Project title:	Biology, epidemiology and control of liverwort infestation of nursery plant containers
Project number:	HNS 126
Project leader:	Prof M J Jeger Department of Agricultural Sciences Imperial College London Wye Campus Wye Ashford Kent TN25 5AH
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Previous reports	None
Key workers:	Mrs Jill England (Principle Investigator) Dr J Rossiter (Glucosinolate phytochemistry) Dr G P Buckley (Liverwort biology and taxonomy)
Location:	Department of Agricultural Sciences, Imperial College London, Wye Campus
Project co-ordinator:	Chris Lane Coblands Nurseries Ltd Trench Road Tonbridge Kent TN11 9NG
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Key words:	Liverwort, *Marchantia polymorpha*, Hardy Nursery Stock

The results and conclusions in this report are based on an investigation conducted over one year. 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 growth (radial expansion), fresh weight, dry weight and number of gemma cups was greater in low light (400 µ mol m^{-2} S^{-1}) than high light conditions (800 µ mol m^{-2} S^{-1}), when grown at both 15 C and 10 C, consistent with year 1 results for liverworts grown at 25 C and 15 C.
- 'Clumping' of liverwort gemmae, observed in dispersal experiments, did not aid establishment or colony growth (fresh and dry weights) when grown on compost.
- Increased gemma cup replenishment was observed with increased occasions of gemmae removal.

Background and expected deliverables

Liverworts 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 a previous 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 (mainly labour costs). Zero tolerance for liverworts in accreditation schemes and government withdrawal of chemical approvals is putting huge pressure on the industry to find alternative, cost effective control measures.

The expected deliverables from this project are to:

- provide comprehensive information on the biology and epidemiology of liverwort infestations
- identify key stages in liverwort life cycles where control interventions, could be most effective
- identify the liverwort species infesting nurseries
- investigate non-chemical means of liverwort control: fungal pathogens, glucosinolate hydrolysis products obtained from seed material and cultural methods
- carry out trials based on the above results to investigate individual and integrated methods of control

Summary of the project and main conclusions

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

An experiment using four Fitotron growth cabinets compared the effect of different temperatures (15 C and 10 C) and light levels (800 µ mol m^{-2} S^{-1} and 400 µ mol m^{-2} S^{-1} or 174 and 87 Watts m^{-2} respectively) on the growth (radial expansion), fresh and dry weight and development of liverworts over 6 weeks.

Dry weight (Figure 1), fresh weight, area, and number of gemma cups were all greater in high temperature than low; and greater in low light than high light after both four and six weeks growth, consistent with year 1 results for liverworts grown at 25 C and 15 C. The only exception was the high temperature treatment where more gemma cups per mm^{-2} were produced in high light than low.

After four weeks there were more gemma cups on male than female gemmalings (a young liverwort developing from a gemma) in high light than low light treatments. In low temperature the gemmalings were particularly small and gemma cups were only produced in the female, low light treatment.

