linked to plant defenses against microbial pathogens (Maruzzella *et al.* 1963, Shrimpton & Whitney 1968, McDowall *et al.* 1988).

It is very probable, therefore, that volatile oils have defensive roles in sweet gale which encompass deterring both vertebrate and invertebrate herbivores in addition to giving protection against pathogens. These defenses would be enhanced by any increase in the volume of oil on the leaf surface. The work reported below was designed to investigate the possibility that sweet gale responds to herbivory by increasing gland density. In year 1 leaf collections from Flanders Moss, made for chemical analysis (Chapter 5), provided material for this investigation. In year 2 the experiment was expanded to overcome problems which became apparent when the first results were analysed.

6.2 Materials & Methods.

6.2.1 Year 1 (1988).

Samples of shoots were collected from the ten plants in groups A and B (see Chapter 5 for a description of the experimental design)

on 13 June 1988. Five mature leaves were selected from each sample. Both upper and lower surfaces of each leaf were viewed under a microscope at x 10 magnification (the area under this field = 2.54 mm^2). The number of multicellular oil glands under each field of view was counted. The average of two counts on either epidermis of each leaf was taken. The mean density for the upper and lower epidermis of each plant was calculated and these means were used to test for differences in oil gland density between groups A and B.

6.2.2 Year 2 (1989).

The twenty most clearly delineated plants in the study site, including the ten used in 1988, were selected. Ten plants (group B) were sprayed with cypermethrin at fortnightly intervals. Ten plants were left to sustain herbivory (group A).

Leaves were collected from each plant on three occasions. The first collection was made on 24 May before any signs of herbivory were apparent. Second and third collections were made on 13 June and 10 July respectively. Signs of herbivory had become apparent in early June. On the latter two collections both damaged and undamaged leaves were collected from group A.

Three of the largest leaves were taken from each sample and oil glands were counted under two fields of view (x 10 mag.) on the upper surfaces only. The mean gland density for each plant and then each group was calculated. This provided two sets of data on 24 May (gps A & B) and three sets on the latter dates (gps $A_{damaged}$, $A_{undamaged}$ & B). The data from each collection was subjected to oneway analysis of variance.

A formula for the relationship between leaf dimensions and leaf

area was derived from empirical observations of length, width and leaf area of 22 leaves ranging in length from 14mm to 40mm. The areas of these leaves were estimated using the simple method of drawing around each leaf on $1mm^2$ graph paper and summing the whole squares plus fractions within the outline.

The length and width of each leaf in the study groups was measured and its area calculated. The mean area of leaves in each group was calculated. These data allowed the effect of herbivory on leaf size to be quantified. They also allowed the mean number of oil glands per leaf in each group to be calculated and the changes in this number between the collection dates to be plotted.

Additional stems were collected from five of the group B plants to analyse the relationship between leaf size and oil gland density. The lengths, widths and glands densities of variously sized leaves were measured. The data were analysed to discern whether gland density and leaf area were correlated.

6.3 Results.

6.3.1 Year 1.

In both groups A and B the oil glands on the upper leaf surfaces were approximately three time as dense as the glands on the lower surfaces (Table 6.1). There also appeared to be a difference between the density of oil glands on damaged leaves and undamaged leaves as the 1987 observation had suggested. However, only the difference between the density on the lower epidermis was significant at the 5% level.

The difference between the groups in gland density on the lower leaf surfaces was highly significant ($p = 0.005$), the plants which

Table 6.1 The effect of capsid bug damage on the gland density on upper and lower surfaces of leaves.

	Gland density (glands/mm^2)		
	Undamaged	Damaged	p
Upper epidermis	5.8	8.0	0.230
Lower epidermis	1.6	3.1	0.005

t-test used to calculate p.

had sustained herbivory exhibiting approximately double the gland density of the controls. The density on the upper surfaces was also greater for group A (mean gp A = 139% mean gp B) but this difference was not significant at the 5% level ($p = 0.230$).

6.3.2 Year 2.

On 24 May, before the plants sustained damage, the mean oil densities for groups A and C were similar (6.8 mm^{-2} & 6.4 mm^{-2} respectively). Once the signs of herbivory had become evident a marked difference could be seen between the mean density of oil glands on damaged leaves and undamaged leaves (Fig 6.1). Damaged leaves exhibited a slight increase in gland density whilst undamaged leaves exhibited a marked reduction (Fig 6.2). The difference between damaged and undamaged leaves was significant on both 13 June ($p < 0.05$) and 10 July ($p << 0.01$).

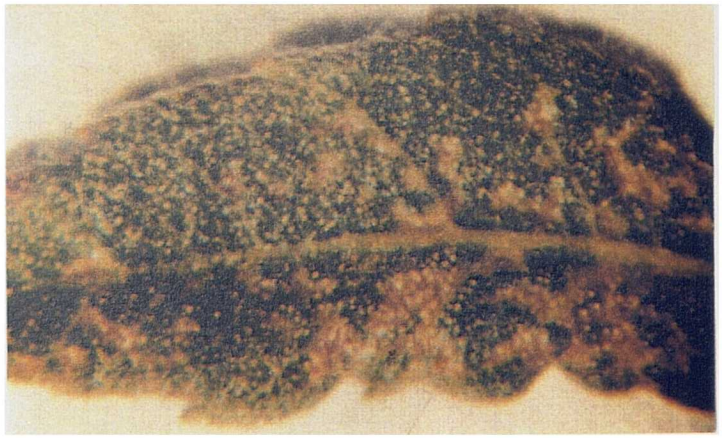
Only the leaves which had sustained damage exhibited an increase in gland density. Undamaged leaves in group A, adjacent to damaged leaves, exhibited a drop in gland density with time which almost exactly mirrored that of group B leaves. The mean gland density of leaves in group B dropped by 41.7% between 24 May and 10 June and this corresponded to a reduction of 43.7% for undamaged group A leaves. The gland density on damaged leaves increased by 26% during this period. The final (13 June) damaged leaf mean density was not significantly different from mean gland density of group A leaves on 24 May.

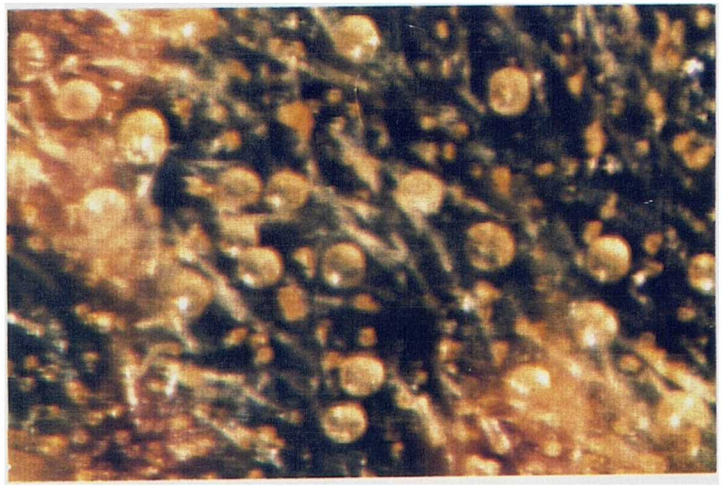
A good relationship was found to exist between leaf dimensions and leaf area. This relationship can be expressed as Area = length x width/1.55. All leaves used to derive this formula fitted the

Figure 6.1 The density of volatile oil glands on undamaged (a) and damaged (b) leaves.

a

b





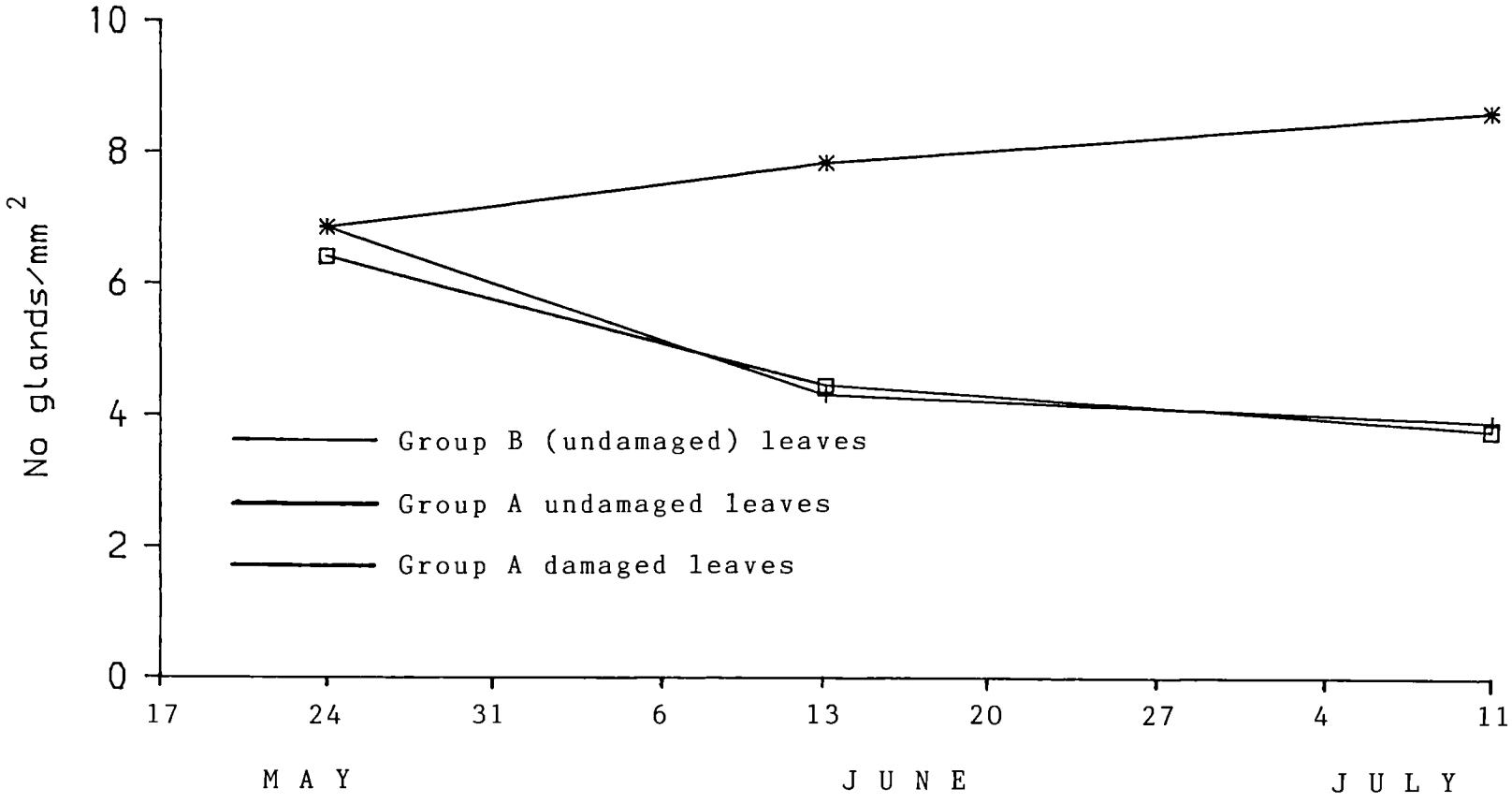


Fig 6.2 The effect of capsid bug damage on the leaf gland density of sweet gale.

model very closely (Fig 6.3).

Figure 6.4 shows the effect that capsid bug damage has on leaf area. It can be clearly seen that the largest leaves of undamaged plants have a considerably greater area than the largest damaged leaves. Undamaged leaves from damaged plants were slightly larger than damaged leaves.

The correlations between leaf area and gland density did not demonstrate a close relationship. In two cases the correlations were significant but the slope was shallow indicating that gland density did not vary greatly with leaf area. In three plants the correlations were extremely poor. In those sets of data where the correlation was significant the underlying pattern was that larger leaves had a slightly greater density of glands (Fig 6.5).

