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The results and conclusions in this report are based on an investigation conducted over three years. The conditions under which the experiment was carried out and the results obtained have been reported with detail and accuracy. However because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results especially if they are used as the basis for commercial product recommendations.

Contents

Grower summary

Headline

Liverwort infestation in container grown hardy nursery stock can be reduced by manipulating irrigation and light modification.

Background and expected deliverables

Liverworts (*Marchantia polymorpha*) growing on the surface of container plant compost are a major problem to the horticultural industry, affecting both protected and hardy nursery stock. According to HDC study HNS 93 the removal of liverwort, moss and weeds is estimated to cost the UK horticultural industry 4% of total production costs, equating to £13 million (mainly labour costs). Zero tolerance for liverworts in accreditation schemes and withdrawal of chemical approvals places pressure on the industry to find alternative, cost-effective control measures.

The expected deliverables from this project are:

- To improve the understanding of the biology and epidemiology of liverwort infestations, enabling research to be targeted towards areas of weakness in the liverwort life cycle.

- Identify key stages in liverwort life cycles where control interventions, especially non-synthetic pesticides, could be most effective.

- Identify the liverwort species infesting nurseries.

- Investigate non-chemical means of liverwort control including fungal pathogens, glucosinolate (GSL) hydrolysis products obtained from seed material and cultural methods.

- Carry out trials based on the results of the investigations considering the use of individual and integrated methods of control.

Summary of the project and main conclusions

Environment - the effect of light and temperature on liverwort growth

Experiments with varying light levels indicated that maximum growth of liverworts occurs in mid range light levels (300 to 400 µ mol m^{-2} s^{-1}), with growth less rapid at either very low or very high light levels.

- The use of shading to reduce light levels may not be appropriate for a vast range of species. However, shading at pot level with mulches or pot covers is a commercial option.

Temperature experiments demonstrated that the rate of growth of liverworts increases with increasing temperatures. However, when subjected to very high temperatures and high light levels, growth ceased and such conditions were found to injure the liverworts.

Lunularic acid (LNA) is a plant growth regulator produced by liverworts. High levels of LNA are known to reduce liverwort growth. Production of LNA has been found to increase in high temperatures and during high light levels, such as those experienced in May and June. Liverwort growth and spread is therefore likely to decline at this time of year.

- This has relevance to growers and indicates that it is prudent to remove liverworts during the summer period when re-growth is likely to be slow.

Epidemiology – liverwort dispersal

Liverworts can reproduce asexually by vegetative propagules (gemmae) produced by gemma cups (circular structures found on the upper surface of the liverwort). The gemmae are released when water splashes into the cup, transporting them away from the parent plant. Liverwort gemmae were dispersed up to 1.6 m during this study, with some gemmae travelling further than the majority, demonstrating liverworts' ability to invade and colonise new areas. It was also found that the more often gemmae are dispersed from gemma cups, the greater the number that are produced to replace them, suggesting the number of gemmae produced could be minimised with fewer overhead irrigation cycles.

Liverwort dispersal was compared when irrigated by nozzle (MP Rotator model 1000 and Dan modular 180^0 spreader), drip and capillary matting systems, with 2-daily or twice-daily irrigation cycles.

- The capillary matting treatment required markedly less water and the area of liverwort present in the capillary matting treatments was less than other treatments.

- There were more pots with established liverwort in the MP Rotator nozzle than the Dan nozzle treatment.

- More liverwort gemmae were dispersed and became established with more frequent irrigation cycles (twice daily).

Glucosinolates

Glucosinolates (GSLs) and their breakdown products, including isothiocyanates (ITCs), are responsible for the distinctive pungent smell and hot taste of cabbages and other green vegetables. The herbicidal effect of selected ITCs on liverwort gemmae was investigated. The results indicated that all ITCs tested had an effect. However, this work is at an early stage and requires further development.

Financial benefits

The cost to the UK horticulture industry of liverwort, moss and weed removal from pots is estimated to be £13 million (mainly labour costs) (HNS 93). Reduction of overhead irrigation provides growers with water and nutrient cost savings. Reduced liverwort infestation and the subsequent reduced need for its removal before crop marketing result in both labour and media (top dressing) savings.

The use of shading at pot level or pot covers to exclude light from the surface of pots is a commercial option.

Action points for growers

Every liverwort present in the nursery should be considered a source of new infestation. The following nursery hygiene procedures would help to reduce liverwort spread:

- Ensure non-cropping and standing down areas are kept free of liverwort.
- Keep growing media covered to prevent contamination.
- Ensure any second hand pots are cleaned before use.

In addition, growers should revise their current irrigation practices:

- Consider using sub or drip irrigation systems, particularly when renewing or upgrading existing systems.
- If using overhead systems, consolidate irrigation cycles from many short applications to fewer longer applications.
- Ensure the surface of the compost dries out between water applications.
- Group plants with similar irrigation requirements to prevent over-watering of plant species that prefer drier conditions.

Other forms of management should also be considered:

- Manipulate shade and light levels to provide environmental conditions unfavourable to maximum liverwort growth. This should be tailored to crop plant requirements, grouping sun loving plants in areas of high sunlight and shade loving plants in areas of low light. Whole plant shading can be provided, but a more practical method is to reduce light at the pot level using pot covers.
- Remove liverwort during long day, high temperature conditions as liverwort re-establishment would be delayed.

Science section

Introduction

Liverworts growing on the surface of container plant compost are a major problem to the horticultural industry, affecting both protected and hardy nursery stock, and are most prevalent in the warm, moist conditions provided in propagation systems, where plants are at their most vulnerable.

According to Horticultural Development Council (HDC) study HNS 93, the removal of liverwort, moss and weeds from pots is estimated to cost the horticultural industry 4% of total production costs, equating to £13 million annually (mainly labour costs). These figures combined with zero tolerance for liverworts in accreditation schemes and government withdrawal of chemical approvals is putting huge pressure on the industry. Chemicals currently in use have a short-lived effect and multiple applications are necessary (Scott and Hutchinson, 2001).

The overall aim of this project was to confirm the identity of the liverwort species investing nurseries, and then to provide information on liverwort biology and epidemiology of infestation currently lacking, enabling future research to be targeted towards areas of weakness in the liverwort life cycle and biology. There was an investigation of non-chemical means of controlling liverwort: fungal pathogens, GSL hydrolysis products obtained from seed material and cultural methods. Glasshouse trials based on the results of these investigations considered the use of individual and integrated methods of control.

Summary of laboratory and growth cabinet results in years 1 and 2

Identification of liverwort species infesting nurseries

HDC members and ADAS consultants were invited to advise of any liverworts growing as a weed in nursery containers that appeared to be different to the commonly reported *Marchantia polymorpha*. No instances were reported in nursery plant containers.

Liverwort culture

Separate male and female liverwort cultures were established on phytagel media in sterile conditions and maintained in growth rooms, and also on compost media, and maintained under glass for use in experiments.

Environment

The effect of light level and temperature on the growth and development of liverwort

During years 1 and 2 growth cabinet experiments compared the effect of light level and temperature on the growth (radial expansion), dry weight accumulation and development of male and female liverwort gemmae over 6 weeks. The light levels used were 800 µ mol m^{-2} s^{-1} and 400 µ mol m^{-2} s^{-1} for both experiments and were achieved by manipulating the shelf height within the cabinets. Growth cabinet temperatures were set at 25 C and 15 C in year 1, and 15 C and 10 C in year 2, to provide a range of results. Equal numbers of male and female gemmae were arranged in separate blocks, growing in individual 10 cm clear plastic lidded pots on phytagel media. Relative humidity was 65% and a photoperiod of 12 hrs was applied. The experiment was a split-split plot design with all treatments other than light levels randomly allocated to cabinets.

Radial expansion was used as a measure of growth as the majority of liverwort growth is two dimensional. For each year results indicated statistically significant effects of both light and temperature on liverwort growth. The conclusions of these experiments were that growth (radial expansion), fresh weight and dry weight were greater when grown under low light than high and greater at high temperatures than low.

Liverwort development was also monitored, with the presence and number of gemma cups on each gemma thallus used as a measurement. In year 1, development was faster at 25 C, where the first gemma cup was produced in week 2 for both light levels. More gemma cups were produced in the low light treatments than high light. However, during week six there was a large increase in the number of gemma cups present in the 15 C treatment, where more were produced overall. In year 2, more gemma cups were produced at 15 C than 10 C and more in high light than low after 4 and 6 weeks, this can be attributed to the small size of the liverwort gemmalings (gemmalings are young liverworts developing from gemmae).

A number of observations were made concerning the morphology and colour of the liverwort gemmalings throughout the experiments. By week 5 those grown in high light at 25 C were a dull green colour; with a number of replicates developing dark brown colouration and reduced relative growth rate. The thallus had a dome-shaped

appearance, rather than growing flat over the surface of the media. When grown under 15 C, low light conditions thalli were flatter and a lighter green colour. These replicates were smaller than those grown at 25 C, high light, but may have had the potential to reach similar levels of growth given a longer growth period. In year 2, where there was no 25 C treatment, none of the replicates developed the dark brown colouration; however some replicates grown at 15 C under high light levels did become domed shape, with little radial growth. Liverworts grown under low light levels were, again, flatter and brighter green in colour.

This work indicates that liverworts grown in high temperature and light conditions suffer damage that causes them to degenerate more quickly than when grown in lower light and temperature conditions.

These results suggest that liverwort would be more prevalent in areas protected from full sunlight and high temperature, and during cooler weather. Shading of propagation areas, along with their high humidity levels, for example, would promote liverwort growth. The implications are that a reduction in shading and humidity could reduce liverwort infestations.

An experiment during year three compared the growth and establishment of liverworts when provided with different levels of shade, both outside and within a polytunnel to investigate these conclusions in nursery conditions.

Lunularic acid

Lunularic acid (LNA) is thought to be an endogenous plant growth regulator involved in growth inhibition, arresting germination of gemmae within gemma cups and aiding drought resistance. It is a natural dihydrostilbene carboxylic acid derivative (Pryce, 1972) growth inhibitor identified as 3, 4'-dihydroxybibenzyl-2-carboxylic acid.

LNA is a leachable inhibitory factor that accumulates within liverworts, becoming effective above a critical level; with increased quantities of the inhibitor found in liverwort species grown in continuous light, and high light intensities (Gorham, 1975). Inhibition is reversible with growth recommencing within 3-4 days of reinstatement of short day conditions with lower temperatures (Valio, 1969; Valio et al., 1969). The presence of lunularic acid in *Marchantia polymorpha* L. was

established by Pryce (1971) and has been detected in all parts, including the rhizoids, with the highest concentrations found at the thallus apex (Gorham, 1977).

LNA has been reported to inhibit growth in higher plants: water cress (*Nasturtium officinale*), timothy grass (*Pleum pratense*) (Nakayama et al., 1996), lettuce hypocotyls (at concentrations normally toxic to *Lunularia* gemmae), and rice coleoptiles. LNA also inhibits rice seed (Hashimoto et al., 1988), *Lactuca sativa* and *Lepidium sativum* germination (Yoshikawa et al., 2002). Wheat and oat coleoptiles sections showed no growth inhibition or promotion due to the inhibitor (Arai et al., 1973; Valio and Schwabe, 1970). No reports have been found relating to any effect of lunularic acid on woody plants.

Several groups have successfully synthesised LNA. However, it is not available commercially and initially proved unobtainable, therefore it was initially necessary to extract and purify a sample from liverwort tissue to use as a standard during HPLC analysis. A sample was subsequently provided by Professor Asakawa of Tokushima Bunri University, Japan.

HPLC analysis techniques were developed to identify optimum procedures for detecting LNA. Fluorescence and UV detection were investigated; the structure of lunularic acid, incorporating phenyl, carboxyl and hydroxide groups suggested that fluorescence would be most appropriate, and as most molecules do not exhibit fluorescence this can be an extremely sensitive tool. Fluorescence excitation and emission curves were produced which identified the optimum detector settings as 300 nm (excitation) and 405 nm (emission), at which LNA produced the maximum peak size.

It proved difficult to find a suitable compound to use as an internal standard, therefore a simplified extraction and detection process was developed, minimising the opportunity for losses of LNA and removing the absolute need for an internal standard. Liverwort tissue was simply crushed with a pestle and mortar and the LNA extracted with an acetonitrile solution. Recovery rates were calculated using serial extractions of LNA from liverwort tissue.

Methods continued to be developed to extract lunularic acid from liverwort tissue with minimum losses, in preparation for HPLC analysis to extract and quantify the

amount of LNA present in liverwort tissues grown under nursery conditions, in different photoperiods and at different lifecycle stages during year 3.

Epidemiology

Liverwort gemma dispersal

Liverworts can reproduce asexually by vegetative propagules (gemmae) produced by gemma cups (circular structures found on the upper surface of the liverwort thallus), and are dispersed when water splashes into the cup, transporting them away from the parent plant. The gemma cups of *Marchantia polymorpha* have sides at an angle of 60-70^0 to the horizontal and together with lentil-shaped gemmae they form an efficient splash-cup mechanism. Water droplets falling into the gemma cup displace water, thrusting gemmae upwards along the cup sides. Dispersal distances of up to two feet by small raindrops have previously been observed (Brodie, 1951).

Dispersal of asexual liverwort gemmae has not previously been characterised within a plant nursery setting, therefore it is unknown how irrigation systems, particularly the overhead sprinkler systems commonly used affect dispersal. Characterisation of the effect of overhead irrigation on gemma dispersal will provide an insight into the effective design and use of irrigation regimes as part of an integrated pest management system.

An experiment during year 1 investigated the dispersal distance of liverwort gemmae by a simple glasshouse overhead sprinkler system, using three sprinkler nozzle sizes and four water pressures (1.5, 2, 2.5 and 3 bar) at two nozzle heights (1 and 2 metres). The nozzles used were Agridor 700 Dynamic Sprayers manufactured by Ein Dor. A half tray with approximately 1/3 covered with established liverwort thallus bearing gemma cups was placed on the ground beneath the nozzle as a gemma source. Collection pots were arranged on the glasshouse floor, water applied to the liverwort via the sprinkler system for 15 mins, and the number of gemmae that had fallen into each collection pot counted. This was repeated three times for each combination of nozzle, water pressure and nozzle height. A measure of relative droplet size for each nozzle was determined using water sensitive paper.

During a preliminary test using the blue nozzle and red dye as a source in place of plant material, with water pressures of 2, 3 and 4 bar, red splash droplets travelled a maximum of 45 cm. However, when using liverworts as a source, gemmae were recorded at a maximum distance of 160 cm, suggesting that whilst some gemmae that landed close to the source may be transported within splash droplets, others were propelled out of the gemma cups and travelled further in air.

Regression analysis was used to produce a dispersal gradient for each treatment, showing the relationship between treatments and dispersal distances obtained. Using a log-linear model, regression curves fitted to gemma dispersal data were highly significant, with F-probability <0.001 for all treatments. Regression coefficients indicated steeper dispersal gradients at 2 and 2.5 bar than 3 and 1.5 bar; steeper dispersal gradients suggest more gemmae were deposited nearer to the source, travelling shorter distances.

Gemmae tended to travel further at the extreme water pressures (1.5 and 3 bar). Using the brown (160 lh^{-1}) and blue (105 lh^{-1}) nozzles the maximum distance travelled by gemmae was greater with the nozzle at 1 m, although the bulk of the gemmae travelled further when the nozzle was at 2 m. Using the grey (60 lh^{-1}) nozzle gemmae travelled further with the nozzle at 2 m. Maximum dispersal distances obtained were 160 cm, far exceeding the distances of 60 cm and 121.4 cm were previously recorded by Brodie (1951) and Equihua (1987) respectively.

Fewer gemmae were dispersed at the two extreme water pressures (1.5 and 3 bar) when the nozzle was set at the lower height; at the 2m nozzle height the two exceptions to this were for the brown nozzle, 3 bar treatment and the grey nozzle, 3 bar treatment. The brown nozzle (160 lh^{-1}) did not operate at 1.5 bar, therefore no results were obtained. The brown nozzle (160 lh^{-1}) was generally the least effective at dispersing gemmae; the main exception occurring at 2 bar with the nozzle at the 2 metre height. The total number of gemmae dispersed was greater at the 2m nozzle height at all water pressures when using the brown nozzle; this was not the case in all treatments using the blue and grey nozzles.

When using the brown nozzle, more gemmae were dispersed with increased nozzle heights, however, there was no clear effect of nozzle height on the number of gemmae dispersed for the grey and blue nozzles as the results were more variable. Distances travelled by the gemmae generally increased with increased nozzle

height. For the blue and brown nozzles some gemmae travelled further when the nozzle was at the lower position, although the bulk of the gemmae did travel further when nozzles were higher. Steeper dispersal gradients were obtained at 2 and 2.5 bar than 3 and 1.5 bar; the exception was the 3 bar, 2m height treatment using the grey nozzle. However, for the blue and brown nozzles although the bulk of the gemmae travelled further when nozzles were higher, a number did further when the nozzle was at the lower position.

More gemmae were generally dispersed using 2 and 2.5 bar water pressure, and these were dispersed closer to the source, suggesting there may be an optimum combination of water pressure and nozzle for dispersing maximum gemmae numbers. This would appear to coincide with the manufacturers recommendation that 2 bar is the optimum operating pressure for this particular nozzle range, as above 2.5 bar and below 1.5 bar water distribution becomes uneven, and damage may be caused to the emitters, so they fall outside the terms of the guarantee (Anon, Undated).

A measure of relative droplet size for each nozzle was obtained using water sensitive paper. The droplet pattern produced was photographed digitally and analysed using Image J software. Distribution charts constructed using this data described the range of droplets sizes produced during each treatment. The number of gemmae dispersed appears to be related to water drop size, not number of drops. Mean drop sizes ranged between 161 to 271 µm diameter; maximum number of gemmae were dispersed by water drops 63 to 236 µm mode diameter (172 to 271 µm mean).

There were exceptions to each trend due to the complex interactions between water pressure, nozzle and nozzle height, all of which impacted on drop size and the number of drops produced; the number of gemmae dispersed appears to be related to water drop size, not number of drops.

These results show that *Marchantia polymorpha* can spread large distances, up to 1.6 m during this experiment, using these vegetative propagules. In each treatment a number of gemmae travelled further than the majority, clearly demonstrating the ability of liverwort to invade and colonise new areas in nursery situations.

Gemma cup replenishment

The gemma dispersal experiment provided information on how gemmae are dispersed by water droplets, but no information was available to indicate how gemma cups are replenished, and whether replenishment was a response to gemma dispersal or removal from the cup. If so, the rate of gemmae replenishment could alter as a consequence of continual emptying, and the time interval between water applications could affect the amount of gemmae available for dispersal. A glasshouse experiment was designed whereby all the gemmae were removed from pre-identified gemma cups and counted. Three treatments were then applied whereby gemmae were removed from cups and counted either every 3 days, weekly or after 4 weeks.

The number of gemmae collected during the initial removal of gemmae was fairly constant across all treatments. For subsequent collections, however, markedly more gemmae were collected during the 3-day treatment than either the weekly or 4-weekly treatments although for the 3-day and weekly treatments the average number of gemmae collected each week declined overall during the course of the experiment. These results were achieved even though a number of the designated gemma cups were degenerating throughout the experiment. This was particularly evident for the 3-day treatment where only 30% of the designated gemma cups were still active, compared to 36% for the weekly treatment and 60% for the 4-weekly treatment, for the final data collection.

The results suggest that when gemmae are removed, more are produced to replenish them; the number of gemmae produced could be minimised by reducing or eliminating overhead irrigation events.

Clumping of gemmae

During both growth and development and gemma dispersal experiments it was observed that gemmae tend to clump together. Why and how they achieve this is largely unknown. Mature gemmae are attached to the parent liverwort by a single-celled stalk. Mucilaginous glands that grow up from the base of the cup among the gemmae (Cavers, 1903) are thought to produce muciliage, imbibe water and swell, breaking the gemmae from their stalks and forcing them out of the cup (Round, 1969). The mucilage may be a mixture of water soluble carbohydrates that holds the gemmae together in clumps (Equihua, 1987). It has been suggested that this is a

lifecycle strategy to ensure gemmae remain close to the parent plant after dispersal, keeping them in an environment known to be safe until conditions are adequate to produce sexual spores that would facilitate long distance dispersal (Equihua, 2005).

An experiment designed to test whether clumping of gemmae is an aid to establishment and subsequent growth of liverwort thalli compared the weight of liverworts established on compost from clumps and freely dispersed groups of three different weight classes of gemmae after four weeks growth. ‘Clumps’ were numbers of gemmae naturally found in tightly masses of gemmae. ‘Groups’ were clumps that were first separated in water and then dispersed in a group. The number of gemmae contained in each clump and group was estimated.

Naturally formed clumps (C) and groups (G) of separated gemmae categorised into three size classes were placed on damp compost in pots arranged in a completely randomised design on a glasshouse bench. Fresh and dry weights were recorded after four weeks, and liverwort establishment was recorded.

Both fresh and dry weights were greater for groups of individual gemmae than clumps in all three size classes. Dispersed groups of gemmae also established more successfully, with 100 % establishment in the small and medium size classes.

The hypothesis for this experiment was that gemmae clump together as an aid to establishment and growth. However, the results obtained under the conditions of this experiment suggest that dispersed groups of gemmae establish better than clumps. The clumps of gemmae used in this experiment were encouraged to form by watering the liverworts from below only, so that gemmae were not dispersed. However, the clumps observed in previous experiments occurred naturally, even with overhead watering, so there may be other factors that influence their formation and that may affect growth and establishment.

Fungal antagonists

Fungal species identified as potential parasites on liverworts were sourced from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands (*Bryoscyphus atromarginata* and six strains of *Phaeodothis winteri*). Four fungal species were isolated from dying liverworts provided by John Atwood (ADAS) and identified by CABI Bioscience, Egham as *Fusarium equiseti, Penicillium velutinum* and

Trichoderma harzianum (2 samples). Specimens of each fungal species were established in culture on either Potato Dextrose Agar (PDA) or Malt Extract Agar (MEA) media for use in experiments. Pathogenicity tests were developed to identify fungal species capable of having a detrimental effect on liverworts. Liverwort thalli were inoculated initially with spore and mycelium suspensions and in later tests with agar plugs bearing fungal growth which proved more successful and easier to apply.

Under the premise of Koch's postulates attempts were made to reisolate fungal strains that infected the liverwort and this was successful with *P. velutinum, Fusarium equiseti, Bryoscyphus atromarginatus*, and *Phaeodothis winteri* CBS 162.31 and 102466.

It was decided not to continue with pathogenicity testing using fungal species that were slow to grow and had little effect on the liverwort, therefore *P. winteri* CBS 182.58, *P. winteri* CBS 429.96, *Penicillium velutinum* and *Trichoderma harzianum* were discarded. *Trichoderma harzianum* did not appear to infect the liverwort; spore inoculations did germinate and began to invade the tissue, but then stopped. Although *P. velutinum* did attack liverwort tissue and cause some collapse, the liverwort was not overcome and continued to develop new growth. *Bryoscyphus atromarginatus* was slow to establish, and had limited effect on liverwort. However, as it has been reported to attack *Marchantia polymorpha* specifically efforts were made to reinvigorate it by passaging it through liverworts.

Fusarium equiseti was the most successful of the species tested, causing severe collapse of liverwort tissue although some samples did exhibit signs of new growth at the edges. Round orange sporodochia developed on the surface of one liverwort which released many macroconidia when crushed. Liverworts inoculated with *P.winteri* CBS 551.63 also collapsed completely. Small, black structures were found growing on the surface of liverworts inoculated with this strain and when examined microscopically were found to be pycnidia, which when crushed released thousands of spores. The most promising of the strains tested were *Fusarium equiseti, Phaeodothis winteri* CBS 551.63 and *P.winteri* CBS 102466.

These pathogenicity tests enabled the selection of the most effective fungal antagonists against liverwort for use in a glasshouse experiment investigating their effect when applied as pre- and post-emergent treatments against liverwort growing on peat-based media.

Glucosinolates

GSLs and their hydrolysis products are responsible for the distinctive pungent smell and hot taste of cabbages and other green vegetables (Taiz and Zeiger, 1998), and are known to have fungicidal (Gamiel and Stapleton, 1993; Lewis and Papavis, 1971; Manici et al., 1997; Smolinska et al., 1997), phytotoxic, bacteriocidal, nematocidal, allelopathic and cancer protective properties (Fahey et al., 2001). Found in dicotyledonous angiosperms, GSLs are predominately produced by members of the order Capparales, particularly Brassicaceae. Some 500 plus non-cruciferous dicotyledonous species are reported to contain GSLs, including members of Limnanthaceae (Fahey et al., 2001; Matthiessen and Kirkegaard, 2006)

The delivery mode and distribution of GSLs in soil includes leaching from the roots of living plants (allelopathy) and diffusion from cover crops ploughed into the soil (biofumigation), each resulting in the breakdown of GSLs (Chew, 1988). Members of the Brassicaceae family are considered poor companion plants, and this is attributed to their allelopathic effects on other plants, whereby GSLs infuse into soil, leaching from roots of living plants or from whole plants ploughed into the soil. One aspect of allelopathy is biofumigation, the beneficial use of *Brassica* species to eliminate soil borne pests (e.g. weeds, fungi, microorganisms, insects, nematodes) through the release of the toxic GSL hydrolysis products, isothiocyanates (ITC's), into the soil (Matthiessen and Kirkegaard, 2006).

Glucosinolate chemistry

GSLs are non-toxic thioglucosides, having a common core comprised of a β-D-thioglucose group with a sulphonated oxime, and a variable side chain ('R' group) derived from an amino acid that largely determines the biological activities of the degradation products (Figure 1) (Brown and Morra, 1999).

GSL hydrolysis (

Figure 2) is catalysed by a myrosinase enzyme (thioglucoside glucohydrolase, EC 3.2.3.1) released following mechanical damage in the presence of water. The thioglucoside linkage is cleaved, yielding D-glucose and an unstable aglycone intermediate (thiohydroximate-*0*-sulphonate) which spontaneously rearranges producing sulphate and one of an array of degradation products. These are primarily

ITCs, thiocyanates, nitriles, or epithionitriles, depending on the 'R' group present on the GSL substrate, pH and availability of ferrous ions (Fe^{2+}). Nitriles and thiocyanates are produced at lower pH's; ITCs in alkaline conditions (Chew, 1988; Gil and MacLeod, 1980). Aliphatic and aromatic GSLs produce ITCs, the most bioactive of the hydrolysis products and more phytotoxic than the corresponding nitriles (Vaughn et al., 2006).

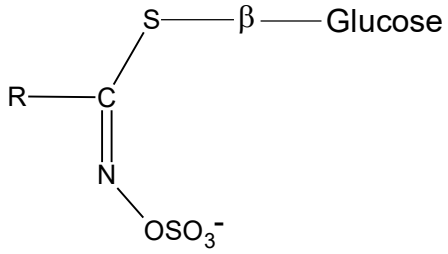


Figure 1. Structure of GSL. R represents the variable side chain. Adapted from Mithen (2001)

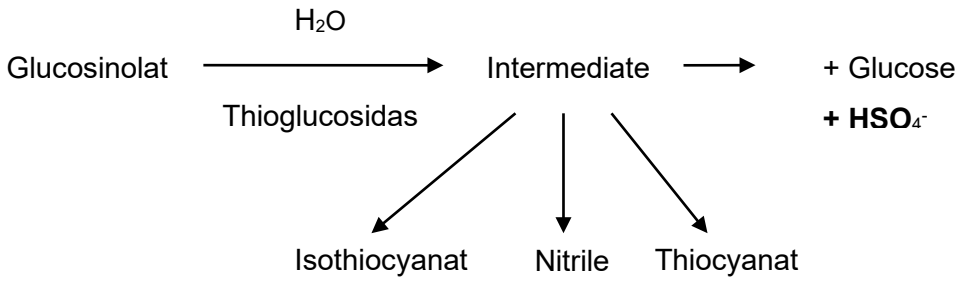


Figure 2. General structure of GSLs and their major hydrolysis products. Adapted from (Vaughn *et al.*, 1996).

There is evidence of the phytotoxic effect of soil-incorporated ITCs on the weeds Palmer amaranth (*Amarathus palmeri*), pitted morning glory (*Ipomoea lacunosa*) and yellow nutsedge (*Cyperus esculentus*) seedling emergence, with 3-methylthiopropyl and phenyl ITCs the most effective (Norsworthy and Meehan, 2005b). Bialy (1990) found ITCs inhibited wheat (*Triticum aestivum* 'Boja') seedling germination, particularly 2-phenethyl ITC and 2-propenyl ITC.

The seedmeal of *Limnanthes alba*, a member of limnanthaceae (

Table 1), contains the GSL glucolimnanthin (Bartelt and Mikolajczak, 1989; Miller et al., 1964), and is known to form primarily 3-methoxybenzyl ITC following hydrolysis (Bartelt and Mikolajczak, 1989). Vaughn (1996) compared the phytotoxicity of meadowfoam GSL hydrolysis products and found that 3-methoxybenzyl ITC (Limnanthin or 3-MBITC) had a phytotoxic effect against both velvetleaf (*Abutilon thiophrasti* Medici) and wheat (*Triticum aestivum* L. 'Cardinal') seedling radicle elongation (Vaughn and Berhow, 2005).

There are also reports of the toxicity of *L. alba* GSL products' to fall armyworm larvae and European corn borer (Bartelt and Mikolajczak, 1989), of soil-incorporated *L. alba* seed meal towards the root-knot nematode *Meloidogyne chitwoodi,* (Santo, 1999), and of incorporated seed meal to the plant pathogen *Plasmodiophora brassicae*, possibly due to the effects of thionin and aglycone allelochemicals (Deuel and Svenson, 1999).

Taxonomic level	Taxonomic group
Class	Dicotyledoneae
Order	Geranialea
Family	Limnanthaceae
Genus & species	*Limnanthes alba*

Table 1. Taxonomy of Limnanthes alba

The general principle of biofumigation has been adopted in the use of *Limnanthes alba* (meadowfoam) seed meal as a mulch, thereby harnessing its pesticidal and growth promotion properties. Svenson and Deuel (2003) recognised the potential of *Limnanthes alba* seed meal as a natural liverwort control and plant growth promoter. In trials seed meal applied as a top dressing on pots containing *Rhododendron* 'Cannon's Double', previously inoculated with liverwort gemmae and thallus, provided excellent control after 30 days, although efficacy reduced after 60 days, and with no evidence of phytotoxicity. Disadvantages of the seed meal were difficulty in application, an unpleasant odour, grass seed infestation and fungal growth on treated pots (Svenson and Deuel, 2000). The experiments were repeated with the seed meal substituted with AlbaGro™, a pelleted product derived from

L.alba seed meal (with the addition of granular sulphate, produced by Natural Plant Products, Salem, Oregon, US) applied to the surface of liverwort-infested pots with improved results. The pellets were more easily applied, and the odour and grass seed problems were removed (Svenson and Deuel, 2001). HDC report HNS 93c (Atwood, 2005) found that incorporating meadowfoam seed meal into compost at 1% an 2% levels provided only short term liverwort control. Currently only the meadowfoam-based product AlbaAide is marketed, and as a fertiliser in the state of Oregan as it does not have an herbicide licence (Martinez, 2006).

In this study, investigations were carried out into *Limnanthes alba*, establishing the GSL profile of the whole plant, previously unpublished, and optimising the extraction of glucolimnanthin and 3-methoxybenzyl ITC from seed meal.

In-vitro bioassays were designed using the predominant ITCs (benzyl, 2-phenylethyl, 2-propenyl and 3-methoxybenzyl) found in the root tissues of the plants grown hydroponically to investigate the effect of ITC s on the growth of liverwort thalli and also cress (*Lepidium sativum*) radicle germination and elongation, to provide a comparison with higher plants. Further bioassays characterised the inhibitory effects of two synthetic herbicides (Metazachlor and Lenacil) on liverwort gemmae.

Plant species (*Diplotaxis tenuifolia, Brassica juncea, Sysimbrium orientale* and *Limnanthes alba*) were selected for their high root GSL content, to provide a range of bioactive products for use in bioassays. These plants were grown in a nutrient film technique (NFT) hydroponic plant growth system, and methods developed to collect and analyse their root exudates from the nutrient solution, based on methods used by Yamane (1992).

Various experimental methods have been described for harnessing plant allelochemicals, including ITC s, by collecting and utilising root exudates to control weeds. Examples are plants grown in soil culture and their root exudates collected either in run-off water (Pope et al., 1985), or the plants transferred to a soilless system for exudate collection (Khan et al., 2002). Alternatively, seedlings have been grown in a hydroponic system with bioactive compounds collected on in-line C-18 columns (Tsanuo et al., 2003). Yamane (1992) collected *Rorippa indica* (Indian cress) plants from the wild and transferred them to a hydroponic system with

continuous root exudate trapping based on that designed by Tang (1982), collecting the ITC s on an in-line column containing XAD-4 resin.

II Experimental results from year 3

Environment

Lunularic Acid

Method development continued for the extraction, analysis and quantification of lunularic acid from liverwort tissue during year three, when the methods were used in a final experiment to characterise lunularic acid content of different liverwort tissues when grown under long and short day lengths, both outside and inside a glasshouse.

Extraction technique method development

Introduction

Grinding of plant material with a pestle and mortar is slow, particularly when preparing large numbers of samples therefore using a TissueLyser (Manufacturer: Qiagen) was investigated. The TissueLyser pulverised plant tissue by shaking samples in Eppendorfs with a steel ball, with each movement referred to as a motion. Several different volumes of extraction solvent were compared to find which extracted LNA most efficiently. The extraction solvent was optimised, as using high concentrations of acetonitrile produced dirty samples which adversely affected the HPLC chromatography, producing variable retention times and unacceptable peak shapes. Extractions using various concentrations of acetonitrile (ACN) and methanol (MeOH) were compared, aiming to extract LNA but not chlorophyll from liverwort tissue. LNA extraction rates were investigated using ACN (60%, 95%) to identify how many extractions were required to obtain maximum LNA from tissue. 95% ACN was used as it was known to extract efficiently (although too green to use regularly), to compare overall extraction rates. A control was also used, comprising 60% ACN but no plant extract, to establish if any peaks were produced by plasticisers from the Eppendorfs during extraction. All solvents were acidified with 0.1% acetic acid.

Method

Samples of liverwort thallus (3 x 25 mg) were extracted six times in 50, 100, 150 and 200 µL acetonitrile + 0.1% acetic acid. For each extraction liverwort tissue was placed in Eppendorfs with solvent, TissueLysed (2 min, 30 motions min^{-1}),

centrifuged (5 min, 12651 rpm) (Eppendorf Centrifuge 5810R with FA45-30-11 Eppendorf adaptor) and the supernatant removed to a vial; supernatants were not combined.

To produce samples free of particles of plant tissue the method was adjusted, reducing the acetonitrile concentration and acidifying the solvent; samples were centrifuged for longer. Samples of liverwort thallus (25 mg) were extracted six times in 100, 150 and 200 µL quantities of solvent (solvent A, H_20 + 0.1% acetic acid, and solvent B, 95% acetonitrile, 60:40), TissueLysed (2 min, 30 motions min^{-1}), centrifuged (10 min, 12651 rpm) and the supernatant removed to individual vials; supernatants were not combined. Samples were analysed by HPLC (Refer to page 93) with 10 µL injection (solvent A, H_20 + 0.1% acetic acid, and solvent B, 95% acetonitrile, 60:40).

Extractions using different solvents (ACN and MeOH) at various concentrations were compared. Samples of thallus tissue (25 mg), taken from a single liverwort, were extracted three times with various solvents (80%, 60%, 40% acetonitrile (ACN); 80%, 60%, 40% MeOH), TissueLysed (2 min, 30 motions min^{-1}) and centrifuged (10 min, 12651 rpm). Supernatant colour after each extraction was recorded; supernatants were then bulked and selected samples (60% and 40% ACN) analysed (HPLC, solvent A, H_20 + 0.1% acetic acid, and solvent B, 95% acetonitrile, 60:40).

To establish LNA extraction rates six samples of liverwort thallus tissue (25 mg) were extracted five times, with 60% and 95% ACN, TissueLysed (2 min, 30 motions min^{-1}), and centrifuged (10 min, 12651 rpm). Supernatants of those extracted in 95% ACN were bulked; samples extracted in 60% ACN were kept separate and analysed (HPLC, solvent: solvent A, H_20 + 0.1% acetic acid, and solvent B, 95% acetonitrile, 60:40). The control sample, comprised of 60% ACN only, was TissueLysed and centrifuged along with the plant samples but contained no plant tissue.

Following the procedure in earlier experiments, 95% acetonitrile was used; however this also extracted too much chlorophyll, adversely affecting the column, therefore the process was repeated with more samples, extracted with the ACN content of the solvent reduced to 65%. The first extraction from each sample of the 65% extractions and one 95% extraction were then run with standards to establish the retention times and peak areas were consistent.

Results

The samples produced were very green and dirty, suggesting the solvent should be adjusted to remove more chlorophyll and centrifuged for longer to improve the separation; the TissueLyser pulverised plant tissue more thoroughly than a pestle and mortar, producing smaller particles of tissue that required a longer centrifuge time. 50 µL solvent quantities proved too small to provide usable samples, so only the larger quantities were used. The extended centrifuge time resulted in improved separation, producing cleaner samples. However, peaks produced were small with inconsistent retention times (standards 11.53 and 11.71 min) when compared with those of previous analyses (standards 8.97 and 9.93 min). Some variation in peak size was expected as the plant material was not grown under controlled conditions.