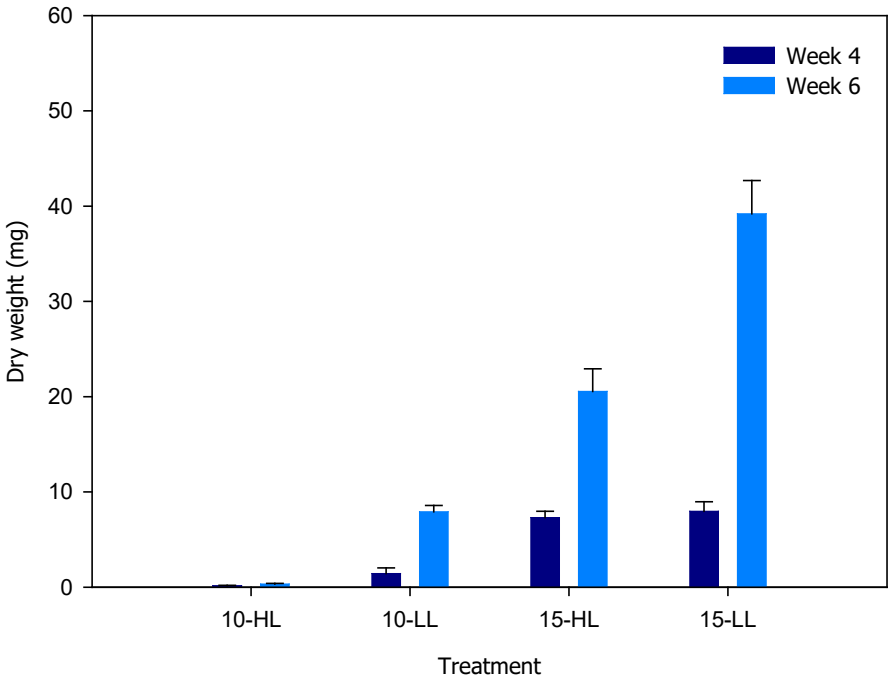


Figure 1. Dry weight of gemmalings after 4 and 6 weeks of growth. HL=High Light. LL = Low Light. Temperature = 15 C & 10 C

Gemma Cup Replenishment

In this glasshouse experiment pots of liverwort were positioned in a randomised design on capillary matting on a shaded glasshouse bench, providing damp, shady conditions. All the gemmae were removed from pre-identified 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 at the end of the experiment (control).

The number of gemmae collected in the initial removal of gemmae was fairly constant across all treatments (Table 1). For subsequent collections, however, more gemmae were collected during the 3-day treatment than either the weekly or control treatments (Figure 2), suggesting that when gemmae are removed, more are produced to replenish them.

Table 1. Initial gemma collection

	No. Gemmae Collected	Average for treatment	Standard errors
3-day	3131	104	21.28
Weekly	3180	106	21.02
Control	3646	122	25.74

For the 3-day and weekly treatments the average number of gemmae collected each week declined overall during the course of the experiment, although this effect was more pronounced for the first three data collections of the 3-day treatment.

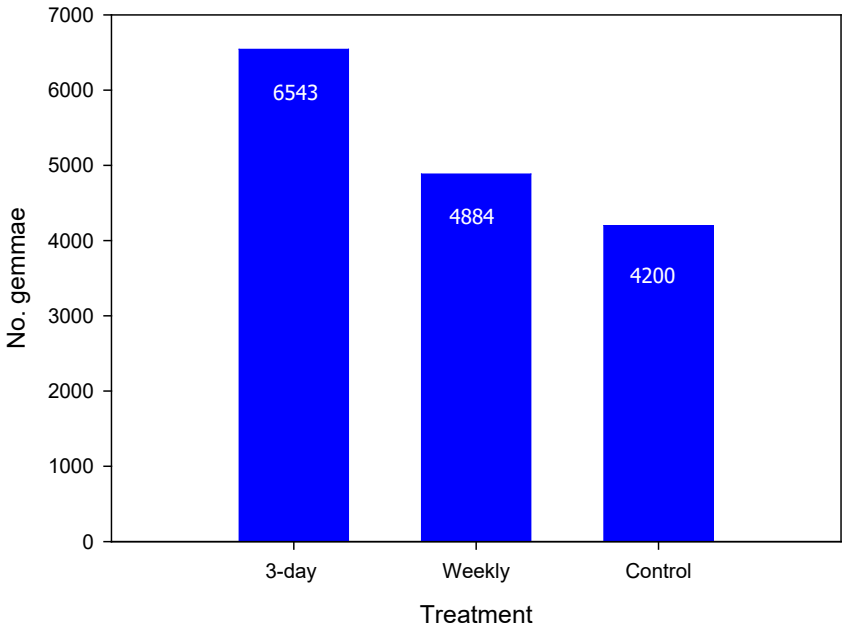


Figure 2. Total number of gemmae collected, excluding initial gemmae count

During the experiment some cups began to degenerate, becoming brown, and produced few, if any, gemmae. Some previously healthy gemma cups became discoloured and surrounded by areas of dieback, possibly due to scorch during a period of hot, sunny weather; other

gemma cups may have been adversely affected by gemma removal. 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 control.