6.4 Discussion

6.4.1 The 1988 results: an indication of induced changes.

It appears from the results of year 1 that lower epidermis gland density increases in response to capsid bug herbivory. It is likely that the differences for the upper surfaces and lower surfaces are part of the same response. In year 1 the lack of significance for the upper surface statistic reflected the large variation in gland density between plants within each group (e.g. the means for B3 & B4 = 0.6 glands mm^{-2} & 7.4 glands mm^{-2} respectively). If the oil has a defensive role then it appears that the plant invests more on defending the upper surfaces of its leaves. This is not surprising as the predominant herbivore in the field experiment, *Lygocoris spinolai*, feeds on the upper surface of sweet gale leaves. Furthermore, opportunist fungal spores are

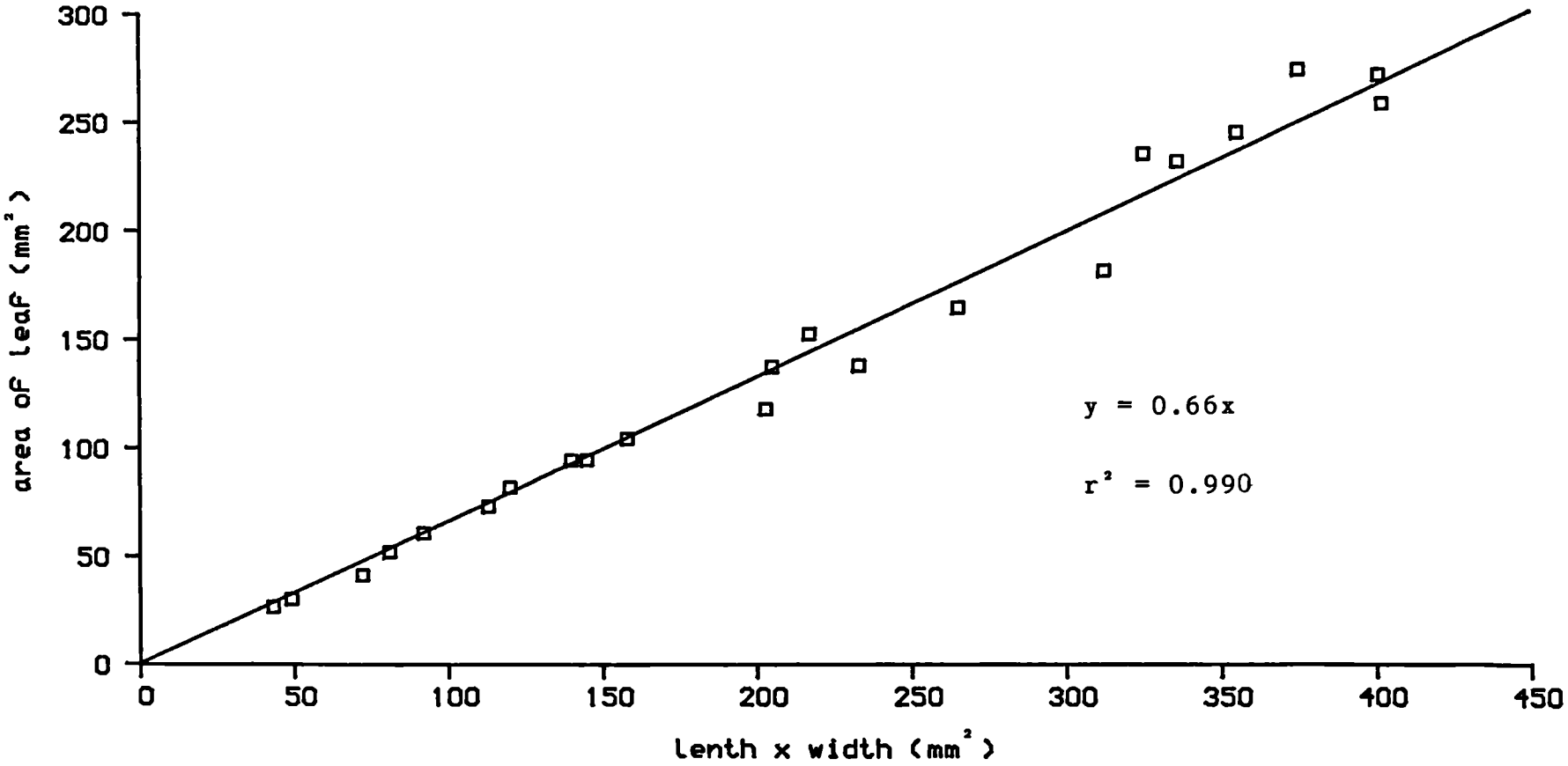


Fig. 6.3 The relationship between the dimensions of sweetgale leaves and the leaf areas.

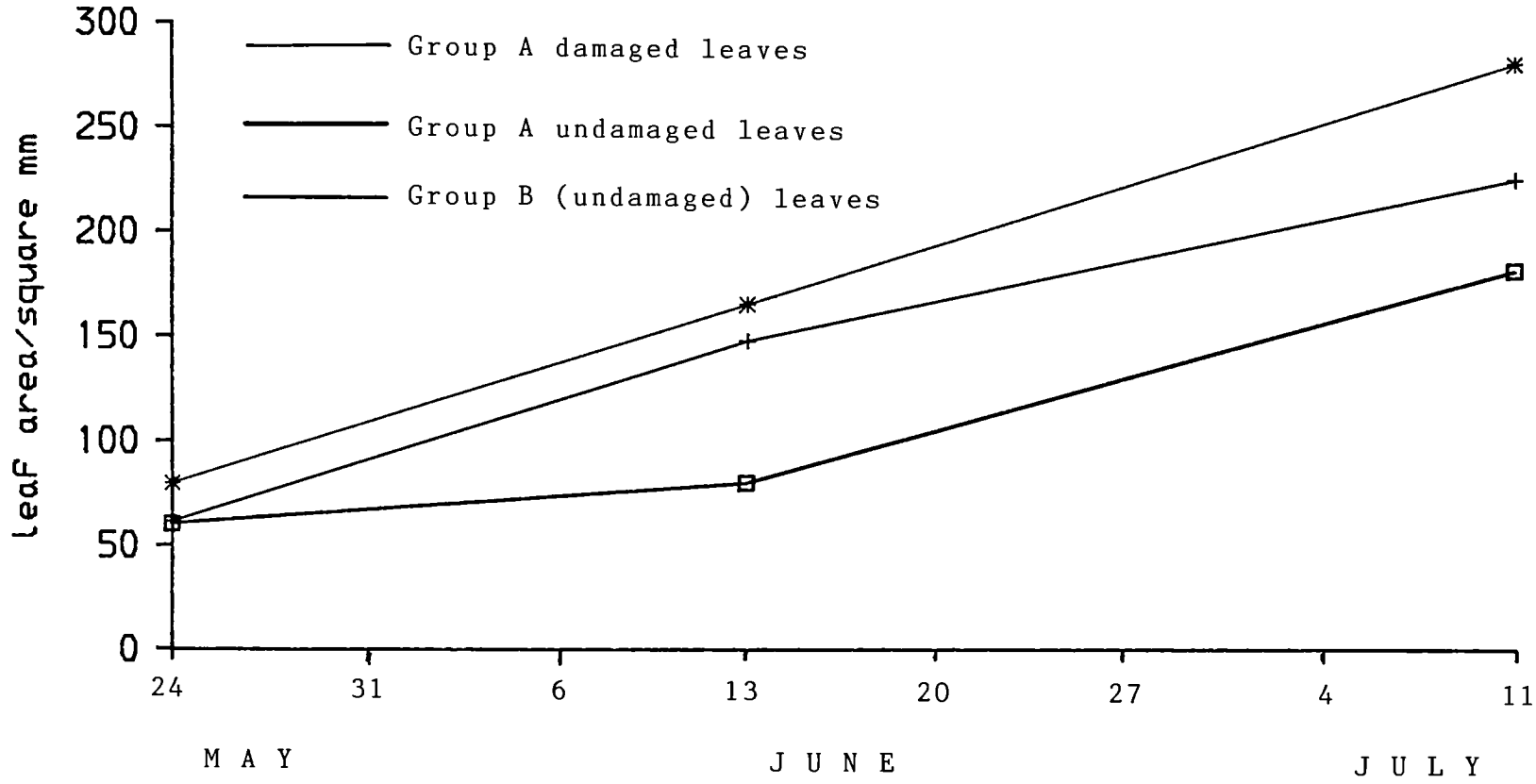


Fig. 6.4 A comparison of the areas of the largest damaged and undamaged leaves from group A and the largest leaves from group B.

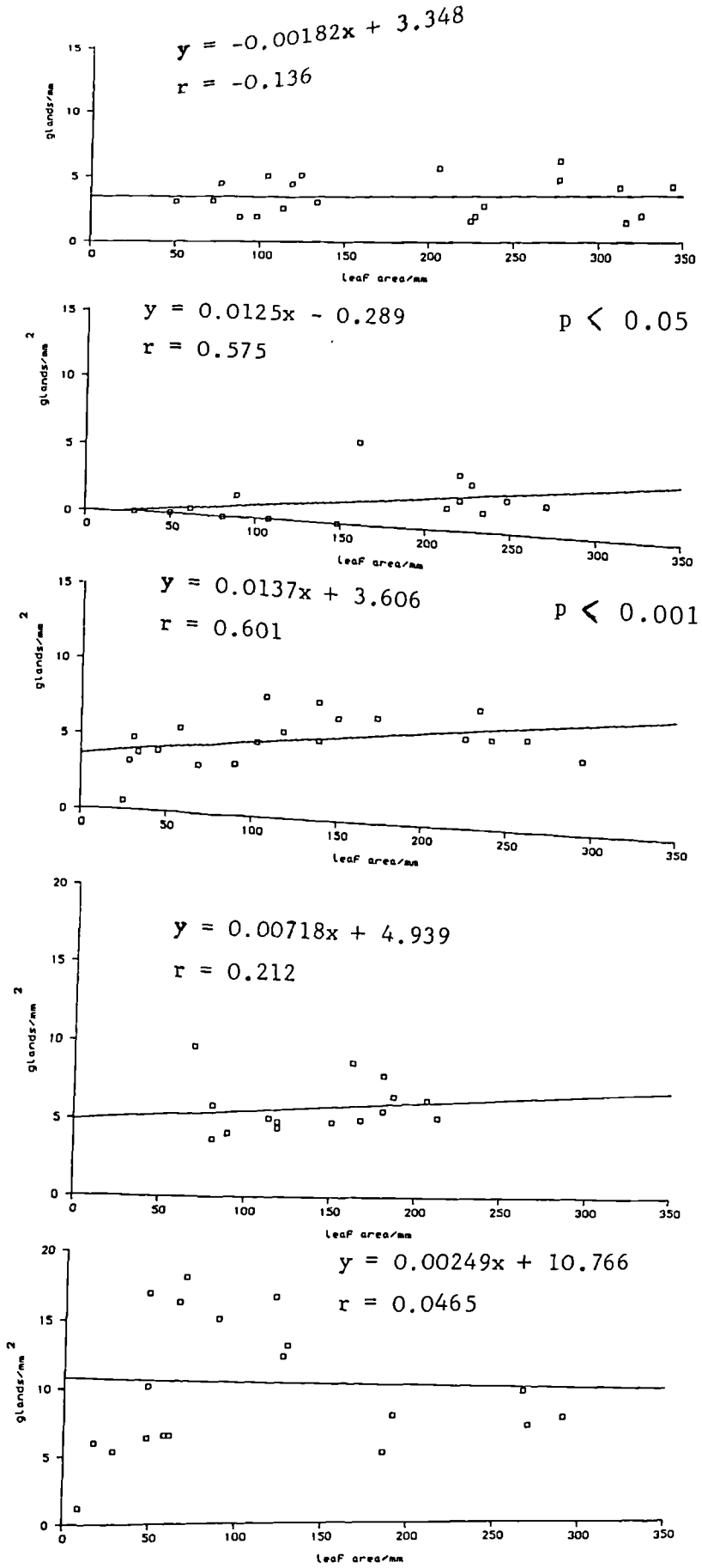


Fig. 6.5 The relationship between leaf area and gland density in five sweet gale plants.

usually carried in the wind (Dickinson & Lucas 1977) and would be expected to fall on possible hosts, either directly or in rain drops. Thus, the plant would be expected to invest more in defending the upper surfaces of leaves.

The results of the year 1 experiment reinforced the previous year's casual observation that herbivory effects oil gland density. They did not, however, provide strong enough evidence of this effect on their own. As the problem appeared to lie in the broad variation for this characteristic within the population the experiment was open to modification by increasing of the number of plants within each group.

A further effect of herbivory, observed while the gland density data were being collected, was that the size of damaged leaves appeared, in general, to be smaller than undamaged leaves. This raises the possibility that the observed differences in gland density between the groups could be explained in terms of variation in leaf size rather than as an independent response to herbivory. If a leaf's total complement of oil glands develop whilst the leaf is immature then leaf growth would be accompanied by a reduction in gland density. Any disruption to growth would halt the reduction in gland density. While this would not necessarily change the defense implications of higher gland density it would discount the possibility that the plant actively responds to herbivory with increased oil gland production.

A further question, not addressed in the year 1 experiment, was whether the relatively high gland density is local to the sites of herbivory or also characterizes undamaged leaves of those plants which had sustained herbivore damage. This had implications for the

interpretation of data in terms of defense against future herbivory and pathogen infection.

Perhaps the major conclusion from the 1988 results was that the subject of leaf gland density is far more complex than initial observations suggested. The experimental approach in 1988 was not sufficiently sophisticated to provide the results necessary to draw concise conclusions or even make any attempt at interpretation beyond conjecture. On the other hand the results did provide grounds for further investigation. A further field experiment (year 2) was required to investigate the aspects of oil gland density discussed above.

6.4.2 A clear example of induced changes.

The results in year 2 clearly demonstrated that herbivory did effect oil gland density. They also demonstrated that herbivory reduced the growth of leaves. Between 24 May and 13 June the damaged leaves exhibited only a slight increase in gland density which corresponded to small increase in size. This result could have been due to damage simply stopping any further leaf development, the increase measured being due to random variation. However, the further increase in gland density by 10 July corresponded to more than a doubling of leaf area. This is equivalent to almost a four fold increase in total number of glands per leaf between 24 May and 10 July. It follows that leaves must have the ability to produce new glands long after leaf burst. This is also reflected in the undamaged leaves of groups B and A where the drop in density of 41.7 - 43.7% corresponded to a 260 - 277% increase in area respectively. This equates to the total number of

glands on these leaves more than doubling.

Since the correlations between leaf size and gland density were significant in only two out of five cases and, when significant, were slightly positive the difference in gland density between damaged and undamaged leaves cannot be ascribed to differences in area. The expected pattern from the correlation results would be that the leaves which had sustained herbivore damage, being smaller, would exhibit a less dense covering of glands than larger, undamaged, leaves.

The difference between group B leaves and damaged group A leaves cannot be explained as a result of cypermethrin application. If insecticide application had produced the result by lowering gland density in group B then group A leaves which had not sustained damage should not have differed significantly from damaged leaves. As it was undamaged group A leaves differed significantly from damaged group A leaves but did not differ from group B leaves.