Table 2. Appearance of extracts of liverwort thallus, comparing solvent and solvent concentration. G = green, LG = light green, Y = yellow, C = clear

	Acetonitrile			**Methanol**		
Extraction	80%	60%	40%	80%	60%	40%
1	G	Y/G	CL/Y	LG	LG	C
2	G	L/G	C	LG	LG	C
3	C	C/Y	C	YG	LG	C
Bulked	LG	Y	C	Y	Y	C

Table 3. HPLC analysis of LNA extracted from liverwort thallus in methanol and acetonitrile (ACN)

Sample	**Fluorescence**	
	Rt	**Peak area**
0.019 mg/ml LNA std	11.89	9935181
40% methanol	-	unmeasurable
60% methanol	-	unmeasurable
40% ACN	-	unmeasurabl

		e
60% ACN	11.98	422842
0.019 mg/ml LNA std	12.45	12567227

The comparison of different solvents and solvent concentrations showed that MeOH removed less chlorophyll from tissue than ACN (

Table 2). For both solvents 40% conc. extracted least chlorophyll; the greatest amount was extracted by 80% ACN. HPLC analysis for samples extracted in methanol and 40% ACN produced small, unmeasurable peaks (Table 3), suggesting that little LNA was extracted. A sizeable peak was produced extracting with 60% ACN, and a smaller peak for 40% ACN. The retention times for both standards and samples was extended beyond 11 min.

100% LNA was recovered from samples after 3 extractions and 98% after 2 extractions (Table 4). The control produced a single peak after 2.48 min, which did not compromise the LNA results.

Table 4. Recoveries for LNA tissue extracted five times with 60% ACN, 0.1% acetic acid

Extraction	Average of 3 samples	% of total LNA extracted
1	3,956,031	85.7
2	573,320	12.4
3	89,248	1.9
4	0	0.0
5	0	0.0

The retention times of the samples were changeable, but moved in tune with the LNA standards. Possible reasons for variations were an adjustment to room temperature, which had become markedly warmer (23 C). The first extraction from each sample of the 60% extractions and one 95% extraction and three LNA standards were re-analysed and produced peaks with similar retention times and symmetrical peaks (Table 5), indicating an acceptable level of consistency.

Table 5. Comparison of HPLC analysis results obtained from selected samples analysed on 16th and 17th November.

	16/11/2005		17/11/2005	
Fluorescence	**Rt**	**Area**	**Rt**	**Area**
	11.0			
60%-A-1	4	4,342,611	8.76	6,056,885
60%-B-1	7.53	4,575,219	8.85	5,979,054
60%-C-1	7.61	2,950,263	8.78	3,961,495
95%-A	7.71	1,732,178	9.08	1,607,969

The effect of day length and environment on lunularic acid content of liverwort grown in nursery conditions.

Introduction

The extraction and sample analysis methods developed were used to investigate the lunularic acid content of liverwort tissue grown in nursery conditions. Two extractions of lunularic acid were carried out during short days (November and December, 2005) and two during long days (May and June, 2006); liverwort samples were taken from Madrona Nursery, Pluckley, and Imperial College Nursery at Wye from inside and outside environments.

Liverwort tissue used for these experiments were thallus edge (TE), thallus centre (TC), archegonia (AR), antheridia (AN), and gemmae & cups (G) and rhizoids (R). The thallus edge was defined as tissue within 2 mm of the outside edge of thallus, and the thallus centre was thallus tissue only with no other structures, such as gemma cups nearby. Archegonia and antheridia tissue included the stalks. Gemmae and gemma cups were analysed together. For the November extractions there was not enough clean rhizoid material collected for analysis. For the December experiment liverwort tissue was collected from different areas of the nursery as the plants used previously were not in good condition and the area was being heated.

Method

Tissue samples (25 mg) were extracted three times in 150 µL 60% ACN with 0.1% acetic acid; each tissue type was replicated three times. Samples were TissueLysed for two mins at 30 motions per min (the TissueLyser shakes samples back and forth, each movement is referred to as a motion), centrifuged for 10 mins (12651 rpm) and supernatants for each sample bulked. Extracts were subjected to HPLC analysis with 20 min runs, 10 µL injections and detected by fluorescence. The system was washed through with 95% ACN prior to the analysis.

As no internal standard was used in these experiments a calibration chart (Figure 3) was constructed to quantify LNA content following HPLC analysis. Approximately 0.1 mg LNA was weighed and using the molecular extinction coefficient the actual LNA concentration was found to be 0.0613 mg ml^{-1}.

The molecular extinction coefficient (ε) of LNA at a UV absorption wavelength of 308 nm = 4200 (Table 6) molecular weight of LNA = 258. The equation used was A_λ =

εcL where A_λ = absorbance wavelength (nm), ε = molar extinction coefficient, c = concentration, L = light path length (1 cm). LNA absorbencies were determined using a Shimazu UV-210-PC UV-VIS scanning spectrophotometer (
Table 1)

Serial dilutions of this standard were analysed by HPLC and the results used to construct the calibration curve, with LNA concentrations adjusted to the actual LNA concentration previously calculated. Using linear regression a curve was fitted to the data (Figure 3), and the regression line used to quantify LNA concentration of peak areas produced by HPLC analysis of liverwort tissue samples.

Table 6. Molecular extinction coefficients of LNA in neutral ethanol at given UV wavelengths (Valio and Schwabe, 1969).

Absorbance wavelength λ_{max} (nm)	Extinction coefficient ε
308	4,200
287	3,600
280	3,300

Table 7. Absorbances of 0.1 mg ml^{-1} LNA in ethanol

Absorbance wavelength (nm)	Absorbance (Å)
308	0.998
287	0.766
280	0.720

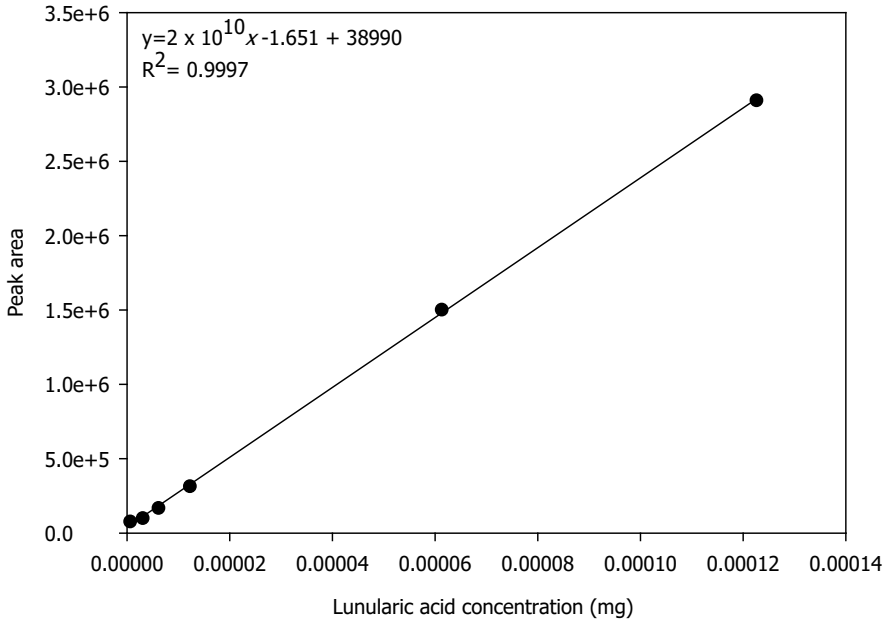


Figure 3. Calibration curve produced for lunularic acid

Results

The analysis of samples proved difficult initially because of the problem finding a suitable internal standard, and although a simpler extraction process was established it was difficult to produce clean samples. Maceration of liverwort tissue using a pestle and mortar was successful, but time consuming for a large number of samples; using the TissueLyser pulverised the tissue into smaller fragments that could not be completely removed and caused difficulties with the HPLC. Thorough cleaning of the system with 95% acetonitrile before putting samples through and changing the guard column alleviated this problem.

There was a trend where greater LNA levels were found in the long day experiments than in short days, in agreement with Gorham (1975) who found that liverwort growth decreases during long days with high light intensities above 5,600 lux due to high levels of lunularic acid (Gorham, 1975). In the outside environment and long day treatments, higher LNA levels were found in May than July (Figure 4); however, for liverworts grown inside higher levels were found in July than May, other than in rhizoids and gemma cups.

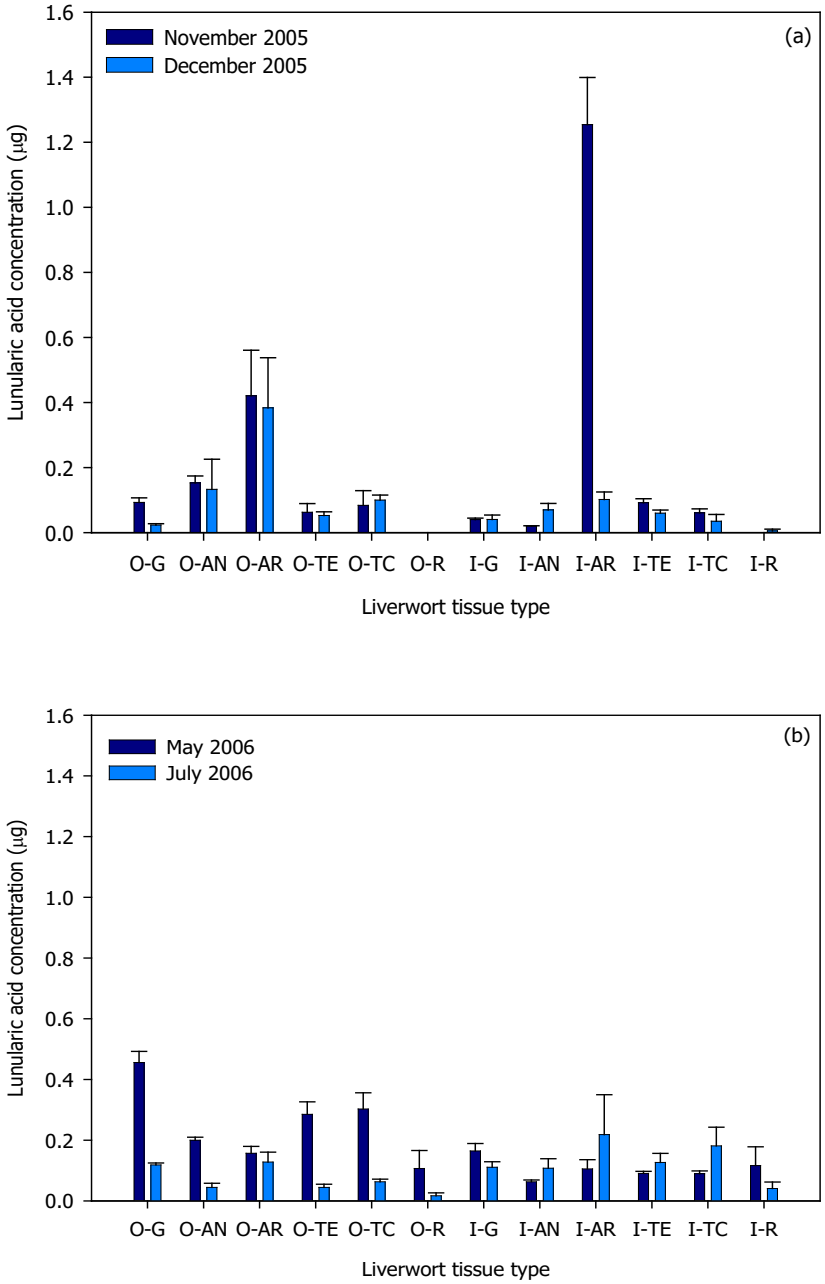


Figure 4. Analysis of lunularic acid content of liverwort tissue. Results are an average of three replicates. O=outside, I=inside. G=gemmae and gemma cups, AN=antheridiophores, AR=archegoniophores, TE=thallus edge, TC=thallus centre, R=rhizoids

With the short day length, lunularic acid levels were more variable than with long day length. Highest levels were found in archegoniophores, particularly in the November, inside treatment; the LNA content of the archegoniophores was markedly greater than the other tissues.

Results for both short and long day experiments were analysed, separately, using ANOVA, and found not to be significant (vr 3.12, $p<0.05$).

Rhizoids were not included in the November 2005 experiments, in the December 2005 LNA was detected in only one of the six samples analysed, but in May and July all rhizoid samples contained lunularic acid.

The effect of shading on liverwort establishment and growth

Introduction

Previous growth and development experiments indicated that for liverwort grown *in-vitro*, radial growth was greater under high light levels (800 ms^{-1} s^{-1}) than low (400 ms^{-1} s^{-1}) with morphological changes occurring to the gemmalings in high light with high temperature as they became brown and started to die, suggesting that high light levels for extended periods may damage liverwort, with least growth at low light levels.

A further experiment, located at Palmstead Nurseries Ltd., Wye, was designed to investigate the effect of light levels on liverwort establishment and growth in nursery conditions, between May 4th and September 1st, 2006. 'Seed' pots of liverworts and pots of liverwort-free compost were placed in shaded tunnels designed to provide different levels of light. Liverwort gemmae were dispersed by the overhead irrigation system and their establishment and growth measured.

It was hypothesised that at high light levels liverwort would initially grow and establish, but in the longer term would be damaged, resulting in reduced liverwort presence; liverwort would establish and grow less in very low light levels. Liverwort would proliferate more in medium light levels.

The aim of this experiment was to investigate the effect of light levels on liverwort establishment and growth and to ascertain whether using shading as cultural practice that could be utilised as an aid to control liverwort.

Method

Treatments

Liverworts were grown within shade tunnels providing three light treatments: 44% and 73% shading, and no shading, with one 2 L 'seed' pot of healthy liverwort and 20 x 2 L pots of compost placed in each shade tunnel. All treatments were replicated twice inside a polytunnel and twice outside, as in the experiment layout (Figure 5); the placement of blocks and treatments were fully randomised.

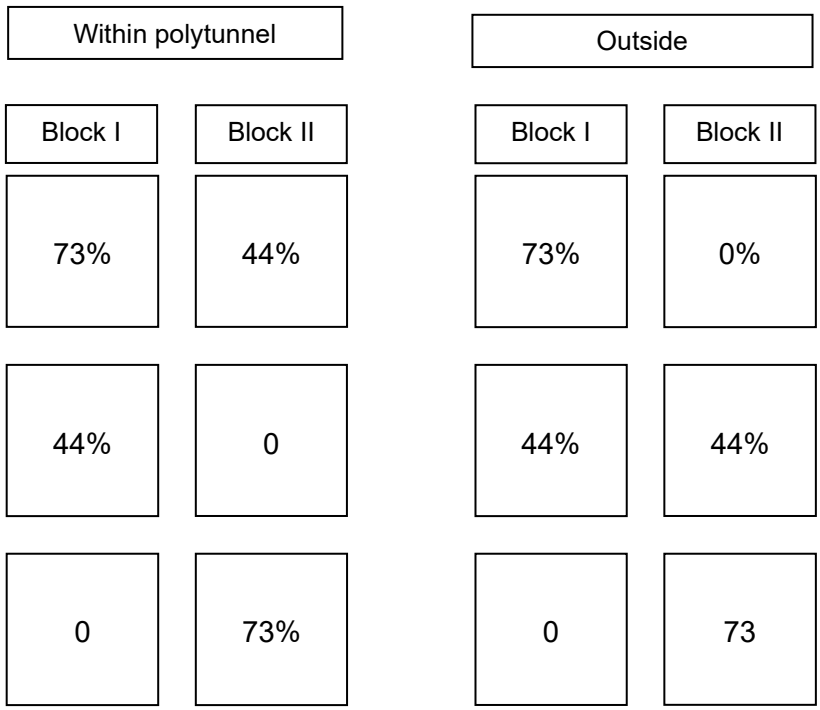


Figure 5. Experiment layout. Each treatment is replicated twice within the polytunnel and outside, and provides 73%, 44% and 0 shading.

Shade tunnels

The shade structures were constructed from galvanised steel hoops, the ends of which were forced into the ground, two per structure placed 1 metre apart with 300 mm between tunnels. They were clad in woven polypropylene shading fabric

(Supplier: Growing Technologies) tied to the hoops to provide stability (Figure 6 and Figure 7).

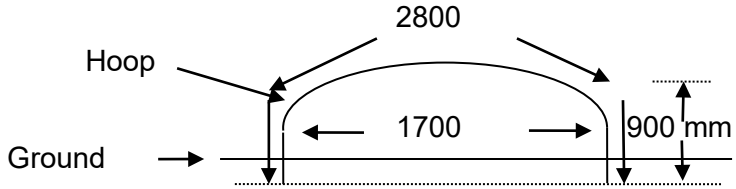


Figure 6. Construction of shading structures. Galvanised steel hoops are forced into the ground and clad with shading fabric.

Figure 7. Shade tunnels (a) outside (b) inside the polytunnel

Compost

Compost was 100% peat (Maxi Bale Natural AOGBF 2.25 m, Kekkilä Oyj, Tuusula, Finland), with added Osmocote Pro (3500 g m^{-3}) and lime as Dolodust magnesium limestone (2000 g m^{-3}), provided in 2 L pots by Palmstead Nurseries Ltd.

Irrigation

The overhead irrigation system at Palmstead Nursery is based on vapour pressure deficit, with water applied in two short 2 min bursts, with a few mins between each, and minimal water application, equating to daily winter and twice daily spring and summer applications.

Data collection

Percentage coverage of each pot by liverwort was recorded after 119 days. A 10 mm grid printed on a transparency was placed over each pot and the area of liverwort coverage recorded.

Supplementary environmental data was recorded at pot level within each shade tunnel: light levels using a Basic Quantum Meter model QMSW-SS (Apogee Instruments Inc. Logan, UT, USA), and relative humidity and temperature using an A1 Hygromer (Rotronic Instruments (UK) Ltd., Crawley, W. Sussex).

Statistical analysis

For statistical analysis the inside and outside treatments were treated as independent experiments as the results of the outside treatments were confounded by uncontrollable environmental conditions (rain) which affected gemma dispersal.

Results

Temperature, relative humidity and light levels were recorded weekly for each treatment, and are presented in Figure 9. Liverwort growth was measured as the percentage area of pot covered.

A range of growth patterns were observed throughout the treatments, a sample of which is shown in Figure 8. The polytunnel itself reduced the light level by approximately 50% (Table 8, treatments O-0 and I-0) so an effect of the lower light levels may be evident in liverwort establishment and growth in treatments with no additional shade applied. The shade tunnels afforded 73% and 44% shade to the treatments (Figure 5), with no shading provided to the controls.

Treatment	Light level (μ mol m^{-1} s^{-1})	Temperature (C)	Relative Humidity (%)
O-0	714.13	23.25	41.22
I-0	338.92	24.49	42.60
O-44	316.08	22.75	46.12
I-44	171.29	24.41	45.77
O-73	127.33	22.72	47.43
I-73	83.25	24.63	46.71

Table 8. Comparison of light levels, temperature and humidity inside the polytunnel and outside for each treatment. Figures are averages of 12 readings with block I and II figures amalgamated. O = outside, I = inside, 0, 44 and 73 = % shade.

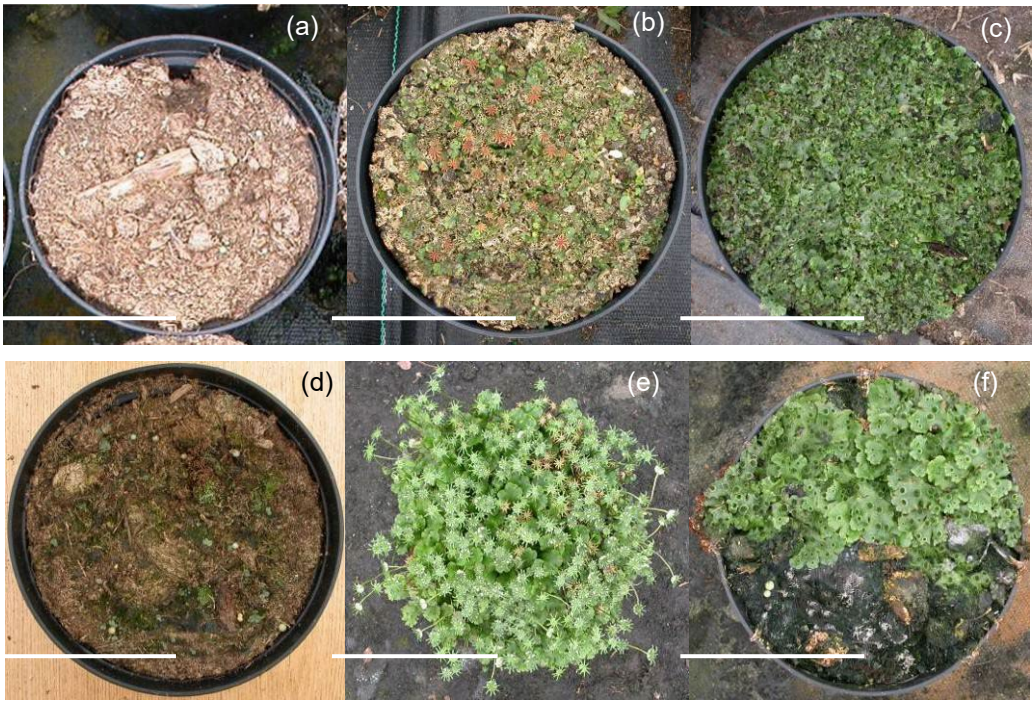


Figure 8. Examples of liverwort growth showing (a) very dry compost (I-73) (b) an area of liverwort die back with new growth (O-0) (c) many small, congested plants (O-73) (d) sparse liverwort establishment (O-0) (e) vigorous growth, (I-44) (f) large gemma cups with many gemmae (O-0). Scale bars = 10 cm. I = inside, O = outside. 0, 44 and 73 = percentage shade.

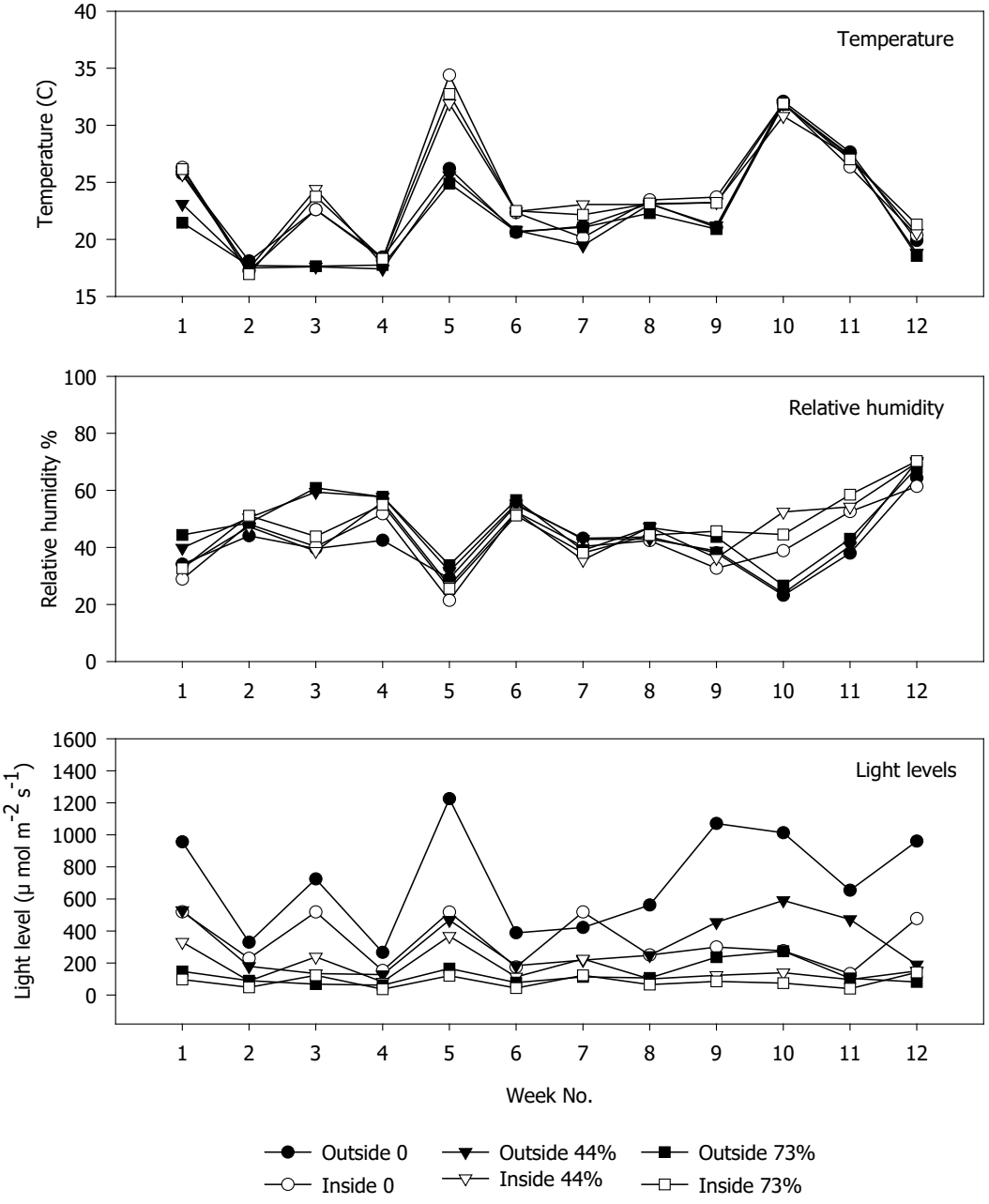


Figure 9. Temperature, relative humidity and light level readings taken from each shade tunnel during the experiment. Legend refers to treatments, with 0 (none), 44% and 73 % shading.

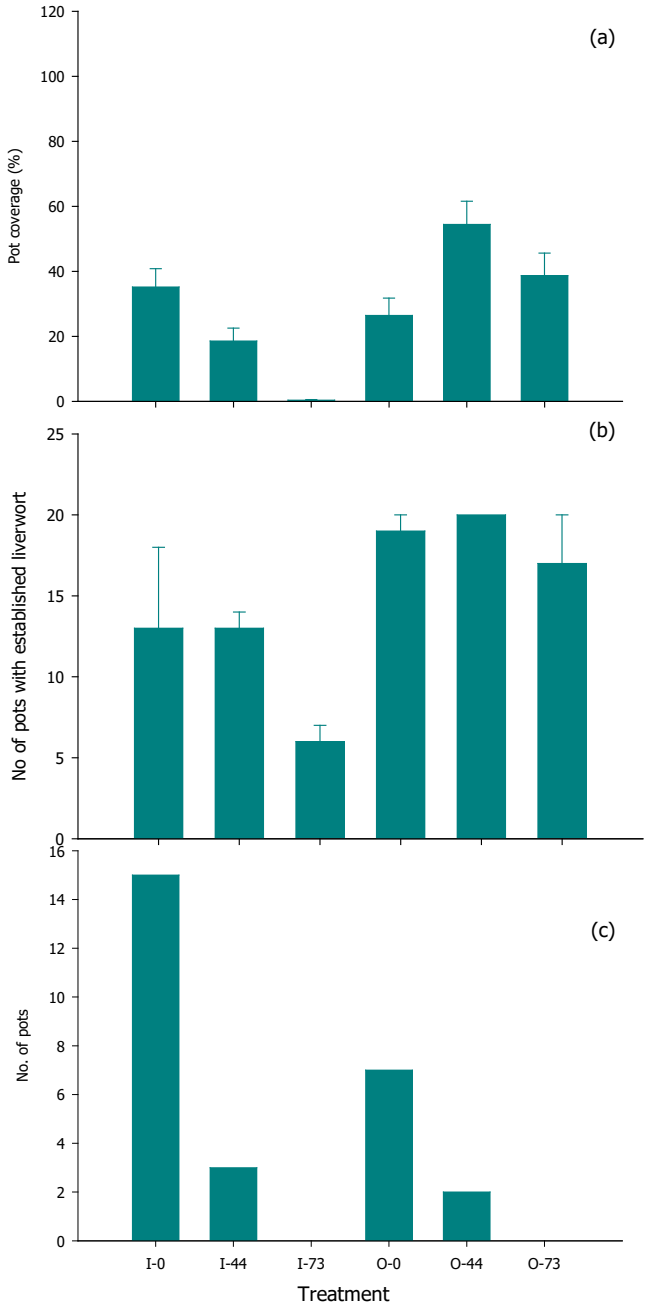


Figure 10. The effect of light level on (a) liverwort growth, expressed as pot coverage, (b) establishment (c) the number of pots from each treatment containing gametophore-bearing liverwort. I = inside, O = outside. 0, 44 and 73 = percentage shade.

Light levels

The effect of light levels on pot coverage (Figure 11 and Figure 10a) was demonstrated with less growth at the highest and lowest light levels, and maximum pot coverage for inside and outside treatments where liverworts experienced mid-range light levels between 300 to 400 ms^{-1} s^{-1} (I-0 and O-44).

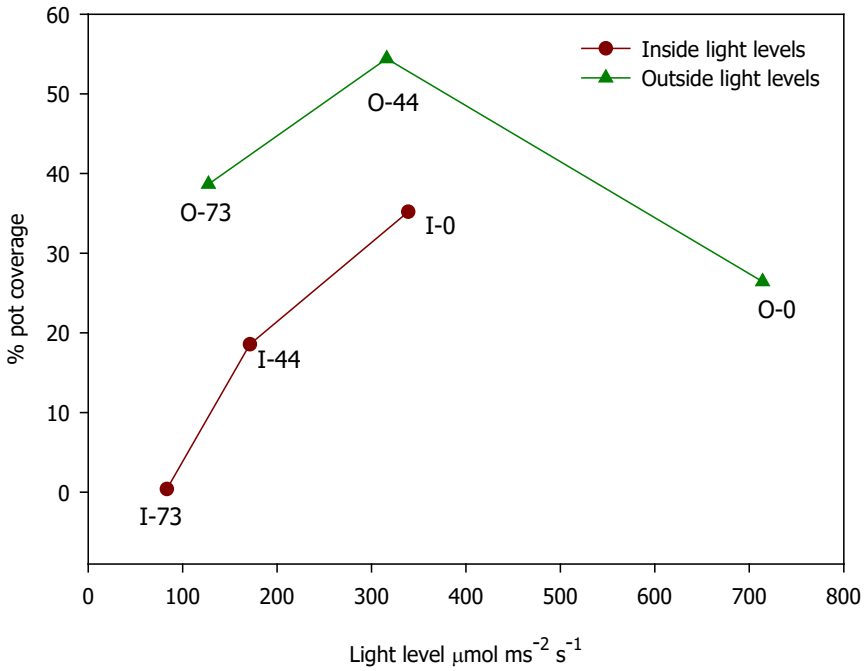


Figure 11. % pot coverage compared to light levels for inside and outside treatments. Light levels are averages of weekly readings for each treatment, taken over the full term of the experiment. I = inside, O = outside. 0, 44 and 73 = % shade.

Mean liverwort area was less inside than out; with average pot coverages of 17.91% and 39.84% respectively. Inside there was less liverwort growth in the 73% than 44% shading treatment; greatest growth was observed with no shading applied. Fewer liverworts established per pot, but these grew larger and more vigorously, with larger thalli than outside treatments, and gemma cups tended to be large containing many gemmae (Figure 8f). In the 73% shade treatments, however, fewer liverworts established and were smaller than other treatments. The compost of the inside, no shade treatment was very dry, typical for Palmstead Nursery; previously established liverwort had died and new colonies were subsequently developing.

Outside, there was less liverwort growth with no shade than with 73%; greatest growth with 44% shade. Treatments tended to be comprised of many liverworts that

were small and congested, possibly the result of the dispersal of a greater number of gemmae than inside due to rainfall. There were fewer gemma cups than inside, containing fewer gemmae, particularly where liverwort establishment was sparse.

The results for the outside treatments were affected by rain, which increased gemma dispersal. None were found to be significant ($F_{2,2}$ = 2.37, P < 0.05), accepting the null hypothesis of no difference in pot coverage as a result of shade level.

For inside treatments, the means of residuals proved problematical, however data was transformed using a logit transformation (Table 9) and then analysis of variance showed that there was a significant effect of light levels on pot coverage by liverwort ($F_{2,2}$ = 39.93, P < 0.05).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
BLOCK stratum	1	0.8282	0.8282	2.37	
BLOCK.*Units* stratum					
SHADE	2	27.9487	13.9743	39.93	0.024*
Residual	2	0.7000	0.3500		
Total 5	**29.4768**				

Table 9. Analysis of variance for inside treatments using logit transformation.

Establishment

Establishment was calculated as the number of pots containing established liverwort (Figure 10b). The results suggest an effect of light level on liverwort establishment that reflects the effect of light on liverwort growth, with less liverwort established successfully inside than out in all treatments.

Statistical analysis (calculated separately for inside and outside treatments) using a Chi-squared contingency test confirmed a significant effect of shade on liverwort establishment for both the inside and outside experiments. The critical value at p=0.05 with two degrees of freedom is 5.99; chi-squared values for outside were 0.113, and for inside 6.46; shading therefore did have a statistically significant effect on liverwort establishment for the inside environment, rejecting the null hypothesis, but not for the outside environment.

Further inspection of each set of calculations revealed that the inside, 73% shade treatment had a greater effect than either 44% shade or no shade; fewer pots

contained established liverwort in the 73% shade treatment. Outside, less liverwort was established in the treatment with no shade, corresponding to the maximum light level, than 44% shade. Inside there was little difference between the 44% and '0' shade treatments, and again fewer pots contained established liverwort in the 73% shade treatment.

Maturity

There appears to be an effect of light level on the sexual maturity of liverworts, with more pots containing liverworts bearing gametophores where no shade was provided, even though one treatment was very dry, had died back, was re-growing and bore no gametophores. No gametophores were produced within the 73% shade treatments either inside or outside.

For the inside treatments the growth trend is reflected in the number of pots of liverwort bearing gametophores (Figure 10c), with fewer gametophores and less growth in treatments with 73% shade provided, suggesting that light levels could affect liverwort maturity; most gametophores and greater growth occurred in the treatments without shade. Where there was greatest growth and more gametophores the liverworts were more mature, and more of them had entered the sexual phase. However, outside the 73% treatment exhibited good pot coverage but no gametophores were produced and pots contained many smaller liverworts.

Statistical analysis using chi-squared contingency test confirmed a significant effect of light on gametophore production. Using the critical value of chi-square at $p<0.05$ from the statistical table, with two degrees of freedom (5.99), chi-squared analysis results for outside were 10.49, and for inside 30.17, therefore the null hypothesis of shade having no effect on gametophore production was rejected for both inside and outside treatments. Further inspection of the calculations revealed that the '0' shade treatment had a greater effect from other treatments, with more gametophores present than either 44% or 73% shade treatments.

Liverwort in the seed pots for the inside treatments died back early in the experiment; some were re-establishing themselves either from an influx of gemmae from other pots or regeneration of surviving tissue. Gemmae from the seed liverworts had dispersed prior to this and newly established colonies developed their own gemma cups and gemmae which were subsequently dispersed. Outside, some seed pots had died, but others were growing vigorously.

The inhibiting effect of extreme high and low light levels on liverwort growth reflects the results of the growth cabinet experiments where liverworts grew less in low light and more, initially, in high light before becoming damaged and dying.

Growers of protected crops could take advantage of these observations, with shade and light levels manipulated to provide light levels outside a critical range favourable to maximum liverwort growth, working towards an economic injury level acceptable to the grower. This could be tailored to crop plant requirements, with plants tolerant of higher light levels grown in reduced shade conditions and shade loving plants grown in low light levels.

Epidemiology

Characterisation of gemma dispersal in different nursery irrigation systems

Introduction

Previous experiments have suggested that liverwort gemma dispersal is affected by the irrigation system (nozzle type, water pressure) and by watering regime; the gemma cup regeneration experiment suggested that by reducing irrigation events less gemmae are produced and therefore available for dispersal.

A large scale experiment, using the irrigation facilities at East Malling Research was designed to test these possibilities, combining different irrigation systems: two different nozzles, drip, capillary matting and no irrigation. Irrigation was provided either every two days or twice daily to the nozzle and drip irrigation systems. Capillary matting required markedly less water to maintain a moist environment. A ‘seeder’ pot of liverwort was placed in each treatment as a gemma source.

As gemmae are dispersed by water droplets, it should be the case that no dispersal would be observed in the capillary matting and drip treatments; no gemmae should establish and the ‘seed’ liverwort would be unlikely to survive summer temperatures in the control treatment with no irrigation. Results of previous experiments suggest that more liverwort gemmae should be dispersed by the irrigation systems, and of these more should be dispersed in treatments irrigated twice daily than every two days.

Method

For each treatment twenty 2 litre pots were prepared with SHL Professional Potting Compost manufactured by William Sinclair Horticulture Ltd (Table 10); along with a 2 litre pot containing healthy liverwort as a gemma source, placed centrally. The position of each treatment, except the capillary matting, was randomly selected; all treatments were replicated twice, arranged in two blocks (Figure 12).