6.4.3 How is gland density controlled?

The results demonstrate that herbivory has a very definite localized effect on oil gland density. This effect is to increase the density of oil glands on the leaf surface to over double that of undamaged leaves. As induction of the leaf volatile oil is a quantitative change all the enzymes associated with the terpene pathway must be involved. Volatile oil glands are apparent on leaves from budburst and volatile production continues regardless of herbivory so the metabolic pathway involved is not initiated in response to herbivory. Herbivory simply increases the rate of oil production.

The change observed includes an increase in the number of leaf glands so there is perhaps a mechanism whereby increased oil production stimulates gland production. An alternative explanation is that herbivory triggers oil production at the same time as gland production. This would require a rather more complex genetic response but would be relatively simple if the genes for oil production and gland production were proximate on the chromosome forming a single operon. This would allow for coordinate induction of the enzymes involved (Stadman 1970). A third, and perhaps the most plausible, explanation, however, is that herbivory triggers new gland production and this in turn triggers the production of volatile oil in the glands without further external stimulus.

6.4.4 What benefit might the increasing gland density provide?

If the response is defensive then it is only the damaged leaves which the plant appears to be defending. It was observed that plants tend to lose leaves which have sustained herbivore damage within four weeks of that damage becoming apparent. These plants go on to produced fresh shoots in place of the lost leaves.

It is not immediately obvious, if the major function of the oil is related to herbivore deterrence, why the plant should invest in greater levels of volatile oils to protect leaves which have already sustained herbivory. If the plant sustains a herbivore attack it might be expected that the plant would invest in defending undamaged tissue against further herbivory in preference to damaged tissue which it would shed within a relatively short period. However, once a leaf has sustained damage the plant would actively retrieve many of the nutrients before the leaf is shed. White (1984) argues that

it is at such times that the tissue is most attractive to herbivores since by mobilizing the nutrients the plant makes them readily available for herbivores to digest. As the plant would be sequestering the available nutrients from a damaged leaf the photosynthate from its remaining viable tissue would be in excess of the leaf's needs and oil production could provide a sink for this surplus. Thus, by increasing oil gland density the plant would defend the nutrient available damaged tissue at very little cost.

An alternative, though not mutually exclusive, explanation is that the oil has a role in defense against plant pathogens. *Lygocoris spinolai* feeds by puncturing the upper epidermis of the leaf and taking the subcutaneous tissue. This leaves a wound which would present access to an opportunist pathogen. The cells in both the upper and lower epidermis die within a few days of attack by which time these necrotic spots typically have a dense covering of full oil glands on them. Often the individual feeding sites are close together leaving a large area of dead leaf with a dense covering of glands. These dead areas could be prone to attack by both pathogens and saprophytic fungi if left unprotected and once infected the fungi could spread to healthy tissue.

In addition to increasing the total volume of oil on the leaf surface an increase in gland density would increase the area of gland membrane, the interface between the oil and the air, thus increasing the rate of oil vapourisation and the oil's partial pressure in the air around the plant. A high partial pressure of oil in the microenvironment of the leaf could affect the growth of fungi on the leaf. The glands are fragile so any further damage to the leaf could easily result in the gland membrane being ruptured

with oil spreading over the wound providing an antifungal coating. It follows that an explanation giving the oil a role as a defense against pathogens fits the results very well. This explanation is open to be tested readily through an investigation of the activity of the oil against possible plant pathogens.

The correlations between mature leaf area and gland density suggest that the plant invests slightly more in oil in larger leaves. In the early shoots the substrate for the oil must come from the plant's stores. Once the leaves are mature local photosynthesis would readily provide the substrate for the oil. Oil gland density would be largely a function of photosynthetic efficiency under these circumstances.

Small mature leaves derive from the earliest growth and are normally located towards the base of a shoot where they are shaded by the larger leaves. Gref and Tenow (1987) found that the needles of Scots pine (*Pinus sylvestris*) growing in shade conditions produce less diterpene resin than needles in sunny positions. In another study Svoboda (1990) found that the oil production of savory (*Satureja hortensis* L.) was dependant on the number of hours of sunlight in the season. Thus, the trend observed of less oil glands on smaller leaves in sweet gale may be explained in terms of the quality of incident light. However, it was observed that the smaller mature leaves largely avoided herbivory so it is possible that the plants do not need to invest as much in protecting them.

6.4.5 Summary.

This experiment has demonstrated that leaves sustaining herbivore damage respond by increasing the oil gland density on the leaf

surface. This response is not observed on neighbouring undamaged leaves. The results cannot be readily explained in terms of the plant investing in defense against further herbivory. However, the results can be explained in terms of the plant investing in defenses against pathogens at the sites of likely infection.

Chapter 7. Bioactivity in the secondary metabolites of sweet gale.

7.1 Introduction.

The results reported in the previous two chapters demonstrate that sweet gale responds to herbivory by rapidly enhanced production of two phenolic compounds throughout the plant and increased production of volatile oil in the immediate vicinity of herbivore damage. One question arising from these observations is what function herbivore induced secondary metabolites have in sweet gale.

In this study the emphasis has been centred on possible defense roles for induced secondary metabolites. As the responses observed were triggered by herbivory it might reasonably be expected that some role exists for the induced compounds in deterrence of further herbivory.

However, in the case of the volatile oil the increase in gland density was restricted to leaves which had sustained herbivore damage. Capsid bugs, the major herbivore on the study plants, which cannot move from plant to plant until they reach the winged adult stage in July, were never found on previously grazed leaves. It appears that once a bug had finished grazing on one leaf it selects an ungrazed leaf on which to feed. Furthermore, the capsid bugs appear to feed without disturbing the oil glands.

If the observed volatile oil induction represented a defense against herbivory the plant would have been expected to increase oil gland density on undamaged leaves adjacent to recently grazed leaves rather than those leaves which had already sustained herbivore damage. However, if the observed induction had a role in defense against pathogens then damaged leaves, which are most susceptible to

pathogen attack, would be primarily defended. The data fits this second hypothesis well.

The threat presented to a plant by herbivores is not dissimilar to that presented by disease (e.g. loss of function in some tissue, loss of biomass and occasional death). Furthermore, herbivores can act as vectors for pathogens and the wounds left by herbivores present a possible site of infection by opportunist pathogens (Dickinson & Lucas 1977).

Plants might be expected to invest in similar defenses against both herbivores and pathogens. In the field of secondary metabolites many compounds have both antimicrobial and antiherbivore activity (Kuc 1976, Russell *et al.* 1978, Sutherland *et al.* 1980, Harborne 1985). The possibility exists, therefore, that rapidly induced chemical responses to herbivore attack may provide protection against associated pathogen attack.

The following experiments were designed to test this proposition. In separate studies the fungal growth inhibitory activities of the volatile oil and peak 44 were measured. The antibacterial activity of the oil was also investigated.

It was not possible to make any predictions about the biological activity of peak 44 since, at the time this work was carried out, its structure was unknown. Volatile oils, however, are well known for their antimicrobial activity (Macht & Kunkel 1920, Maruzzella 1962, 1963a, Morris *et al.* 1978, Christiansen & Ericsson 1985, Deans & Richie 1987, McDowell *et al.* 1988).

Fungi isolated from leaves in the field were used as it was considered important that the compounds isolated from the sweet gale population on Flanders Moss should be tested against strains of

fungi which may actually present a challenge to those plants. Fungi from other sources would be highly unlikely to be pathogenic to sweet gale since pathogen adaptations to a particular plant gene for resistance almost always result in the pathogen strain being less competitive in the absence of the resistance gene (Vanderplank 1984). Sweet gale invests heavily in constitutive defenses so it is unlikely that it would be susceptible to alien fungi. Clearly sweet gale's induced defenses could not be an adaptation to fungi not found near the plant. The investment by sweet gale in a system of induced responses can best be explained as a defense against pathogens and herbivores able to overcome the plants constitutive defenses.

Myrica gale is not known to succumb to many diseases (Table 2.2). Only two pathogens: *Ramularia destructiva* Plowr. & Phill. and *Septoria myricae* Trail have been reported from populations in Scotland (Foister 1930, Grove 1935). There are no reported bacterial pathogens. This small species list may be due to a lack of interest in the species in the past by mycologists in Britain. The species list of pathogens in the United States of America (Pirone *et al.* 1960), where there is considerable interest in sweet gale as an alternative host for pathogens of Douglas Fir, is larger (*Diplodia sp., Ramularia molinoides* [= *R. destructiva*], *S. myricae, Cronartium comptoniae* Arth. and *Gymnosporum ellissii* [Berk.]Farl.) but cannot be regarded as large when compared with many other plants (e.g 30 fungal species from *Vaccinium oxycoccus* L., 31 from *Betula* sp., 36 from *Rubus* sp. [Westcott 1960]).

Fungal pathogens penetrate plant tissue in a number of ways (Dickinson & Lucas 1977). Some enter through natural openings such

as stomata (e.g. *Puccinia graminis* Pers.on wheat) or sites where the protective layers are thin or absent (e.g. *Plasmodiophora brassicae* Woron on brassica root hairs). Other pathogens have the ability to enter directly by penetrating the phylloplane (leaf surface) directly (e.g. *Bremia lactucae* Regel on lettuce). However, these modes of entry are relatively specialised and often require optimal conditions for the fungal spores in the phyllosphere or rhizosphere (immediate environment of leaves/roots). By far the most frequent, and often only, avenue of entry for pathogens is via wounds. Wounding can be a consequence of natural growth (i.e. leaf abscision), adverse weather and all forms of mechanical damage including herbivore damage. Pathogen damage itself can provide avenues of entry to secondary infections which can be more destructive than the primary infection.

In addition to simply wounding leaves and leaving them open to pathogen attack herbivores can actively transport pathogens to the plant. It is probable that viral infections always require such vectors. Some fungal associations with herbivores are well documented (e.g. *Ceratocystis ulmi* [Buism.]C.Moreau and the Elm bark beetle) although a herbivore may act as vector by simply being contaminated with the pathogen through having come into earlier contact with infected tissue. Although few diseases depend upon insect dissemination infection is often associated with herbivore activity. In cacao (*Theobroma cacao* L.) damage by two species of capsid (*Sahlbergella singularis* Hagl. and *Distantiella theobroma* Dist.) is associated with infection by a fungal pathogen (*Calonectria rigidiuscula* [Benk & Br.]Sacc.) which, in cases of severe capsid infestation, can cause canopy die-back (Carter 1973).

The rhizosphere for sweet gale is unlikely to support many micro-organisms as it is an acidic solution of tannins and phenolic compounds. The roots of sweet gale spread laterally through the *Sphagnum* moss within a few centimetres of the surface. *Sphagnum* mosses are known to be mildly antiseptic and were used as dressings for wounds during the Great War (N. MacHatton, pers. comm.). Sweet gale is, therefore, far less susceptible to pathogen invasion through the roots than plants which grow in less hostile soils. An exception to this is the infection of sweet gale roots by *Frankia*. In this case, however, a close relationship exists between host and infectant and this is mutualistic.

Very little disease was evident in the plants of the study group. Where leaf spot, consistent with the description of *R. destructiva* (Foister 1930), was observed it was restricted to very few leaves and did not appear to cause the plant as a whole any major problems.

The presence of an antimicrobial volatile oil in glands on the leaf surface would provide the plant with a defense against disease when the integrity of the leaf epidermis had been broken. Leaves present vulnerable tissue to wounding because they are delicate, compared to woody material, and many herbivores prefer leaf tissue. Clearly, wounding of leaves which are covered with fragile oil glands would often result in the rupture of some glands liberating oil which would flow over the wound. Any antimicrobial activity in the oil in this situation would be of great benefit to the plant. It is interesting that the greatest oil gland density is on the upper surface of leaves since oil liberated from these glands would flow into wounds.

In addition to directly coating wounds the oil vapour would be present in the air close to the leaf surface known as the phyllosphere. The vapours of many essential oils have antimicrobial activity and fungi are particularly susceptible to this source of inhibition (Maruzzella 1963b). The partial pressures of oil constituents in the phyllosphere would be proportional to the sum of the surface areas of the leaf glands. Increasing the number of glands would also reduce airflow at the leaf surface and help to maintain the high partial pressure of the oil. Thus, if the oil is antifungal the density of leaf surface glands could affect the plant's susceptibility to fungal invasion whether or not the glands were ruptured.

The oil is a complex mixture. It differs quite considerably from the volatile oil reported from sweet gale in other parts of the world (Chapter 4). This is most clearly demonstrated by the high proportion of sesquiterpenes in the Flanders Moss population, of which ß-elemenone and germacrone are the major components. One question that arises is whether the high proportion of sesquiterpene is an adaptation to local biological pressures. This question required an investigation into the activity of the separate monoterpene and sesquiterpene fractions.

7.2 Isolation of fungi.

7.2.1 Materials and methods.

Twenty sterile universal bottles with sloped potato dextrose agar (PDA) were taken to Flanders Moss on 4 October 1988. Five types of leaf material were collected. These were (a) whole leaves showing no signs of herbivore damage, (b) leaves which were clearly

damaged by insect herbivores, (c) dying leaves, (d) green leaves exhibiting blackening around the edges and (e) the red/brown tissue of diseased leaves.

Two samples of each were collected using sterile tweezers. Each piece of leaf material was washed in sterile Ringer's solution before being placed on a PDA slope. A drop of Ringer's solution from each of the washings was also spotted onto a separate slope. The slopes were incubated at 25°C for two weeks.

Fungi were isolated from the resultant mixed colonies. Pure strains of each species were grown on PDA. Spores were harvested from each strain and rinsed in Ringer's solution. The spore suspensions were stored at 4°C until required.