Peat grade (mm)	Proportion (%)
0-10	70
3-15	30

Table 10. Peat content of SHL Professional Potting Compost. N:P:K ratio:1:1.2:2

For the nozzle and drip irrigation application water was applied either twice daily or every two days. For the 2-day treatment water was applied for 60 mins at 10 pm, and for the twice daily treatment it was applied for 15 mins at 6 am and 10 pm, therefore pots in each treatment received a total of 60 mins irrigation during each two day time period. For the capillary matting it was essential to prevent the capillary matting from drying out, thereby maintaining good capillary action between the growing medium in the pot and the matting. In preliminary tests, irrigation was applied for 1 min on 6 occasions in each 24 hr period. However, this resulted in the growing medium being too wet and was reduced to 1 min at 6 am and 6 pm.

	Nozzle 1		**Nozzle 2**		**Drip**		**Capillary matting**	**Control**
Block I	2-day (2-D)	Twice daily (TWD)	2-day (2-D)	Twice daily (TWD)	2-day (2-D)	Twice daily (TWD)	Twice daily (CM)	None
Block II	2-day (2-D)	Twice daily (TWD)	2-day (2-D)	Twice daily (TWD)	2-day (2-D)	Twice daily (TWD	Twice daily (CM)	None

			)		

Figure 12. Experimental design: all treatments are replicated once in each block, with drip and overhead irrigation applied with two time schedules, every two days or twice daily.

The overhead irrigation systems used two nozzles, MP Rotator® model 1000 and Dan modular 180^0 spread (Supplier: Revaho Ltd) arranged as in Figure 16, supported on 60 cm risers.

The MP Rotator® sprinkler, manufactured by the Walla Walla Sprinkler Company, USA, delivers adjustable arcs and radii of droplets in individual rotating streams, with flow changes proportional to the area being covered to match the original precipitation; it is advertised as having a low application rate and high uniformity.

The capillary matting bed construction consisted of a layer of plywood with a wooden frame covered with a waterproof black plastic membrane overlapping the frame to prevent water run-off and to provide a smooth, level base. Within the frame was layered Florimat 2 capillary matting (Supplier: Flowering Plants Ltd) to hold water and an upper layer of micro-perforated polythene to keep the matting clean whilst still allowing free water movement, (Figure 14a). Water was supplied to the matting via Netafim™ trickle tape with 20 cm hole spacing (Figure 14b). The beds provided were on a slope, so the most level area of each block was selected for the capillary matting, rather than being randomly allocated, therefore this treatment was used for observation only, and was not included in statistical analysis.

Drip irrigation consists of a feed pipe with micro tubes with a dripper on the end held into each pot with a stake connected to; this system provides water direct to the growing medium, with no wastage due to water falling outside the pot. Although an efficient system, it can take more time to set up than overhead or capillary matting systems.

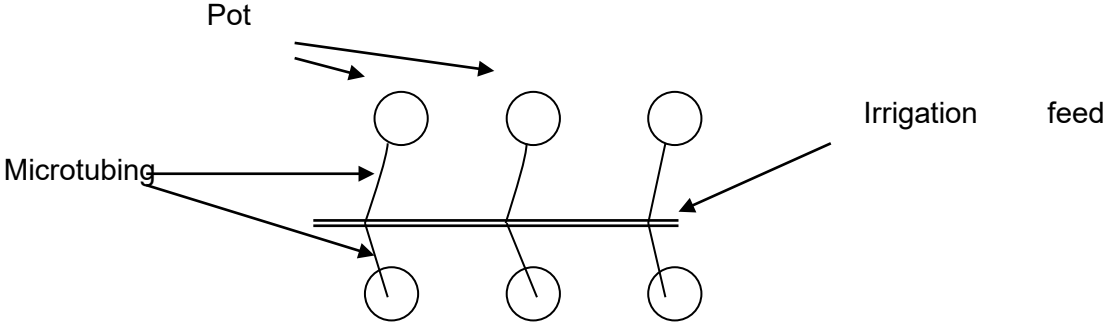


Figure 13. Example drip irrigation arrangement with microtubes connecting the feed pipe with a dripper held in position in each pot with a stake.

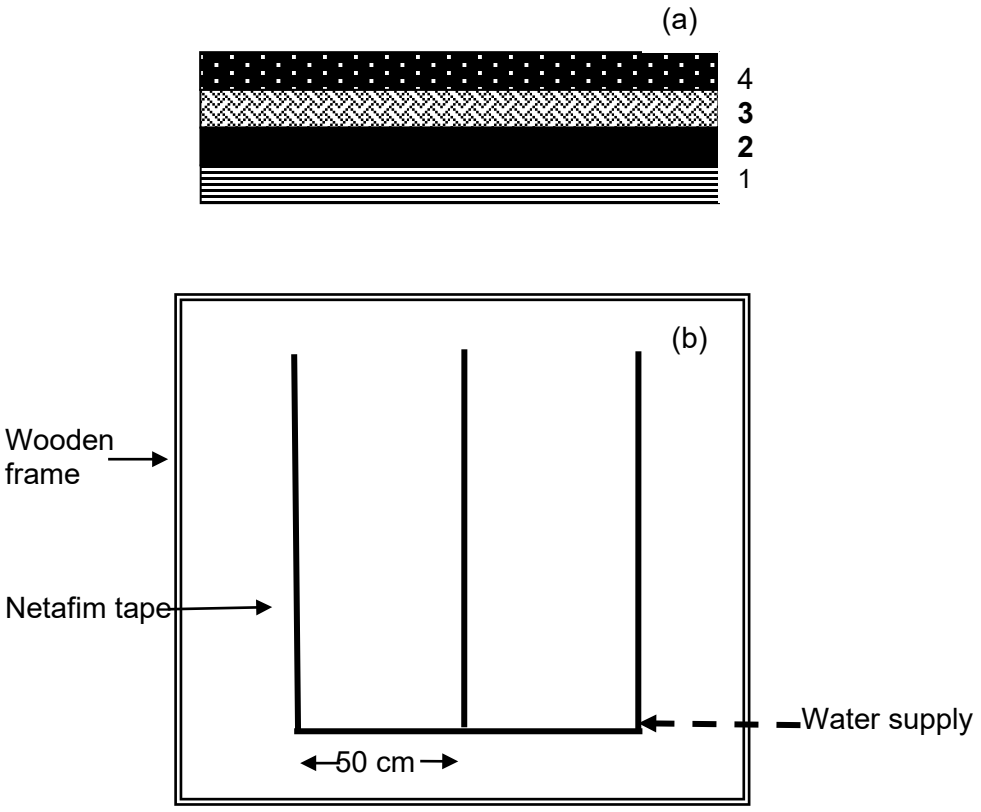


Figure 14. Capillary matting construction (a) Layers comprising the base: 1- plywood, 2- black plastic waterproof membrane, 3-capillary matting, 4- perforated plastic (b) Layout of Netafim tape, positioned between layers 2 and 3, and water supply.

Irrigation timing was controlled with two Orbit Sunmate automatic garden watering systems, one each for the 2-day and twice daily treatments; and a Kompernass Watering Computer KH 4038 (Supplier: City Irrigation Ltd) for the capillary matting.

Two beds, (block I and block I), were constructed from Mypex laid on grass and edged with concrete blocks. A Spanish tunnel construction protected the beds from rain whilst allowing free air movement. Screens were constructed from clear plastic supported by nylon rope connected to the tunnel supports and road irons to protect treatments from contamination from neighbouring sprinkler irrigation, down the side of the tunnel and at each end, providing protection from the prevailing weather conditions (

Figure 15 to Figure 17).

Figure 15. Spanish tunnel construction: (a) side view (b) treatment area with pots protected by plastic screens (c) screen attachments using elasticated tarpaulin ties and balls (d) view of general layout.

Data collection

The number of gemmae dispersed was measured by positioning 10 collection pots per treatment for the duration of a full irrigation cycle, 2 days, and the number of gemmae collected recorded.

Final results taken were the surface area of the pot covered by liverwort and the number of liverwort infestations present. A measure of relative droplet size for each nozzle was obtained using water sensitive paper (Supplier: Syngenta Crop Protection AG), card with a coating that stains dark blue in contact with water, those used were 76 x 52 mm. Three cards per treatment were individually exposed to droplets produced by each treatment, mid-flow, and then placed in a box with silica gel to prevent humid air from affecting the droplet stain sizes.

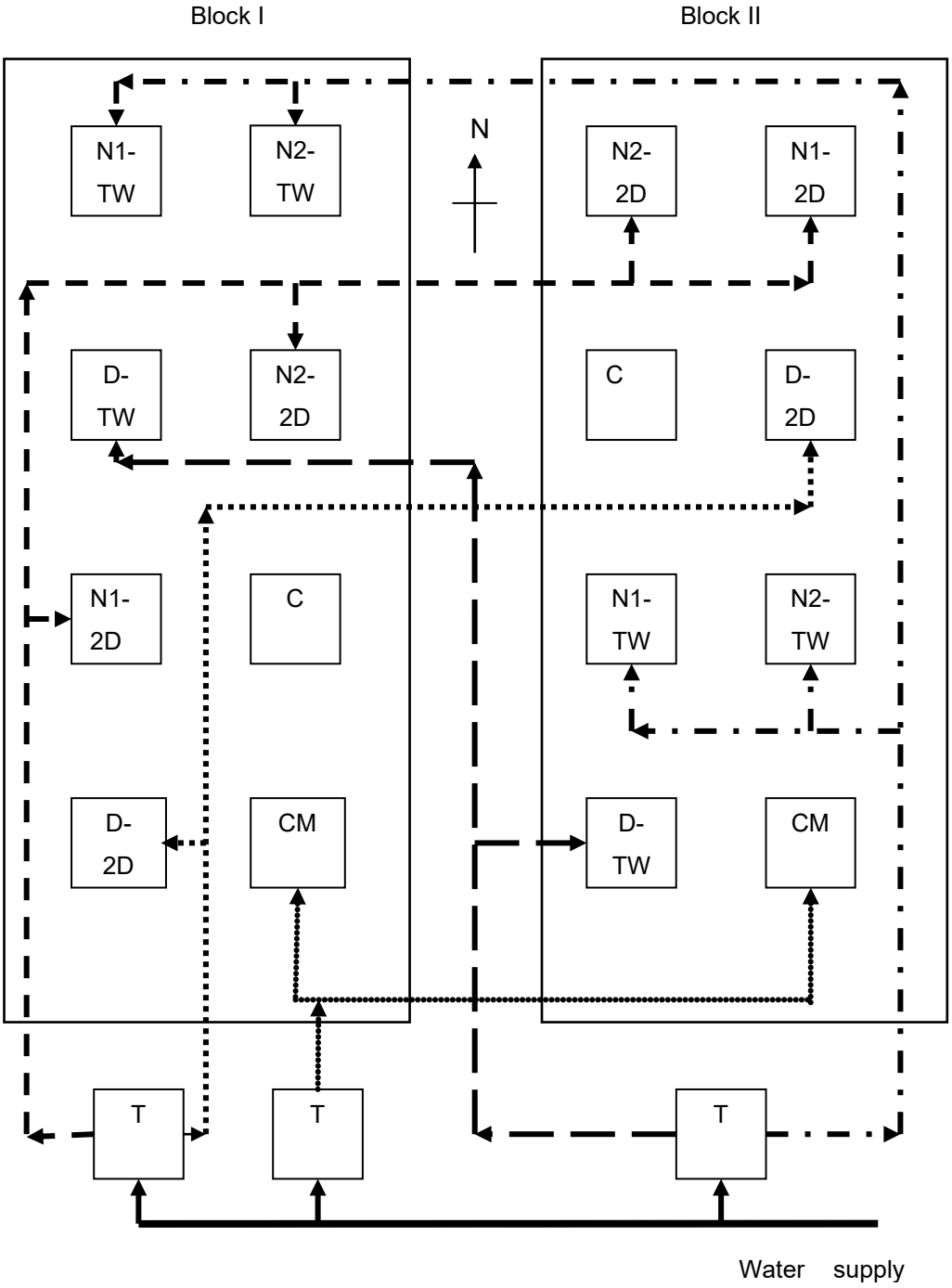


Figure 16. Layout of treatments showing irrigation design. T=timer, N=nozzle, C=control, CM=capillary bed, D=drip, TWD=twice daily, 2D=2-daily. Dotted lines indicate pipes connecting mains water supply, timers, sprinklers and drippers and capillary matting.

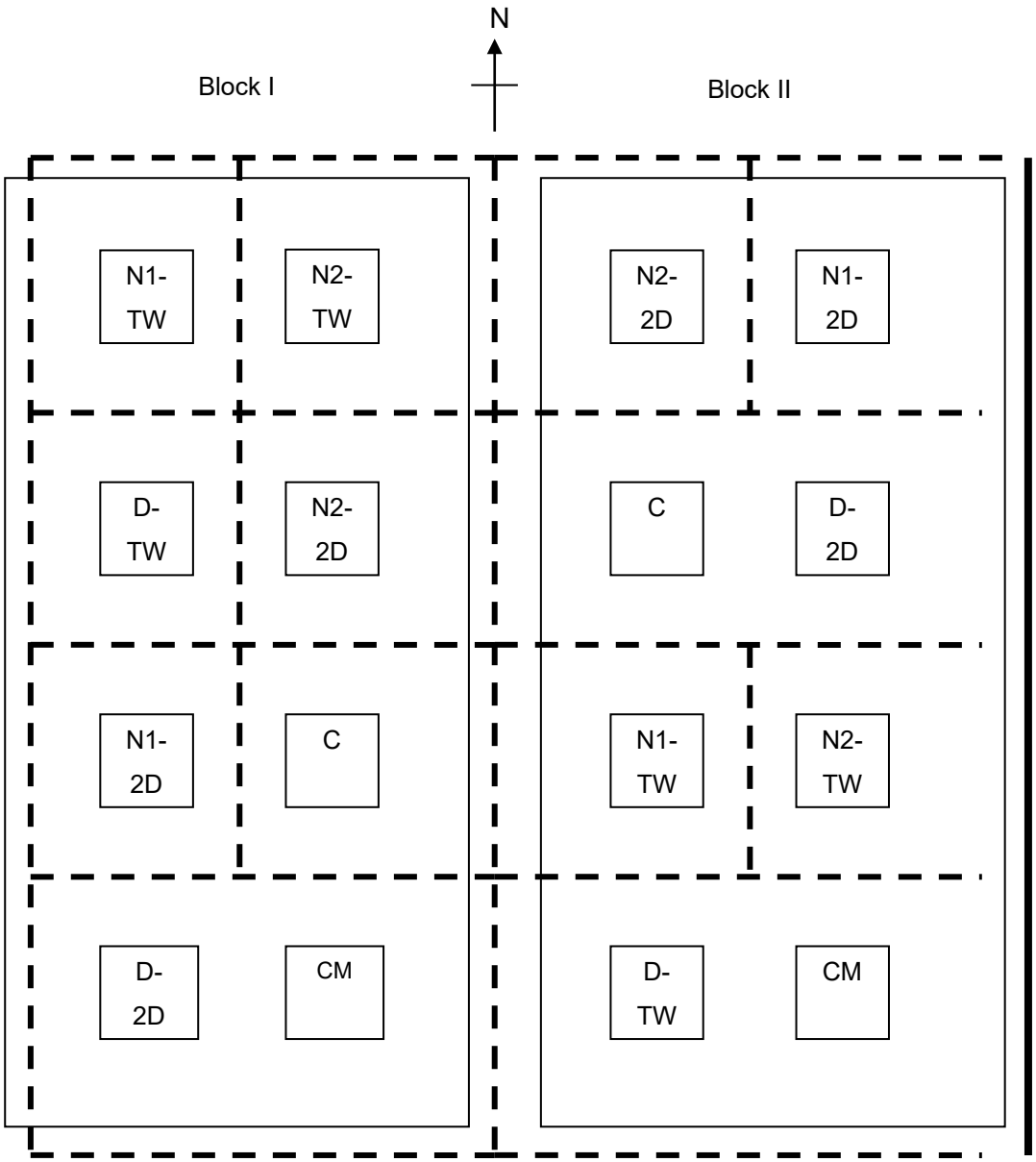


Figure 17. Layout of treatments showing irrigation design. T=timer, N=nozzle, C=control, CM=capillary matting, D=drip, TWD=twice daily, 2D=2-daily. Dotted lines indicate position of clear plastic screens. The solid line to the right indicates the position of the neighbouring polytunnel.

Results

Seed pots

The liverwort in the seed pots for the control treatments, with no water applied had died before the first dispersal results were collected, eight days after they were put in place. The seed liverwort in the Block II, nozzle 1, 2-day treatment had died by 56 days. Some dieback was observed on the Block II, drip, twice daily treatment and

Block II, drip, 2-day treatment. After 56 days the seed pots were still healthy in the remaining treatments, with the majority bearing gametophores. In order to prevent spore dispersal archegoniophores were removed.

Gemma distribution within pot and relative to the source

Observations were made relating to the distribution pattern of gemmae; in the Block II, nozzle 2, 2-day and block 1, nozzle 1, 2-day treatments more gemmae were established in pots nearest to the seed pot, whilst for the remainder of the treatments they appeared randomly distributed, showing no clear trend.

Some gemmae had established in the drip treatment, and although some of these were near to the dripper, others were randomly distributed, suggesting the drippers were not involved in their distribution.

Where treatments had many gemmae established in a pot they were small and congested, however where a small number of liverworts had established the liverwort thalli had grown larger. This pattern of growth was also observed in the shading scale up experiment.

Number of gemmae dispersed

A measure of the number of gemmae dispersed was made by counting the number of gemmae that fell into collection pots on five occasions (Figure 18 b). A trend soon emerged, with no gemmae collected in any of the drip, control or capillary matting treatments. With the nozzles, gemmae were collected in all but both replications of nozzle 2, twice daily. More gemmae were collected in the nozzle 1, twice daily than 2-day treatment.

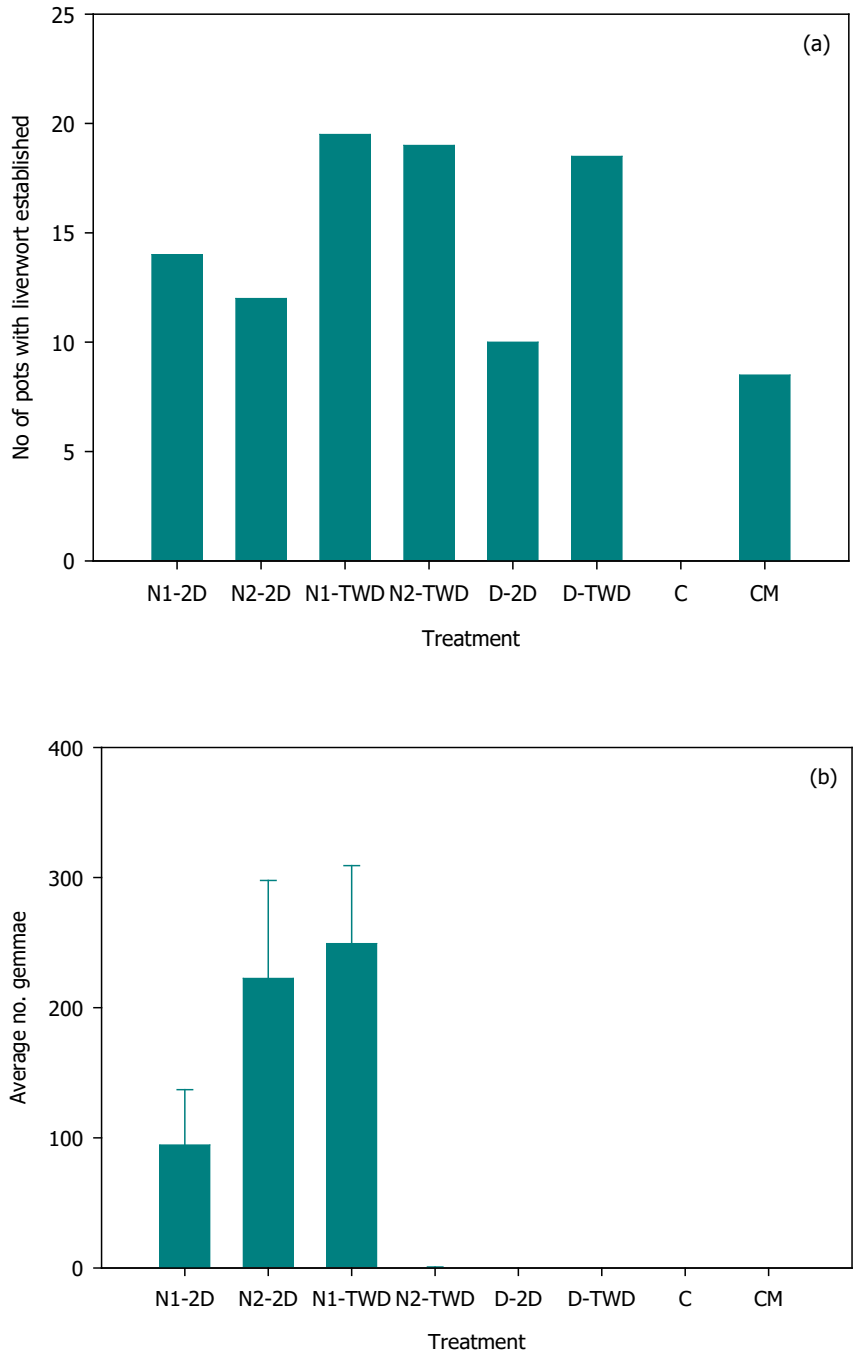


Figure 18. Liverwort infestation after 56 days: (a) Liverwort establishment, measured as the number of pots with more than one gemma present. Results for each treatment are the average of forty pots arranged in two blocks. (b) Number of gemmae dispersed. Results for each treatment are an average of the number of gemmae collected in pots during one two day irrigation cycle. N1 = nozzle 1, N2 = nozzle 2, D = drip irrigation, C = control (no irrigation), CM = capillary matting irrigation. 2D = irrigation every two days, TWD = irrigation twice daily.

Liverwort establishment

The number of pots with any liverwort established was recorded (Figure 18a). It had been expected that no liverworts would be dispersed or established in the capillary matting, drip and control treatments; however, this was not the case. These could have come from a number of sources, spores produced either by liverworts used in the experiment, or growing elsewhere on the site and present in the air, contamination by gemmae from other treatments, or gemmae from seed pots when they were placed in their positions. Care was taken to prevent such contamination by erecting screens between treatments removing gametophores from seed liverworts and by careful handling of pots. A greater number of pots contained established liverwort in the nozzle and drip treatments irrigated twice daily than every 2-days, with more established in the nozzle 1 than the nozzle 2 treatments.

The amount of liverwort in each pot was measured using two methods (Figure 19) counting the number of liverwort colonies present and recording their surface area and the same trends are exhibited in each. More liverwort was present in the twice daily than 2-day treatments of the nozzle 1 and drip treatments. Results for nozzle 2 were almost equal: the average liverwort area was greater for the 2-day (1.84 cm^2) than the twice daily (1.82 cm^2) treatment, and conversely the number of colonies was greater in the twice daily (445) than the 2-day (533) treatment. The area of liverwort present in the capillary matting treatments were less than all other treatments except for the drip, 2-day and the control. However, the number of liverworts established was less than all treatments other than the control.

Statistical analysis using chi-squared contingency test confirmed a significant effect of dispersal treatment on establishment ($P = 0.001$). Further investigation of the results indicated the greatest difference was found between the control and the other treatments; large differences were also found between the twice daily and 2-day applications for both nozzles and the drip treatments. Differences between application methods were smaller than differences between application timing.

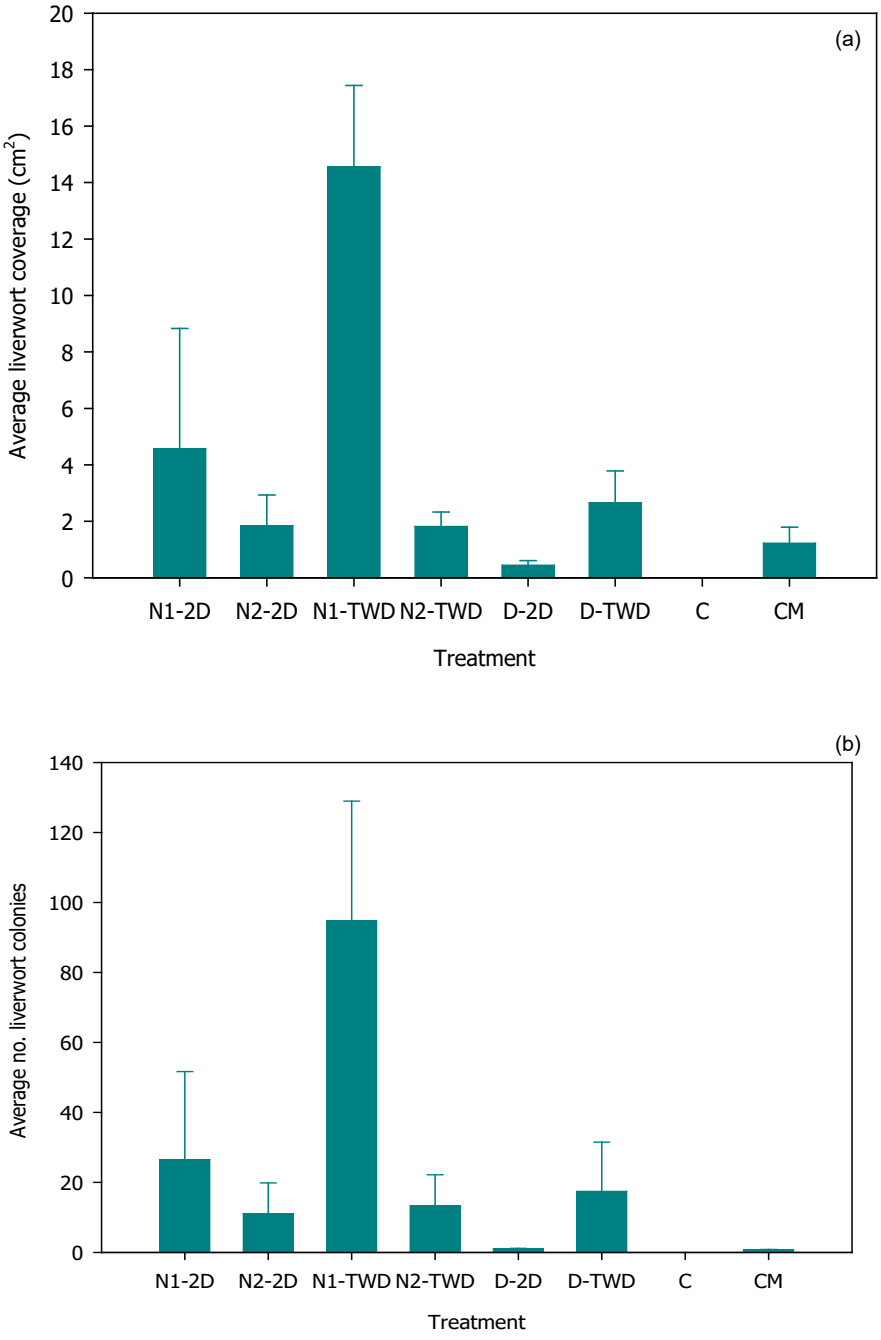


Figure 19. Liverwort infestation after 56 days. (a) Liverwort area. (b) No. of liverwort colonies. Results for each treatment are the average of forty pots arranged in two blocks. N1 = nozzle 1, N2 = nozzle 2, D = drip irrigation, C = control (no irrigation), CM = capillary matting irrigation. 2D = irrigation every two days, TWD = irrigation twice daily.

These results reflect those of the gemma cup replenishment experiment where more gemmae were produced in gemma cups from which gemmae were removed in the 3-day than either the weekly or 4-weekly treatments.

Droplet size distribution graphs were constructed for each nozzle treatment (Figure 21), and overall graphs for the MP Rotator® model 1000 nozzle and Dan modular 180^0 spread nozzles (Figure 20). The greatest number of droplets was produced by the Dan modular nozzle, with a mean diameter of 341 µm diameter, compared to 546 µm diameter droplets produced by the MP Rotator nozzles. The greatest mean number of gemmae dispersed by the nozzle treatments was 60.68 by the MP rotator and 12.25 by the Dan modular nozzles. This suggests that overall the number of gemmae dispersed is dependant on droplet size rather than number of droplets, in accordance with that of the previous gemma dispersal experiment; the mean diameter of those droplets was 161 to 271 µm, smaller than those produced by either the Dan modular nozzles (341 µm) or the MP Rotator (546 µm). The overall mode of droplets produced by the Dan modular nozzles was 160 µm, and of the MP Rotator 182 µm, both falling within the size range of the mode droplet diameters produced by nozzles in the previous experiment. Had smaller droplet sizes been produced during this experiment it may have resulted in a greater number of gemmae being dispersed.

	2-day	**Twice daily**
N1	554	539
N2	249	433

Table 11. Mean droplet sizes (µm) produced by nozzle 1 (MP Rotator model 1000) and nozzle 2 (Dan modular 180^0 spread).

When the data for timing of application and nozzle type are considered separately the trends are less clear (Table 11): the greatest number of gemmae are dispersed by the N1, twice daily, and the least by the N2, twice daily treatments.

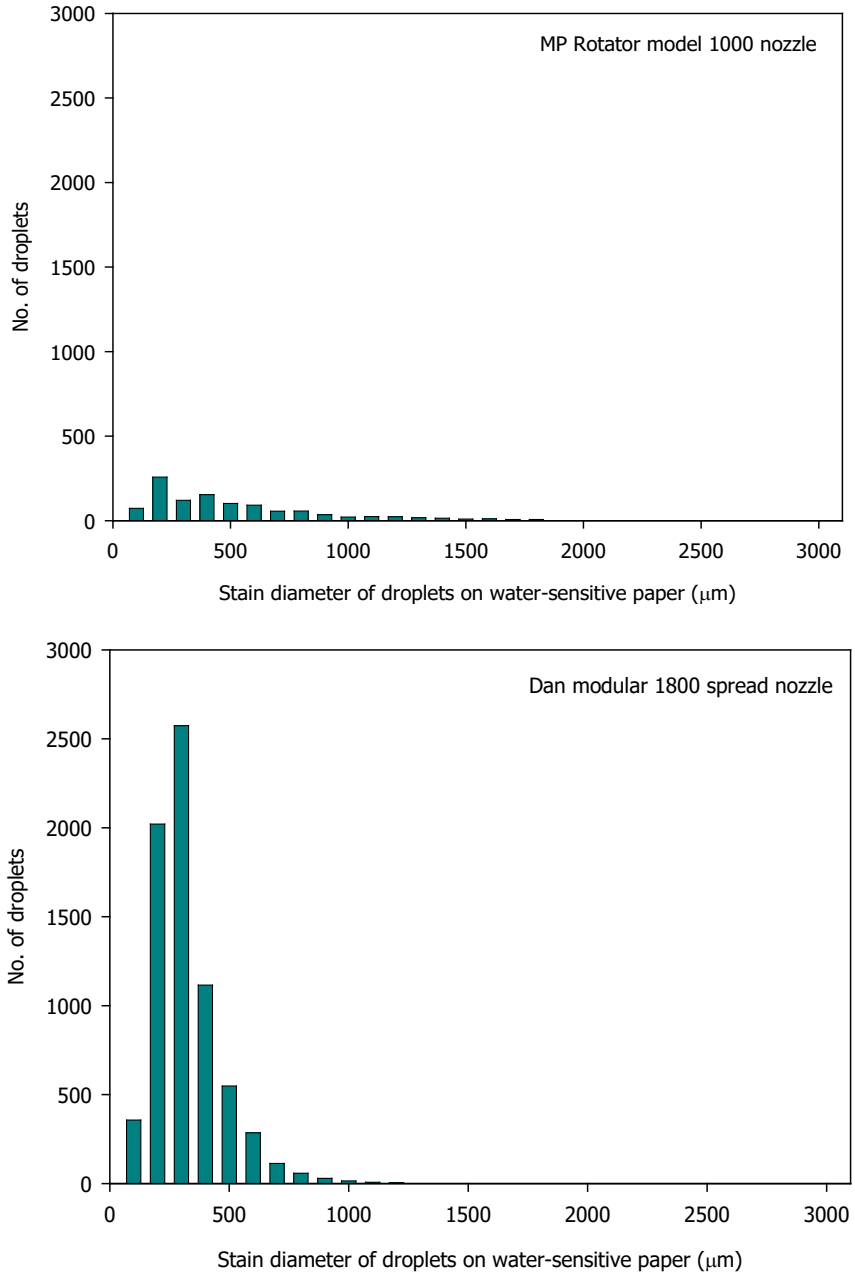


Figure 20. Characteristic size distributions of droplets produced by the MP Rotator® model 1000 and Dan modular 180^0 spread nozzles.

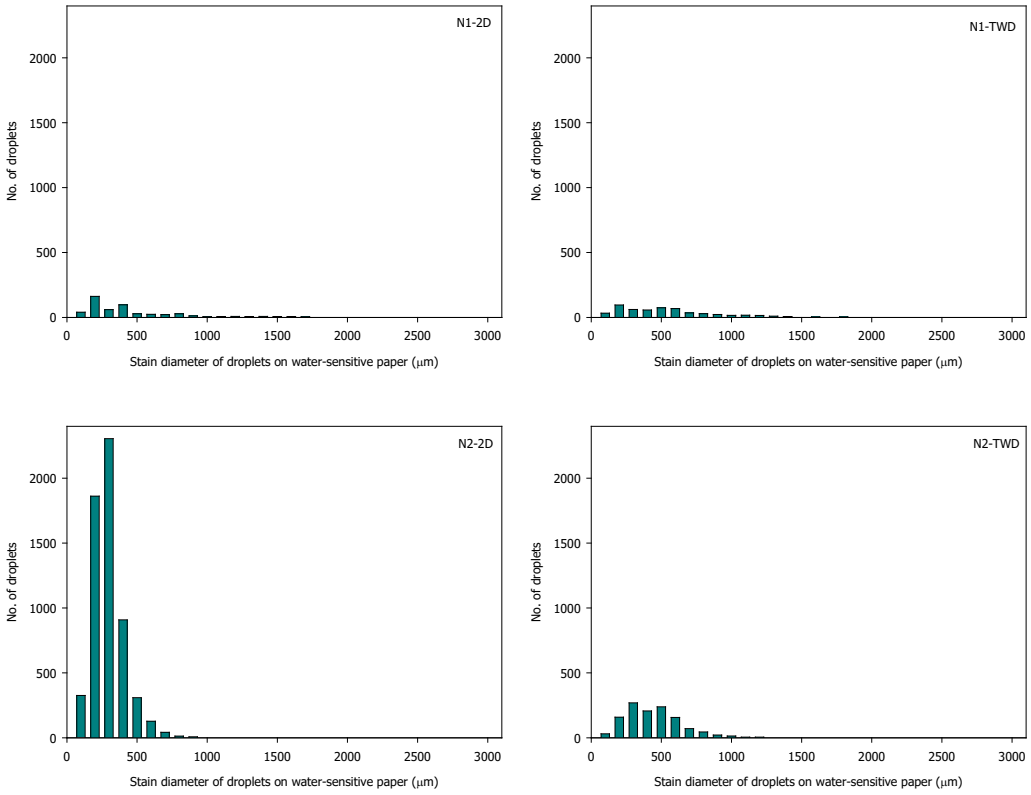


Figure 21. Characteristic size distributions of droplets produced by nozzles. N1 = MP Rotator model® 1000 nozzle, N2 = Dan modular 180^0 spread nozzle, I = block 1, II – block 2, 2D = irrigated every two days, TWD = irrigated twice daily.

Fungal antagonists

Fungal glasshouse experiment

A glasshouse experiment was designed to investigate the effect of *Fusarium equiseti* and *Phaeodothis winteri* on liverwort when applied pre and post emergently to liverwort; the species selected were those that had the greatest effect on liverwort in earlier laboratory tests. A successful pre-emergent mycoherbicide would combat liverwort prior to gemma cup formation on liverwort thalli, minimising spread and colonisation of new areas; post-emergent treatments would act on existing infestations to prevent further spread, and killing the liverwort.

Sterilised compost was used for the fungal treatments to eliminate interference by other fungal species. Three different controls were used (Table 12): sterilised compost; unsterilised compost to compare any effects of the sterilisation process;

and sterilised compost with formulation but no fungal inoculation, to compare any effect of the formulation.

Control	Treatment
Control 1 (C1)	Unsterilised compost only
Control 2 (C2)	Sterilised compost only
Control 3 (C3)	Sterilised compost with formulation only
Fungal application (F)	Formulation with fungal inoculum

Table 12. Controls and fungal treatments

For the pre-emergent treatment the formulation was prepared with and without fungal inoculation, incorporated into sterilised compost and liverwort gemmae dispersed over the surface. For the post-emergent treatment liverwort gemmae were established on sterilised and unsterilised compost to produce healthy, vigorous colonies ready for inoculation with a combination of spores and mycelium.

An appropriate inoculation carrier was required for the pre-emergent treatment. A method was adapted from that of Blok and Bollen (1997), who used a soil meal culture containing potting compost amended with 5% oatmeal, autoclaved for 30 mins on two consecutive days, inoculated with fungus and then incubated at 23 C for 2-3 weeks.

Method

Compost sterilisation

700 g quantities of Imperial College compost media, comprised of a peat and grit substrate with base fertilisers, were autoclaved at 126 C for 30 mins in unsealed bags, six at a time to allow maximum steam penetration, and then immediately sealed.

Fungal species

Fungal species used for this experiment were *Fusarium equiseti* and a mixture of four isolates of *Phaeodothis winteri*, all of which showed vigour and had exhibited parisitising potential against liverwort in laboratory bioassays (Table 13).

Fungal species	No. fungal plugs
Phaeodothis winteri, 102466, 102483, 551.63, 162.31	3
Fusarium equiseti	6

Table 13. Fungal species used for pre and post-emergent treatment, and inoculum quantities used for pre-emergent treatment.

Post-emergent treatment

Liverwort preparation

Seed trays (19 x 23 cm) containing 700g autoclaved and unsterilised compost were each inoculated with liverwort gemmae: 5 gemma cups were placed with 10 ml sterile water in a centrifuge tube, agitated to separate and suspend the gemmae, and dispersed evenly over the compost; any remaining gemmae were rinsed out onto the compost with a further 10 ml water. The gemmae were then left to establish for eight weeks in the glasshouse (Table 14).

Parameter	Setting
Day length	16 hrs
Supplementary lighting	400 watt MBF/U
Temperature, 24 hrs	18 C
Vent temperature	21 C
Irrigation	Rainwater

Table 14. Glasshouse environmental conditions

Spore inoculum preparation

A small amount of sterile distilled water was poured over a Petri dish of sporulating fungi. A flame sterilised inoculating loop was used to scrape the surface of the fungi, releasing spores, and the fluid poured into a centrifuge tube, centrifuged for 2.5 mins at 3000 rpm, the water poured off and the spores and mycelium re-suspended in sterile distilled water. The number of colony forming units (spores and mycelium fragments) was estimated using a haemocytometer, the samples re-centrifuged, the supernatant removed and fungal material re-suspended in the carrier, a solution of 0.05% (0.5 mL) Tween 20 and 0.01% (0.1 g) glucose in 1 L sterile distilled water to achieve the required concentrations (Table 15); the glucose provided nutrients and

the Tween 20 helped to separate the fungal material establishment, aiding even dispersal.

Fungal species	Colony forming unit concentration
Phaeodothis winteri 551.63, 163.91, 102466, 102483	3.86 x 10^6
Fusarium equiseti	1.0 x 10^6

Table 15. Post-emergent treatment fungal inoculum concentration

A second application of spores was carried out after 3 weeks (Table 16) with inoculum applied to one randomly selected tray per cabinet of the post-emergent treatments: I-post-Fe-f1, II-post-Fe-f3, I-post-Pw-f2, II-post-Pw-f3 as the liverwort appeared to be outgrowing the fungal infection initially observed.

Fungal species	Colony forming unit concentration
P. winteri 551.63, 163.91, 102466, 102483	1.93 x 10^6
Fusarium equiseti	1.64 x 10^6

Table 16. Post-emergent treatment fungal inoculum concentration – 2nd inoculation

Post-emergent treatment application

For the post-emergent fungal treatments, 20 ml quantities of fungal inoculum of either *Fusarium equiseti* or *Phaeodothis winteri* were applied as a spray onto each tray of established liverwort. For control 3 (Table 12), sterilised compost with formulation, 20 ml quantities of carrier (glucose and tween 20 in distilled water) without fungal inoculum was applied. Controls 1 and 2 did not require any additions to the compost.

Pre-emergent treatment

Inoculum preparation

Two pre-emergent formulations were prepared using oatmeal and bran as a nutrient supply: Sixteen conical flasks, eight containing 70 g oatmeal finely ground in a food processor, and eight containing 35 g flaked bran (bran was bulkier than ground

oatmeal), all with 300 ml water were autoclaved for 20 mins at 121 C on two consecutive days to ensure sterility. Fungal plugs, taken using a No. 3 cork borer, were added to half the flasks of formulation as detailed in Table 13, two flasks each of oatmeal and bran per fungal group, and incubated on a shaker (Innova™ 4330 refrigerated incubator shaker, New Brunswick Scientific) at 120 rpm, 23 C for 7 days. 75 ml sterile water was added to each flask and incubated for 14 days, then 100 g sterilised compost was added to each flask to bulk up the inoculum and incubated for a further 12 days.

It was clear from the large amount of mycelium found that both *P. winteri* and *F. equiseti* had proliferated more in the oatmeal formulation, and this was used for the experiment. 70 g quantities of formulation, with and without inoculum (fungal mycelium was broken into small pieces by hand) were evenly incorporated into 700 g sterilised compost to form six trays each of control 3 and fungal treatment for each fungal species.

Both pre- and post-emergent fungal treatments were placed in a humid environment prior to transference to growth chambers for 24 hrs to encourage the fungi to establish.

Liverwort gemmae were applied to the surface of the inoculated compost as for the post-emergent treatment, using 5 gemma cups in 10 ml sterile water in a centrifuge tube, dispersing them evenly over the compost and then rinsing any remaining gemmae out onto the compost with a further 10 ml water.

Experimental design

Eight growth cabinets were prepared with a base of perforated polythene covered with capillary matting. They were set at 23 C, using natural daylight and day length, and watered daily. Three trays each of controls 1 to 3 and the appropriate fungal treatment were placed in each growth cabinet. Blocks, cabinets and tray positions were randomly assigned (Figure 22).

The growth cabinets were fitted with fans to circulate air, providing an even temperature, therefore each tray was fitted with a clear lid (supplier: William Sinclair Horticulture) to prevent contamination; trays therefore required careful handling during data collection, with lids removed from one tray at a time, once removed from the growth cabinet.

Data collection

An A4 size grid of 1 cm squares on clear plastic was placed over trays of liverwort and used as a template to draw liverwort and dieback precisely. An outline of liverwort and dieback areas were drawn, scanned into a computer and the area calculated using ImageJ digital image analysis.

Area of liverwort coverage and dieback were recorded weekly, initially for 5 weeks and then a final assessment after 11 weeks. Possible contaminants present on the compost surface were re-isolated from trays onto PDA.

Block 1

Post emergent, Fusarium equiseti			
C2	F	F	C1
C1	C3	C3	C3
C2	F	C1	C2

Pre emergent, Fusarium equiseti			
C1	F	F	F
C1	C3	C3	C3
C1	C2	C2	C2

Post emergent, Phaeodothis winteri			
C1	C2	C1	C1
F	C3	C3	C3
F	F	C2	C2

Pre emergent, Phaeodothis winteri			
C1	C1	C1	F
F	F	C3	C3
C2	C2	C2	C3

Block 2

Pre emergent, Phaeodothis winteri			
C1	F	F	C1
C1	F	C2	C3
C2	C3	C3	C2

Pre emergent, Fusarium equiseti			
C1	C3	C3	C2
F	C1	C1	F
C2	C3	F	C2

Post emergent, Fusarium equiseti			
C1	C3	F	C2
F	F	C3	C3
C1	C2	C2	C1

Post emergent, Phaeodothis winteri			
F	C3	C3	C3
C1	C1	F	C1
F	C2	C2	C2

Figure 22. Experimental layout of randomly assigned controls and treatments, each 12 tray set contained within one growth chamber. Block 1=glasshouse 1; block 2=glasshouse 2; C1=control 1, unsterilised compost; C2=control 2, sterilised compost only; C3=control 3, sterilised compost with formulation only; F=fungal treatment applied in formulation.

After four weeks post-inoculation, reisolations from fungal growth in the *P. winteri* and *F. equiseti* treatments were made onto potato dextrose agar (PDA), incubated and identified. At week 5 tissue samples with lesions were taken and examined microscopically. Liverwort tissue was soaked in 95% methanol in a water bath at 65 C for approximately 30 mins to remove chlorophyll, dipped in chloralhydrate to clear the tissue, and then lactophenol tryphan blue stain before mounting onto

microscope slides. To make slides semi-permanent tissue was mounted in 50% glycerol (Mansfield, 2005).

Results

Although the compost was sterilised and efforts were made to produce uncontaminated inoculum, it was not practical to keep the experiment completely sterile. However, the aim was to test the effect of fungal treatments in conditions reflecting those found in nurseries, albeit with temperature control and minimising cross-contamination of treatments and controls. Post-emergence treatments were prepared using sterilised compost to mitigate the effects of other fungal species in the compost, so that any infections observed should be due to those applied in the treatments; however they were subject to contamination from air-borne fungal spores prior to inoculation and from run-off water via the capillary matting throughout the experiment.

Five days post-inoculation, fungal mycelium was observed growing over the compost surface in the pre-emergence trays. Liverwort gemmae had germinated and liverwort thalli were growing vigorously (Figure 23), and by 21 days *F. equiseti* mycelium could be seen colonising the liverwort thallus (Figure 24). Fungi were successfully reisolated from inoculated treatments and (Table 17), except from the post -emergence *F. equiseti* treatments (II-post fe-f).

Figure 23. Pre-emergence treatments (a) II-pre pw–f (b) II-pre-fe-f, 5 days post-inoculation. I= block 1, II = block 2, pre = pre-emergence, post = post-emergence, fe = *F. equiseti*, pw – *P. winteri*, f = fungal treatment.

Figure 24. Liverwort thallus colonised with *F. equiseti* mycelium. Pre-emergence treatment (II-pre-fe-f) 14 days post-inoculation. II = block 2, pre = pre-emergence, fe = *F. equiseti*, f = fungal treatment.

Table 17 Record of successful reisolation of fungi from fungal treatments. I= block 1, II = block 2, pre = pre-emergence, post = post-emergence, Fe = *F. equiseti*, Pw – *P. winteri*.

Treatment	Date	
I-Pre Fe	01/06/06	30/07/06
II-Pre Fe	01/06/06	30/07/06
I-Pre Pw	01/06/06	30/07/06
II-Pre Pw	01/06/06	30/07/06
I-Post Fe	01/06/06	30/07/06
II-Post Fe	-	-
I-Post Pw	01/06/06	-
II-Post Pw	01/06/06	30/07/06

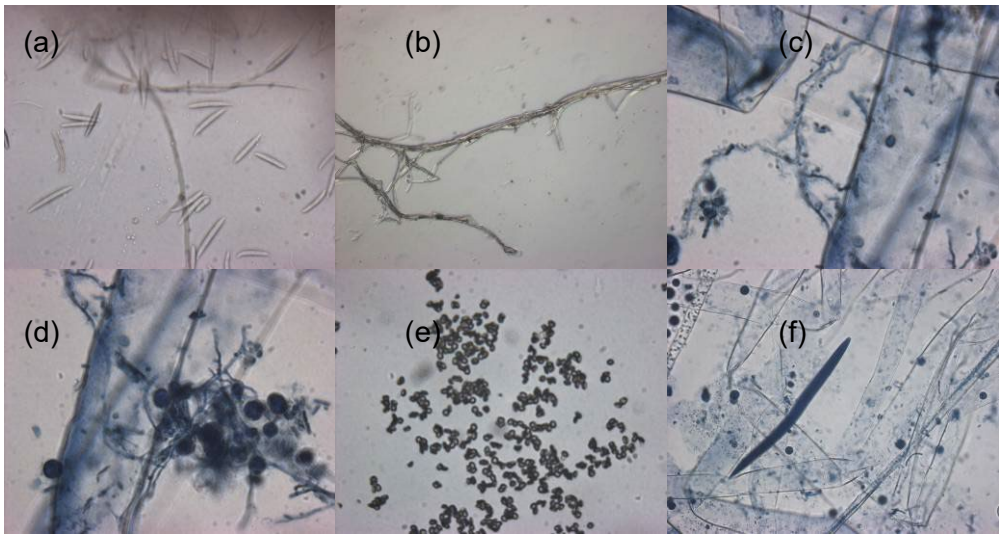


Figure 25. Microscopic analysis of infected liverwort tissue 5 weeks post inoculation. *F. equiseti* (a) macroconidia x400 (b) mycelium, x200 and *P. winteri* (c) and (d) mycelium wrapped around rhizoids x400 (e) spores x400 (f) an unidentified nematode among liverwort rhizoids x400

Microscopic analysis (Section 6.2.4) of infected liverwort tissue indicated presence of *F. equiseti* and *P. winteri* in their respective treatments. The *F. equiseti* was identifiable from its macroconidia (Figure 25a) although the mycelium appears to be growing around liverwort rhizoids rather than penetrating (Figure 25b). Similarly, spores and mycelium of *P. winteri* were identified around liverwort (Figure 25c-d). An unidentified nematode was also observed (Figure 25f) which may have been introduced with the unsterilised control treatment.

After 11 weeks liverwort growth was extremely vigorous in most treatments (Figure 26a) and outgrew the trays so the lids no longer fitted closely. However, in a minority of treatments the liverwort did not cover the compost (Figure 26b). Although efforts had been made to use sterile compost in controls 2 and 3, because the trays had been exposed in the glasshouse whilst the liverwort established they were unlikely to be sterile at the start of the experiment, and fungal mycelium was present in some (Figure 26c). Fungal mycelium was present within the gemma cups of a number of treatments, across both fungal species and all controls, in pre- and post-emergence treatments, indicating some cross contamination had occurred (Figure 27a-c).

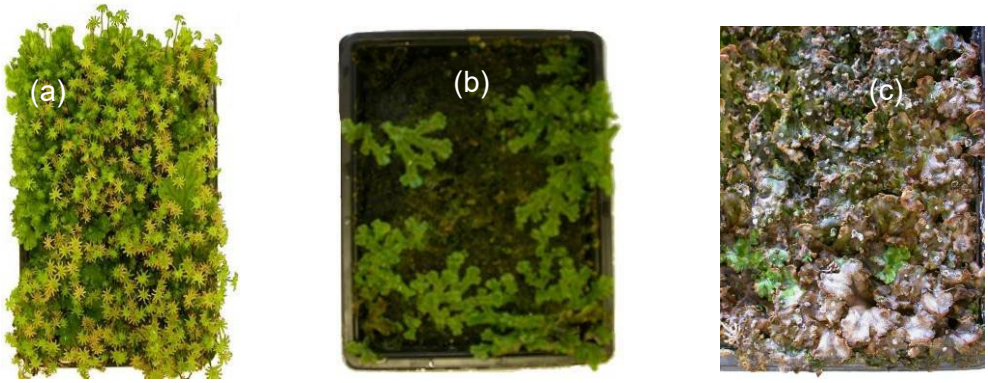


Figure 26. Liverwort growth after 11 weeks (a) II-pre fe-C3 (b) II-post pw-C3 (c) area of contamination, I-post pw-C2. I= block 1, II = block 2, pre = pre-emergence, post = post-emergence, fe = *F. equiseti*, pw – *P. winteri*, f = fungal treatment, C2=control 2 sterilised compost only, C3=sterilised compost with formulation.

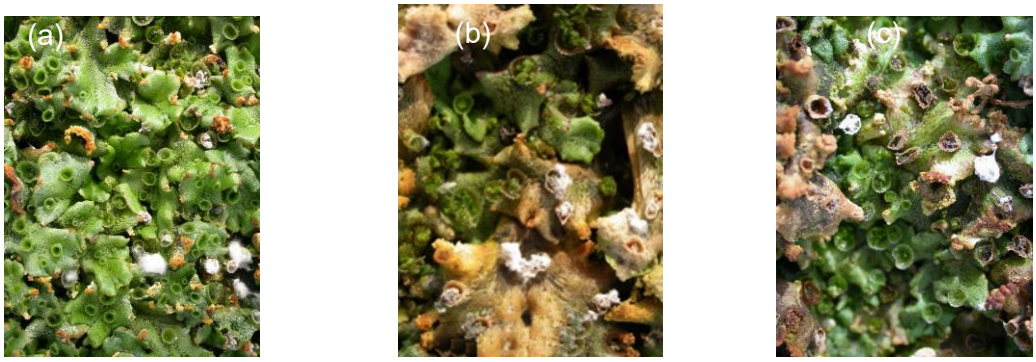


Figure 27. Liverworts with gemma cups infested with fungal mycelium (a) II-post pw-C3 (b) I-pre fe-f (c) II-post pw-f. I= block 1, II = block 2, pre = pre-emergence, post = post-emergence, fe = *F. equiseti*, pw – *P. winteri*, f = fungal treatment, C3 = control 3, sterilised compost with formulation,

The data for this experiment is presented and analysed separately for total, healthy (total area less dieback area), and dieback areas of liverwort. Analysis was by analysis of variance (ANOVA) and mean separations where appropriate, using Genstat for Windows, 8th Edition. The results of the analyses are summarised within the text and ANOVA tables are presented in Appendix 4.

Pre-emergence *P. winteri* treatment

For the pre-emergence *P. winteri* treatment, at week 3 (Figure 28) the total ($v.r._{3,3}$= 14.97, P<0.05) and healthy areas ($v.r._{3,3}$= 21.98, P<0.05) of liverwort were significantly less when grown in controls 1 and 2 (unsterilised and sterilised compost) than when treated with the fungus formulation; there was no dieback by this stage. By week 5, least liverwort dieback was observed, with control 2 (sterilised compost) and then the fungal treatment. Greatest dieback occurred using control 3 (sterilised compost with formulation). By weeks 5 and 11 (Figure 29) there was no significant difference in liverwort area between the *P. winteri* treatment and any of the controls; at week 11 the greatest dieback occurred in the fungal treatments, but again this was not significant.

Pre-emergence *F. equiseti* treatment

For the pre-emergence *F. equiseti* treatment the total area of liverwort was significantly less at week 3 ($v.r._{3,3}$= 17.27, P<0.05) and significantly less at week 5 ($v.r._{3,3}$= 46.74, P<0.05)(Figure 30), and healthy liverwort area was significantly less at weeks 3 and 5, when grown in control 2 (sterilised compost) than when treated with the fungal formulation or controls 1 (unsterilised compost) and 3 (sterilised compost with formulation). However, two of the six liverworts grown in control 1 were markedly smaller than the remainder, at 7822 mm^2 and 6614 mm^2 compared to the mean (of six liverworts) of 17,043 mm^2 at week 5, and this may be distorting the results. Figure 30 represents growth with the outliers removed. Dieback at week 5 was least with control 1. As with the pre-emergence *P. winteri* treatment, greatest dieback was observed with control 3, suggesting the formulation had the greatest effect on liverwort dieback. However, closer inspection of the data revealed that for *F. equiseti*, within control 3 only one liverwort exhibited any dieback (10,892 mm^2) in week 5 and again this seems to be distorting the figures, and the small areas of dieback present in control 3 in weeks 3 and 4 were no longer apparent. By week 11 total and healthy areas of liverwort were not significantly different among the treatments (Figure 29), although dieback area of the *F.equiseti*-treated liverwort was greater than the controls ($v.r._{3,3}$= 5.36, P<0.05).

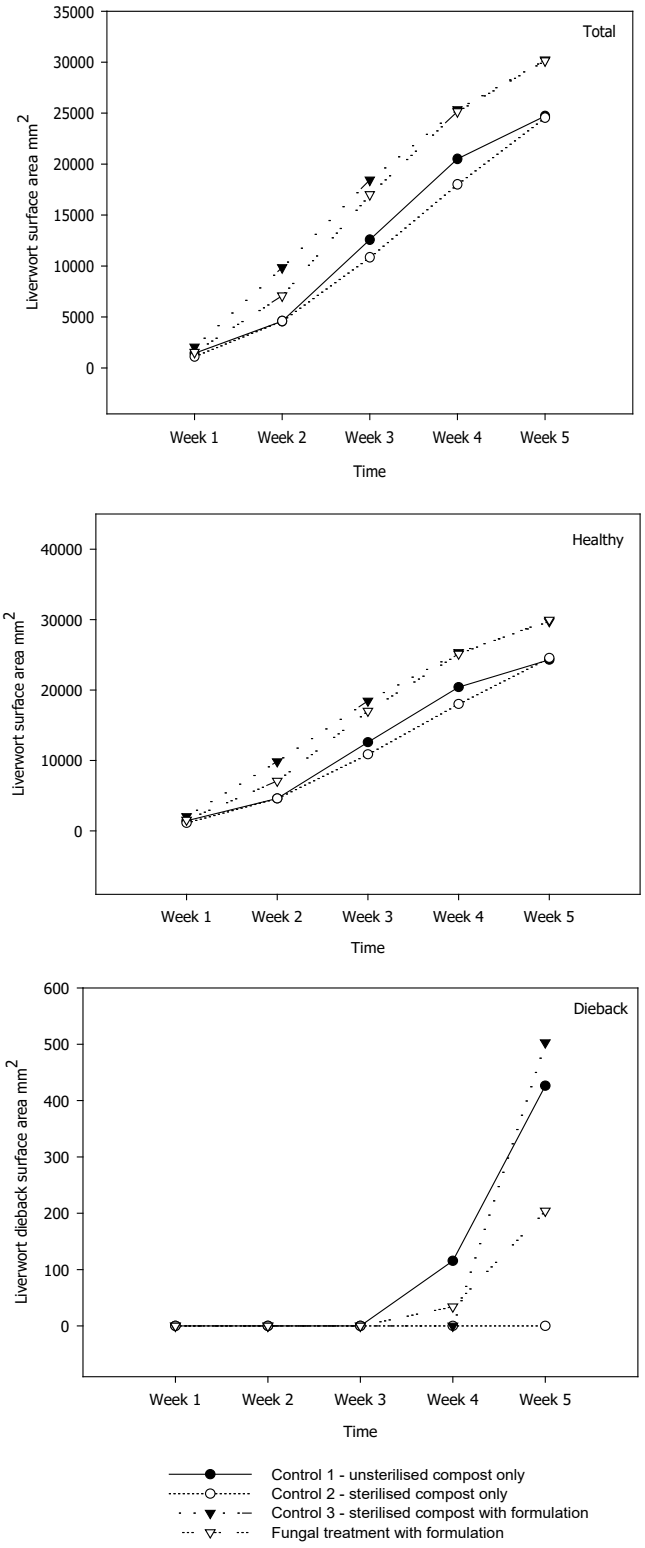


Figure 28. Areas of liverwort treated pre-emergence with *P. winteri*

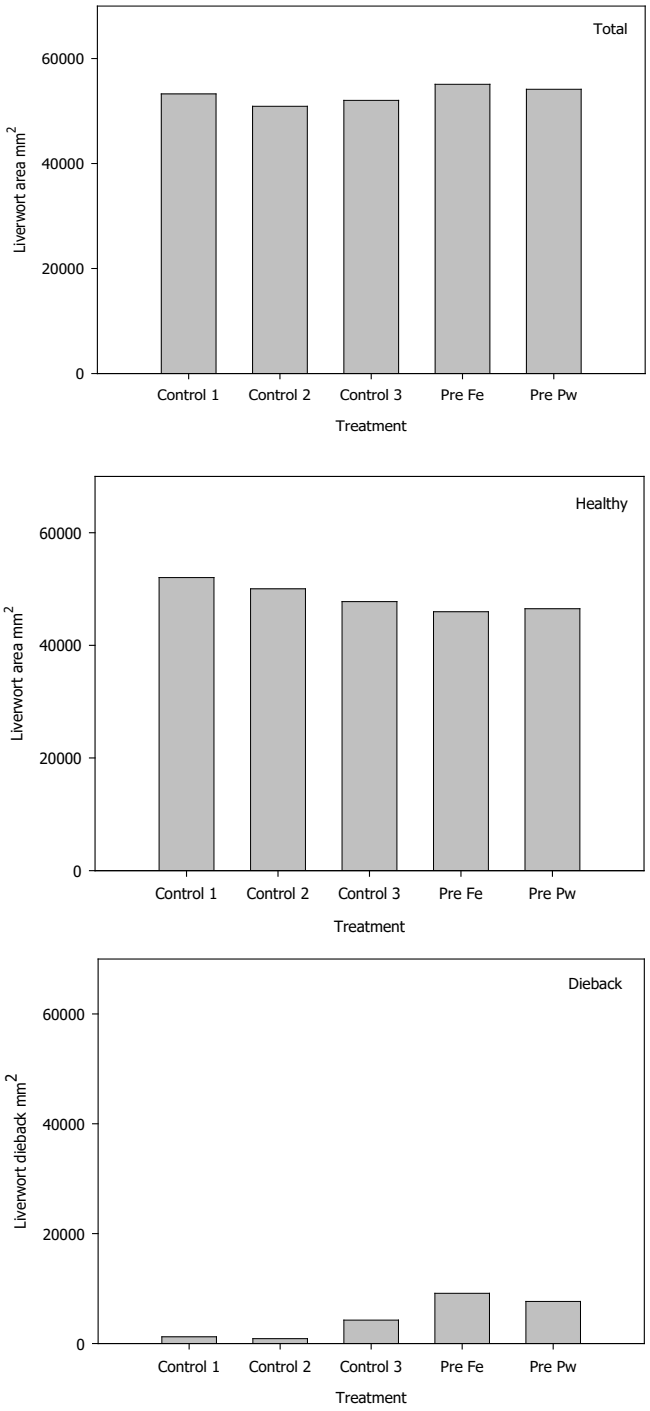


Figure 29. Areas of pre-emergence treated liverwort after 11 weeks. Control 1 = unsterilised compost only. Control 2 = sterilised compost only. Control 3 = sterilised compost with formulation. Pre Fe = pre-emergence *F. equiseti* treatment. Pre Pw = pre-emergence *P. winteri* treatment.

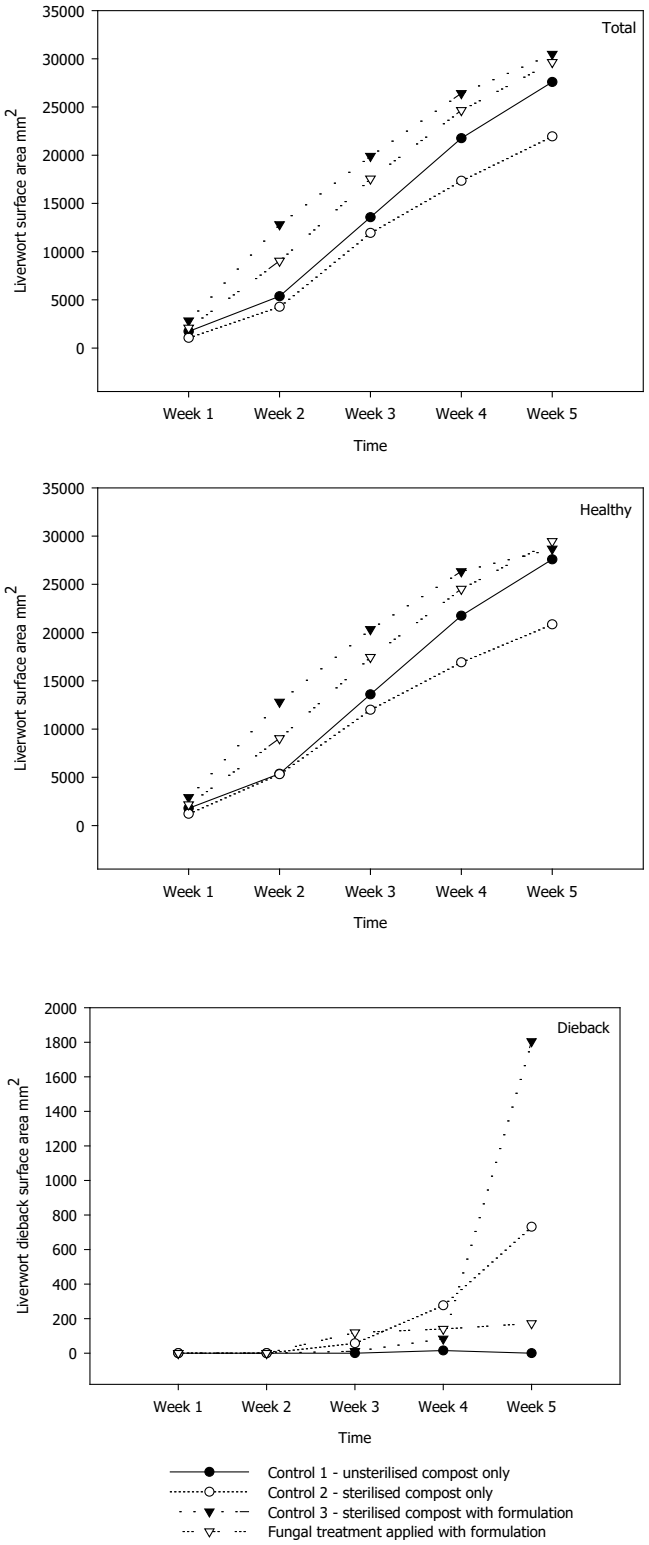


Figure 30. Areas of liverwort treated pre-*emergence* with *F. equiseti*. Total and healthy liverwort areas represented with two outliers removed.

Post-emergence *F. equiseti* treatments

For the post-emergence *F. equiseti* treatment, at week 3 the total ($v.r._{3,3}$= 18.86, P<0.05) (Figure 31) and healthy ($v.r._{3,3}$= 15.79, P<0.05) (Figure 32) liverwort areas were significantly less when grown in unsterilised compost than the *F. equiseti* and sterilised treatments. There was no significant difference between dieback areas of liverwort grown in any treatments, including the unsterilised compost ($v.r._{3,3}$= 2.60, P<0.05)(Figure 33).

At week 3 one randomly selected fungal-treated tray from each block was inoculated for a second time; therefore for statistical analysis and graphs, figures are averages of one tray per block for '2 application' treatments, and two trays per block for '1 application' treatments.

By week 5, for the post-emergence *F. equiseti* treatment both the total ($v.r._{3,3}$= 63.89, P<0.05) (Figure 31) and healthy ($v.r._{3,3}$= 83.08, P<0.05) (Figure 32) liverwort area of control 1 (unsterilised compost) was significantly ess than when treated with 1 application of fungal inoculum. For those trays treated with two fungal applications, although liverwort growth was less than with a single fungal application, total and healthy liverwort area was still greater than with control 1 (unsterilised compost). There was no significant difference between control 1 when compared with 2 fungal application treatments for either total ($v.r._{3,3}$= 3.39, P<0.05) or healthy ($v.r._{3,3}$= 1.21, P<0.05) liverwort area. There were no significant differences in dieback area for any of the treatments (Figure 33). Although liverwort dieback was greatest for the fungal treatment with the second fungal application (Figure 33b), this was not found to be significant ($v.r_{3,3}$ = 0.92, P<0.005) and was due to a single tray of liverwort with a dieback area over 1000 mm^2 at 8375 mm^2.

By week 11, there was no significant difference in Figure 34), healthy (Figure 35) or dieback (Figure 36) liverwort areas with one or two post-emergence *F. equiseti* applications. With one fungal application total liverwort area was least in control 1 (unsterilised compost) and greatest with the fungal application; and with a second fungal application least in control 1 (unsterilised compost) and greatest in control 3 (sterilised compost with formulation), indicating an effect of the second fungal application, albeit not significant. This trend was reflected with healthy liverwort area (Figure 35) where liverwort area was least in control 1 (unsterilised compost) with both one and two fungal applications. When comparing fungal treatments, liverwort

area was less with two fungal applications than one, again indicating an effect of the second fungal application.

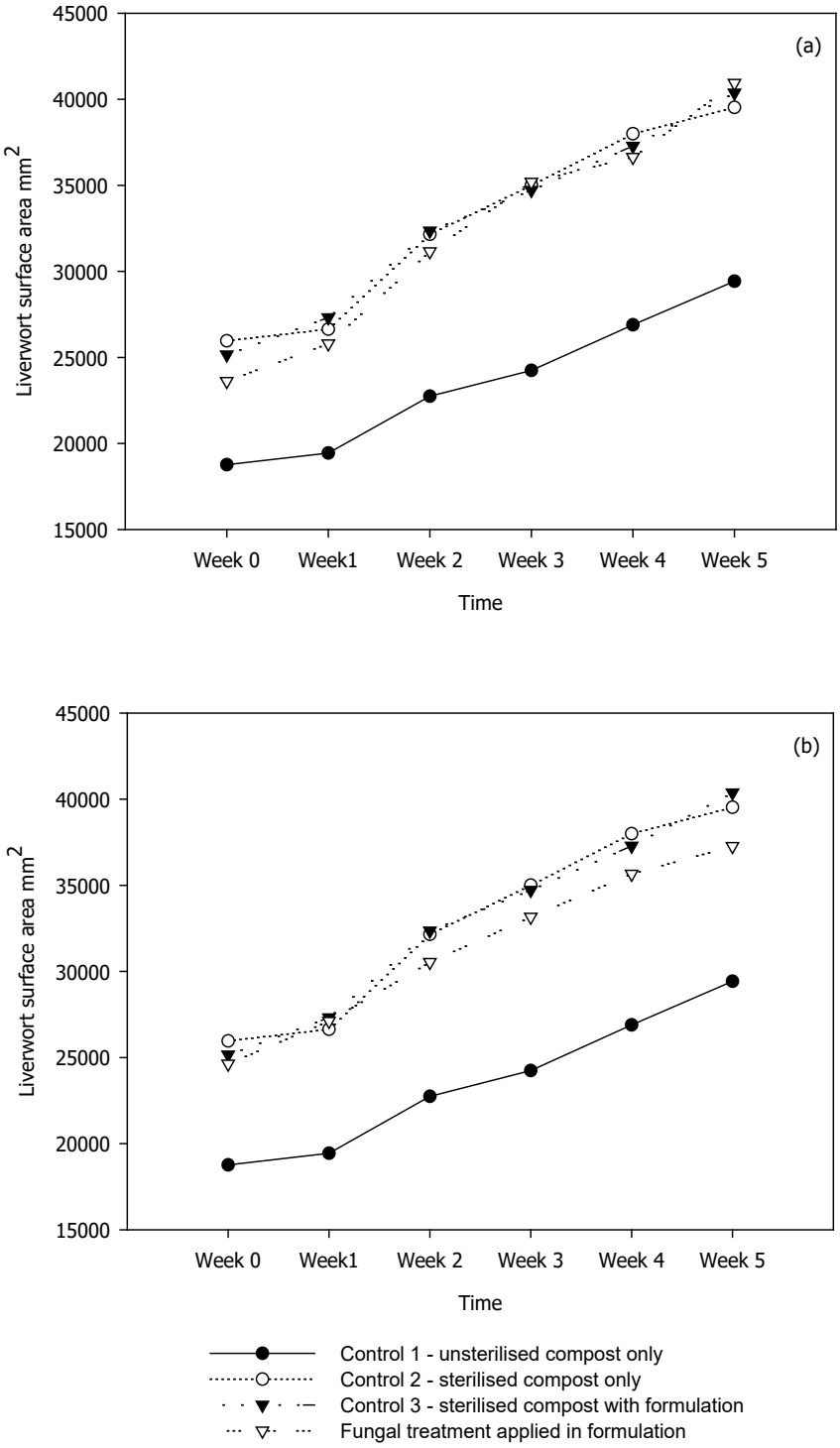


Figure 31. Growth curve of total liverwort area of liverwort subjected to (a) one and (b) two applications of *F. equiseti*

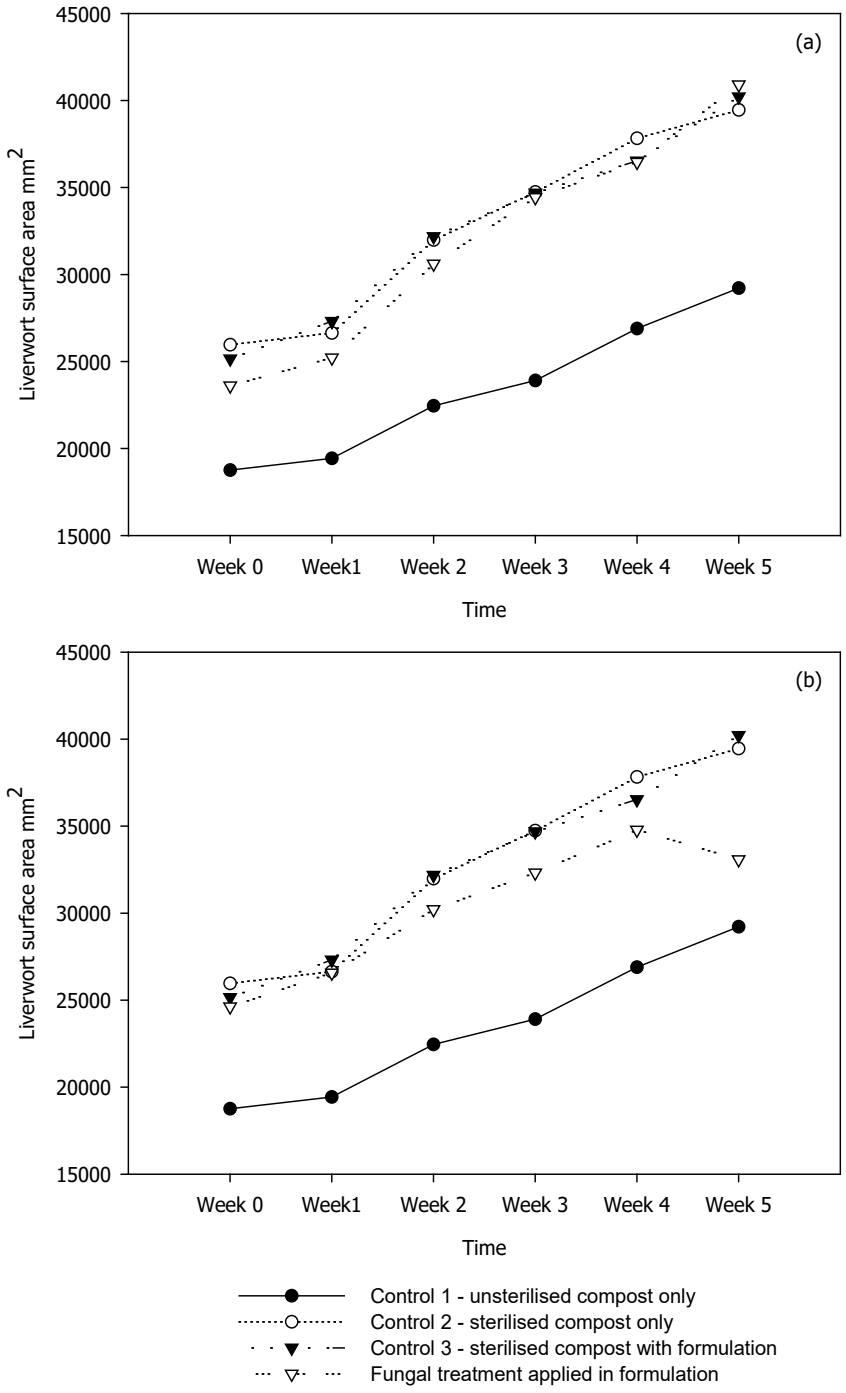


Figure 32. Growth curve of healthy liverwort subjected to (a) one and (b) two applications of *F. equiseti*