Where possible the fungi were identified by Dr. S. Deans of the West of Scotland Agricultural College. Those fungi which could not be readily identified but were considered to be of interest were sent to the Commonwealth Mycological Institute at Kew (London) for identification. Fungi which were not thought likely to have any association with sweet gale (i.e. common contaminants) were discarded.

Each fungi was plated out from the spore suspensions onto PDA mixed with blended sweet gale leaves (39.5g PDA : 27g leaf : 1 l H_2O). The ability of the fungi to grow on this medium was recorded.

7.2.2 Results.

Ten fungi were isolated from sweet gale material, of which eight species were identified (Table 7.1). No fungi were isolated from the undamaged leaves or their washings. No cultures were recorded from the herbivore damaged leaves but a cream yeast

Table 7.1 Species of fungi isolated from sweet gale leaf tissue and leaf washings collected on Flanders Moss.

	Source (figure refers to whether species isolated from 1 or 2 leaves).
Alternaria alternata (Fr.) Keissler	Insect-damaged leaf washings
Apiospora montagnei Sacc.	Diseased leaf tissue (x 2)
A pergillus niger van Tiegham	Senescing leaf washings
Epicoccum nigrum Link	Diseased leaf tissue (x 1)
Fusarium sporotrichioides Sherb.	Senescing leaf washings
Penicillium citrinum Thom.	Diseased leaf washings
Penicillium spinulosum Thom.	Senescing leaf (x 2)
Trichoderma harzianum Rifai	Senescing leaf (x 1)

developed from one of the washings. Five species of fungi were isolated from the dying leaves: *Penicillium citrinum* Thom., *Trichoderma harzianum* Rifai and *Fusarium sporotrichioides* Sherb. from the leaves plus *Aspergillus niger* van Tiegham and a cream yeast from the washings. Two species were isolated from the leaves with black edges: *P. citrinum* from the leaves and a pink yeast from the washings. *Epicoccum nigrum* Link was isolated from one of pieces of diseased leaf tissue together with *Penicillium spinulosum* Thom. and *Apiospora montagnei* Sacc. *A. montagnei* and *E. nigrum* were also isolated from the other piece of diseased leaf tissue. *Alternaria alternata* (Fr.)Keissler, *P. spinulosum* and *F. sporotrichioides* were isolated from the washing of one piece of diseased leaf. The yeasts were discarded. *T. harzianum*, *F. sporotrichioides* and *A. montagnei* were the only fungi to grow successfully on the leaf/PDA plates.

7.3 Fungal growth inhibition, bacterial growth inhibition and antiherbivore tests.

7.3.1 Methods.

7.3.1.1 Antifungal tests for the volatile oil.

Instrumentation: Carlo Erba HRGC 5300 with MFC 500 console using flame ionisation detection and on column injector. Column: SE30 (10% on chromosorb A) (2m x 9mm). Gilson 303 HPLC ternary gradient system controlled by Gilson 'HPLC Manager' run on an Apple IIe microcomputer. Shimadzu SPD-6AV UV spectrophotometric detector. Column: Techsphere S5 Octadecylsilicate 1 (25cm x 9mm). Trivector Trio integrator.

The activity of the volatile oil as a fungal growth inhibitor

was tested at various concentrations against eight species of the isolated fungi (Section 7.2). In each test the fungi were grown in the presence of steam distilled oil. Oil, at a range of concentrations (0.005%-1.0% v/v) was added to 10ml of malt broth containing 50 µl of spore suspension (approx 5 x 10^5 spores ml^{-1}) in a 100 ml conical flask. At the lower concentrations (0.005%-0.2%) the oil was made up to 50 µl with pentane for dispensing accuracy. In the control flasks the oil was replaced by liquid paraffin to confirm that any activity was due to the chemical characteristics of the oil rather than purely physical factors such as forming a film over the broth. The flasks were agitated at room temperature for five days. Three replicates were used in early experiments (*A. alternata, A. niger* & *P. spinulosum*) and ten replicates were used in all further experiments.

The resultant cultures were treated with sodium hypochlorite before being filtered through preweighed glass fibre filters (GF/C 5.5 cm). The filters plus fungi were dried for 24 hours at 60°C and reweighed. The dry weight of fungus in each flask was calculated and the results subjected to oneway analysis of variance (ANOVA). The significance of differences between treatment means was calculated by the least significant difference method (Sokal & Rohlf 1981).

A further set of experiments were used to attempt to locate the active fractions of the oil. In these experiments *P. citrinum* was used as the test organism. The oil was split into monoterpene and sesquiterpene fractions using semipreparative gas liquid chromatography. Steam distilled oil (0.25 ml) was injected on column at 80°C and the oven temperature was ramped to 250°C at

10° min^{-1} and maintained at 250° for 10 min. The monoterpene fraction was collected between 0 and 10 min and the sesquiterpene fraction was collected between 10 and 28 min. The fractions were collected in pentane cooled to -70° in acetone and dry ice. A second sesquiterpene fraction was prepared with semipreparative high performance liquid chromatography using the method of Morin *et al.* (1986) (Appendix A). The antifungal activity of each fraction was investigated using the growth inhibition assay outlined above. The composition of each fraction was ascertained by analytical GLC using the conditions described in Chapter 4.2.2.

The activities of the major constituents of the monoterpene fraction were also measured. Limonene, 1,8-cineol, α-pinene, ß-pinene, α-phellandrene and myrcene were used for the tests. These were not separated from the oil but were taken from laboratory supplies. The effect of 0.1% of each compound on fungal growth was investigated using the above method.

7.3.1.2 Antifungal tests for peak 44.

Peak 44 was tested against two species which did not successfully grow on the leaf/PDA plates, *E. nigrum* and *P. citrinum*, and the three species which did grow successfully on the medium, *F. spinulosum, T. harzianum* and *A. montagnei*. In the first experiment the effect of 50 μg ml^{-1} peak 44 on the growth of *P. citrinum* was tested by adding 50 μl of the flavonoid in methanol (10 mg ml^{-1}) was added to 10 ml of malt broth plus 50 μl spore suspension in a 100 ml conical flask. In the controls 50 μl methanol replaced the flavonoid solution. The flasks were agitated at room temperature for five days. In the later experiments two

concentrations (50 μg ml^{-1} & 100 μg ml^{-1}) were tested against the remaining fungal species. Five replicates were used in each test.

Fungal growth was measured, after treatment with sodium hypochlorite, using the dried filter technique described above. Results were analysed using analysis of variance and the significance of differences between treatments was ascertained by the least significant difference technique (Sokal & Rohlf 1981).

7.3.1.3 Antibacterial tests.

The antibacterial tests plates (Isosensitest agar Oxoid CM) were seeded with 25 species of bacteria. Three wells (4mm diam.) were punched in each plate. 50 μl of oil was placed in each well and the plates were incubated at 25°C. Inhibition zones were measured using Vernier callipers (Mauser). No attempt was made to isolate bacteria from the field and standard culture bacteria were used.

7.3.1.3 Antiherbivore tests.

In addition to being tested for antimicrobial activity peak 44 was sent to Dr M. Simmonds of Kew Gardens, London to be tested for antiherbivore activity. Two species of insect, *Spodoptera littoralis* Boisd. and *Heliothis viriscens* Fabr. were used in these tests, the report of which appears in Appendix B.

7.3.2 Results.

7.3.2.1 The volatile oil.

In all cases the oil proved to significantly inhibit fungal growth at 0.1% v/v (Table 7.2). In general the inhibitory effect of

Table 7.2 Inhibition of fungal growth in malt broth by a range of sweet gale volatile oil concentrations. Fungal growth is expressed as a percentage of control growth.

	Concentration of oil in treatment								
Test organism	0.005%	0.01%	0.05%	0.10%	0.20%	0.50%	1.00%		
Altenaria alternata	n.d.	90	93	80a	77b	45b	23c	n = 3	F = 41.95
Trichoderma harzianum	n.d.	94	90	66c	55c	49c	34c	n = 10	F = 132.06
Aspergillus niger	n.d.	116	74a	67a	86a	63b	n.d.	n = 3	F = 34.57
Epicoccum nigrum	n.d.	48c	39c	18c	17c	12c	n.d.	n = 10	F = 39.93
Penicillium spinulosum	n.d.	80	55b	66a	47b	18b	n.d.	n = 3	F = 18.20
Apiospora montagnei	41c	22c	20c	23c	n.d.	n.d.	n.d.	n = 10	F = 214.93
Fusarium sporotrichioides	n.d.	n.d.	n.d.	63c	33c	n.d.	n.d.	n = 10	F = 250.95
Penicillium citrinum	n.d.	n.d.	n.d.	32c	n.d.	22c	n.d.	n = 10	F = 283.27

a: $p < 0.05\%$, b: $p < 0.01\%$, c: $p < 0.001$
(probabilities refer to difference from control)
n.d. = no data

the oil was proportional to concentration although one result, in which 3 replicates were employed (*A. niger*: 0.01%), went against this trend. Although the oil inhibited the growth of all fungi tested the degree of inhibition differed markedly. *A. montagnei* was the most susceptible exhibiting greater than 50% inhibition at 0.005% oil in the medium. *T. harzianum, A. alternata* and *A. niger* were least susceptible to the inhibitory properties of the oil although the growth of each of these was inhibited at the higher concentrations of oil (0.1% - 1.0%).

7.3.2.2 Antifungal activity of the oil fractions.

The monoterpene fraction from the GLC (Fig 7.2) proved to consist of monoterpenes in similar proportions to those apparent in the whole oil (Fig 7.1). The GLC sesquiterpene fraction (Fig 7.3) differed markedly from the HPLC sesquiterpene fraction (Fig 7.4). The GLC fraction contained predominantly ß-elemenone whereas the major peak in the HPLC fraction was germacrone. In the steam distilled whole oil ß-elemenone was not a major compound. Its presence in the GLC fraction must have been due to the thermal decomposition of germacrone (Reichart *et al.* 1989). However, as ß-elemenone was a major constituent of the leaf gland oil of many plants early in the summer its occurrence in the GLC fraction enabled its antifungal properties to be assessed.

Each oil fraction proved to have activity against *P. citrinum* at 0.1% v/v (Table 7.3). The whole oil inhibited growth to 32% of the control. The monoterpene fraction restricted growth to 38% of control and this was significantly less inhibition than the whole oil and the GLC sesquiterpene fraction ($p < 0.01$). The GLC

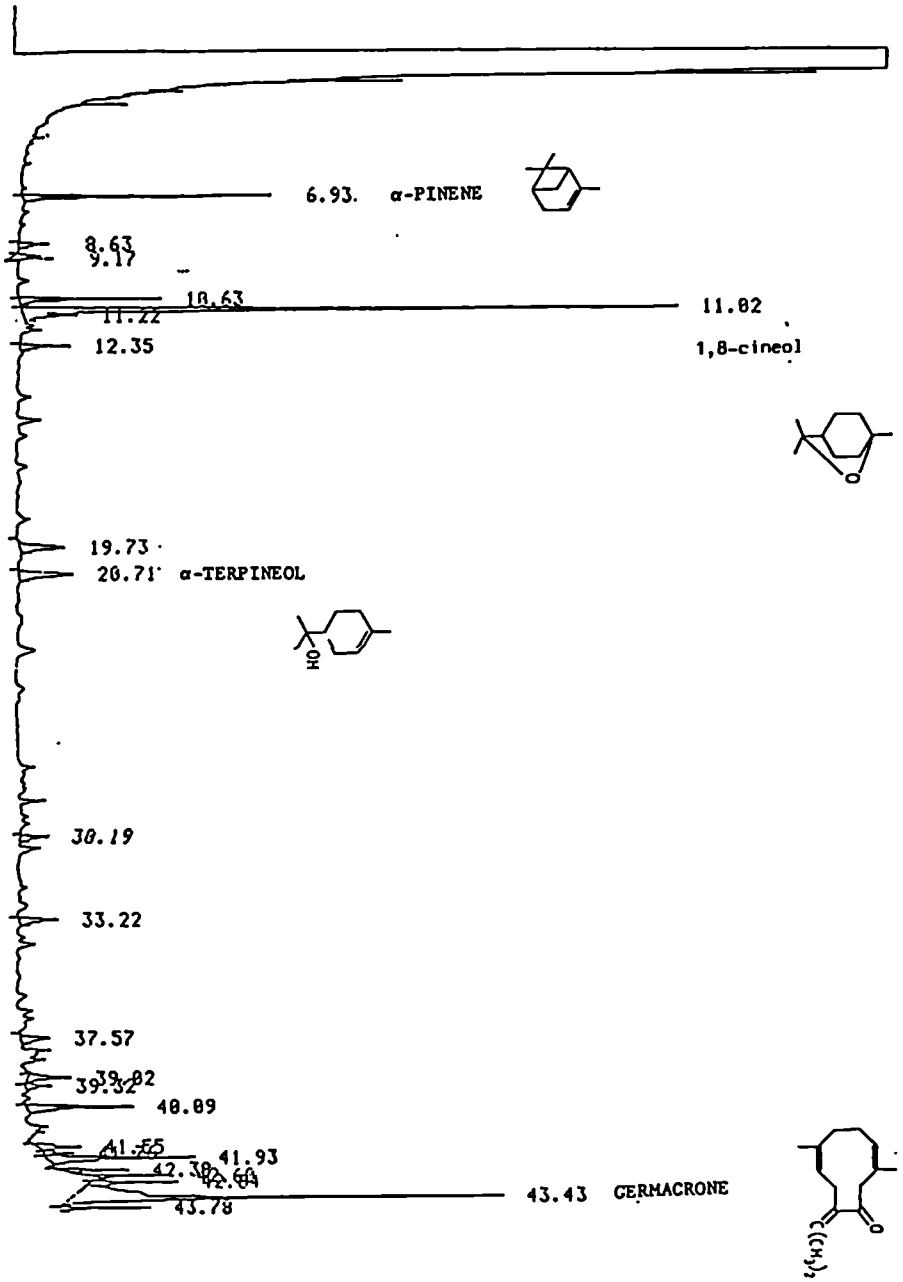


Figure 7.1 The volatile oil of sweet gale extracted by steam distillation.