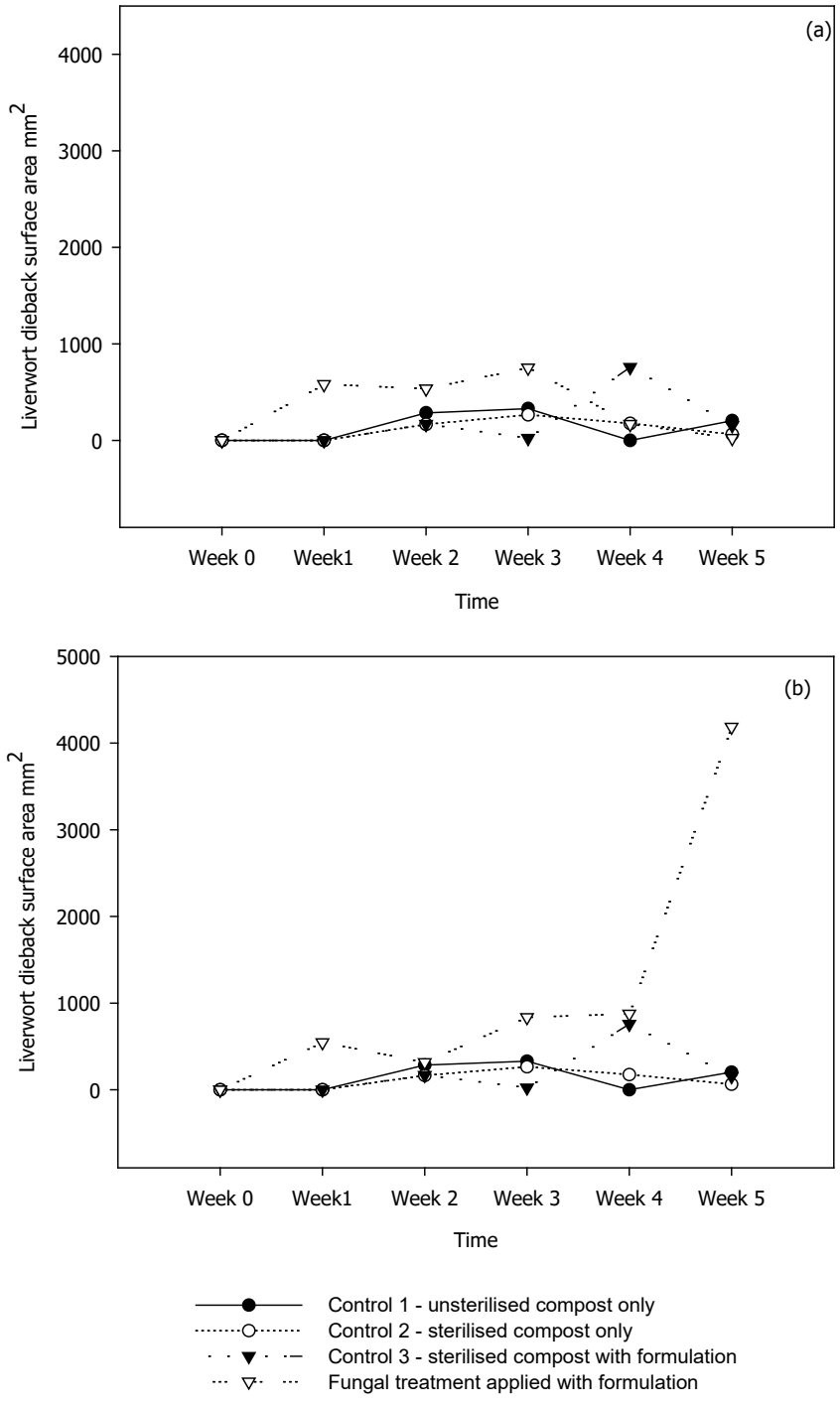
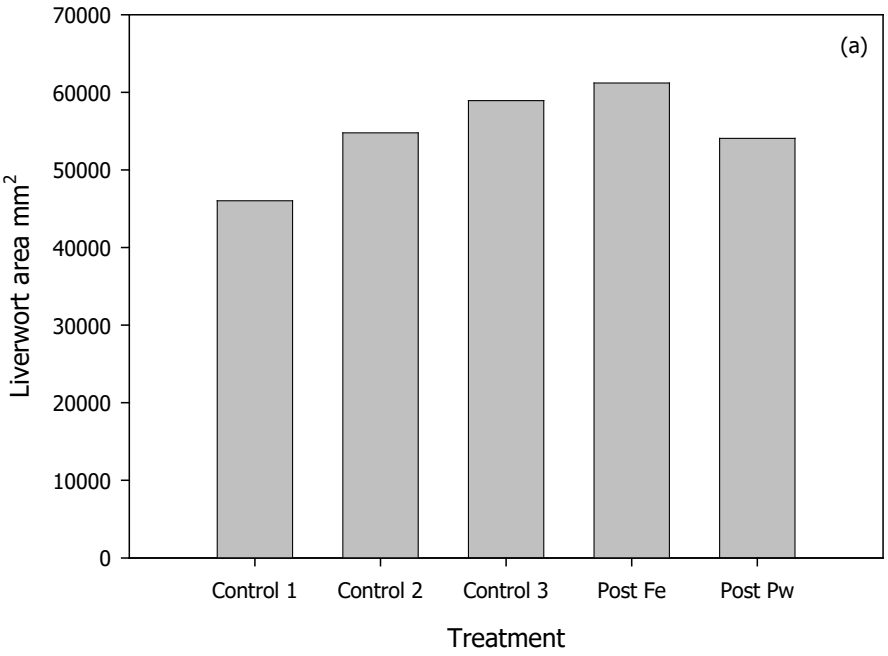


Figure 33. Growth curve of liverwort dieback, subjected to (a) one and (b) two applications of *F. equiseti*



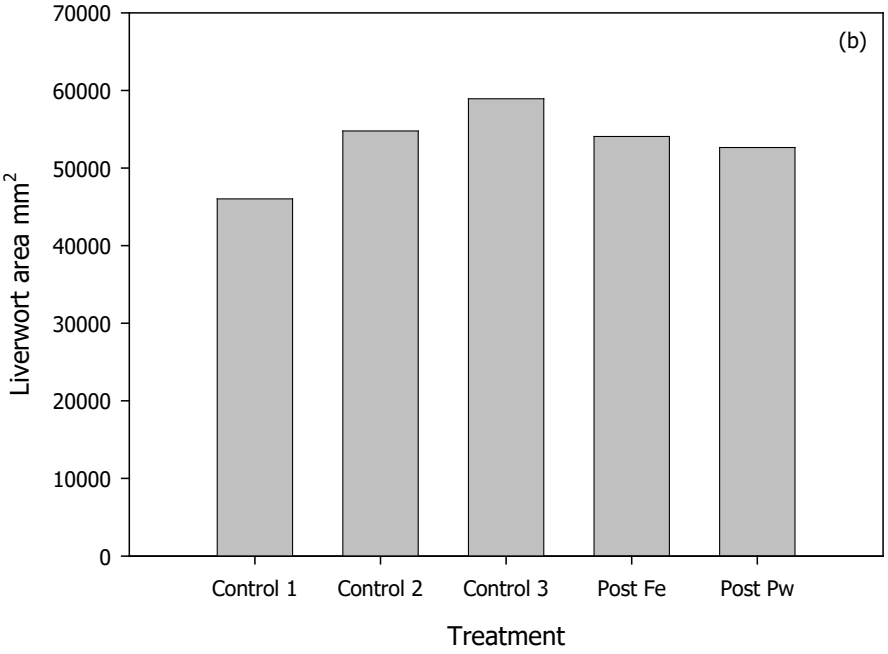


Figure 34. Total area of liverwort subjected to (a) one and (b) two applications of fungal inoculum, after eleven weeks. Fe=*Fusarium equiseti*, Pw=*Phaeodothis winteri*. Control 1 – unsterilised compost only, control 2 - sterilised compost only, control 3 sterilised compost with formulation.

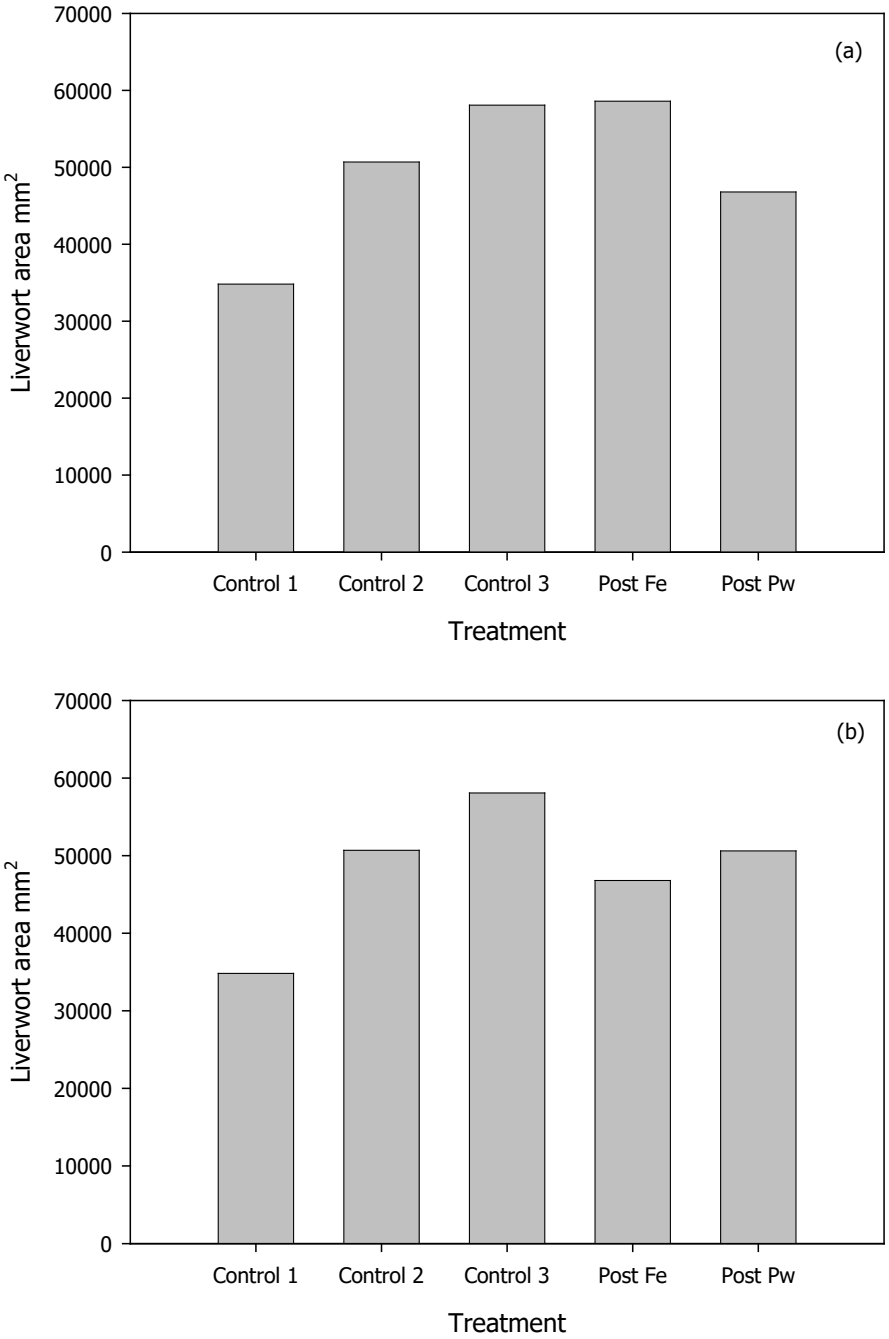


Figure 35. Area of healthy liverwort subjected to (a) one and (b) two applications of fungal inoculum, after eleven weeks. Fe=*Fusarium equiseti*, Pw=*Phaeodothis winteri*, Control 1 – unsterilised compost only, control 2 - sterilised compost only, control 3 sterilised compost with formulation.

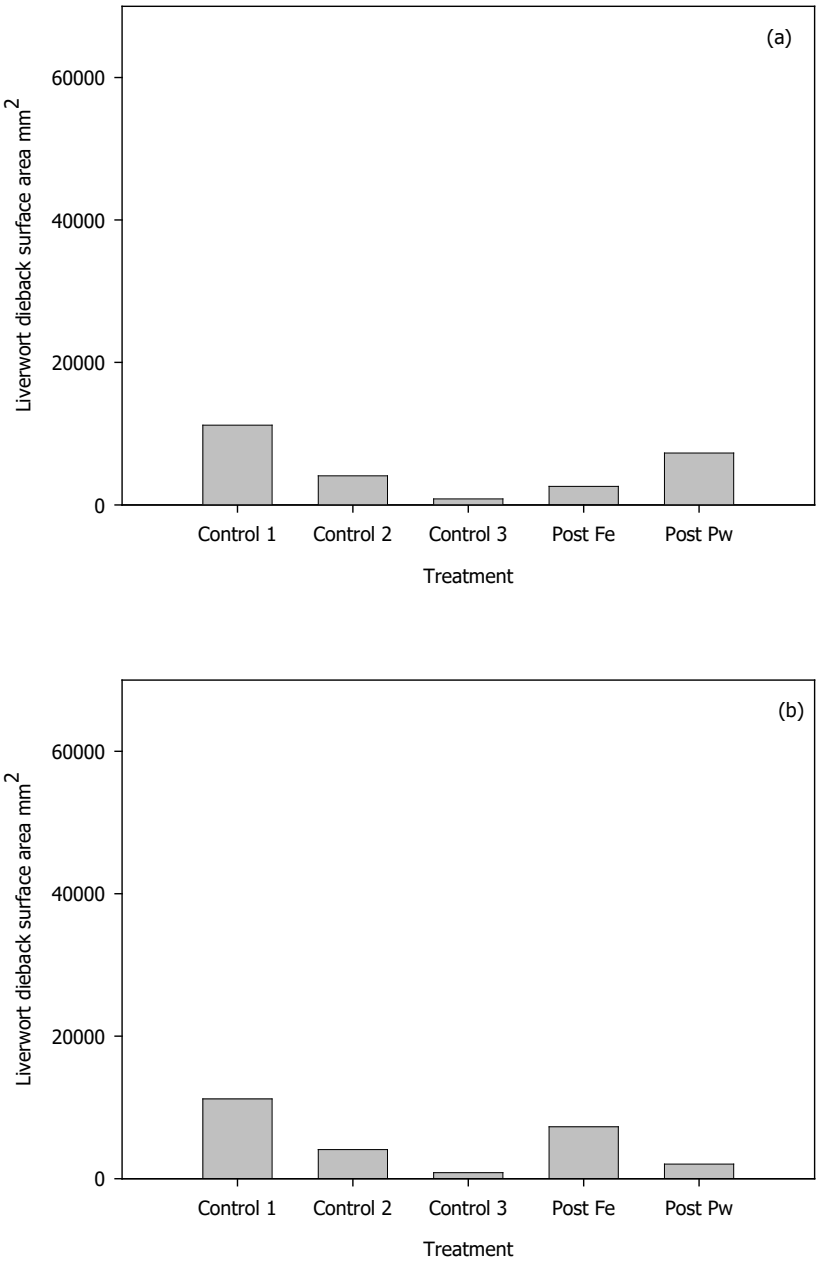


Figure 36. Area of dieback of liverwort subjected to (a) one and (b) two applications of fungal inoculum, after eleven weeks. Fe=*Fusarium equiseti*, Pw=*Phaeodothis winteri*, Control 1 – unsterilised compost only, control 2 - sterilised compost only, control 3 sterilised compost with formulation.

Post-emergence *P. winteri* treatments

After 3 weeks, for the post emergence treatment of *P. winteri* there was a significant difference between the total ($v.r._{3,3}$= 11.96, P<0.05) (Figure 37) and healthy ($v.r._{3,3}$= 11.92, P<0.05) (Figure 38) liverwort areas compared to control 1 (unsterilised compost), with the liverworts in the control smaller than those treated with fungal inoculum. This effect was also seen with the post-emergence *F. equiseti* treatments, strongly suggesting that unsterilised compost is more effective than the fungal treatments at suppressing liverwort growth. There were no significant differences between dieback areas of fungal treatments and controls ($v.r._{3,3}$= 0.73, P<0.05) (Figure 39). As with the *F. equiseti*, a second inoculation of one tray from each block was carried out in week 3, and the effect of this was observed in week 5. Total liverwort area was significantly less in control 1 (unsterilised compost) than the fungal treatment with either one ($v.r._{3,3}$= 11.82, P<0.05) or two ($v.r._{3,3}$= 9.80, P<0.05) fungal applications (Figure 37) However, there was no significant difference in healthy liverwort area (Figure 38) or dieback area (Figure 39) between the fungal treatment and controls with one or two fungal applications. With two fungal applications (Figure 39b) the dieback area peaked during week 3, but then decreased in size suggesting that the liverwort had overgrown the area of dieback. This trend was also seen between weeks 3 and 4, but the dieback area was increasing again by week 5 (Figure 39a).In the final assessments, at week 11, the total liverwort area of control 1 (unsterilised compost media) was significantly less than the fungal treatment with two fungal applications when data was transformed using natural logs ($v.r._{3,3}$= 16.64, P<0.05) (Figure 34); there were no significant differences observed with one fungal application. No significant difference was observed between healthy (Figure 35) or dieback (Figure 36) liverwort areas with either one or two fungal applications; however, the healthy liverwort area was less with one fungal application than two, indicating no benefit in the second application. Dieback area was greater with one and two fungal applications than control 3 (sterilised compost with formulation) suggesting a greater effect with fungal treatment than with formulation alone.

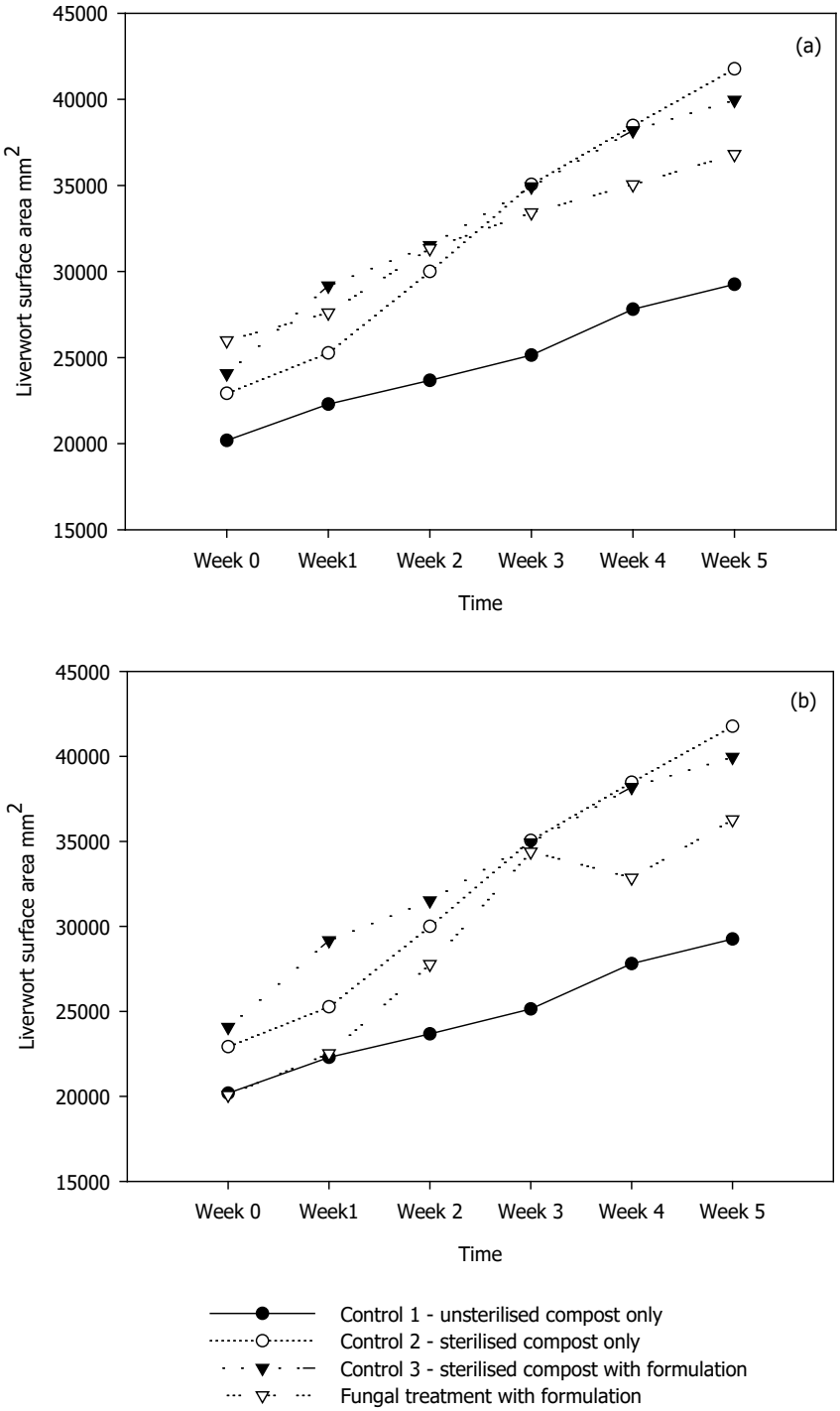


Figure 37. Growth curve of total liverwort area of liverwort subjected to (a) one and (b) two applications of *P. winteri.*

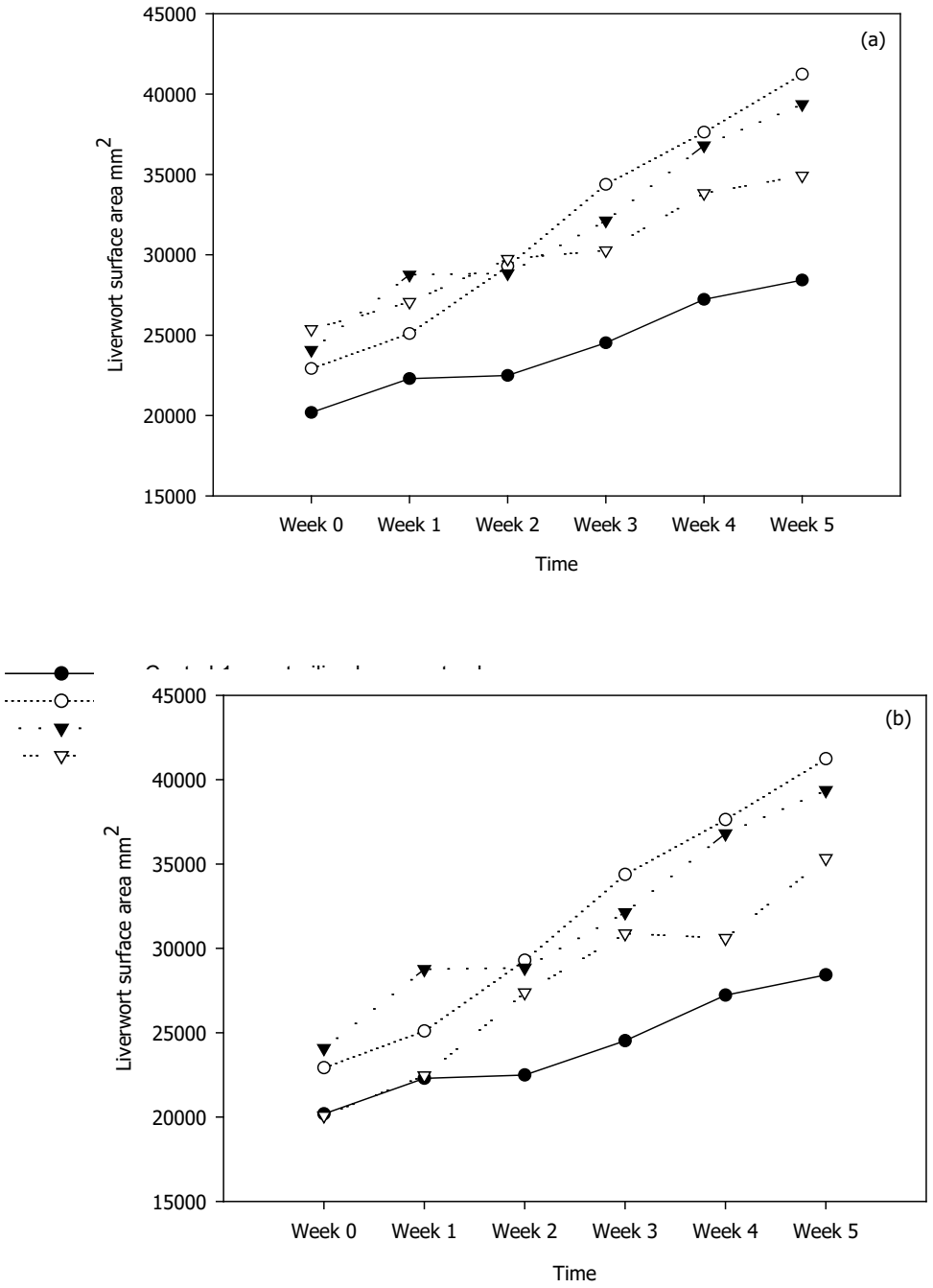


Figure 38. Growth curve of healthy liverwort subjected to (a) one and (b) two applications of *P. winteri*

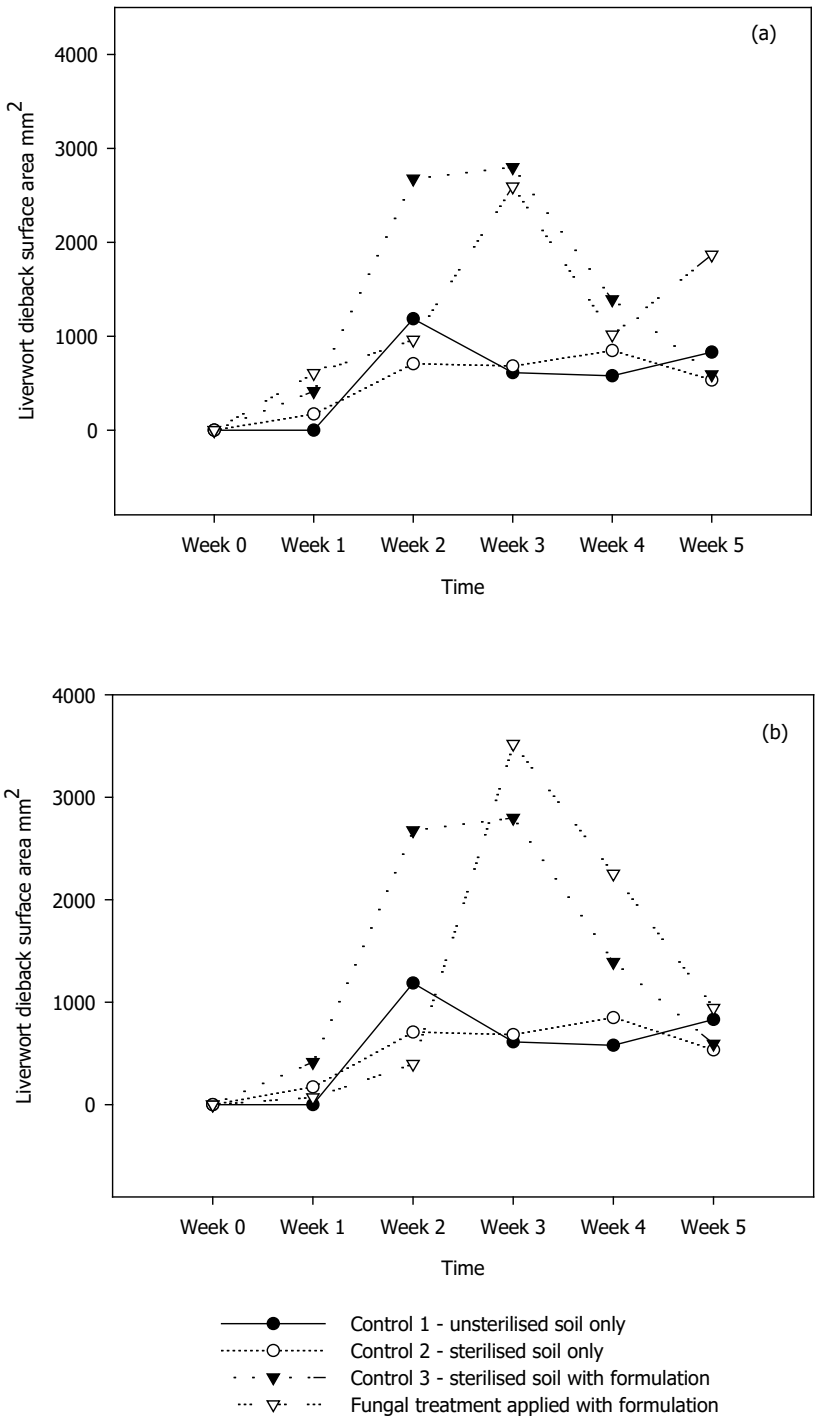


Figure 39. Growth curve of liverwort dieback subjected to (a) one application (b) two applications of *P. winteri*

In conclusion, the laboratory experiments gave an initial indication of the strong effect of *P. winteri* species, and *F. equiseti* in particular, on liverwort and provided information that enabled the selection of the fungal species most likely to be successfully in subsequent experiments.

Pre-emergence treatments

At week 5 in the pre-emergence treatments for *F. equiseti* control 2 (sterilised compost) appeared to limit liverwort growth (total and healthy liverwort area) more than either the fungal treatments or other controls, however the removal of apparent outliers reduced the extent of the growth limitation. There was marginally greater liverwort growth in treatments incorporating formulation (control 3 and the fungal treatment). Control 1 (unsterilised compost) also appeared to have a greater growth-limiting effect than the fungal treatment. For *P. winteri*, however, both control 1 (unsterilised compost) and control 2 (sterilised compost), neither of which contained formulation, had a similar limiting effect on liverwort growth that was greater than the fungal treatment or control 3 (sterilised compost with formulation), both of which did contain formulation. The total and healthy liverwort area of control 2 in the pre-emergence *F. equiseti* growth cabinets was less than in the corresponding controls in the *P. winteri* cabinets, although their treatment was the same. Examination of the liverwort areas indicated two replicates of control 2 (sterilised compost) were small at 7,822 and 6,614 mm^2 compared with the overall average of 17,043 mm^2, suggesting that this effect was not due to sterilisation of the compost. By week 11, however, the fungal treatments (*F. equiseti* and *P. winteri*) both limited the healthy liverwort area more than any of the controls, and the dieback area was greater in the fungal treatments than any of the controls, suggesting the fungal treatments had an increasing effect on the liverwort, although this effect was not significant.

The effect of the formulation in pre-emergence treatments

At week 5 liverwort area was greater in treatments containing formulation (with and without fungal inoculum) than without (controls 1 and 2) for both *F. equiseti* and *P. winteri*, however these differences were not significant. Liverwort growth was not limited by the fungal inoculum, with minimal difference between formulation only and formulation with fungal inoculum. Dieback area was greater in the sterilised compost applied with formulation (control 3), for both fungal species, however this effect is not evident in the total liverwort areas. For *F. equiiseti* only one tray of liverwort exhibited any dieback, distorting the results. By week 11 healthy liverwort area was less in all treatments containing formulation (including the fungal treatments) than

controls 1 and 2, although the difference was not significant. When considering both fungal treatments, this could suggest that the formulation either slightly mitigated the liverwort growth-limiting effect of the sterilised and unsterilised compost or promoted liverwort growth.

The effect of unsterilised compost (control 1) in post-emergence treatments

In the glasshouse experiment the most significant effects were found in the post-emergence treatments for both fungal species, where the unsterilised compost (control 1) consistently reduced liverwort growth without necessarily causing any dieback. Autoclaving of compost in the post-emergence treatments of this experiment resulted in increased liverwort area, suggesting that sterilisation removed a growth-restricting factor. Production of peat-based compost used by the horticulture industry involves the harvesting, milling and drying of peat, and during this process the aeration is improved and consequently the number and activity of microorganisms is increased (Kuster, 1972). It is unclear from this experiment whether microorganisms in the compost caused the reduction in liverwort growth, whether they provided conditions particularly conducive to fungal health and vigour, or if some other factor contributed to the liverwort's ability to tolerate fungal pathogens was involved. A similar effect was observed in an experiment looking at the effect of mulches and growing media amendments on liverwort and moss infestation, where incorporating 10% (v/v) unsterilised loam in the growing media reduced liverwort infestation, although there was considerable infestation by other weeds (Atwood, 2005). The reisolated fungal species, including *F. equiseti*, were found on dying liverwort growing in unsterilised loam and its progress had been observed for a number of weeks in the nursery, with the fungal species growing over and killing the liverwort (Atwood, 2004).

The effect of the second fungal application in post-emergence treatments

In the post-emergence treatment with two fungal inoculations, for *F. equiseti* total and healthy liverwort area was reduced by the second fungal application, and had a greater limiting effect on liverwort growth than either control 2 (sterilised compost) or control 3 (sterilised compost with formulation). After 5 weeks the difference in total liverwort area due to control 1 was highly significant with one fungal application but not significant with two as liverwort growth was reduced due to the second inoculation. An increased amount of dieback was observed due to the second fungal application.

For *P. winteri* there was little observable difference due to the number of fungal inoculum applications suggesting that either the inoculum did not survive or the liverwort vigour did not allow it to proliferate. For *P. winteri*, the dieback area observed in treatments with two fungal applications peaked in week 3 and reduced over the next two weeks, suggesting overgrowth by liverwort.

By week 11, when comparing fungal species, the total and healthy liverwort area was greater when treated with one fungal application of *F. equiseti* than *P. winteri*. The second fungal application increased the effect of *F. equiseti*, so that total and healthy liverwort area were both reduced, however for *P. winteri* the total liverwort area was similar whether treated with one or two applications of *P. winteri*. Control 1 (unsterilised compost) had a greater limiting effect on total and healthy liverwort than any of the other treatments. Dieback area at week 11 was greater with the two fungal applications for F*. equiseti*, and with one fungal application for *P. winteri*, with the greatest amount of dieback found in control 1.

In the post-emergence treatments the liverwort was already growing vigorously when inoculated, therefore the fungal inoculants would have required greater growth and vigour to produce any observable effect on the liverwort. Different management of the fungal inoculation could have yielded better results, as greater spore densities and longer dew periods are reported to increase biocontrol efficacy with older plants. Naseema (2001) found that maximum infection of *F. equiseti*, when used to against water hyacinth (*Eichhornia crassipes* (Mart) Solms), increased from 62.7% to 89.7% by increasing the spore concentration from 10^9 to 10^{11} spores ml^{-1}.

In summary, there was some, but variable, evidence for fungal antagonism having some effect on liverwort growth. In pre-emergence tests total and healthy liverwort area were greatest in treatments containing formulation (control 3 or the fungal treatment), which could suggest a growth promoting effect of the formulation. However, by week 11 this effect had disappeared and healthy liverwort area was least, and dieback area greatest in the fungal treatments (both species), showing evidence of some effect of fungal antagonism.

In the post-emergence tests total and healthy liverwort areas were least with control 1 (unsterilised compost), sustained throughout the experiment, and which may suggest a growth-inhibiting effect of microflora that were removed by sterilisation of the compost. There is some evidence for a growth-limiting effect of two applications

of *F. equiseti* after 5 weeks and which was still observed in the healthy and dieback areas after 11 weeks.

Ideal growth conditions are similar for liverwort and fungi hence there are many close associations reported. Conditions provided for this experiment had to include high humidity to encourage the fungi to persist long enough to establish either in the compost or on the liverwort; these conditions also allowed the liverwort to establish and grow vigorously. Other limitations of this experiment were the size of the growth cabinets, which meant that each could accommodate a limited number of replicates. A further treatment where inoculum was applied to unsterilised compost would have identified any effect of the sterilisation procedure on fungal establishment.

Young plants are more vulnerable than older plants (Hallett, 2005; Morin *et al.*, 1998), therefore it was expected that the pre-emergence treatments would be more successful in controlling liverwort growth, preventing the gemmae from developing into large colonies. Once a dense mat of liverwort has built up it is far more difficult to eradicate, with chemical herbicides often requiring repeated applications.

The appearance of established liverwort successfully treated by fungal antagonists would be an unsightly brown, decomposing mass of liverwort and fungi which would still require removal prior to marketing. A pre-emergence treatment would be easier and more cost-effective to apply as it could be incorporated in the compost rather than applied as a separate task, and as it would prevent liverwort from establishing it would remove the need for attention prior to sale.

Glucosinolates

Introduction

In this section of work glucosinolates (GSLs) and their hydrolysis products, isothiocyanates (ITCs) were investigated for their effect on liverwort gemmae. Methods were developed for the extraction and identification of 3-methoxybenzyl GSL (glucolimnanthin) and 3-methoxybenzylITC from *L. alba* seed meal, and the GSL profile of the whole plant established. *L. alba* was used as previous research (Svenson and Deuel, 2000) indicated some success in controlling liverwort using *L. alba* seed meal as a mulch. Although the GSL glucolimnanthin (Figure 40) and its hydrolysis products were previously known (Bartelt and Mikolajczak, 1989; Vaughn *et al.*, 1996), the full GSL profile of *L. alba* has not been elucidated.

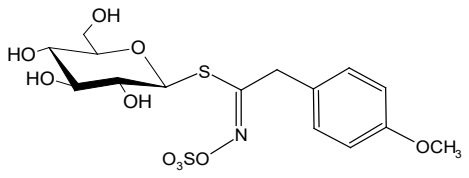


Figure 40. Chemical structures of glucolimnanthin.

Various experimental methods have been described for harnessing plant allelochemicals, including ITCs, by collecting and utilising root exudates to control weeds. Examples are plants grown in soil culture and their root exudates collected either in run-off water (Pope *et al.*, 1985), or the plants transferred to a soilless system for exudate collection (Khan *et al.*, 2002). Alternatively, seedlings have been grown in a hydroponic system with bioactive compounds collected on in-line C-18 columns (Tsanuo *et al.*, 2003). Yamane *et al* (1992) collected *Rorippa indica* (Indian cress) plants from the wild and transferred them to a hydroponic system with continuous root exudate trapping based on that designed by Tang and Young (1982), collecting the ITCs on an in-line column containing Amberlite XAD-4 resin (Supplier: Sigma-Aldrich). Amberilite is a hydrophobic polyaromatic compound used to remove small hydrophobic compounds from a solution, for example organic substances from aqueous systems and polar solvents (Sigma-Aldrich, 2006).

Plant species (Figure 41) were selected for their high root GSL content (Fahey *et al.*, 2001; Kirkegaard, 1998), to provide a range of bioactive products for use in

bioassays; their taxonomic detail, germination conditions and root GSL profiles are summarised in Table 18). These plants were grown in a nutrient film technique (NFT) hydroponic plant growth system, and methods were developed to collect and analyse their root exudates from the nutrient solution, based on methods described by Yamane *et al* (1992). *L. alba* plants were also grown in this system to provide clean plant tissue for GSL and ITC extraction and bioassays.

In vitro bioassays were designed using the predominant ITCs (benzyl-, 2-phenylethyl-, 2-2-propenyl- and 3-methoxybenzyl-) (Figure 42). found in the root tissues of the plants grown hydroponically (Table 18). These were used to investigate the effect of ITCs on the growth of liverwort thalli and on cress (*Lepidium sativum*) radicle germination and elongation to provide a comparison with higher plants.

As an indicator of the potential usefulness of ITCs in controlling liverwort comparative bioassays were carried out using two herbicides, Lenacil and Metazachlor (Figure 43), found within different herbicide groups and currently recommended for use with liverwort (Atwood, 2005).

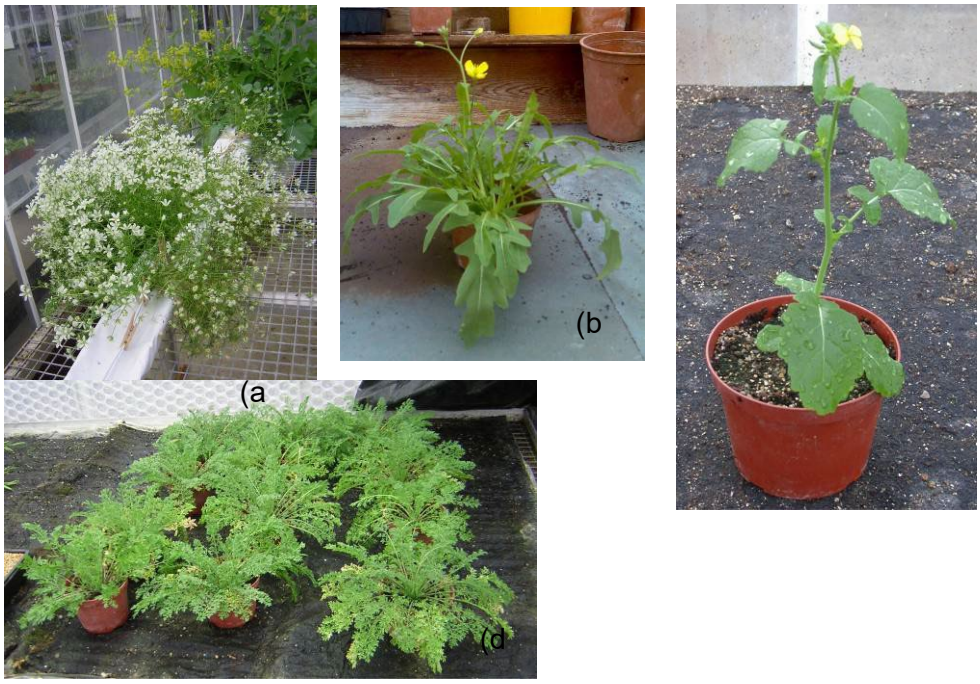
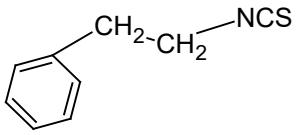
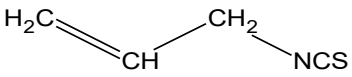


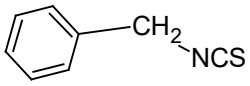
Figure 41. Plant species used in GSL experiments (a)*Limnanthes alba* (b) *Diplotaxis tenuifolia* (c) *Sisymbrium orientale* (d) *Brassica juncea*.



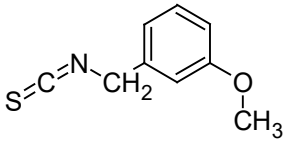
2-PhenylethylITC



2-PropenylITC

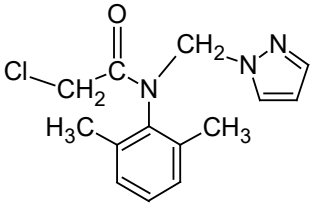


BenzylITC

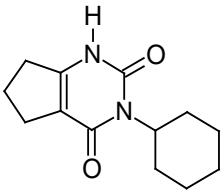


3-MethoxybenzylITC

Figure 42. Chemical structures of ITC s used in bioassays



Metazachlor ($C_{14}H_{16}ClN_3O$)



Lenacil ($C_{13}H_{18}N_20_2$)

Figure 43. Chemical structures of herbicides used in bioassays

Plant details				Root glucosinolate profile		
Plant	**Plant family**	**Seed source***	**Germination conditions**	**Chemical name**	**Trivial name**	**Glucosinolate classification**
Brassica juncea L. Czern. (Brown or Indian mustard) Herbaceous perennial	Brassicaceae	John Rossiter, Imperial College	2 mm deep, 15-25 C	2-phenylethyl	Gluconasturtiin, Phenethyl	Aromatic
				2-propenyl	Sinigrin, 2-propenyl	Aliphatic - Olefin (straight chain, double bond)
				3-butenyl	Gluconapin	Aliphatic - Olefin (straight chain, double bond)
				4-methoxy-3-indolylmethyl	4-Methoxyglucobrassicin	Indole
				3-indolylmethyl	Glucobrassicin	Indole
				1-methoxy-3-indolylmethyl	Neoglucobrassicin	Indole
				Benzyl	Glucotropaeolin	Aromatic
				4-pentenyl	Glucobrassicanapin	Aromatic
				4-hydoxy-3-indolylmethyl	4-Hydroxyglucobrassicin	Indole
Diplotaxis tenuifolia D. DC (Perennial wall rocket) Herbaceous perennial	Brassicaceae	Herbiseeds	None	Benzyl	Glucotropaeolin	Aromatic
				4-methylsulphinylbutyl	Glucoraphanin	Aliphatic - AlkylThioAlkyl - $CH_3SO(CH_2)_4$-X
				p-hydroxybenzyl	Glucosinalbin	Aromatic
				2-phenylethyl	Gluconasturtiin, Phenethyl	Aromatic
				3-Indolylmethyl	Glucobrassicin	Indolyl
Sisymbrium orientale L. (Indian hedge mustard) Herbaceous annual	Brassicaceae	Herbiseeds	5mm deep	2-phenylethyl	Gluconasturtiin, Phenethyl	Aromatic
				3-butenyl	Gluconapin	Aliphatic - Olefin (straight chain, double bond)
				p-hydroxybenzyl	Glucosinalbin	Aromatic
				1-methoxy-3-indolylmethyl	Neoglucobrassicin	Indole
				4-methoxy-3-indolylmethyl	4-Methoxyglucobrassicin	Indole
				3-indolylmethyl	Glucobrassicin	Indole
Limnanthes alba Hartw . ex Benth. (Meadowfoam). Herbaceous winter annual	Limnanthaceae	Massey University	12 C, dark			

Table 18. Details of selected plant species; root GSL profiles are listed in order of abundance. Root GSL profile for *Limnanthes alba* is unknown (Fahey et al., 2001; Kirkegaard, 1998).

Lenacil, introduced in 1964, is a residual, selective, systemic herbicide (Tomlin, 2000), one of three belonging to the uracil group of herbicides, which are comprised of a uracil nucleus with various chemical groups substituted. Uracils are carried to the root zone by water, absorbed by the roots and move through plants in the transpiration stream (Ware and Whitacre, 2004). Lenacil's mode of action is to block photosynthetic electron transport at the photosystem II receptor site. It is used to control annual grass, broad-leaved weeds in various field crops, ornamental plants and shrubs, applied pre-planting or pre-emergence (Tomlin, 2000). Residual activity is reduced in soils with high organic matter; moist conditions are required to sustain residual activity, dry conditions reduce efficacy. A number of products are available with Lenacil as the active ingredient in the UK, with Clayton Lenacil 80W (MAPP No. 09488) approved for use in ornamental plant production. It is supplied as a wettable powder containing 80% w/w Lenacil (Pesticide Safety Directorate, 2006; Tomlin, 2000; Whitehead, 2006).

Metazachlor, introduced in 1982, is a residual, selective chloroacetamide (syn. acetanilide) herbicide absorbed by plant hypocotyls and roots, with activity dependent on root uptake (Tomlin, 2000; Whitehead, 2006). Chloroacetamides are meristematic growth inhibitors, with inhibition of long chain fatty acids the predominant mode of action (Ware and Whitacre, 2004). Metazachlor is used pre- and early post-emergence, either incorporated pre-planting or surface applied; effectiveness is reduced on soils with over 10% organic matter content. It is approved for use in brassicas, nurseries and forestry, where it is used to control winter and annual grasses and broad-leaved weeds in fruit and vegetable crops and ornamental plants and shrubs. A number of products are available for use in ornamental plant production with Metazachlor as the active ingredient, e.g. Butisan S (Pesticide Safety Directorate, 2006).

It was considered that the GSL hydrolysis product 3-methoxybenzylITC, found in *L. alba* seed meal, was the active substance that reduced liverwort presence on the compost surface in experiments carried out by Svenson and Deuel (2000). Vaughn *et al* (1996) had previously found 3-methoxybenzylITC to be toxic against velvetleaf and wheat seedlings. One aim was, therefore, to extract this ITC from *L. alba* seed meal, identify it and apply it to liverwort gemmae in bioassays to measure the direct effect on their growth. Three other selected ITCs (benzyl, 2-propenyl and 2-phenyethyl) and the herbicides Metazachlor and Lenacil were similarly applied to

liverwort gemmae and their effects measured. Additionally the previously unknown GSL profile of all *L. alba* tissues was established.

ITCs have been collected from plant root exudates using an in-line column containing XAD-4 resin (Tang and Young, 1982). It was hypothesised that these methods could be used to collect ITCs, which could then be tested for herbicidal effect against liverwort gemmae. The objectives were to grow selected plants with known high levels of root GSLs in a hydroponic system, collect and identify the ITCs produced and then apply them to liverwort gemmae in laboratory bioassays.

General methods

Hydroponic growth of plant species

A nutrient film technique (NFT) hydroponic system was designed using black guttering for the NFT channel to contain plants (Figure 44, Figure 45). Nutrient solution was pumped around the system from a 20 litre reservoir by a submersible pump via plastic tubing, into one end of the channel, over the plant roots and then returned to the reservoir, keeping the solution aerated. Seedlings were grown in 3 cm rockwool blocks which rested in the nutrient solution. The guttering, rockwool and reservoir were wrapped in co-extruded black on white plastic film, with the white layer to the outside to reduce algal growth.

Feeding regime

Genesis Formula, manufactured by Green Air Products a proprietary hydroponic nutrient solution fertiliser concentrate system was used (supplier: Growth Technology), supplied as 2 parts: Microbase providing nitrogen, phosphorus, potassium and micronutrients; and Grow 2 providing nitrogen and potash (Table 19). Both Grow 2 and Microbase were applied at 3.78 ml L^{-1} to provide optimum growth conditions; the nutrient solution was changed weekly.

As the plants were growing in alkaline rockwool, which raises the pH, the nutrient solution was adjusted to pH 5.8 using ‘pH Down’ (81% phosphoric acid), manufactured by Essentials Hydroponics (supplier: Growth Technology). Using the recommended nutrient doses an acceptable electric conductivity (E.C.) of 1.2 was

obtained. E.C. is a measure of nutrient concentration which, if too high can result in root dehydration, and if too low in lack of growth.

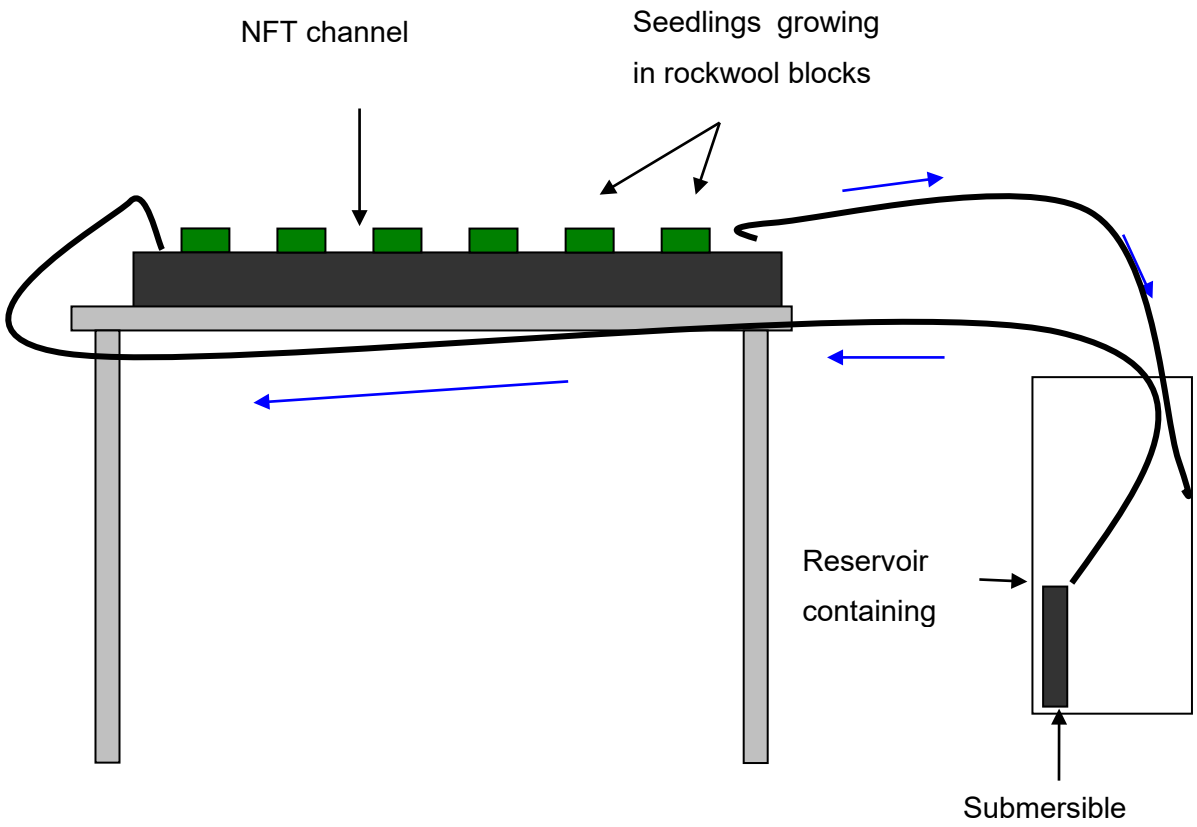


Figure 44. Diagram of hydroponic system. Nutrient solution is pumped from the reservoir by the submersible pump to the end of the NFT channel, flows down the channel over the plant roots and back into the reservoir in the direction of the blue arrows.

As the plants were growing in alkaline rockwool, affecting the pH, it was adjusted to 5.8 using 'pH Down' (81% phosphoric acid), manufactured by Essentials Hydroponics (supplier: Growth Technology). Using the recommended nutrient doses an acceptable electrical conductivity (E.C.) of 1.2 was obtained.

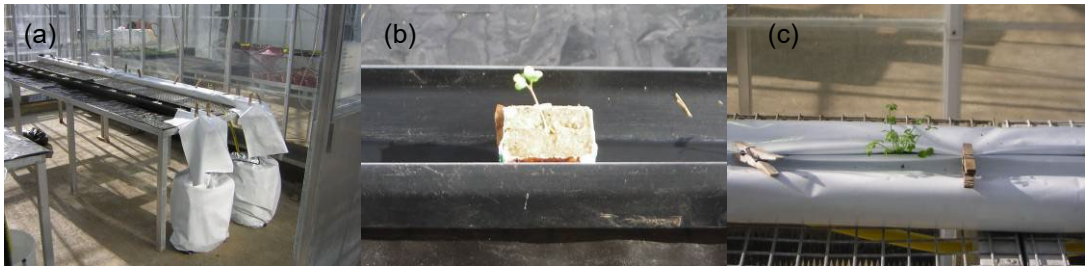


Figure 45. Hydroponics system set up: a) two NFT channels with their nutrient reservoirs, b) NFT channel containing a *Brassica juncea* seedling in its rockwool block, c) a *Limnanthes alba* seedling with its block and NFT channel covered in light omitting plastic

Microbase B. N:P:K 3:10:19	
Component	**%**
Total nitrogen	3
Available phosphate (P_2O_5)	10
Soluble potash (K_2O)	19
Total magnesium (Mg)	3
Sulphur (S)	4
Boron(B)	0.03
Cobalt (Co)	0.002
Copper (chelated) (Cu)	0.02
Iron (chelated)(Fe)	0.2
Manganese (chelated)(Mn)	0.05
Molybdenum (Mo)	0.003
Chlorine (Cl)	≤.12
Zinc (chelated)(Zn)	0.05
Grow nutrient 2. N:P:K 15:00:08	
Component	**%**
Total Nitrogen	15
Ammoniacal nitrogen	3
Nitrate nitrogen	12
Soluble potash (K_2O)	8
Calcium (C)	8

Table 19. Genesis Formula nutrient solution components

Seed germination

Rockwool blocks were soaked in water, a well made in the centre and two seeds sown in each. They were then placed in a growth chamber with the correct germination conditions for the plant species (Table 18). Once germinated the weakest seedling was removed from each rockwool block, the block transferred to the NFT system (Figure 45), and fed with nutrient solution at half the required concentration for the first week.

M51C media

Stock solutions of the macros, micros, vitamins and potassium iodide were prepared in quantities to produce the final concentrations indicated (Table 20). The remaining ingredients, except phytagel, and stock solutions were combined, dissolved in 500 ml of distilled water, stirred and made up to 1 litre with more water; pH was adjusted to 5.8 using sodium hydroxide and hydrochloric acid as necessary. Phytagel was then added, heated until dissolved and autoclaved.

Stock solutions	Ingredients	M51C (mg L^{-1})	MS with vitamins (mg L^{-1})	MSMC (mg L^{-1})
	NH_4NO_3	400	1650	1650
	KNO_3	2,000	1900	1900
Macro's	$MgSO_4.7H_2O$	370	180.54	180.7
	KH_2PO_4	275	170	170
	$FeSO_4.7H_2O$	0	0	27.8
	$MnSO_4.H_2O$	10	16.9	16.9
	$ZnSO_4.7H_2O$	2	8.6	8.6
	H_3BO_3	3	6.2	6.2
Micro's	$NaMoO_4.2H_2O$	0.25	0.25	0.25
	$CuSO_4.5H_2O$	0.025	0.025	0.025
	$CoCl_2.6H_20$	0.025	0.025	0.025
	$NaH_2PO_4.H_20$	0	0	148
	Nicotinic Acid	1	0.5	0
	Thiamine.HCl	10	0.1	0.4
Vitamin's	Pyridoxine.Hcl	1	0.5	0
	MyO-Inositol	100	100	100
	Glycine	0	2	0
	KI	0.75	0.83	0.83
	$CaCl_2.2H_2O$	300	332.2	332.2
	EDTA-FE	40	36.7	37.26
	N-Z Amine (A)	200	0	0
	L-Glutamine	292	0	0
	Sucrose	20000	20000	20000
	Phytagel	3000	3000	3000
	Adenine hemi-sulphate	0	0	80
Hormones	Kinetin	0	0	1
	NAA	0	0	0.1

Table 20. *in-vitro* liverwort cultivation media components

Buffer preparation

Buffer solutions were prepared as follows:

Tris buffer – pH 7.0, 8.0 and 9.0:

100 ml 0.1 M Tris(hydroxymethyl)methylamine (Tris) (MWt 121.14 g mol^{-1}) in milli-Q water, the pH adjusted to 7.0 with 5 M hydrochloric acid.

Acetate buffer – pH 5.0 and pH 4.0

100 ml 0.1 M glacial acetic acid (Mwt = 60.05 g mol^{-1}) in water, the pH adjusted with 5 M NaOH.

Phosphate buffer - pH 6.0

0.2 M solutions of NaH_2PO_4 (MWt = 119.98 g L^{-1}) and Na_2HPO_4 (MWt = 141.96 g L^{-1}) were each made up to 100 ml with water, mixed (6.1 ml: 43.9 ml, NaH_2PO_4:Na_2HPO_4) (Dawson *et al.*, 1969) to obtain pH 6.0.

Gas chromatography (GC-MS)

Gas chromatography – mass spectrometry (GC-MS) separates compounds into its components in the vapour phase, ionises and then subjects them to analysis by mass spectrometry. The compound of interest has to be relatively volatile, forming a stable vapour or convertible to derivatives that are.

Gas chromatography (GC) equipment and operating conditions (Table 21 andTable 22) were standard for all ITC analysis, however, samples were injected with either a 1:3, 1:5 or 1: 50 split depending on ITC abundance, adjusted to prevent peak broadening by overloading the column.

GC data was analysed using MSD ChemStation Build 75, 26 August 2—3. Version G1701DA D.01.00. Agilent Technologies. Spectra were compared with known standards or by computer comparison with the Wiley 275 mass spectra library.

GC	GC System	HP 6890
	Detector	HP 5973 mass selective detector
	Column	Agilent HP-5ms
	Column dimensions	30m x 250 µm internal diameter 0.25 µm film thickness
	Carrier gas	Helium

Table 21. Gas chromatography equipment

Method 3:1 split	**Temperature parameters**	Initial tempterature	40 C, equilibration time 0.5 mins 40 C, then held for 5 mins
		Temperature increases	5 C min^{-1} to 180 C 10 C min^{-1} to 280 C
		Final oven temp	280 C, held for 10 mins
		MSD transfer line heater	290 C
		Injection volume	µL
		Run time	53 mins
Method 50:1 split	**Temperature parameters**	Initial temperature	125 C, equilibration time 0.5 mins 125 C, then held for 5 mins
		Temperature increases	10 C min^{-1} to 280 C
		Final oven temp	280 C, held for 5 mins
		MSD transfer line heater	290 C
		Injection volume	1.0 µl
		Run time	25.50 mins
Method 5:1 split	**Temperature parameters**	Initial temperature	50 C, equilibration time 0.5 mins 50 C, then held for 5 mins
		Temperature increases	5 C min^{-1} to 180 C 10 C min^{-1} to 280 C
		Final oven temp	280 C, held for 10 mins
		MSD transfer line heater	290 C
		Run time	51 mins
		Injection volume	1.0 µl

Table 22. Gas chromatography methods

High performance liquid chromatography (HPLC) system

High pressure (or high performance) liquid chromatography (HPLC) was developed from gas chromatography techniques. A solvent, into which the sample to be analysed is injected, is pumped through a sorbent-packed column under high pressure. Compounds that adsorb weakly onto the surface of the solid stationary phase pass down the column faster than a more strongly absorbed solute, causing the required separation; the polarity of the mobile and stationary phases can be adjusted to improve sample separation. The small size of the sorbent particles provides resistance to the solvent flow; hence the mobile phase has to be pumped through the system. The retention time (Rt) taken for a solute to pass through the column is characteristic for the solute in the specific chromatography conditions used.

Adsorption chromatography, specifically reverse phase, was used in this project:, having a non-polar stationary phase and polar mobile phase (e.g. acetonitrile or methanol with water), more non-polar compounds are retained on the column for longer than less polar compound.

Systems can be operated isocratically or using a gradient: isocratic elution uses a mobile phase of constant composition; gradient elution uses a mobile phase with pre-determined changes to its composition during separation and is used when the range of retention times of solutes being analysed is too long. It reduces the retention time of molecules so they can be eluted in a reasonable time.

HPLC alone cannot provide enough information to identify a compound; for this it is combined with mass spectrometry (LC-MS, liquid chromatography, mass spectroscopy), which separates gas phase ions according to their mass to charge ratio (m/z value).

For HPLC samples should ideally be dissolved in the same solvent as used for the mobile phase to improve efficiency and peak shape. If different solvents are used, compounds which move into the different mobile phase move down the column at different rates than those in the sample solvent, spreading the time over which the compound is detected and producing a tailed peak.

Reversed phase HPLC analysis techniques were used for lunularic acid and GSL sample analysis. The equipment used was the same in each case, with the solvent system, delivery programs and detectors varied to separate and detect the compounds being analysed.

HPLC equipment was arranged as Figure 46. Solvents were degassed with helium, and then passed via pumps A and B, where they were combined and pumped through the automatic injector. The sample to be analysed was injected into the system and was carried by the solvent flow through the chromatography column where it was separated before passing through the detectors. A Phenomenex SphereClone 5µ ODS (2), Size 250 mm x 4.6 mm x 5 µmicron, reverse phase column was used, at 35 C. Solvent flow rate was 1 ml min^{-1}.

Compounds can be detected using a number of methods, the most commonly used are UV absorbance detectors as they detect a wide range of compounds that absorb

UV radiation, e.g. alkenes, aromatics and others with multiple bonds between carbon and oxygen, nitrogen or sulphur. A UV detector (Waters 486 tuneable absorbance detector) was used for the GSL analysis. A fluorescence detector (Merck/Hitachi F-1050 fluorescence spectrophotometer) was used for lunularic acid analysis. As most molecules do not exhibit fluorescence this can be an extremely sensitive tool; the structure of lunularic acid, incorporating phenyl, carboxyl and hydroxide groups suggested that fluorescence detection would elicit a good response.

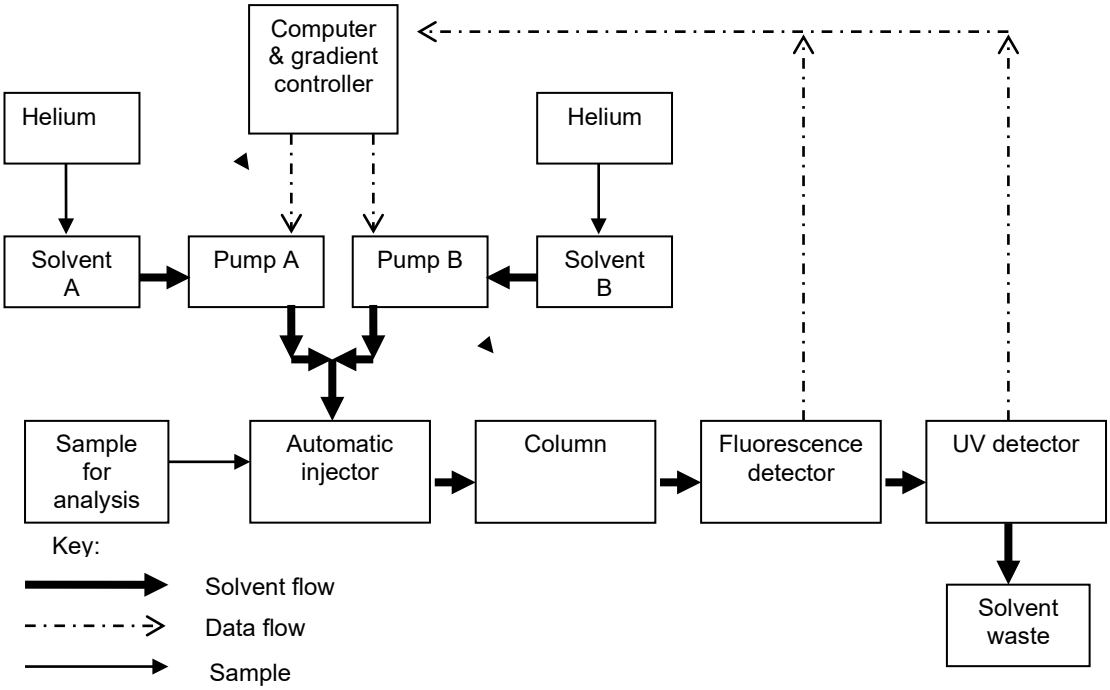


Figure 46. Layout of equipment used for HPLC analysis. Solvents A and B were degassed with helium, then passed through pumps A and B, where they were combined and pumped through the automatic injector. The sample to be analysed was injected into the system and was carried by the solvent flow through the chromatography column where it was separated before passing through the detectors.

GSL extraction from Limnanthes alba seed meal

Introduction

L. alba seed meal has been shown to have an effect on liverwort proliferation in nursery containers (Svenson and Deuel, 2000). To examine if GSLs present in the seeds could have caused this inhibition, methods were developed to isolate and identify any GSLs; the GSL hydrolysis products were then used in bioassays to determine any effect on liverwort gemma growth. Seeds of *L. alba* were defatted to

remove the oil and volatile components, and the GSL content extracted, using a known method (Thies, 1988), quantified and identified.

Method

Defatting seeds

L. alba seed (150 g) was ground until fine using a coffee grinder and weighed. It was defatted using Soxhlet apparatus with petroleum ether (40-60 C bp) and a few anti-bumping granules added. The ground seed was placed in a cellulose extraction thimble, the top plugged with cotton wool and placed into the glass Soxhlet chamber and, with the condenser operating, sufficient heat applied to allow the petroleum ether to gently reflux. When all the oil had been extracted the thimble was removed and left for the seed powder to dry thoroughly.

GSL extraction

For the GSL extraction, defatted seed meal (18.2 g) was extracted in methanol (150 ml, 4 hrs) with a Soxhlet extractor, and the methanol evaporated until nearly dry under reduced pressure (Büchi Rotavapor RE120, 35 C). The extract was twice dissolved in water (10ml) and the concentrates transferred to a conical flask (50 ml). 0.5 M Lead acetate (1 ml) and 0.5 M barium acetate (1 ml) were added to the extract, mixed, allowed to stand at 4 C and centrifuged for 30 min (3,500 rpm). The supernatant was decanted through a column of 1g dry weight Sephadex DEAE-A-25, formiate form (Supplier: Sigma-Aldrich), in a tube of 12 mm internal diameter, 100 mm long, and washed through twice with 10 ml water (discarding the run off solution each time); twice with 5 ml formic acid:i-propanol:water, 3:2:5; three times with water (10 ml); and then eluted with 12.5 ml 0.5 M potassium sulphate (K_2SO_4) in 5% isopropyl alcohol, allowing the eluate to drop into 12.5 ml ethanol. The eluate was filtered in a No. 3 sintered glass funnel to remove the potassium sulphate, agitated and cooled for 10 min at +4 C. It was centrifuged, the supernatant collected and then evaporated to nearly dry under reduced pressure (Büchi Rotavapor RE120, 35 C). After drying *in vacuo* over phosphorus pentoxide a sticky, glassy solid was obtained which did not crystallise.

Results

18.2 g seed meal produced 504 mg of residue, and further drying reduced this to 260 mg of solid. This equates to 14.3 mg g^{-1} (32.5 µmol g^{-1}) seed meal, providing a yield of 1.4%, greater than the 265 nmol g^{-1} obtained by Vaughn *et al* (2006).

Although Vaughn also extracted the GSL in methanol, he agitated the seedmeal in boiling 70% methanol for 15 min before filtering, a 16-fold reduction in compared with the method used in this experiment.

GSL extraction from Limnanthes alba plant tissue

Introduction

GSLs were extracted from different plant tissues harvested from *Limnanthes alba* plants (grown hydroponically) using the method described by Heaney (1986), and then analysed and identified.

Method

Plant material

Plant material of *L. alba* was removed from plants grown in the hydroponics system, different tissues separated, weighed (Table 23) and freeze dried.

Plant tissue	Fresh weight (g)
Stem	16.4
Buds	2.5
Roots	7.6
Leaves	12.2

Table 23. Fresh and dry weights of *Limanthes alba* tissue.

Extraction of GSLs from plant tissue

Freeze dried plant material was ground until very fine using a coffee grinder. Quantities of stems, leaves, buds and rhizoids (3 x 50 mg) were weighed and placed into Eppendorfs, with an anti-bumping granule in each and a hole made in each lid to release vapour. A quantity of the GSL sinalbin (*p*-hydroxybenzyl GSL) was used as an internal standard (2 mg ml^{-1}). GSLs were extracted using 80% (v/v) methanol (1 ml) at 85 C for 10 min. 20 µl sinalbin standard was then immediately added to each Eppendorf, and the lid closed.

Eppendorfs were centrifuged for 5 min (13,000 rpm, Eppendorf centrifuge 54141) and the supernatant transferred to clean Eppendorfs. The extraction was repeated with 80% (v/v) methanol (1 ml) at 85 C (5 min) and centrifuged (5 min). The supernatant was collected and combined with the first extraction. The methanol was evaporated to almost dry using the Techne Dri-Block DB-3A sample concentrator at 60 C and samples were made up to 1 ml with water.

Removal of proteins

Proteins contaminating GSL samples were precipitated with 30 µM $Pb(OAc)_2$ and 30 µM $Ba(OAc)_2$ per sample, mixed, left to stand (5 min), centrifuged (5 min, 13,000 rpm) and the supernatant removed.

Preparation of ion exchange columns

Ion exchange columns (DEAE Sephadex A25) were prepared, for the plant tissue extracts and external standards, using Sephadex DEAE A-25 (2-(Diethylamino)ethyl-Sephadex) gel, formiate form (Supplier: Sigma-Aldrich), supplied in powder form and stored as a stock solution in 20% ethanol. The stock was shaken well, approx. 15 ml removed, washed twice to remove the ethanol by shaking it in a centrifuge tube with plenty of water, leaving it to settle and the upper, water layer removed. An equal quantity of water was added to the gel (50/50), mixed well, then pipetted (1.2 ml) into each graduated ion exchange column, the water draining away to leave 0.5-0.6 ml gel in each. The gel in each was washed twice with 1 ml water, leaving a smooth, level surface to the gel. One column was prepared for each sample plus two for external standards.

Load samples onto columns

The supernatant was pipetted onto the gel columns; the two external standard columns were loaded by pipetting sinalbin standard (2 mg ml^{-1}, 20 µL), mixed with water (200 µL) for ease of even application, onto each of the two columns.

Each column was washed twice with water (1 ml) and twice with 0.02 M acetate buffer (pH 5.0, 0.5 ml). Sulphatase (75 µl) was added to each column to desulphate the GSLs bound to the column, left overnight, eluted 3 times with water (0.5 ml), collecting the eluate which was transferred to 2 ml Eppendorfs, frozen to -20 C and freeze dried. Samples were resuspended in water (200 µl) prior to HPLC analysis.