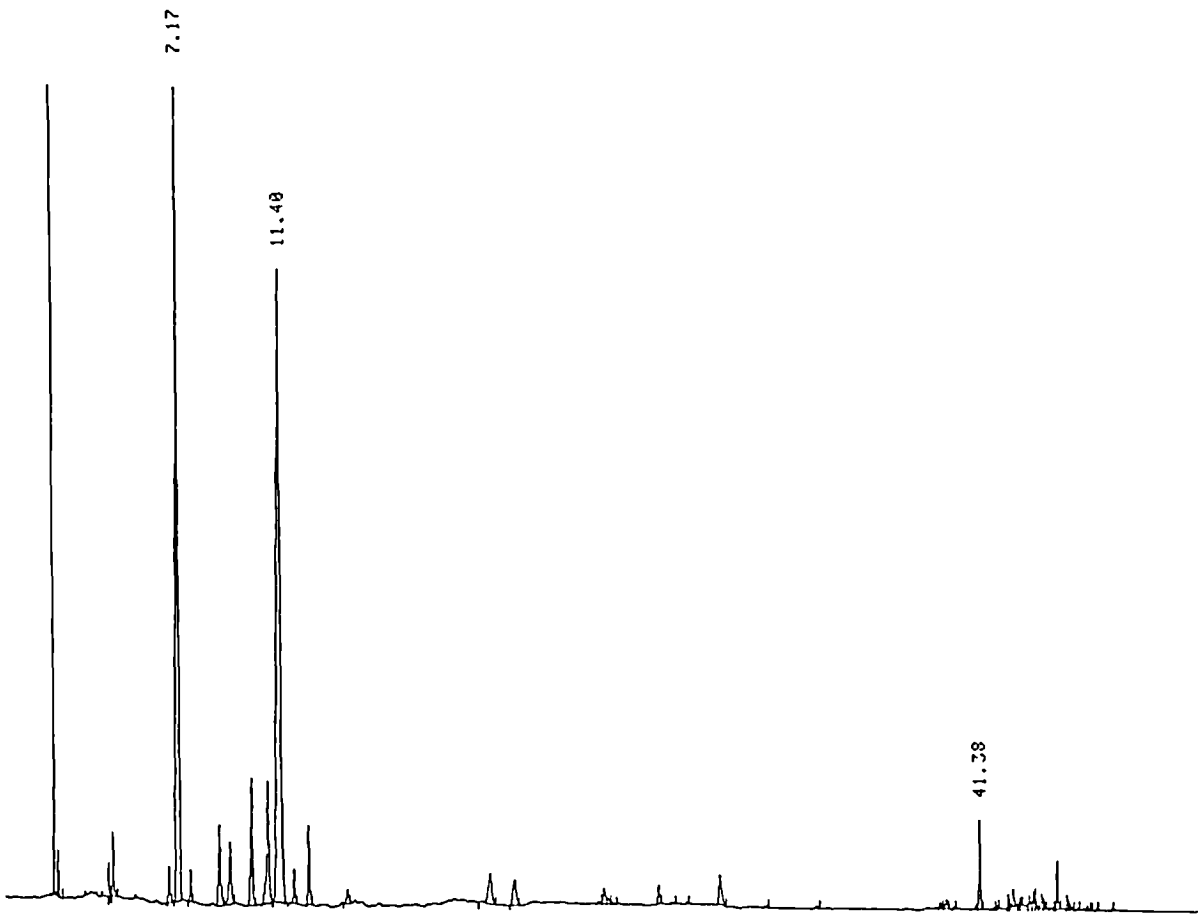


Figure 7.2 The monoterpene fraction of sweet gale volatile oil, prepared by semipreparative GLC.

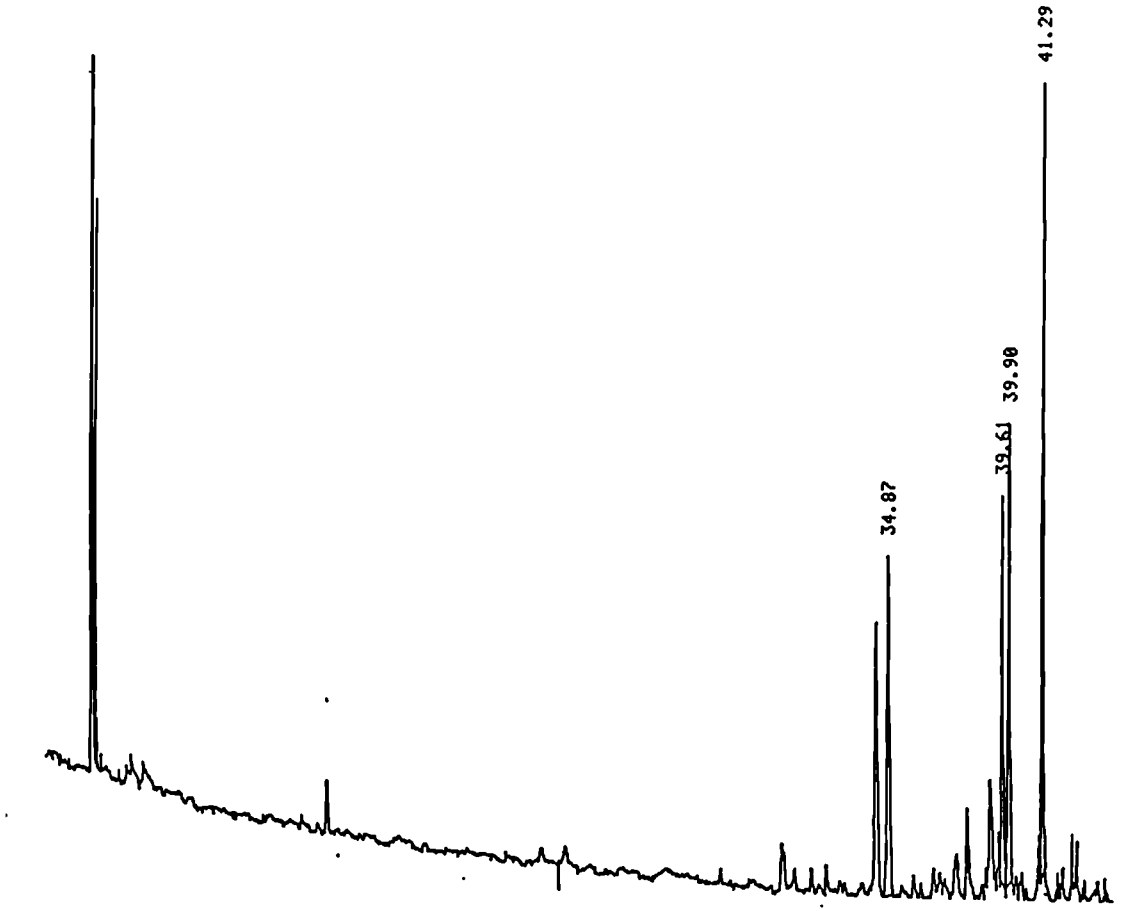


Figure 7.3 The sesquiterpene fraction of sweet gale volatile oil, prepared by semipreparative GLC.

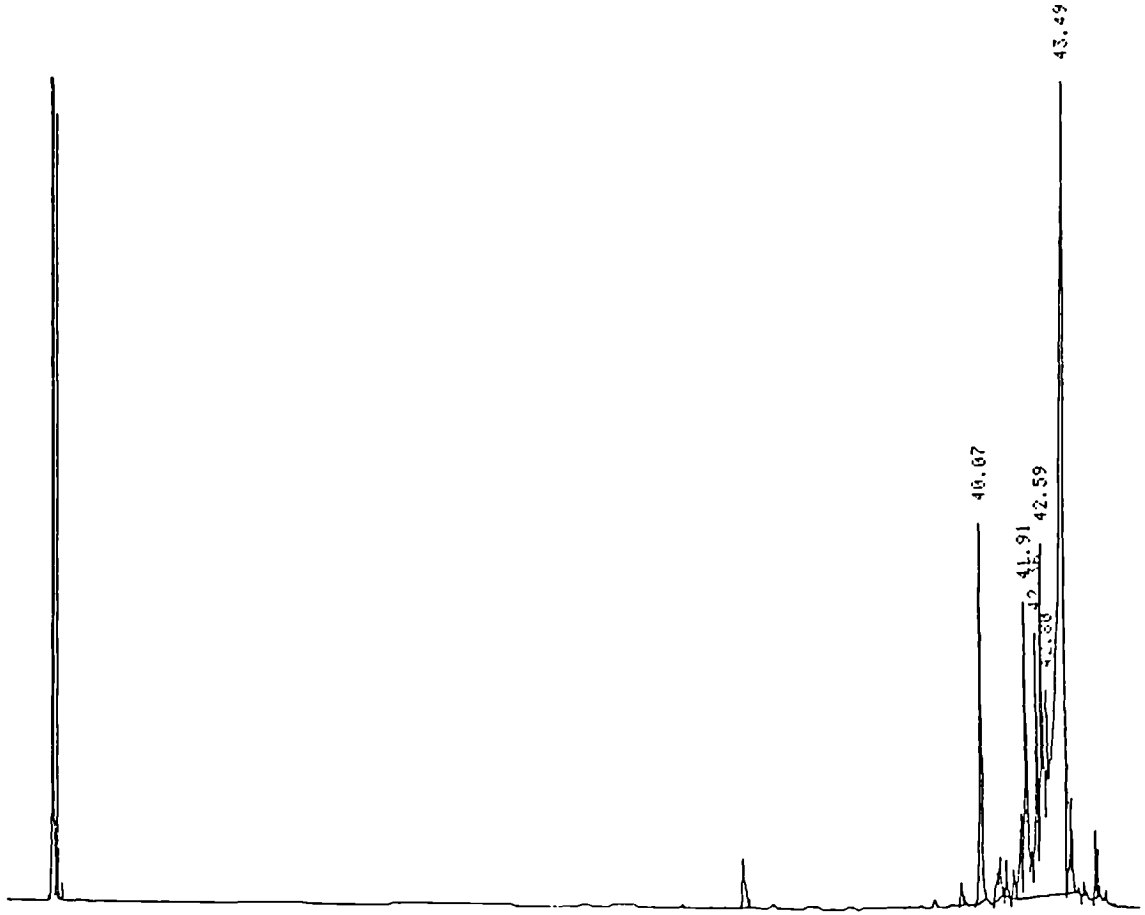


Figure 7.4 The sesquiterpene fraction of sweet gale volatile oil, prepared by semipreparative HPLC.

Table 7.3 Growth inhibition of P. citrinum by the volatile oil of sweet gale and three oil fractions (0.1% v/v) expressed as a percentage of control growth.

Volatile oil (whole)	GLC fraction A (monoterpenes)	GLC fraction B (sesquiterpenes + β-elemenone)	HPLC fraction B (sesquiterpenes + germacrone)	
32	38a	35a	n.d.	F = 67.7
31	n.d.	n.d.	29	F = 0.64

a: growth inhibition by fraction significantly different to that of whole oil ($p < 0.001$).

sesquiterpene fraction also inhibited growth to a significantly lesser degree than the whole oil. The HPLC sesquiterpene fraction restricted growth to 29% of control. This fraction was tested separately to the GLC fractions and was not significantly different to the whole oil.

At a concentration of 0.1% v/v each of the individual monoterpenes proved to significantly inhibit the growth of *P. citrinum*. It can be seen from Table 7.4 that α-terpineol was most active monoterpene almost completely inhibiting growth at 0.1% v/v. However, the laboratory α-terpineol is a racemic mixture so is not strictly comparable with α-terpineol in the volatile oil. Limonene was slightly more active than the GLC monoterpene fraction. α-Pinene exhibited similar activity to the GLC monoterpene fraction. The other monoterpenes proved to inhibit fungal growth less than the GLC monoterpene fraction.

Fungi which produced pigment in the control flasks were observed to have noticeable reductions in pigment, or be pigment free, in the mediums containing inhibitory concentrations of the whole oil. When the individual monoterpenes were tested for activity it was observed that certain monoterpenes reduced pigment to a far greater extent than others (Fig 7.5). Fungi grown in medium with limonene and with α-phellandrene exhibit this reduction most clearly. Limonene was observed to inhibit both growth and pigment formation to a greater extent than other monoterpene treatments. However, α-phellandrene was observed to inhibit pigment production to a greater extent than α-pinene and the GLC monoterpene fraction yet inhibited growth less. On the other hand, ß-pinene and 1,8-cineol did not noticeably affect pigmentation although they

Table 7.4 Inhibition of P. citrinum by single monoterpenes (0.1% v/v) expressed as a percentage of control growth, in which controls were grown in malt broth plus oil fraction A (0.1% v/v).

Compound	% control growth
alpha-pinene	95.61
beta-pinene	136.00b
myrcene	122.47a
alpha-phellandrene	129.00b
limonene	77.32b
1,8-cineol	138.61b
terpineol	4.34c

Probabilities refer to difference from controls
($F = 32.81$; a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$).

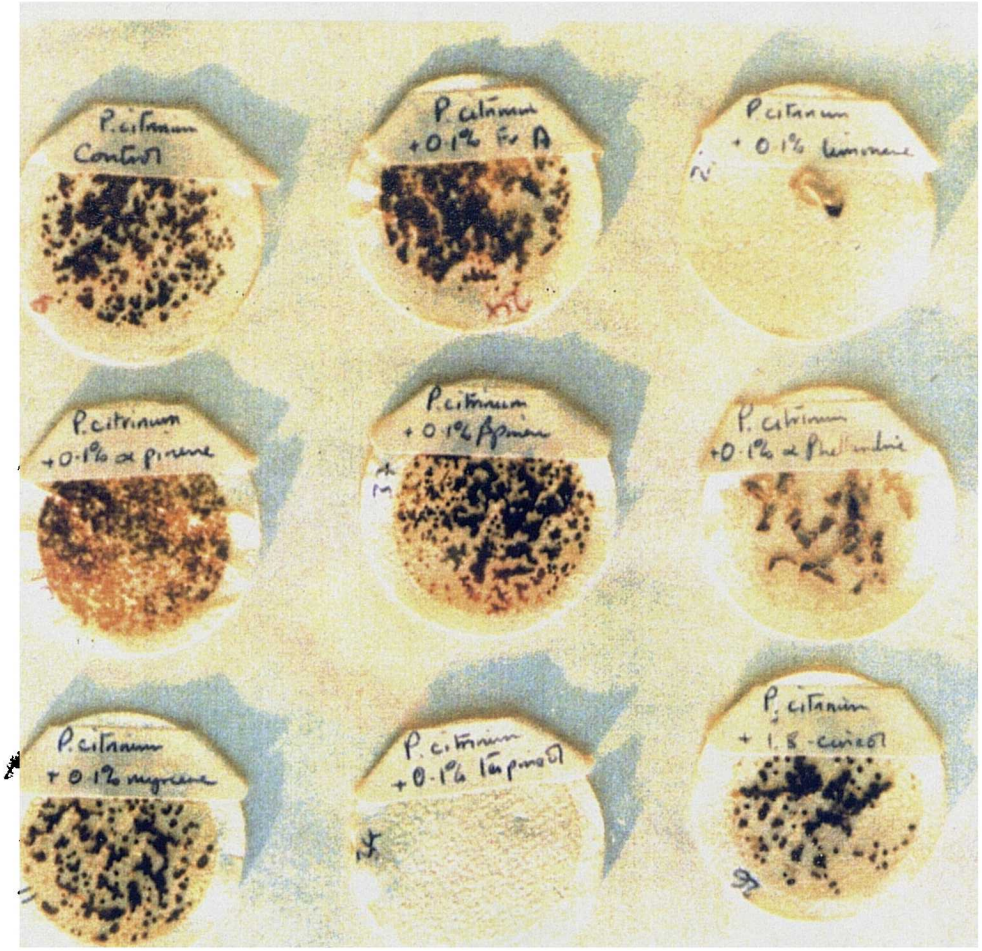


Figure 7.5 The effect of different monoterpenes on the growth of P. citrinum.

inhibited growth significantly. It was not possible, in the framework of this experiment, to quantify these observations.

7.3.2.4 The antifungal activity of peak 44.

Peak 44 was found to inhibit both *P. citrinum* and *E. nigrum* quite markedly at the concentrations tested (Table 7.5). The three species which could grow on leaf/PDA medium were inhibited to a smaller extent. The growth of *T. harzianum* was not significantly different to that of the control although the treatments did appear to depress growth slightly. The growth of both *F. sporotrichioides* and *A. montagnei* was significantly inhibited by peak 44, *A. montagnei* exhibiting a greater susceptibility to the flavonol.

7.3.2.4 The antibacterial activity of steam distilled oil.

The oil was active against 8 of the 25 bacterial species (Table 7.6). The most susceptible species was *Brocothorix thermosphacta* and *Clostridium sporogenes* also exhibited considerable inhibition. The other 6 species were inhibited to a similar degree.

7.3.2.5 The antiherbivore activity of peak 44.

In the antiherbivore tests peak 44 proved to be a mild phagostimulant of *S. littoralis* and to have no influence on the mortality or development of *S. littoralis* or *H. viriscens*.

7.4. Reinfection of healthy plants.

7.4.1 Methods.

A further experiment was set up to try and identify whether fungi isolated from the leaf spots and fungi able to grow on

Table 7.5 Inhibition of fungal growth in malt broth by by peak 44, expressed as a percentage of control.

	50ug/ml	100ug/ml	
Apiospora montagnei	n.d.	52.55	$p < 0.0001$
Epicoccum nigrum	25.82	5.90	$F = 211.15$
Fusarium sporotrichioides	87.81	n.d.	$p = 0.006$
Penicillium citrinum	9.24	n.d.	$p < 0.0001$
Trichoderma harzianum	95.84	94.40	$F = 1.38$
			$n = 5$

n.d. = no data.

Table 7.6 Inhibition of bacteria by sweet gale volatile oil.

Organism	Source	Mean diameter of inhibition zone (mm).
Acinetobacter calcoacetica	NCIB 8250	0.0
Aeromonas hydrophila	NCTC 8049	8.8
Alcaligenes faecalis	NCIB 8156	0.0
Bacillus subtilis	NCIB 3610	6.8
Beneckea natriegens	ATCC 14048	0.0
Brevibacterium linens	NCIB 8456	0.0
Brocothrix thermosphacta	Sausage meat	22.7
Citrobacter freundii	NCIB 11490	0.0
Clostridium sporengnes	NCIB 10696	11.2
Enterobactera erogenes	NCTC 10006	0.0
Erwinea carotovora	NCPPB 312	0.0
Escherichia coli	NCIB 8879	0.0
Flavobacterium suaveolens	NCIB 8992	6.2
Klebsiella pneumoniae	NCIB 418	0.0
Lactobacillus plantarum	NCDO 950	0.0
Leuconostoc cremoris	NCDO 543	0.0
Micrococcus luteus	NCIB 8165	0.0
Moraxella sp.	NCIB 10762	0.0
Proteus vulgaris	NCIB 4175	0.0
Pseudomonas aeruginosa	NCIB 950	7.0
Salmonella pullorum	NCTC 10704	7.8
Serratia marcescens	NCIB 1377	6.2
Staphylococcus aureus	NCIB 6571	0.0
Streptococcus faecalis	NCTC 775	0.0
Yersinia enterocolitica	NCTC 10460	0.0

leaf/PDA plates were pathogenic. *E. nigrum, F. sporotrichioides, T. harzianum* and *A. montagnei* were used in this experiment.

Thirty plants were removed from Glasgow Botanic Gardens to the West of Scotland Agricultural College on 17 April 1989. On 27 July the twenty five plants were selected and split into five groups of five plants. Each plant was covered with a white plastic bin liner fastened around the stem to increase the relative humidity in the phyllosphere (adapted from Hammerstein, pers comm). The plants in each group were sprayed, through apertures in the bags, with 20ml from one of the spore suspensions. The control group were sprayed with Ringer's solution.

The plants were left bagged for seven days and then inspected for disease. They were left with the bags removed for a further seven days and reinspected.

7.4.2 Results.

None of the bagged plants developed signs of disease. At the end of the first week three of the plants sprayed with *F. sporotrichioides* exhibited small growths of the fungus on the leaf surfaces but these did not persist.

7.5 Discussion.

7.5.1 Oil from ruptured glands, an antiseptic coating for wounds.

The volatile oil of *Myrica gale* had clear inhibitory properties against a broad spectrum of fungal species. This provides strong support for the hypothesis that the plant invests in oil as a defense against fungal pathogens. Since the oil is stored in glands on the surface of the leaf its role is likely to be in preventing

infection from occurring. If this defense fails and a pathogen penetrates the leaf tissue it is difficult to see how the oil could further affect the course of disease, except possibly by excluding secondary infection.

It is not clear what mechanism of inhibition is in action. The oil could reduce growth in at least two ways. By reducing germination of spores growth would be correspondingly reduced although hyphal growth from spores which did germinate would continue until nutrients became limiting. Thus, in tests using a fixed volume of medium (i.e. in which growth would be nutrient limited) the growth of fungi in treatment flasks would be delayed but the final weight of fungus would be similar for controls and treatments. Only total inhibition of germination would stop growth and this clearly did not happen. The oil might also act after germination by inhibiting the growth of hyphae. In this case, if growth occurred, it would be more gradual. Without measuring growth at intervals it is not possible to distinguish between these varieties of inhibition. Taking interval measurements is relatively simple for single cell cultures since the optical density of the culture can be measured without disturbing growth. Hyphal growth, however, is not amenable to this method.

The observation that pigment production was reduced, even when inhibition of biomass production is slight, suggests that the oil may affect fungal metabolism without necessarily inhibiting growth. Fungal pigments are believed to have a defensive role (Bloomfield & Alexander 1967, Bull 1970) and it is possible that in the presence of growth inhibitors in the medium the fungus diverts energy and nutrients away from the pigment pathways. However, the individual

monoterpene tests provided some evidence that reduced pigment does not necessarily relate to reductions in growth. There is no reason why the oil could not act by independently reducing spore germination, reducing hyphal growth and reducing pigment formation.

Both monoterpenes and sesquiterpenes have antifungal activity. It is not immediately obvious why the plant invests in a complex volatile oil when all fractions investigated proved to be active growth inhibitors. One explanation is that the different components have differing antifungal activity so the whole oil provides an array of defenses. A second possible explanation for the complexity of the leaf gland oil lies in the fact that the sesquiterpenes exhibit greater activity than the monoterpenes. However, they are less volatile and they can be extremely viscous when pure. Germacrone quite readily crystallizes at low temperatures. Thus, in the event of glands being ruptured due to leaf chewers such as tortricids, the high proportion of monoterpenes present will allow the oil to spread readily. The monoterpenes will then evaporate more rapidly than the sesquiterpenes leaving a high concentration of the more active sesquiterpenes, predominantly germacrone, on the leaf.

It was suggested in Chapter 4.4 that germacrone production in some members of the Flanders Moss population of sweet gale may be a recent adaptation which confers an advantage over the more common investment in ß-elemenone. The ability of the HPLC oil fraction to inhibit fungal growth more than the GLC oil fraction lends support to this supposition. This, in turn, adds weight to the argument that the volatile oil has a major role in disease resistance although, as evidence, it is extremely speculative.

7.5.2 Increasing gland density, how does it help?

The possible explanations of the mode of action of the oil argued above fit the data quite well in respect of defense of leaves where there has been herbivore damage and there is an associated pathogen threat. However, it has been seen that the plant responds to herbivore damage by increasing the density of oil glands on the damaged leaf surface. Since the plant increases the density of oil glands in response to herbivory, in general once the herbivore has left the leaf, the mechanism of further defense is unlikely to involve the herbivore rupturing the oil glands. Furthermore, in the study group on Flanders Moss the major herbivore was *Lygocoris spinolai*, a capsid bug, which feeds without disturbing the oil glands, so for those glands to be an effective form of defense they would have to act without being ruptured. Such a mode of defense would help to explain the herbivory induced increase in gland density.

The monoterpenes are more volatile than the sesquiterpenes. In the situation where no glands are ruptured the increase in oil gland density, with corresponding increase in oil gland area, would lead to an increase in the partial pressure of oil, predominantly monoterpenes. The results demonstrated that the monoterpenes have antifungal activity so raising the partial pressure of the monoterpenes would be likely to create a phyllosphere hostile to fungal spore germination and growth. α-Pinene, a major component of the oil, exhibited strong antifungal activity. As a major component the partial pressure of α-pinene would be high in the phyllosphere. The same, however, cannot be said of the terpineols which, although exhibiting the strongest activity, are not major

components of the oil. The laboratory α-terpineol was so active that even as a minor constituent of the oil α-terpineol, which increase in late summer, would add to the overall activity. It is reasonable to assume that an increase in gland density would actively inhibit infection even in the absence of gland rupture.

The ability of the oil to inhibit fungal growth clearly varies between the species of fungi. It is surprising that the species isolated from the diseased tissue, *A. montagnei* and *E. nigrum*, both proved to be highly susceptible to low concentrations of oil. However, until the end of the growing season the diseased tissue was only observed on one of the plants. This plant (C2) had been kept free of herbivores and consequently had a relatively low oil density (3 glands mm^{-2}). In this case infection must have occurred without herbivore vectors and the absence of a dense covering of oil glands would have left the plant susceptible to fungal infection.

At the end of the growing season, when the plants were in the process of shedding leaves, many more plants were observed to exhibit signs of disease. At this time close inspection of the oil glands revealed many were opaque and almost all had lost much of their earlier turgidity. This suggests that the plants had ceased to produce oil allowing for the gradual collapse of glands through unreplaced loss of oil by evaporation. By this stage of the summer the oil had already lost much of the sesquiterpene fraction so the loss of monoterpenes would leave a high concentration of flavanones in the leaf glands, perhaps resulting in their opaque appearance. If high gland density on damaged leaves acts by increasing the partial pressure of the oil, especially monoterpenes, in the phyllosphere this mechanism of defense would cease to be effective

once the monoterpenes being lost ceased to be replaced. This would account for the apparent ease with which some fungi, especially the comparatively resistant *T. harzianum*, invade leaf tissue in late autumn. The plants would not be expected to invest in defenses at this time since the leaf tissue is no longer of any importance. Indeed, by this time of year the incident light is low and the average temperature has dropped so photosynthesis, which supplies the carbohydrate for oil production, would be greatly reduced.