Preparation of glucolimnanthin standard

Glucolimnanthin previously extracted from *L. alba* seeds (Section 5.3.1) was used as a standard. Glucolimnanthin (2.38 mg) was dissolved in water (1.5 ml) to give a concentration of 1.59 mg ml^{-1}. Two ion exchange columns were prepared as before, omitting the barium acetate step as the sample was already protein-free. A sample (0.75 ml, containing 1.19 mg glucolimnanthin) was loaded onto each column, washed twice with water (1 ml), twice with 0.02 M acetate buffer (pH 5.0, 0.5 ml),

sulphatase (75 µL) added and left overnight. The next day the sample was eluted 3 times with water (0.5 ml), collecting the eluate in vials for HPLC analysis.

HPLC analysis

Samples were analysed for GSL content using HPLC equipment previously described (Page 9). Samples were injected (20 µl) and analysed using a gradient solvent system (Table 24with a 42 min run time at 35 C with 1.5 ml min^{-1} flow rate. GSLs were detected using the UV detector set at λ 230 nm.

Table 24 Solvent gradient conditions used for HPLC analysis. Solvent A = water, Solvent B = 20% acetonitrile

Time	Flow	% A	% B
Initial	1.50	99	1
1.0	1.50	99	1
21.0	1.50	1	99
34.0	1.50	1	99
36.0	1.50	99	1
42.0	0.00	99	1

Results

Recovery rates (Table 25), comparing peak sizes of the internal and external sinalbin standards were above 80% for all tissues except the roots (average 41%), possibly due to losses during the methanol boiling stage.

Unusually, glucolimnanthin was the only GSL detected throughout the plant; most plant species have a more complex GSL profile. The GSL peak was confirmed as glucolimnanthin, with retention times averaging at 17.08 mins, compared to 17.25 for the glucolimnanthin standard, which was then used to calibrate the GSL peak sizes.

Tissue type	Glucolimnanthin content (nmol mg^{-1} dry weight) ± SD	Recovery %	R_t (min)
Stem	86.9 ±1.27	100	17.01
Root	40.9 ±1.04	39	17.14
Leaf	78.8 ±1.15	99	17.06
Bud	79.5 ±2.85	82	17.09

Table 25. Glucolimnanthin content of *L. alba* tissue. All values given are an average of three samples. Retention time (R_t) of glucolimnanthin standard was 17.25 mins.

Extraction and identification of glucolimnanthin hydrolysis products from seed meal using GSL hydrolysis time course assay: Preliminary experiment

Introduction

A small scale preliminary experiment to extract ITC s from seed meal was carried out, using a method adapted from Vaughn and Berhow (2005). An appropriate time scale for the extraction was established and the identity of the compounds extracted was confirmed.

Method

50 mg samples of ground, defatted *L. alba* seed meal was placed into Eppendorfs with buffer (250 µl Tris pH 7.0, 0.1 M) (Section 5.2.2), mixed thoroughly and incubated at 35 C. The Eppendorfs were removed at predetermined time intervals, dichloromethane (DCM) (1 ml) added, mixed thoroughly for 30 seconds to form an emulsion, and centrifuged (Eppendorf 54141 centrifuge, 13,000 rpm) for 3 min. The DCM was removed with a syringe, transferred to a clean Eppendorf containing a small amount of anhydrous magnesium sulphate (to remove water), mixed and centrifuged again (2 min, 13,000 rpm). Supernatant (500 µL) was removed for analysis. Samples were removed from the water bath at hourly intervals: 0, 1, 2, 3, 4 and 5 hrs. As this was a preliminary experiment samples were not replicated. Samples were analysed by GC-MS using a 5-1 split, and repeated using a 50-1 split due to high abundance of the primary compound detected (Table 22).

Results

A peak, detected by GC-MS analysis (Figure 47a) which appeared in the 0 hr sample, was larger in the 1 hr sample and subsequently reduced, producing a degradation curve (Figure 49) suggesting the molecule was unstable at pH 7.0. The compound extracted was identified by mass spectography (Figure 47b) as 3-methoxybenzylITC (C_9H_9NOS) (limnanthin) by comparison with mass spectral data presented by Vaughn and Berhow (2005).

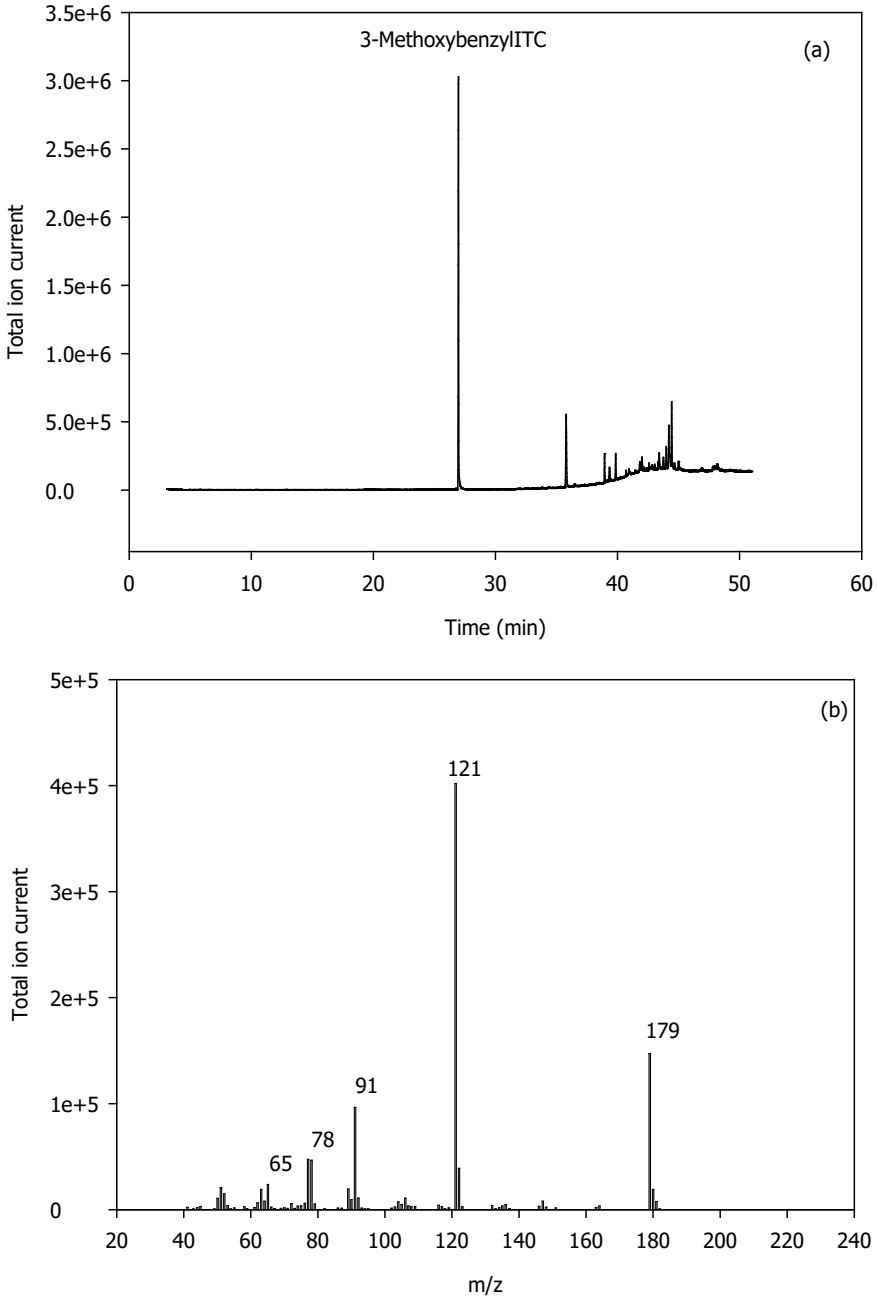


Figure 47 (a) Chromatogram and (b) mass spectrum (EI) of 3-methoxybenzylITC. Extracted from 50 mg *L. alba* seed meal in 1 ml DCM at pH 7.0.

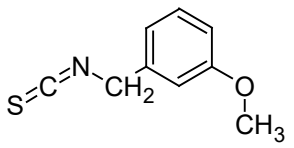


Figure 48. 3-methoxybenzylITC .

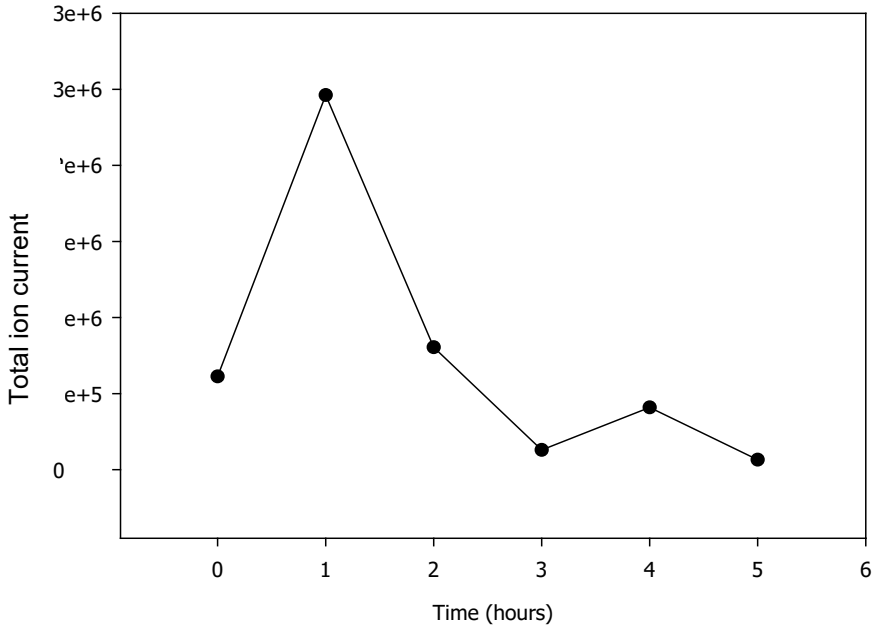


Figure 49. Preliminary glucolimnanthin degradation curve produced during a time course assay at hourly intervals, pH 7.0, extracted with DCM (1 mL)

Optimisation of glucolimnanthin hydrolysis products extraction

Introduction

The preliminary experiment was repeated with reduced time intervals, providing results at more time points during the initial 1.5 hrs to characterise more precisely the glucolimnanthin degradation curve.

Method

The preliminary experiment (Page 99) was repeated with eight extractions at 20 min intervals, at pH 7.0 with two samples at each time point to further characterise glucolimnanthin hydrolysis. Time points were chosen to ensure that complete GSL degradation could be observed.

Results

The maximum amount of GSL product was present after 40 mins, sharply declining thereafter (Figure 50).

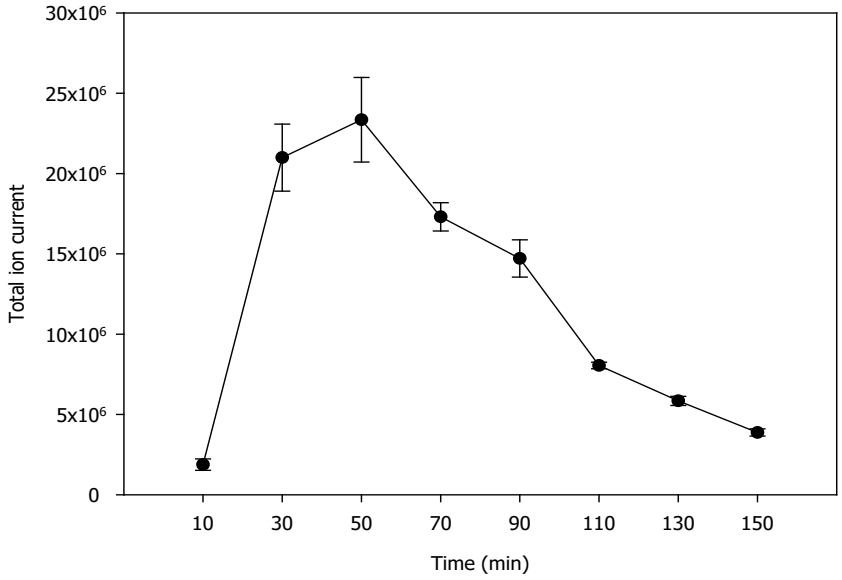


Figure 50. Glucolimnanthin degradation curve produced during a time course assay, samples taken at 20 min intervals, pH 7.0, extracted with DCM (1 mL)

Optimisation of 3-methoxybenzyl ITC extraction from seed meal

Introduction

Degradation of GSLs produces different products and in different concentrations dependent on pH, for example producing nitriles at low pHs, and ITCs in neutral conditions (Gil and MacLeod, 1980). This experiment was designed to identify an optimum pH for ITC extraction from glucolimnanthin.

Method

This time course experiment used the previous method with six extractions at hourly intervals (0, 1, 2, 3, 4, 5 hrs), two samples were taken at each time point and at pH 4.0, 5.0 6.0, 8.0 and 9.0, 30 samples in total; pHs were adjusted using 0.1 M acetate, 0.2 M phosphate and 0.1 M Tris buffers.

Results

Samples were analysed using GC-MS (50-1 split method) (Table 22), producing the degradation curves shown in (Figure 51). Additional GSL products were detected at lower pHs (4.0 and 5.0); less at pH 7.0, 8.0 & 9.0, with a second peak appeared in pH 7.0, 8.0 and 9.0, identified as 3-methoxybenzylamine, ($C_8H_{11}NO$)(Figure 52 to Figure 54).

At lower pHs, 4.0, 5.0, 6.0, 9.0 hydrolysis reactions took place immediately prior to degrading; the reaction was slower at pH's 7.0 & 8.0. Previous analysis suggested maximum ITC is produced after 40 mins at pH7.

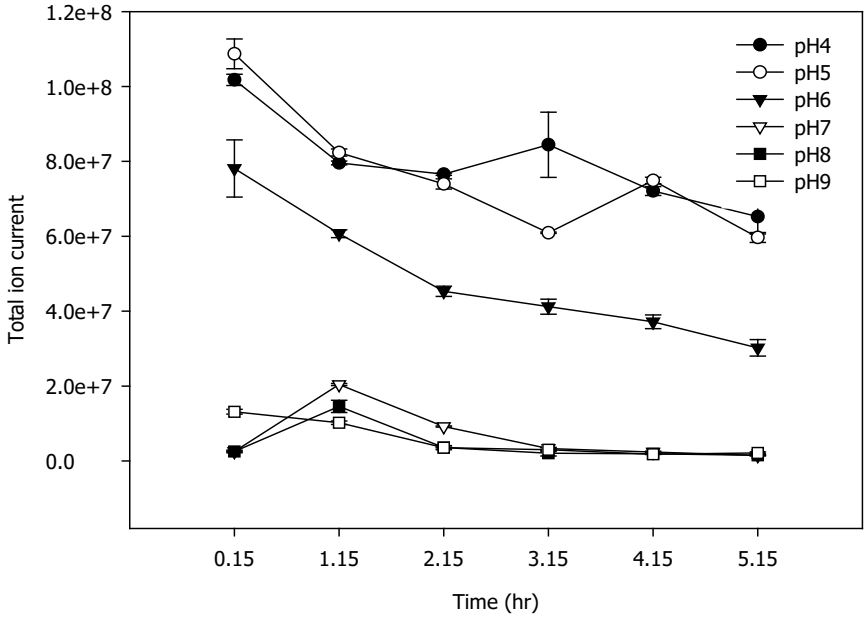


Figure 51. Degradation curves for 3-methoxybenzylITC extracted from *L. alba* seed meal at pH 5.0, 6.0, 7.0, 8.0, 9.0, at hourly time intervals. 50 mg seed meal extracted with DCM (1 mL). Samples were analysed by GC-MS, 50-1 split method

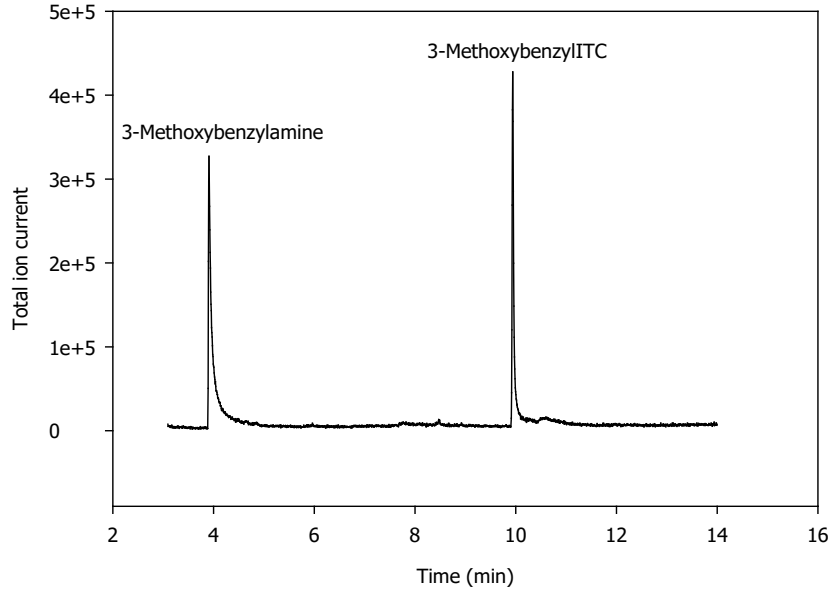


Figure 52. *L. alba* GSL hydrolysis products extracted from seed meal at pH 8.0 for 4 hrs, analysed by GC, 50-1 split (Table 22). 50 mg seed meal was extracted with 1 ml DCM.

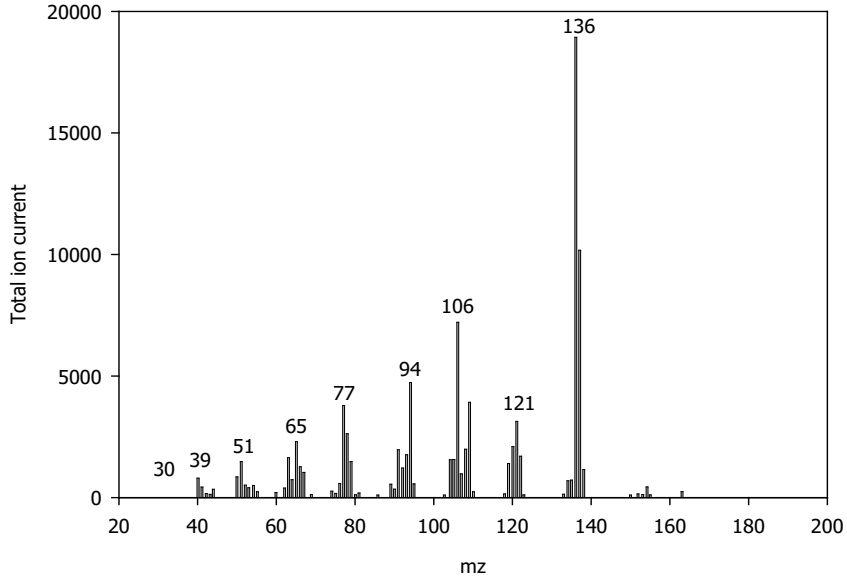


Figure 53. Mass spectrogram (IE) of 3-methoxybenzylamine, GSL hydrolysis product extracted from *L. alba* seed meal at pH 8.0 after 4 hrs, analysed by GC-MS, 50-1 split (Table 22). 50 mg seed meal was extracted with DCM (1 mL)

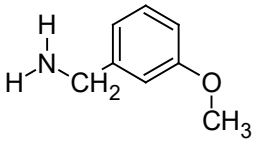


Figure 54. 3-methoxybenzylamine.

Scale up of ITC extraction and purificaton

Introduction

To produce larger quantities of pure 3-methoxybenzyl ITC for use in bioassays the extraction process was scaled up, bioactive fractions identified and separated, producing purified ITC. The pH 5.0 acetate buffer was used, as this produced the maximum amount of ITC.

Method

L. alba seed meal (40g) in 0.1 M acetic acid buffer (200 ml, pH 5.0) (Page 91) was agitated (100 RPM, 37 C for 1 hr), transferred to a separating funnel (1 L), extracted with DCM (400 ml) (ratio of 2:1 with the buffer) and centrifuged (MSE Hi-spin 21, 10,000 rpm) for 15 min. The seed meal was extracted three times, collecting the lower DCM layer each time. Extracts were bulked, dried over anhydrous magnesium sulphate, mixed on a stirrer (10 min) and filtered under pressure (Hartley funnel). DCM was removed under reduced pressure (Büchi Rotavapor RE120, 35 C) and the extract stored (-20 C).

Thin layer chromatography (TLC) techniques were used to identify the bioactive fraction of the extract. Using a sheet of Silica Gel 60 $_{F254}$ TLC aluminium sheet (Merck catalogue no. 1.05554.0001) cut to 3 cm x 9 cm, a drop of ITC extract was dissolved in a small amount of hexane and a small drop placed in the centre of the sheet approximately 1 cm from the bottom and the position marked. The plate was developed (solvent, hexane, 10 ml) until the solvent front almost reached the top of the TLC sheet. It was removed, dried, viewed under ultra violet light and the extract front marked.

As the extract front moved little the TLC was repeated using hexane:ethyl acetate solvent mixtures with ratios of 60:40, 80:20, 90:10 and 95:5, aiming for a retention factor (R_f) of around 0.3 until the correct proportions were found. The retention factor describes the distance travelled by a compound: molecules that move further up the TLC plate have a higher R_f value.

A Teledyne Isco CombiFlash® RETRIEVE ™ purification system with an Isco RediSep™ 40g normal phase silica column, with an optimum flow rate of 40 ml min^{-1}, volume 48 ml was used to purify the extract. Hexane:ethyl acetate (1 litre, 95:5) solvent was prepared and used to prime and equilibrate the CombiFlash purification system using 3 x column volumes of solvent. The extract was dissolved in solvent (4 ml), injected into the purification system and 35 x 15 ml fractions were collected. A TLC plate was spotted with drops from fractions 1-35 and viewed under ultraviolet light. ITC was found in fractions 6-15, with greater concentrations in the darker spots of fractions 9-12.

Further TLC was carried out using the crude extract produced previously and fractions 6-15 with the 95:5 hexane:ethyl acetate solvent. Fractions 9-12 again contained the greatest amount of ITC, the position confirmed by the R_f value. Fractions 9-12 were bulked, the solvent removed under reduced pressure (Büchi Rotavapor RE120, 35 C), diethyl ether (1 ml) added to the flask to dissolve the ITC and then transferred to a vial. The ether was removed with dry argon.

Results

The NCS group is polar and interacts with the silica, moving a shorter distance up the TLC sheet than a less polar molecule would. The ideal R_f value of around 0.3, was produced with a solvent ratio of 95:5 hexane:ethyl acetate (Table 26).

Hexane	EtOAc	Distance to extract front (mm)	Distance to solvent front (mm)	Rf
100	0	10	64	0.2
95	5	23	65	0.4
90	10	29	65	0.5
80	20	36	60	0.6
60	40	38	48	0.8

Table 26. Movement of extract in various solvent ratios during TLC.

780 mg green residue was extracted from 40 g seed meal, which was then purified to obtain 372 mg pure ITC, equating to 9.3 mg g^{-1} (57 µmol g^{-1}). Previously glucolimnanthin 32.492 µmol g^{-1} GSL was extracted from 18.2 g seed meal.

Preliminary bioassay to investigate the effect of ITCs on liverwort gemma growth

Introduction

To test the effect of ITCs (benzyl-, 2-phenylethyl-, 3-methoxybenzyl- and 2-propenyl- on liverwort growth an *in vitro* bioassay was developed whereby ITCs of varying concentrations were incorporated into M51C nutrient media (Table 20), from which they were taken up by individual gemmae laid on the surface. They were incubated for fourteen days and their radial growth measured using image analysis techniques. The ITCs needed to be dissolved in solvent prior to incorporation into the media, however earlier work indicated some sensitivity of gemmae to alcohol; therefore a preliminary experiment investigated gemma tolerance of ethanol. The methods used were developed by Dornbos (1990) who found that agar bioassays required smaller quantities of the bioactive compound than filter paper bioassays, partly due to the size of the cells used compared to Petri dishes, providing a more sensitive system.

Methods

Previous liverwort growth experiments (Table 27) indicated that liverwort gemmalings grow to approximately 5-6 mm in 3-4 weeks; therefore 25 ml well plates were an appropriate size to use.

Week	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6
Gemmaling diameter (mm)	1.0	2.9	5.1	6.8	7.3	8.3

Table 27. Gemmaling diameter mm when grown at 25 C, 400 µ mol m^{-2} s^{-1}.

M51C media was autoclaved in 5 x 100 ml quantities, cooled slightly, ethanol added as shown in Table 28 and mixed by pouring back and forth into a sterilised jar four times. 2 ml aliquots were pipetted into each well, 5 wells per treatment, replicated four times. One gemma was placed on the media in the centre of each well and incubated in growth rooms (20 C, 8 hr day). Plates were digitally photographed after 14 days and gemma areas calculated using ImageJ software.

Treatments					
% Ethanol	Control	0.1%	1%	2.5%	5%
Volume ethanol (ml) /100 ml media	0	0.1	1	2.5	5

Table 28. Treatments used in preliminary experiment.

Results

The ethanol reduced gemma growth in all treatments, markedly more so in the 2.5% and 5% treatments; 0.1% ethanol was used in subsequent bioassays (Table 29, Figure 55).

Treatment					
% Ethanol	Nil	0.1%	1%	2.5%	5%
Gemmaling area after 14 days	4.6	2.1	1.8	0.6	0.6
Gemmaling area as a percentage of control	-	45.5	39.0	13.8	13.5

Table 29. The effect of ethanol on liverwort gemma growth after 14 days.

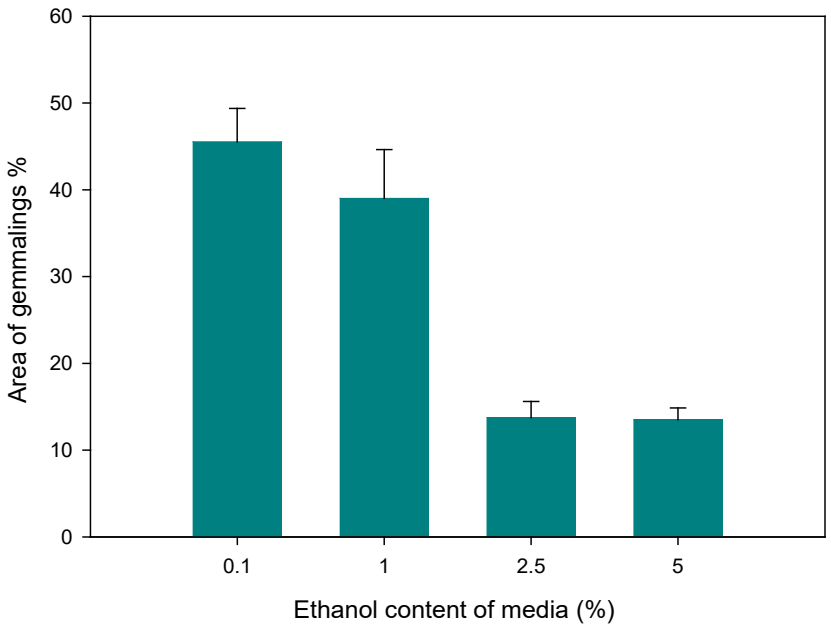


Figure 55. Preliminary investigation into the effect of ethanol on gemmaling growth (radial expansion). Gemmaling areas shown are a percentage of the control.

Bioassay investigating the effect of ITCs on liverwort gemma growth

Introduction

The bioassay method (Page 106) was used to test the effect of different concentrations of ITCs (benzyl-, 2-phenylethyl-, 3-methoxybenzyl- and 2-propenyl-) on liverwort gemmaling growth (radial expansion). Each ITC was dissolved in 0.1% ethanol prior to incorporation into autoclaved M51C media.

Method

ITC (100 mg) was dissolved in ethanol (1 ml), and then four 10-fold serial dilutions each of 100 and 50 mg ml^{-1} ITC, zero ITC (alcohol only) alcohol were prepared. The control contained media only, no ITC or alcohol.

12 x 50 ml aliquots of media were autoclaved and maintained at 50 C in a water bath. 50 µl of ITC (to achieve 0.1% ethanol) was added to each, mixed and pipetted (2 ml) into each well. 5 wells were used for each treatment and replicated 4 times.

Results

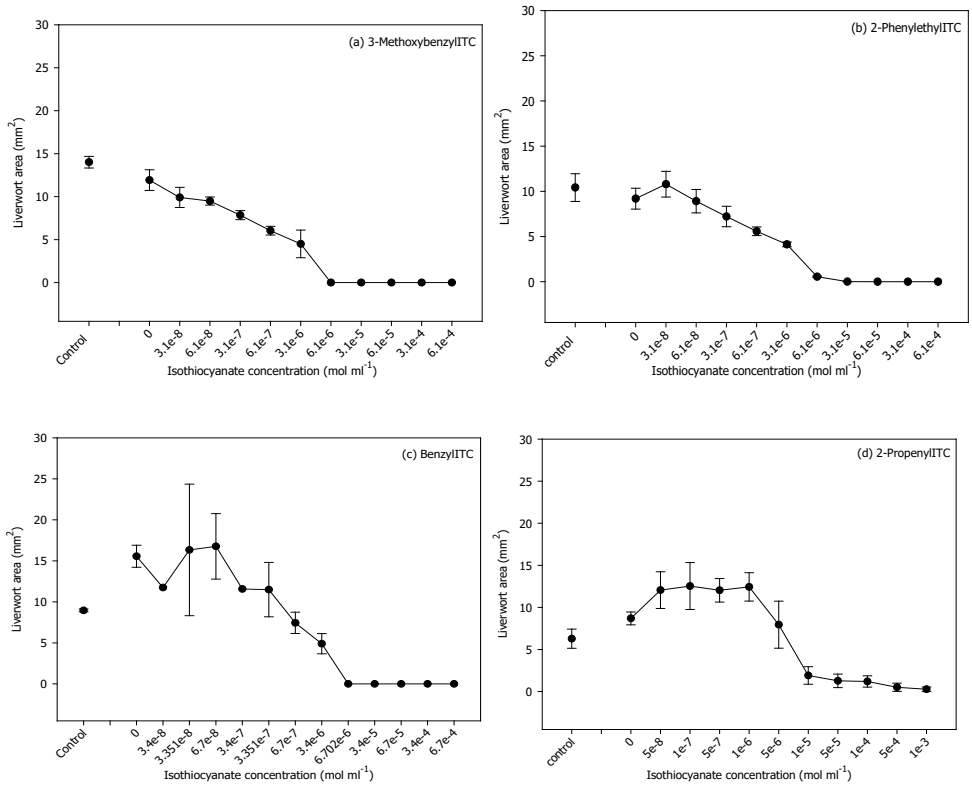


Figure 56. Dose-response curves of ITC concentration and gemmaling growth (radial expansion). Control: treatment with no thiocyanate or alcohol

ITC	ED_{50} (mol ml^{-1}) (±SD) Values and SDs x 10^{-3}
3-Methoxybenzyl	2.6 (±0.098)
2-Phenylethyl	3.1 (±0.106)
Benzyl	3.9 (±0.084)
2-Propenyl	0.3 (±0.129)

Table 30. Estimated ED50s and standard errors of ITCs applied to liverwort gemmae, obtained using probit analysis. ED50 is the effective dose where 50% of the gemma population has an area <1.5 mm^2.

Whilst low doses of 2-propenyl (0 to 0.5 µg ml^{-1}), benzyl (0 to 0.05 µg ml^{-1}) and 2-phenylethyl (0.005 µg ml^{-1}) promoted gemmaling growth compared to the control, all ITCs except 2-propenyl reduced growth almost to zero at concentrations of 1.0 µg ml^{-1} and above (Figure 56). These results were reflected in the estimated ED_{50} figures, with greater doses of 2-propenyl ITC required to limit growth of 50% of the gemma population to an area of 1.5 mm^2 (Table 30).

Herbicide bioassays

Introduction

Further *in-vitro* bioassays were carried out to investigate the effect of the herbicides Lenacil and Metazachlor (Figure 43) (Supplier: Sigma-Aldrich) on the radial growth of liverwort gemmae as a general comparison with ITCs.

Method

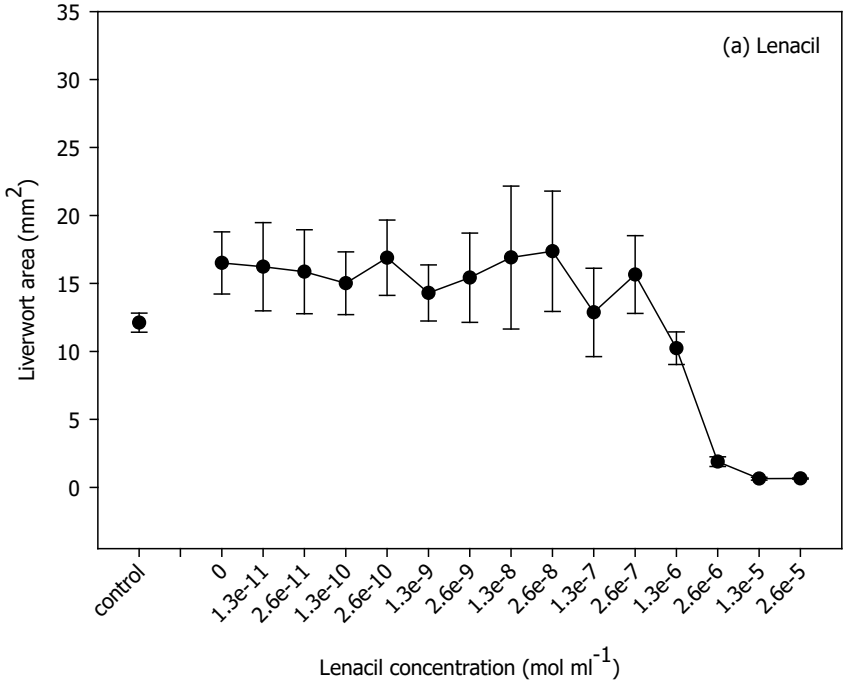
Lenacil is not soluble in ethanol or water, therefore a stock solution was prepared in dimethyl sulphoxide (DMSO), dissolving 100 mg Lenacil in 18.26 ml DMSO; the solubility of Lenacil in DMSO is 6 mg ml^{-1}, DMSO density is 1.1. Treatments used were 5 x 10-fold serial dilutions each of 6 and 3 mg ml^{-1} Lenacil, zero Lenacil (DMSO only), and a control containing media only, no Lenacil or DMSO.

Metazachlor (100 mg) was dissolved in ethanol (1 ml), then 4 x 10-fold serial dilutions each of 100 and 50 mg ml^{-1} Metazachlor, zero Metazachlor (alcohol only), and a control containing no Metazachlor or alcohol were prepared. Bioassays were prepared (Section 5.3.7), using 1 µl herbicide ml^{-1} media.

Results

Metazachlor produced a higher estimated $ED_{50,}$ than either Lenacil or the ITCs, however this does not fully describe its effect on gemma growth. Doses as low as 0.05 µg ml^{-1} reduced the average gemma area to 2.78 mm^2, and gradual growth reduction continued, reaching an average of 1.13 mm^2 with a dose of 100 µg ml^{-1} (Figure 57).

Similarly, the estimated ED_{50} for Lenacil was slightly higher than the majority of ITCs, although it produced a sharp growth reduction response at lower doses, between 0.06 and 0.6 µg ml^{-1} compared with 0.1 and 1.0 µg ml^{-1}. This suggests that these herbicides may produce a great enough effect on liverwort gemma growth to reduce liverwort infestation to below the economic injury level at lower doses than ITCs used in these bioassays (
Table 31). Selecting an ED_{50} standard that reflects the economic injury level would be more realistic in any future bioassays.



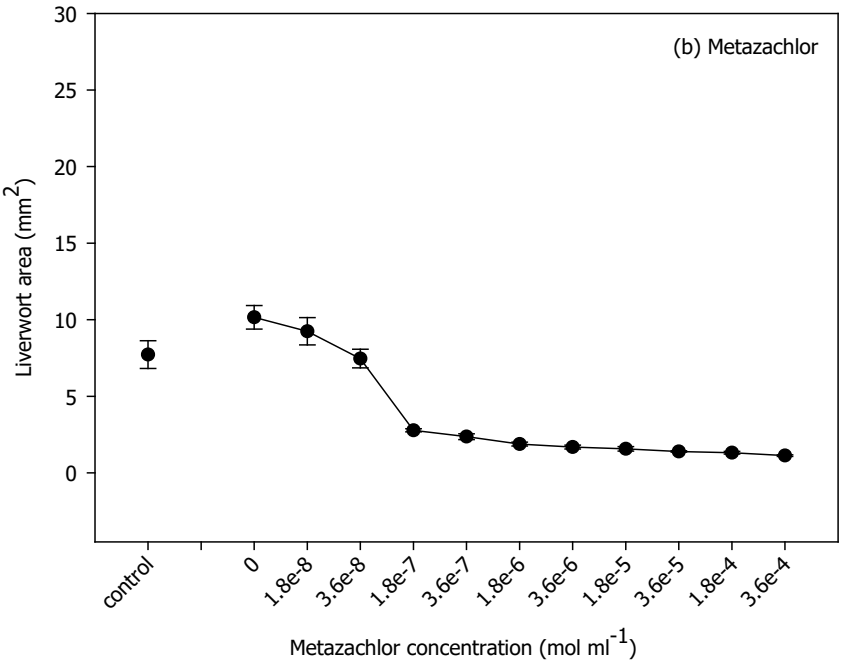


Figure 57. Dose-response curves of herbicides a) Lenacil and b) Metazachlor and liverwort gemma growth (radial expansion) expressed as a percentage of the control. The control treatments contained no solvent (DMSO or alcohol) or herbicide.