It is perhaps not too surprising that the oil has limited antibacterial activity since bacteria are, in general, far less often pathogenic to plants and specifically are not known as pathogens of sweet gale. However, the leaf glands were found to contain myrigalon-B (peak 54: Chapter 3.3.4) and 4,4,6-trimethyl-2-(ß-phenylpropionyl)cyclohexane-13,5-trione (Chapter 4.3) which are not present in the steam distilled oil. Myrigalon-B has been demonstrated to have antibacterial activity although little if any antifungal activity (Malterud & Faergi 1982). The presence of antifungal terpenes and antibacterial dihydrochalcones within the glands concurs with the glands having a role in defense against microbial pathogens.

7.5.3 A possible problem of interpretation!

The major herbivore at the study site was a capsid bug and this simplifies interpretation of the data since only one herbivore is involved. However, it was observed that in other areas of Flanders Moss the major herbivores were lepidopteran larvae (see Chapter 2). Furthermore, sawfly larvae were also recorded from sweet gale on the moss. It is quite possible, therefore, that the observed responses

to capsid bug herbivory had evolved against a background of pressures associated with all types of herbivore.

To attempt to explain the resulting phenomena with reference to just one species of herbivore may well be misleading. This is not to say that the initial hypothesis, invoking high partial pressures of terpenes in the immediate vicinity of the leaf surface as a possible defense against fungal infection, is wrong. It is more likely that the hypothesis highlights just one of the possible advantages the oil gland induced response confers on the plant.

7.5.4 A role for peak 44.

The growth experiments using leaf/PDA medium clearly showed that the leaf material inhibited growth of most fungi isolated from sweet gale on Flanders Moss. As *A. montagnei* grew successfully on this leaf/PDA medium and yet was extremely susceptible to low concentrations of oil the inhibition of fungi on the leaf agar cannot be ascribed to oil from the leaves. This is not surprising since the leaf/PDA medium was sterilised at 121°C at which time much of the oil would have evaporated. A different growth inhibitor or set of inhibitors, therefore, must exist in the leaves to explain the inhibitory properties of the leaf/PDA medium. The malt broth growth inhibition test results strongly suggest that peak 44 was accounting for the inability of most fungal species to grow on leaf/PDA medium. All three species which grew on leaf/PDA exhibited far smaller responses to peak 44 than the two species which failed on the leaf/PDA medium.

Peak 44 was observed to have activity at low concentrations against all fungi tested. The way that peak 44 production increased

in response to herbivory differed from that of oil gland density in a number of ways. The levels of peak 44 were initially very low and increased dramatically in response to herbivory. The difference between damaged and undamaged leaves did not persist throughout the season because undamaged leaves exhibited a corresponding increase in peak 44 concentration within two to three weeks of the rise in damaged leaves.

The results of the antifungal tests support the hypothesis that peak 44 is involved in defense against pathogen invasion. However, flavonoid glycoside synthesis and storage are strictly compartmented within plant cells, the final product commonly being stored in the vacuoles (Hrazdina & Wagner 1985). Peak 44, therefore, would be unlikely to affect initial inoculum as the pathogen would be in the tissue before encountering the compound. It could, however, affect the rate of spread of the pathogen and the severity of the disease.

The characteristics of the diseased spots indicate infection by *Ramularia destructiva* (Foister 1930, Ellis & Ellis 1985). This was not isolated, possibly because initial isolations were carried out on PDA which may favour the growth of more common saprophytes. *A. montagnei*, which was isolated from diseased tissue, was able to grow on leaf/PDA and could grow in medium containing peak 44 which suggests the strain isolated may be specifically adapted to sweet gale tissue. It is common on plant material in swampy habitats and is known to have cellulolytic activity (Domsch *et al.* 1980). However, it has not been recorded as a pathogen and did not cause disease when applied to healthy plants. Its isolation from both pieces of diseased leaf suggests that it maybe a secondary invader. *E. nigrum* was also isolated from diseased tissue yet was strongly

inhibited by peak 44 and failed to grow on leaf/PDA. *E. nigrum* is a common saprophyte (Kirk pers. comm.) and is regarded as a secondary invader of diseased plants (Arsvoll 1975). Since it is susceptible to inhibition by peak 44 it would not be able to infect healthy tissue but could grow in the dying tissue at the disease site.

The other two species which grew successfully on leaf/PDA (*T. harzianum & F. sporotrichioides*) were both isolated from dying leaves. Both are known to be saprophytic (Domsch *et al.* 1980) and *F. sporotrichioides* is occasionally weakly parasitic (Booth 1971). Birch (*B. pendula*) growing on Flanders Moss beside the sweet gale occasionally exhibited leaves supporting a clear infection of *Fusarium* sp. which may have been the source of *F. sporotrichioides* spores on sweet gale leaves. Both *F. sporotrichioides* and *T. harzianum* may well have a saprophytic role early in the leaf decomposition succession which would require the ability to degrade peak 44. Of all the fungi investigated *T. harzianum* appeared to be least susceptible to the chemical defenses of sweet gale. Members of the genus *Trichoderma* are known to have resistance to low concentrations of artificial fungicides and to be antagonistic to pathogenic fungi (Dickinson & Lucas 1977, Westcott 1960). Hutchinson and Cowan (1972) recorded that *T. harzianum* emits volatile compounds capable, under laboratory conditions, of inhibiting the growth of other fungi. If this is true in the field then the presence of *T. harzianum* on leaves immediately prior to leaf drop, when the levels of flavonoids fall, might inhibit infection by pathogens and could be beneficial to sweet gale.

When tested on insects peak 44 exhibited no detrimental effects whatsoever and proved to stimulate feeding. The tests were not

carried out on species which utilise sweet gale as a source of food so it would be unwise to attach too much weight to these results. However, they do indicate that peak 44 is unlikely to have a role in deterring insect herbivores.

7.5.5 Another antimicrobial role for the essential oil.

The antimicrobial activity of the volatile oil may also have a role in deterring herbivory. Terpenes, in particular oxygenated terpenes, have been demonstrated to deter grazing by ungulates (Scholl *et al.* 1977) and this correlates with their ability to reduce ungulate digestion (Oh *et al.* 1968, 1969, Nagy & Tengerdy 1968) by depressing gut microbial activity. This type of activity may well explain why sweet gale escapes significant vertebrate grazing.

7.5.6 Summary.

To summarise, the chemical defenses of *Myrica gale* induced by herbivory have clear antifungal activity. Although these compounds also exist as constitutive defenses and the oil, if not peak 44, may have a role in deterring herbivory, it is difficult to explain induction in terms of defense against herbivores. The evidence, therefore, supports the hypothesis that the primary purpose of herbivory induced enhancement of the volatile oil and peak 44 is to protect the plant from microbial, especially fungal, pathogens. The roles of the oil and peak 44 are not equivalent but, rather, they are complementary. The oil provides protection at the leaf surface, but, when this fails to inhibit infection peak 44 restricts the growth of pathogens.

Chapter 8. Final discussion.

8.1 The constitutive defenses of Myrica gale: why so strong?

Sweet gale invests heavily in constitutive secondary metabolites indicating that its need for chemical defenses is great. The need for this investment becomes clear when the ecology of the species is examined. Its association with *Frankia* sp. allows it to adopt more competitive characteristics than most other plants typical of wetlands (Chapter 2). The plant exhibits rapid growth in early summer and the new tissue is characterised by high proportions of nitrogen relative to other wetland tolerant plants (Small 1972). Sweet gale might, therefore, be expected to provide a prime source of nitrogen rich food to all herbivores in wetlands.

Sweet gale, however, is avoided by vertebrate herbivores (Linklof *et al.* 1974, Jackson 1977, Sprent *et al.* and personal observation) and does not support a large variety of insect herbivores (Chapter 2). This indicates the presence of some effective defences. The high density of volatile oil glands on the youngest leaves (Chapter 6), which are richest in nitrogen (Sprent *et al.* 1978), almost certainly plays a major role in deterring herbivores, especially vertebrates, which are active prior to budbreak. Sweet gale's investment in flavonoids may also play a major role in herbivore deterrence. The major flavonoid, myricetin-3-galactoside (peak 30), is not induced by herbivory but the observed sharp rise in leaf concentration of this compound coincided with the onset of capsid bug herbivory. This suggests that peak 30 has a role closely linked to insect herbivory. Many insects feed by inserting their proboscis into the leaf and thereby avoiding defenses stored in leaf surface glands. Flavonoids, stored in cell

vacuoles, could provide protection against many of these herbivores. Flavonoids are known to have deleterious effects on the larval growth of many insects (Harborne 1985) although flavonoids rarely have the same effect on all insects. Peak 30 may deter feeding by most insect herbivores on Flanders Moss although *Lygocoris spinolai* is clearly able to metabolise it. It is possible that peak 30 has some deleterious effects on *L. spinolai* so it may have a role in controlling the population of this insect.

Flavonoids and terpenoids are also both known to have antimicrobial activity (Friend 1985, Morris *et al.* 1978) and this activity in the plant's constitutive defenses would benefit the plant. It was argued in Chapter 1 that adaptations to biotic stress leading to investment in secondary metabolites as a defense against both herbivores and pathogens would be of selective advantage to the plant. However, in view of the high proportion of nitrogen in the tissues of sweet gale it is likely that these compounds are primarily involved in deterring herbivores.

8.2 Does sweet gale specifically defend nitrogen?

If a relatively high percentage of nitrogen in the tissues makes sweet gale attractive to herbivores it is possible that the associated investment in chemical defenses has developed specifically to protect nitrogen. Just as the resource allocation hypothesis predicts that plants will invest in chemical defenses which utilise the most abundant resources, the plant will benefit from defending the least abundant resources. It was demonstrated in Chapter 2 that the conditions which favour sweet gale are largely determined by its association with *Frankia sp.* due to the high

proportion of the plant's nitrogen which is derived from this source. The importance of a tight nitrogen economy, therefore, cannot be overemphasised.

There is some evidence that nitrogen is specifically defended. Sweet gale leaves have the highest proportion of nitrogen at budburst and this coincides with the highest density of oil glands on undamaged leaves. Furthermore, as leaf nitrogen diminishes the volatile oil changes in composition (Chapter 3) indicating a possible change in its role. Finally, once leaves have been damaged they are readily shed and replaced suggesting that sweet gale can sustain tissue loss.

8.3 Induction in sweet gale in response to herbivory.

It seems likely that a major role of constitutive chemical defenses is in deterring herbivory and, although it is highly conjectural, this role may be intimately associated with the nitrogen economy of the plant. This study has demonstrated that, in addition to constitutive defenses, both terpenes and a flavonoid are induced in sweet gale by herbivory. The question that arises is whether these induced chemicals have a similar role to constitutive defenses.

It was proposed in Chapter 1 that a major role for induced defenses could be to deter feeding by specialists adapted to detoxify constitutive defenses. Although most herbivores do not utilise sweet gale as a food source of preference there are a number of insect herbivores which are adapted to feed on the plant (Chapter 2, Table 2.4). Only three insect species present in Scotland are specific to sweet gale and none of these were identified from the

Flanders Moss population. However, the plants in the study group supported a population of *L. spinolai* which accounted for more than 90% of the herbivory. This species feeds on five other hosts, none of which were growing in the immediate vicinity of the sweet gale. It remains flightless until reaching maturity in mid-summer so the instars effectively specialise exclusively on sweet gale. Feeding by *L. spinolai* induces leaf gland production and kaempferol-3-(2,3-diacetoxy-4-*p*-coumaroyl)rhamnoside (peak 44) production. The induced compounds might be assumed, therefore, to have a role in deterring further capsid herbivory.

Major differences exist between the induction of peak 44 and the induction of leaf glands in sweet gale. Peak 44 induction could be described as qualitative since other flavonoid glycosides did not respond to herbivory. Furthermore, herbivory only acts to advance peak 44 production since sweet gale produces it regardless of herbivory once the first leaves reach maturity. Induction of leaf glands can be described as quantitative as the glands contain a complex mixture of terpenes and flavonoid aglycones none of which exhibited individual changes in response to herbivory. The increase in gland density was restricted to the specific sites of herbivory and there was no increase in gland density on undamaged leaves, comparable to peak 44, at any time. The differences between peak 44 induction and gland induction may indicate separate roles for these phenomena.

However, there is no evidence that either peak 44 or the volatile oil provide any defense against capsid bugs. Peak 44 was not shown to have any antifeedant properties, although the feeding tests (Chapter 7) were not carried out on capsid bugs. Harborne

(1989) points out that flavonoids can have deleterious effects on some insect species yet not effect others. However, the ability to metabolise flavonoids is usually a sign of adaptation to the host (Tahvanainen *et al.* 1985b) and the peak 44 tests were carried out on generalist feeders which would have been extremely unlikely to have encountered peak 44 previously.

High densities of volatile oil glands are present on the young leaves prior to herbivory yet clearly do not deter capsid bugs from feeding. The oil undergoes no qualitative change in response to herbivory and the quantitative response is restricted to leaves which have sustained herbivory. This induction cannot, therefore, defend adjacent undamaged leaves which are in the greatest danger from the flightless capsid bugs early in the summer. In the light of these results other possible roles for sweet gale's induced responses must be considered.

8.4 A role of induction in sweet gale.

The question as to what benefit accrues to the plant through induction was addressed, in some respects, by the establishment of a program of antifungal tests. This part of the study demonstrated that both peak 44 and the volatile oil inhibit the growth of all fungi isolated from the study plants. It seems, therefore, that herbivore induced chemical changes in sweet gale defend the damaged leaves against fungal invasion.

Leaf wounds are likely sites of fungal invasion (Dickinson & Lucas 1977) so wound induced antifungal compounds would be of benefit to the plant. This benefit, however, is rather greater than might immediately seem apparent since plant pathogens do not elicit

immediate phytoalexin responses (Kuc 1976). Pathogens are able to suppress induced responses by secreting compounds which inhibit the plant enzymes capable of degrading fungal cell walls (Albersheim & Valent 1974). Since many elicitors of phytoalexin induction are released during fungal cell wall degradation (Albersheim & Valent 1978, Keen & Legrand 1980, Darvill & Albersheim 1984) inhibition of this process immediately after infection results in a delayed response. Herbivores, however, have not been demonstrated to inhibit an induced response.

Herbivore induced antifungal compounds in sweet gale leaves render the ability of pathogens to suppress phytoalexin responses redundant at wound sites where the probability of infection is greatest. This is an extremely interesting conclusion since it clearly implies that sweet gale has evolved herbivore induced responses as prophylactics. It follows that the plants least well defended and, therefore, most at risk from infection by pathogenic fungi are those which have not sustained herbivory. On Flanders Moss the only plants to exhibit symptoms of disease before autumn were those kept free of herbivores (Chapter 2).

A similar phenomenon, in which mite damage induced changes in cotton inhibited the vascular wilt fungus (*Verticillium dahliae*), has previously been observed (Karban *et al.* 1987). However, in cotton inoculation with the pathogen also reduced the plant's palatability to the mites. It would appear in this case that the plant had evolved an induced defense against both herbivores and pathogens.

8.5 What triggers the induction of secondary metabolites?

The simplest possible trigger for the responses to herbivory observed is simple wounding. Edwards and Wratten (1982) have found that wounding deters further herbivory. By demonstrating increased transcription of PAL, CHS and HRGP genes in response to wounding Lawton and Lamb (1987) provided a genetic basis for wound induced phenolic accumulation. One probable effect of physical wounding is the production of ethylene, a plant stress hormone. Ethylene has been demonstrated to stimulate production of mRNA for PAL, CCL and CHS (Ecker & Davis 1987). The results of the present study (Chapter 5) suggest that extensive leaf wounding in sweet gale rapidly induces increases in all phenolics but that these increases are not persistent.

Induction of all phenolics in sweet gale is consistent with the stimulation of PAL, CCL and CHS. However, herbivory did not induce general increases in leaf phenolics. Similarly, Hartley (1987) found that wounding did not elicit the same response as herbivory. Furthermore, it has been shown that inoculation of plant tissue with fungal mycelium has a far greater induction effect on PAL, CCL and CHS mRNA activity than does simple wounding (Grisebach 1985). It is probable, therefore, that there is something other than physical wounding, associated with herbivory, which caused induction of secondary metabolites in sweet gale.

Herbivore saliva is a possible trigger. Capsid bugs may inject some enzyme solutions into the leaf when feeding and this would be very likely to elicit such a response. However, there has been little work on what triggers herbivore induced responses.

Work on phytoalexin elicitors, however, is extensive (Friend 1985). In addition to fungal cell walls (see above) plant cell walls also release phytoalexin elicitors (Hahn *et al.* 1981, Darvill & Albersheim 1984) These endogenous elicitors are released on plant cell death and possibly account for the delayed phytoalexin response observed after pathogen invasion. The damage associated with capsid herbivory , in which the insect inserts its proboscis into the leaf and sucks out mesophyll tissue (Chapter 2), may well be accompanied by the injection of saliva and partially digestive plant cells and would release endogenous phytoalexin elicitors into the leaf.

Plant cell wall fragments have been demonstrated to elicit induction of proteinase inhibitors (Walker-Simmons & Ryan 1986), which can reduce the palatability of plants to herbivores (Broadway *et al.* 1986). Plants can, therefore, respond to herbivory by producing defenses specifically aimed at herbivores but there is no evidence of this in sweet gale.

It is possible that the response observed following herbivory in sweet gale is, in part, a response to non-pathogenic micro-organisms which either are carried by the herbivore or enter the wound produced by the herbivore. There is no easy way of eliminating this possibility since an experiment to investigate it would require insects with sterile mouthparts in sterile conditions. However, no herbivores, or wounds, are likely to be aseptic in the field so either mechanism will benefit the plant.

Darvill and Albersheim (1984) have demonstrated that fungal cell wall and endogenous elicitors can interact synergistically to produce a greater response than either would independently. They suggest that this may account for the difference between purely

physical damage and fungal invasion. If this is true in all plants it becomes difficult to explain the response of sweet gale to herbivory in terms of endogenous elicitors since artificial damage would be expected to have the same effect. It may be that herbivory releases different endogenous elicitors to physical damage. Miller *et al.* (1986) observed the same response, elevation of monoterpenes in lodgepole pine (*Pinus contorta*), to inoculation with four different stimuli: bark beetles, blue stain fungus, a pectic fraction of tomato leaves and a fungal cell wall fraction. They suggest that a common recognition-defense system may exist. This is clearly a field which require further research.

8.6 Non-defensive roles for sweet gale secondary metabolites.

Sweet gale is adapted to nitrogen deficient environments. However, it is not a typical stress tolerant species but exhibits competitive characteristics. To achieve this competitive advantage sweet gale does not purely rely directly upon its association with *Frankia* sp. Schwintzer (1983) calculated that 43% of the annual nitrogen requirement comes directly from nitrogen fixation. It was argued in Chapter 2 that much of the residual nitrogen requirement must be derived from the soil. Since the soil is inherently poor in all nutrients the source of this nitrogen must be decaying litter. Sprent *et al.* (1978) estimated that the leaf litter alone contains 3g N m^{-2}. By shedding leaves rich in nitrogen sweet gale effectively transforms the immediate area from a nutrient poor habitat to a moderately productive one. However, there are a number of problems associated with nitrogen rich litter.

Firstly, since *Frankia* sp. has a high oxygen requirement sweet gale grows in moving water (Chapter 2). Under these conditions most of the nitrogen rapidly liberated from litter by decay would quickly be leached from the immediate environment of the plant. Furthermore, *Frankia* does not fix nitrogen efficiently when there is a high nitrogen concentration in the environment of the roots so a low nitrogen concentration would be ideal. These problems do not arise as the litter is broken down very slowly (Richard *et al.* 1982) so the nitrogen release is gradual and sustained. These conditions allow sweet gale to absorb nitrogen from the soil as it is released without suppressing nitrogen fixation.

Richard *et al.* (1982) suggest that cuticle thickness may account for the slow rate at which litter decays by reducing the rate at which macro-invertebrates can utilise the litter.. However, Allard and Moreau (1986) argue that macro-invertebrates are unlikely to be important in litter decomposition. They further state that a near optimal carbon:nitrogen ratio for microbial decomposition, as given by Alexander (1961), exists in sweet gale litter. They suggest that the acidity of the soil water may account for the slow rate of decomposition. This acidity is largely due to the phenolic acids in the litter. The secondary metabolites, however, almost certainly have a more direct effect on litter decomposition since both the volatile oil and peak 44 are present in leaves as constitutive secondary metabolites when the leaves are shed. These compounds have demonstrated to inhibit the growth of saprophytes such as *Trichoderma harzianum* and *Penicillium* spp. Thus, these compounds are very likely to reduce the rate at which the litter decays.

It could be argued that the mid-season input of leaf litter as a direct consequence of herbivory (see Chapter 2), when temperatures are ideal for fungal growth, might lead to an uncontrolled flush of nitrogen later in the season when it would not be readily taken up by the plant and, therefore, probably lost in the ensuing winter rains. By increasing the levels of antifungal compounds in these leaves prior to shedding them the plant may delay the release of nitrogen until the following year. Temperatures are lower, and falling rapidly, at the end of the season when leaves unaffected by herbivory are shed, so very little fungal activity would be expected until the next growing season when the constitutive secondary metabolites within the litter regulates decay. Thus, the induction of volatile oil may have more than one role. This argument is, at best, conjectural and it can only be applied to the increase in leaf volatile oil gland density as the concentration of peak 44 is similar in all leaves before any are lost.

By creating moderately fertile conditions in the soil sweet gale creates a second problem since opportunistic non-stress tolerant species could, theoretically, become established. The fact that sweet gale is not found away from the specific conditions described in Chapter 2 suggests that it cannot compete very well in other moderately productive sites. Once a sweet gale stand has been in existence for a few years, therefore, it might be expected that other, more vigorous species, would establish and take advantage of the conditions to overgrow the sweet gale. A study of the site, however, indicates that this does not happen. Only birch (*Betula pubescens* and *B. pendula*) grow amongst the sweet gale on Flanders Moss. A possible reason for this apparent lack of succession is

that some volatile oils can inhibit the growth of competitor seedlings (Heisley & Delwiche 1983). The chemistry of the sweet gale litter may, therefore, inhibit the growth of competitors.

8.7 Conclusion.

Herbivory has been demonstrated to induce two types of chemical change in the leaves of *M. gale* on Flanders Moss. Leaves exhibited a quantitative response to herbivory by increasing the density of surface volatile oil glands without changing the composition of the oil; and leaves exhibited a qualitative response by producing a single flavonol glycoside (kaempferol-3-[2,3-diacetoxy-4-*p*-coumaroyl]rhamnoside). Sweet gale may also produce 7-hydroxy-5-methoxy-6,8-methylflavanone in response to herbivory but this study did not demonstrate this conclusively.

The composition of sweet gale volatile oil has been studied frequently in the past. However, this study revealed that the oil of sweet gale on Flanders Moss differs, in many respects, from the oils of other populations, especially in regard to the relative importance of ß-elemenone and germacrone. It has been shown that the composition of the oil changes during the growing season and this may be related to the plants' changing defense requirements. Furthermore, there is some evidence that local pressures may presently be selecting for plants with the ability to produce germacrone as well as ß-elemenone.

In addition to the volatile oil the leaves were found to contain a variety of flavonoids. One compound, kaempferol-3-(2,3-diacetoxy-4-*p*-coumaroyl)rhamnoside, was novel and a recently developed NMR technique (heteronuclear multiple bond correlation)

proved to be extremely useful in the elucidation of the compound's structure. A second flavonoid, 7-hydroxy-5-methoxy-6,8-methyl flavanone, was isolated from sweet gale for the first time. Both of these flavonoids were isolated and identified because they were highlighted by the induction experiment. This represents a fairly unusual route to the isolation of new bioactive natural products (Waterman 1990).

Both the quantitative and qualitative induced secondary metabolites of sweet gale have antifungal activity. This indicates that the plant responds to herbivory by defending itself against the threat of pathogen invasion. The two classes of compound implicated could have different but complementary roles in this defense, the volatile oil reducing the chance of invasion at the leaf surface and the flavonoids providing a barrier to fungal growth within the leaf. These compounds may also function by deterring further herbivory but there is no evidence to support this hypothesis.

In addition to induced defenses, sweet gale invests heavily in constitutive compounds, especially terpenoids early in the growing season. The terpenoids may provide protection against vertebrate herbivores. A few weeks after budbreak, when insect herbivores become active, leaves increase their investment in flavonoids, especially myricetin-3-galactoside, possibly to deter insect herbivory.

The chemical ecology of sweet gale cannot be dissociated from the nitrogen economy of the plant. Through its association with *Frankia* sp. sweet gale produces nitrogen rich tissues and nitrogen rich litter. This requires a high investment in chemical defenses to protect the plant and, possibly, to control litter decomposition.

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Appendix A.

Monique Simmonds P.Waterman 10/88

Compound PK 44

Antifeedant activity. Choice test

Glass fibre discs (GFD Whatman GF/A 2.1 cm diameter) were used as the test substrate. The discs were made palatable by the addition of 100ul sucrose solution (0.1M). A 100ul aliguot of a solution containing the test compound was applid to the treated disc (T) and a 100ul aliquot of the solvent applied to the control disc (C). When dried, the weighed discs were presented as a pair (CvT) to individual larvae in a Petri dish for up to 8h so that never more than 50% of any disc was eaten. The discs were reweighed and the Antifeedant Index calculated. The Index identifies both phagostimulants (-ve values) and antifeedants (+ve values). Positive values greater than 75% indicate very potent antifeedants.
No-choice bioassay:- compound applied to GFD in combination with sucrose. Experiment terminated when larvae exposed to the control disc had eaten 50%

Insect:- Spodoptera littoralis
n = 20

		Antifeedant Index mean(sem)	No Choice % eaten
Conc.	1000 ppm	-25.8(19.44)	62.1
	100 ppm	-31.3(15.65)*	44.0

The results of the choice bioassay indicate the compound is a phagostimulant, the effect is significant at the lower concentration tested. However, in the no-choice assay larvae eat more of the disc treated with the higher concentration.

Developmental effects:-

The compound did not increase mortality or influence the development of Spodoptera littoralis or Heliothis virescens when applied topically (3µg/larva) to larvae, or cannulated (2µg/larva) into the gut.

Appendix B.

The method of Morin *et al.* (1986) for HPLC semi-preparative separation of terpenoids.

Instrumentation: Gilson 303 binary HPLC with Shimadzu UV detector set at 220 nm. Column: Sperisorb ODS 2 (5) (25cm x 0.9cm).

Sample preparation: Each sample of oil was dissolved in a 1:1 mixture of the eluent (acetonitrile/water, 7:1) and tetrahydrofuran (50mg oil/100ml solvent). Injection volume: 0.8ml.

Conditions: flow rate: 3 ml min^{-1}.
Solvet A: acetonitrile, solvent B: water.
Time 0 min: 70% A, 30% B; time 20 min: 95% B; time 25 min: 70% A, 30% B.

Sample recovery: terpenoids recovered from fractions by liquid-liquid extraction into diethyl ether/ pentane (1:1), dried over anhydrous sodium sulphate and the solvent was removed by rotary evaporation at 30°C.