Herbicide	ED_{50} (□g ml^{-1})	s.e
Lenacil	0.981	0.162
Metazachlor	4.475	0.124

Table 31. Estimated ED_{50}s and standard errors of herbicides applied to liverwort gemmae, obtained using probit analysis. ED_{50} is the effective dose where 50% of the gemma population has an area <1.5 mm^2.

Cress ITC bioassays using Petri dishes

Introduction

A further bioassay was developed to investigate the phytotoxicity of ITCs (benzyl-, 2-propenyl-, 2-phenylethyl- and 3-methoxybenzyl-) on a higher plant species, cress (*Lepidium sativum*), to confirm whether there would be a comparable effect on germination and growth. A method was adapted from that used by Kasama (2003), with seeds incubated on filter paper in petri dishes and fed with the bioactive compound dissolved in distilled water, recording seed germination and radicle elongation.

Method

ITC (100 mg) was dissolved in ethanol (1 ml), then 4 x 10-fold serial dilutions each of 100 and 50 mg ml^{-1} ITC, zero ITC (alcohol only), and a control containing no ITC or alcohol were prepared. Each of these was then made up into a 10 ml solution prepared using 10 µl ITC in sterile distilled water; these solutions were fed to the cress seeds. This was repeated for each of the ITCs used.

10 seeds were placed on filter paper (Whatman 1, 90 mm) in each Petri dish, fed with ITC solution (2 ml) on the first day and sterile distilled water thereafter for 14 days as required maintaining moist, humid conditions. Petri dishes were arranged in a completely randomised design, with two replications, on laboratory benches out of direct sunlight in ambient conditions. Radicle lengths were measured using digital image analysis techniques and the number of seeds that germinated was recorded.

Results

Little effect of these ITCs on cress seed was observed, with average radicle lengths and germination rates similar at all concentrations of ITC used (Figure 58 and Figure 59). This contrasts with the results observed when the same ITCs were applied to

liverwort gemmae, when thallus radial growth was reduced with greater concentrations of ITC. This suggests these ITCs could potentially be applied as a herbicide over higher plants affecting their growth.

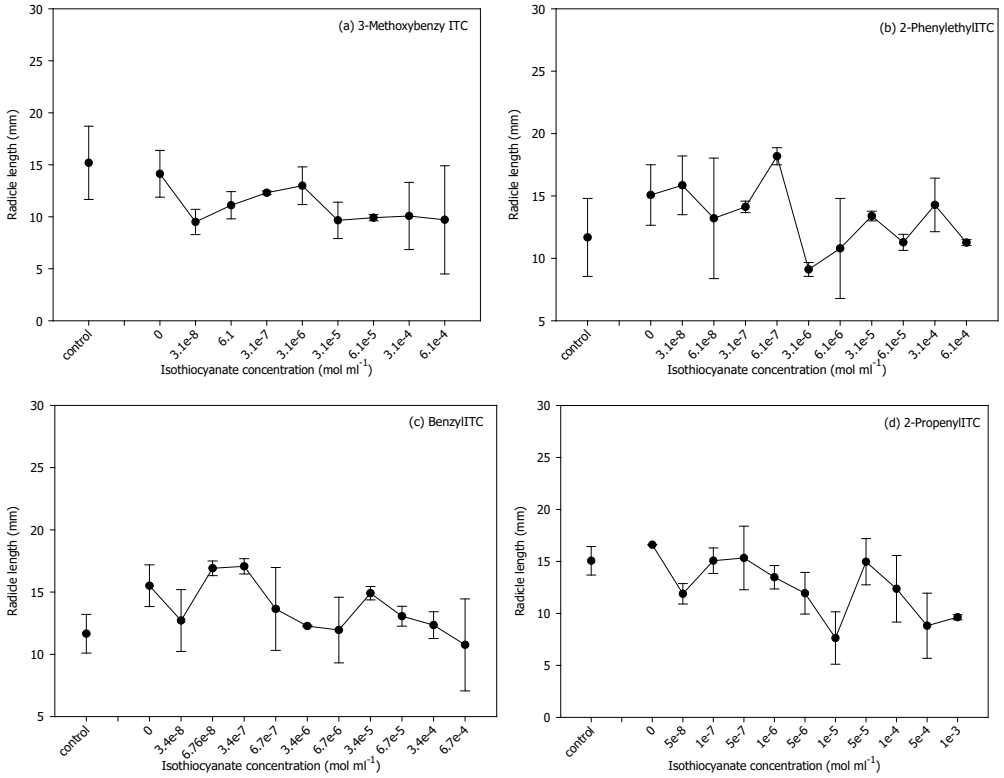


Figure 58. Dose-response curves of ITCs and cress (*Lepidium sativum*) radicle elongation. The control treatment contained no alcohol or ITC.

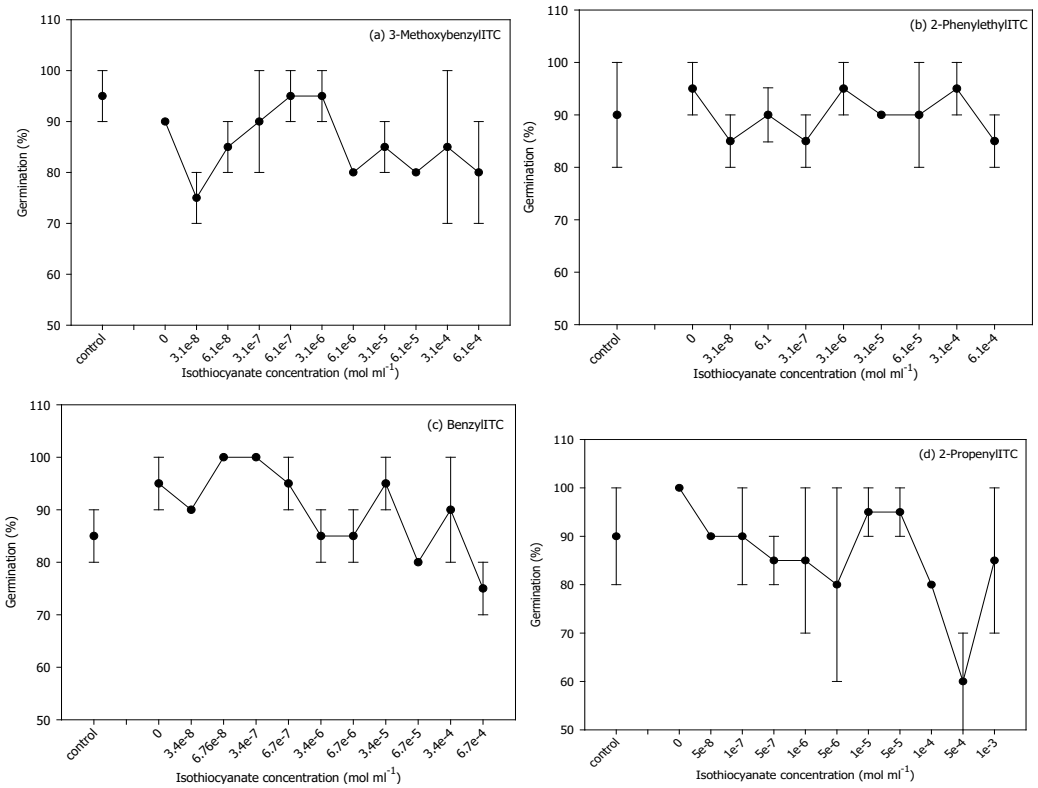


Figure 59. Dose-response curves of ITCs and cress seed (*Lepidium sativum*) germination (%). The control treatment contained no alcohol or ITC.

Preliminary investigation into ITC collection using Amberlite resin.

Introduction

Yamane (1992) successfully trapped *ω*-methylsulfonylalkylITC from root exudates using Amberlite XAD-4 polymeric resin adsorbent (Supplier: Sigma-Aldrich), designed to collect hydrophobic compounds. It was considered that from this a non-destructive method for continuous ITC collection could be developed without resorting to complex extraction protocols; simple lab-based bioassays could then be designed to measure their effect on liverwort thallus growth.

A preliminary experiment using ITC standards was developed adapting Yamane's method for collecting and identifying ITCs from the four test plants (Table 18). Samples were eluted from the column and analysed by GC-MS.

Method

A mixture was prepared containing 10 mg of each ITC (2-propenyl-, benzyl-, 3-methoxybenzyl- and 2-phenylethyl-) in distilled water (2.5 L) shaken well to dissolve and left to settle.

A trap column was prepared using a Pharmacia XK26 column filled with Amberlite XAD-4 resin polymeric adsorbent (15 ml) (Sigma-Aldrich). The resin was mixed with enough distilled water to produce a slurry, put into the column and washed with methanol (200 ml), then 200 ml distilled water using an Eyela micro tube pump MP-3 (manufactured by Tokyo Rikakikai Co. Ltd) to circulate the solutions.

The ITC solution was pumped through the resin, collecting approximately 1 litre. The resin was removed from the trap column into a funnel over a side-arm conical flask and vacuum filtered. The resin was washed with distilled water (200 ml), and the ITC's eluted with acetone (100 ml), collecting the filtrates separately. Water was removed from the acetone filtrate with sodium sulphate, stirred (20 min) then filtered. It was then concentrated to 5 ml (Techne Dri-Block DB-3A sample concentrator) and subjected to GC-MS, 3-1 method (Table 21 andTable 22) analysis.

Results

All four ITCs were detected in the samples (Figure 60), their identity confirmed by comparison of their mass spectra with spectral data published by Spencer and Daxenbichler (1980) and Vaughn and Berhow (2005), confirming the methods were appropriate for the collection and elution of these ITCs from solution.

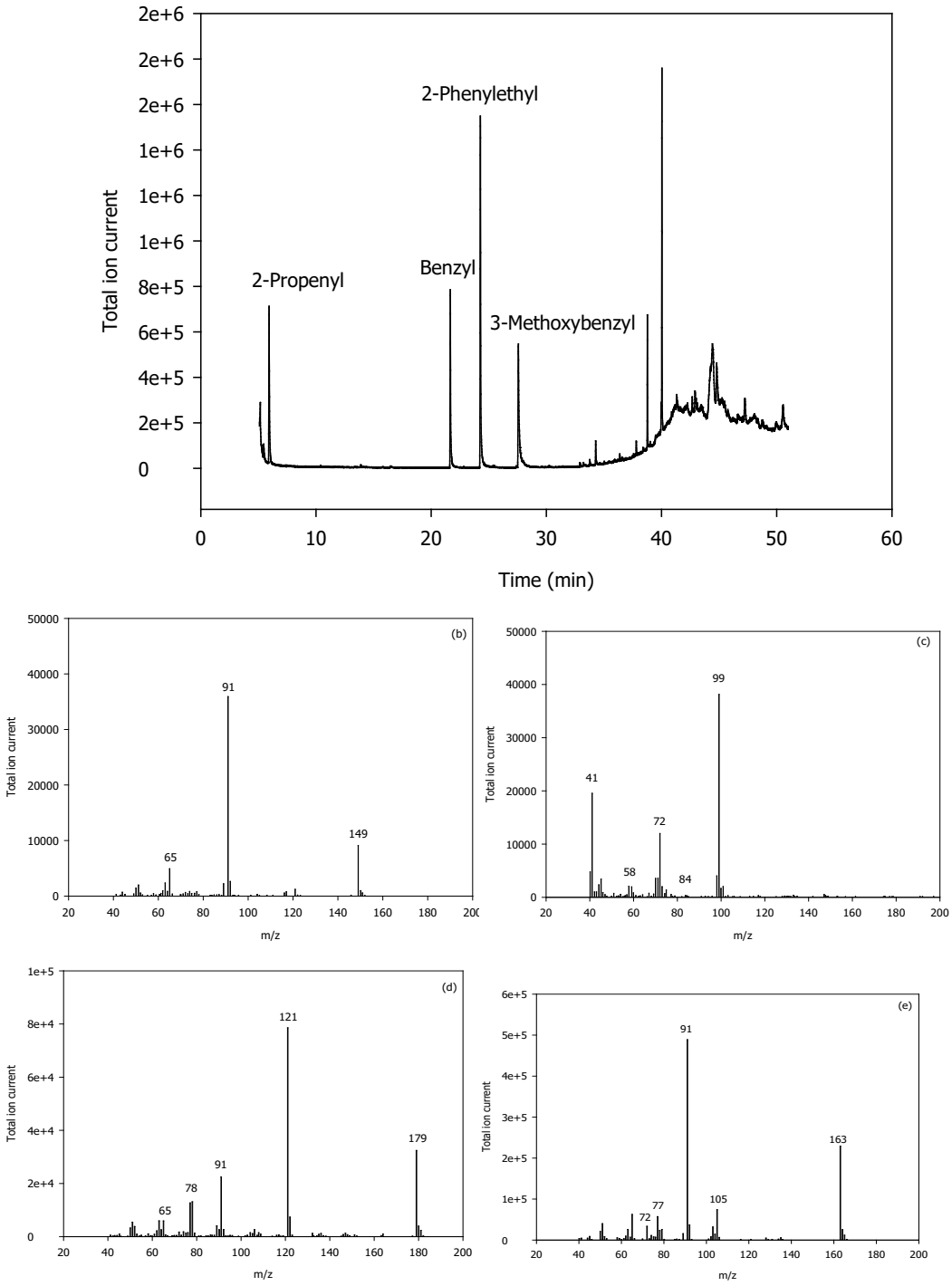


Figure 60. Chromatogram of the four standard ITCs used (a) and their mass spectra: (b) benzyl (c) 2-propenyl (d) 3-methoxybenzyl (e) 2-phenylethyl

Collection of root exudates from the glasshouse hydroponics system

Introduction

The method developed proved successful for ITC trapping and elution and was adapted for the collection of ITCs exuded by plant roots direct from the hydroponic growing system (Page 87) onto the trap column. *D. tenuifolia* was used for this section of work as the *L. alba* plants had deteriorated and been replaced with fresh plants which had young, less developed root systems at this time.

Method

An in-line filter was constructed using a glass tube filled with glass wool at either end and with acid washed sand (BDH Lab supplies, 0.1-0.3 mm grain size, product no. 330945E) between (Figure 61) to prevent plant debris from blocking the plastic tubing.

Figure 61. In-line filter construction

Equipment used in the preliminary experiment was prepared (Figure 62) with the in-line filter attached, and with the open end positioned in the irrigation stream within the root zone of *Diplotaxis tenuifolia*. Nutrient solution containing root exudates was pumped through the trap column (27 hrs). Nutrient solution containing root exudates was pumped through the trap column (27 hrs). ITCs were eluted from the Amberlite resin, concentrated to 5 ml (Techne Dri-Block DB-3A sample concentrator) and subjected to GC-MS analysis, 3-1 method (Table 22).

Samples were purified by diluting with 15 ml distilled water, extracting twice with an equal quantity of dichloromethane (DCM) by shaking, allowing it to settle and the lower, DCM, layer removed. Extracts were bulked, magnesium sulphate added to remove any water, filtered, DCM removed to a small volume by rotoevaporation, and then concentrated to approximately 200 µL. Samples were again subjected to GC analysis using the 3-1 split method (Table 22).

Figure 62. ITC extraction equipment

To quantify the amount of ITCs in the sample a calibration curve was produced. The sample was dried down to remove the remaining DCM and re-dissolved in DCM (300 µL). A standard was prepared by dissolving quantities of benzylITC, 3-methoxybenzylITC and 2-phenylethylITC (Table 32) separately in acetone (1 ml). 100 µL of each was then dissolved in acetone (700 µL) and mixed with acetone to produce: 1:250, 1:500, 1:750, 1:1,000 and 1:2,000, 1:4,000, 1:6,000, 1:8,000 and 1:10,000 dilutions. The standards were analysed by GC-MS, 3-1 split method (Table 22). Graphs were constructed using peak areas, subjected to linear regression and used to quantify the ITCs present.

ITC	Weight (µg)
Benzyl	16.0
2-Phenylethyl	12.5
3-Methoxybenzyl	13.3

Table 32. ITC weights used for standards

Results

The data produced from the GC-MS analysis of the ITC standard solutions was used to construct a linear regression, showing the relationship between peak area and ITC quantity, providing values for α and β. The fitted equation was $y = \alpha x + \beta$, where x = quantity of ITC, y = peak area, α = intercept and β = slope; calculated weights of the ITCs are shown (Table 33).

ITC	Weight (µg)
3-Methoxybenzyl	220
Benzyl	5
2-Phenylethyl	94

Table 33. Quantities of ITCs extracted from root exudates o *D. tenuifolia*.

GC-MS analysis initially contained numerous peaks probably produced by plasticisers from the plastic equipment used in the hydroponics system, and no ITCs were identified. Following purification benzylITC, 2-phenylethylITC and 3-methoxybenzylITC were found in the samples (Figure 63), and their identities confirmed by comparison with spectral data published by Spencer and Daxenbichler (1980) and Vaughn and Berhow (2005). However, as glucolimnanthin had not previously been identified in *D. tenuifolia* roots its hydrolysis product 3-methoxybenzylITC was not expected to be found in this sample and was a suspected contaminant.

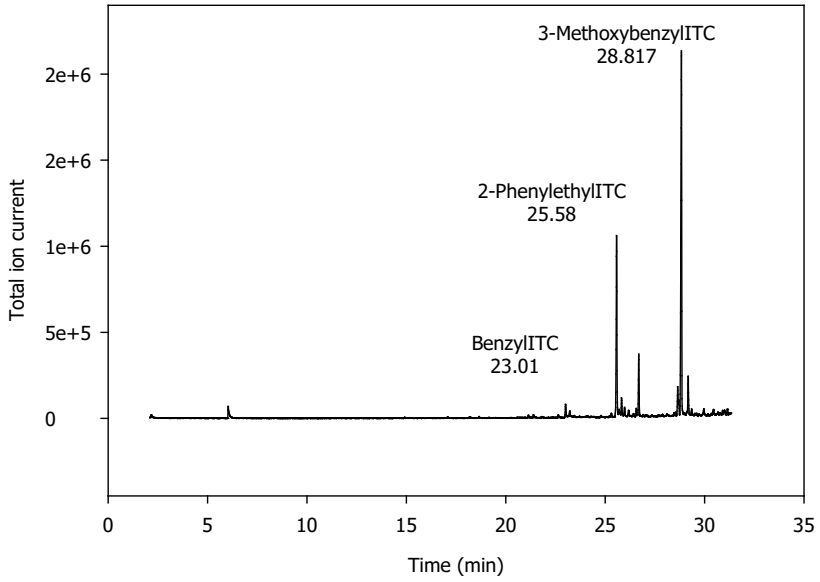


Figure 63. *Diplotaxis tenuifolia* root exudates chromatograph, analysed by GC, 3-1 split method (Table 22).

Collection of root exudates from plants grown in glass containers

Introduction

As plasticisers were detected in the root exudate samples collected direct from the hydroponics system (Page 87), mature plants of *Diplotaxis tenuifolia* were removed to glass vessels and the collection process repeated, followed by GCMS analysis.

Method

3 plants of *Diplotaxis tenuifolia* were suspended in fresh water in a glass chromatography tank. Collection equipment set up was as previously used with the filter again placed in the plant root zone; root exudates were collected over 24 hrs (Figure 61 andFigure 62). The roots of the same plants were then damaged by squeezing between forceps and root exudates, eluted and concentrated as previously.

Samples were diluted with distilled water (15 ml), and extracted twice with an equal quantity of dichloromethane (DCM) by shaking, allowed to settle and the lower (DCM) layer removed. Extracts were bulked, anhydrous magnesium sulphate added to remove any water, filtered and DCM removed under reduced pressure (Büchi

Rotavapor RE120, 35 C), concentrated to approx. 200 µL and subjected to GC-MS analysis, 3-1 method (Table 22) analysis.

Results

None of the ITCs found in the earlier samples were detected in these samples, suggesting the compounds were contaminants as suspected.

GSL extraction from Diplotaxis tenuifolia root tissue

Introduction

The previous experiment seemed to confirm the ITCs previously detected in *D. tenuifolia* root exudates were contaminants. To verify this GSLs were extracted from root tissues and identified on the premise that without GSLs in the root tissue, no ITCs could have been produced in the root exudates.

Method

Roots from *D. tenuifolia* used in the root exudate extraction were washed with water, freeze dried, ground in a coffee grinder and the GSLs were then extracted from a 50 mg sample as described (Page 96) along with 2-propenyl GSL and benzyl GSL standards.The sample and standards were analysed by HPLC (Page 96), however the peaks produced by the sample did not correspond to those produced by the standards. The sample was then subjected to LC-APCI MS/MS and UV (230 nm) analysis for identification.

Roots from *D. tenuifolia* used in the root exudate extraction were washed with water, freeze dried, ground in a coffee grinder and the GSLs were then extracted from a 50 mg sample as described in Section 5.3.2 along with 2-propenyl GSL and benzyl GSL standards. The sample and standards were analysed by HPLC (Page 117), however the peaks produced by the sample did not correspond to those produced by the standards. The sample was then subjected to LC-APCI MS/MS and UV (230 nm) analysis for identification.

Results

Chromatographs and spectra (Figure 64) were analysed and compounds identified by comparison with known standards or by computer comparison with the Wiley 275 mass spectra library. Of these compounds only 4-methylsulphinylbutyl and *p*-hydroxybenzyl have previously been identified in *D. tenuifolia* roots by others (Table

18). The GSLs relating to the ITCs identified in root exudates were not found. This could suggest that either they were contaminants or as the plants' condition had deteriorated by this time, the GSLs were no longer present.

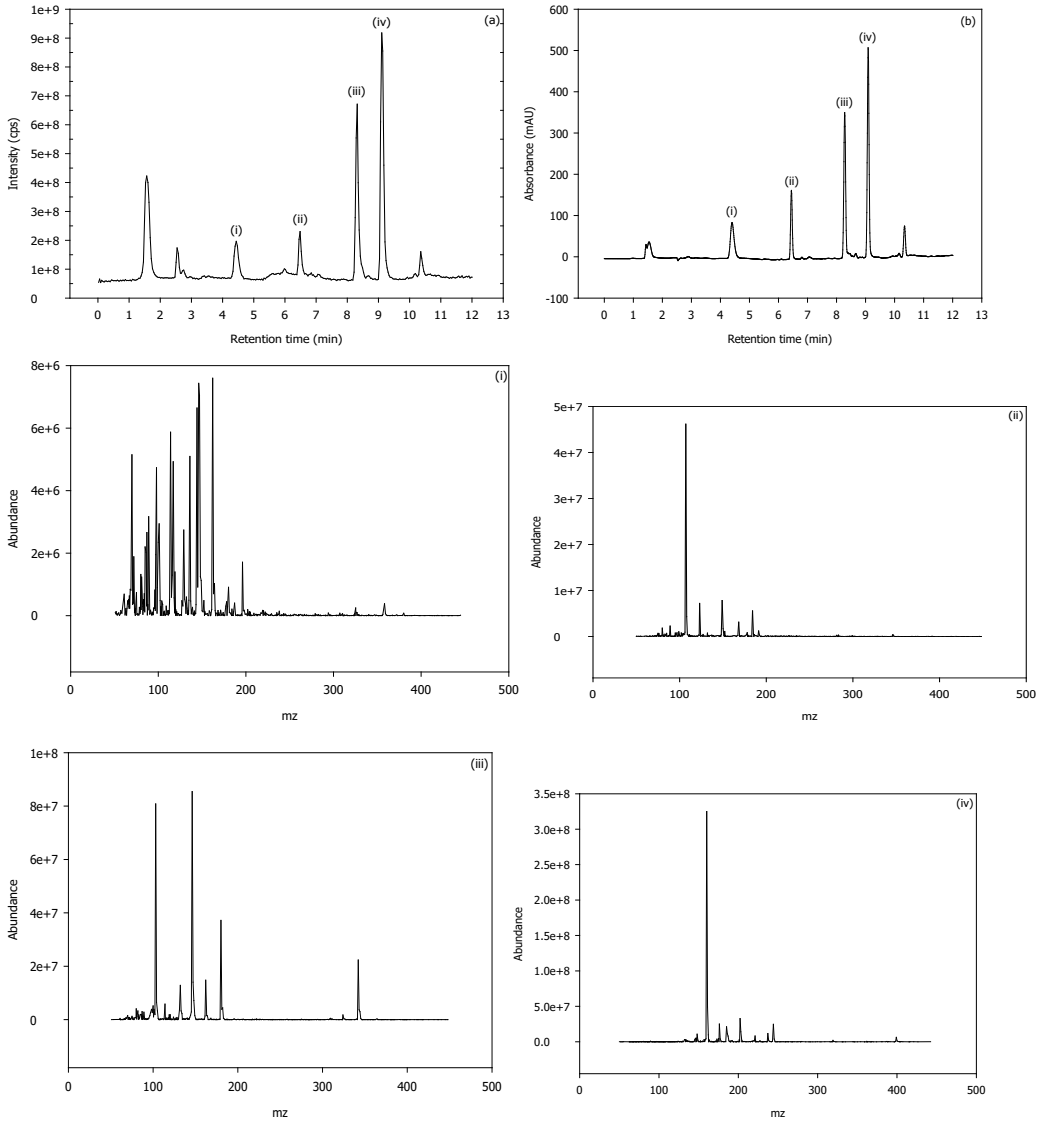


Figure 64. Analysis of GSL content of *Diplotaxis tenuifolia* root tissue: chromatographs analysed by (a) total ion current (90-400 atmospheric mass units) and (b) detected by UV absorption at 230 nm wavelength. Chromatogram peaks and mass spectra (EI) refer to GSLs: (i) 4-methylsulphinylbutyl (ii) *p*-hydroxybenzyl (iii) 4-methylthiobutyl (iv) 4-methoxy-3-indolemethyl.

Discussion

The GSL work in this study was undertaken primarily to identify whether selected GSL hydrolysis products have a herbicidal effect on liverwort. Three main approaches were taken: bioassays investigating the effect of four ITCs, including that found in *L. alba*, on liverwort gemma growth; an investigation into the GSLs

present in *L. alba* seed meal, and the direct harvesting of ITCs from the root exudates of plants grown hydroponically.

Phytotoxicity

The results of the bioassays using both ITCs and herbicides against liverwort gemmae did show a phytotoxic effect, with estimated ED_{50} values showing that 3-methoxybenzyl ITC (Limnanthin) was the most potent of those tested and it was effective at lower concentrations. ED_{50}s for Metazachlor and Lenacil were higher than for the ITCs, suggesting that more herbicide than ITC would be required to be effective against liverwort gemmae. The cress seeds were tolerant of the ITCs at the concentrations used; compounds toxic to target weeds without harming crop plants could suggest a potential use as a selective herbicide that could be applied over crop plants.

ED_{50} values obtained suggest that of those tested 2-propenylITC was the least toxic towards liverwort and is also the most volatile. According to Sarwar (1998) application method should be geared to ITC characteristics, applying volatile ITCs in a headspace and water soluble ITCs in agar media to gain greater inhibitory effects, and this could provide an explanation for the marked difference in toxicity of 2-propenylITC. Losses could also have occurred during incorporation with warm media. The aliphatic ITCs tend to be more volatile than aromatic as volatility decreases with increased molecular weight (Norsworthy and Meehan, 2005a).

Norsworthy and Meehan (2005b) found aromatic ITCs more phytotoxic towards annual dicotyledonous weeds and suggested this is due to the greater stability of their structures provided by their conjugated ring, and therefore greater contact with the weed before degrading. It is the variable side chain found on GSLs that is thought to determine the biological activity of ITCs (Brown and Morra, 1999). Vaughn *et al* (2006) found that aliphatic ITCs with short chain R-groups, such as 2-propenylITC, were amongst the most inhibitory; according to Sarwar *et al* (1998) the shorter the chain length the greater the toxicity of ITCs, when used against soil-borne fungal pathogens, and suggests such a trend occurs within each ITC class.

This hypothesis was not borne out by this research, where 2-phenylethylITC (ED_{50} = 0.513 ug ml^{-1}) was marginally more toxic than benzylITC (ED_{50} = 0.583 ug ml^{-1}), however the values obtained were similar; had there been greater disparity in their structure a more definitive difference may have been observed.

Low ITC concentrations can have a stimulatory effect on plants, as found in these bioassays. Norsworthy and Meehan (2005a) found that phenyl and 2-phenylethylITC had a stimulatory effect on sicklepod emergence at lower concentrations, becoming toxic at higher concentrations, and this effect was observed with benzylITC and 2-propenylITC, and also the two herbicides Metazachlor and Lenacil.

Limnanthes alba

The GSL profile of *L. alba* was established, and identified a single GSL, glucolimnanthin, present throughout the plant. In other plants the profile can be widely variable, with most species producing a range of GSLs in each tissue, e.g. nine have been identified in the roots of *B. juncea* (Figure 41).

The GSL glucolimnanthin was extracted from *L. alba* seed meal, with yields of 32.492 µmol g^{-1} seed meal (1.43%), and the quantity of limnanthin (3-methoxybenzylITC) extracted was 56.992 µmol g^{-1} seed meal (0.93%). These values compare favourably to quantities of 265 nm glucolimnanthin g^{-1} *L. alba* seed meal, and 65 nm limnanthin extracted from a mixture of 0.5 g seed meal and soil by Vaughn *et al* (2006), the variance possibly due to differences in the extraction method. GSL levels obtained from the remainder of the plant ranged between 79 and 87 nmoles mg^{-1} except for the roots, where the recovery dropped to 39%, compared with over 82% for other tissues.

Although ITCs are generally produced in pH 7.0 conditions (Chew, 1988), in the extraction optimisation experiment more limnanthin was produced in acidic conditions (pH 4.0 and 5.0) and least in neutral to alkaline (pH 7.0, 8.0 and 9.0) conditions; Vaughn and Berhow (2005) found a 0.1 M HCL crude extract from *L. alba* produced the greatest amount of 3-methoxybenzylITC, although it was also present in extractions in pH 7 and 10 buffers.

Hydroponics

The method for collecting and analysing ITCs direct from a solution was successful in principle, however when used to collect ITCs direct from the root exudates of *D. tenuifolia* growing in the hydroponics system the samples were contaminated. Further GSL analysis of the root tissue did not identify any of the expected ITCs. Whilst four plant species were grown hydroponically for root exudate collection only

D. tenuifolia had great enough root mass for ITC exudate collection as the others had succumbed to powdery mildew infection (*B. juncea*, *S.orientale*, and *L. alba*), outgrown the equipment (*B. juncea*) or their general condition deteriorated. Replacement plants were not sufficiently developed to be used.

The intention of this section of work was to develop a system whereby ITCs could be harvested simply from root exudates, thereby providing a continual supply and avoiding destructive methods requiring complex ITC extraction techniques. Time constraints did not allow for further development of this work within this project. Improvements could easily be made to the design of the hydroponics system to suit the root mass produced by plants; the narrow width of the plastic guttering used resulted in the roots blocking the water flow on occasions, and free flow is essential to the supply of optimum levels of nutrients and oxygen to the roots. The plastic construction materials caused complications with GC-MS analysis as samples were contaminated, with detectable plasticisers appearing in the chromatographs and requiring analysis before discounting them as compounds of interest.

Proprietary nutrient solutions were used in this hydroponics system. However, by designing a bespoke nutrient solution based on analysis of the local water and plant species requirements, plant growth could be optimised and individual components altered as required. It is suggested that GSL production varies under different fertilisation (Al-Khatib and Boydston, 1999; Josefsson, 1970; Ju *et al.*, 1982) and pH conditions, and according to plant age and life cycle stage (Clossais-Besnard and Larher, 1991); manipulation of the hydroponic growing environment would allow ITC and other bioactive compound levels in root exudates to be characterised in optimal and sub-optimal conditions, as would the application of other stresses such as mechanical root damage.

Conclusions

Every liverwort present in the nursery should be considered a source of new infestation, therefore following strict nursery hygiene procedures, such as ensuring non-cropping and standing down areas are kept free of liverwort, keeping growing media covered to prevent contamination, and ensuring any second hand pots are cleaned before use will help to reduce liverwort spread.

The environment experiments show there is a clear effect of light and temperature on liverwort growth, and these results could be used by growers to help manage liverwort control. Where appropriate to plant growth, shade and light levels could be manipulated to provide environmental conditions unfavourable to maximum liverwort growth, working towards an economic injury level acceptable to the grower. Providing sub-optimal environmental conditions for the liverwort would ensure that it does not proliferate as widely by limiting gametophore production and consequently spores. This should be tailored to crop plant requirements, with plants tolerant of higher light levels grown in reduced shade conditions and shade loving plants grown in low light levels.

The gemma dispersal experiments give a clear indication that by revising current irrigation practices growers can work towards reducing liverwort spread. Use of sub or drip irrigation systems, particularly when renewing or upgrading existing equipment; factoring water and reduced labour cost savings into installation estimates would provide immediate financial benefit to growers. Growers who use overhead systems could consolidate irrigation events from many short to fewer longer applications, ensuring the surface of the compost dries out between water applications.

These results complement the conclusions of HDC report HNS 107 that found that sub-irrigation can provide cost savings of 25-35% over well designed overhead irrigation, improve water use efficiency, uniformity of water distribution and plant quality (Burgess, 2003). Reduction of overhead irrigation events would immediately provide growers with water cost savings. Reduced liverwort infestation and the consequent reduced need for its removal before crop marketing contains implicit labour and media (top dressing) savings.

Grouping of plants by their light and water requirements would help to incorporate these findings into general nursery practice. This would allow less irrigation to be applied to those species requiring less water, reducing liverwort (less overhead irrigation) and establishment (dryer compost surface). Light levels could also be managed in this way, so that light levels could be applied to a group of plants with similar light requirements, rather than managing for the most sensitive plant from a group with a range of different light level requirements for healthy growth.

The fungal antagonists did show some promise in infecting liverwort in glasshouse experiments, however, this did not transfer well to glasshouse experiments. The conditions appropriate to fungal infection and colonisation of hosts are also ideal for liverwort. However, *Fusarium equiseti* is a soil saprophyte that has been observed attacking liverwort in a nursery situation, and therefore may establish with either a dryer or shorter humidity treatment, and further investigation could provide a method of successfully infecting liverwort with this antagonist.

GSL hydrolysis products did prove to have a herbicidal effect on liverwort gemmae, comparable to two herbicides; more work is now required to develop an application system that could transfer this to glasshouse experiments.

The ITC collection system did prove effective when used with ITC standards, however the key issue is growing plants within a hydroponic system and harvesting ITCs from root exudates when they are at peak conditions and this needs to be improved. The hydroponics apparatus would need to be constructed from non-plastic materials to eliminate problems with plasticisers during GC analysis. Further work would establish this as a non-destructive, practical system for ITC collection without the need for complex extraction protocols, which could then be used as a model for other GSL work in addition to liverwort control.

Technology Transfer

An article in the HDC News announced the start of this project, and two further articles were published in the HDC News, in the February 2004 and February 2005 issues.

Posters were presented at annual Post Graduate Symposia held at the Department of Agricultural Sciences, Imperial College London, Wye Campus on 20th June 2004 and June 2005, and a presentation given in November 2006.

Posters were presented at the XIII European Weed Research Society Symposium, Bari, Italy in June 2005, and the North Easter Weed Science Society conference in Providence, Rhode Island, US in January 2006.

Glossary

Subject	Description	Reference
Antheridiophore	Structure bearing antheridia	(Lawrence, 2000)
Antheridium(a)	Organ in which male gametes are produced	(Lawrence, 2000)
Archegoniophore	Structure bearing archegonia	(Lawrence, 2000)
Archegonium(a)	Organ in which female gametes are produced	(Lawrence, 2000)
Azeotrope	A mixture of two substances that boil at the same specific temperature so they may be distilled off. Butan-1-ol can therefore be used to remove acetic acid	(Lawrence, 2000)
Conidioma	Any fungal structure bearing conidia	(Lawrence, 2000)
Conidiophore	Hypha or cell bearing conidiogenous cells	(Holliday, 1998)
Conidium (pl. conidia)	Asexual fungal spore	(Holliday, 1998)
Dioecious	Plant with male and female structures on separate plants	(Lawrence, 2000)
Gametophore	Structure bearing the spore on which the archegoniophore and antheridophore	(Lawrence, 2000)
Gametophyte	Haploid, gamete-forming phase	(Lawrence, 2000)
Gemma cup	Cup shaped structure bearing gemmae	(Lawrence, 2000)
Gemma(e)	Lenticular vegetative propagule (haploid) that develops into a thallus	(Lawrence, 2000)
Gemmaling	Young, recently germinated gemma	(Lawrence, 2000)
Macroconidium (pl, macroconidia)	Larger conidium of a fungus that may also have microconidia e.g. *Fusarium spp.*	(Holliday, 1998)
Mycotoxicosis	Poisoning by toxins produced by fungi	(Lawrence, 2000)
Pycnidia	Flask-shaped, globose structires lined with conidiogenous cells	(Webster, 1980)
Saprophyte	Organism that gains nutrition from dead or decaying organic matter	(Holliday, 1998)
Thallus (pl. thalli)	Liverwort body, not differentiated into leaf or stems, which in *Marchantia polymorpha* bears circular gemma cups	(Lawrence, 2000)

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