This experiment aimed to investigate how gemma cups are replenished following dispersal of gemmae and whether replenishment could be affected by irrigation regime. The results observed indicate that increased gemma cup replenishment occurred with increased gemmae removal occasions. These results along with those of the gemma dispersal experiment of year 1 suggest when overhead irrigation is used, it can act as a major factor in the spread of liverwort by gemmae, and suggest therefore that the number of gemmae produced could be minimised by reducing or eliminating overhead irrigation applications.

Clumping of gemmae

This experiment was designed to test whether clumping together of gemmae is an aid to establishment and growth of liverwort colonies. The weight of liverwort established by clumps of gemmae were compared to the weight of freely dispersed groups of gemmae after four weeks growth.

The experiment was replicated three times (a total of 90 pots), with pots arranged in a randomised design on capillary matting on a shaded glasshouse bench. Fresh and dry weights of liverworts were recorded after four weeks and an establishment rate calculated.

Both the fresh and dry weights (Figure 3) were greater for groups of individual gemmae than clumps in all three size classes assessed (small, medium, large). For groups of gemmae this effect increased with increased class size. Groups of gemmae also established more successfully, with 100 % establishment in the small and medium size classes (Figure 4). Clumping of gemmae, as observed, therefore does not lead to enhanced establishment.

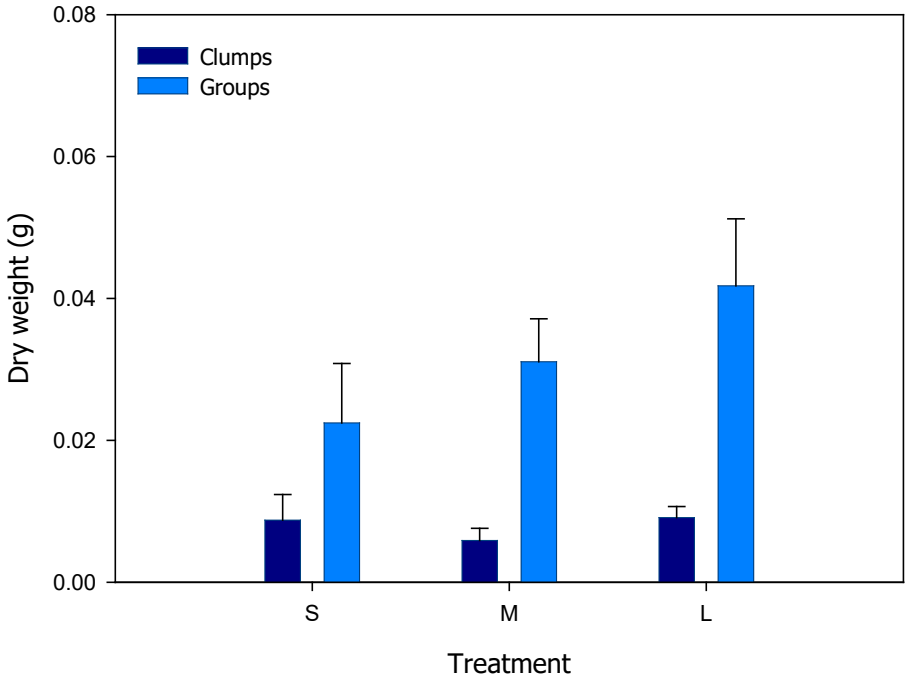


Figure 3. Dry weights of small (S = 26-50mg), medium (M = 101-125mg) and large (L = 176-200mg) groups and clumps of gemmae.

Fungal antagonism

The fungal species *Bryoscyphus atromarginata* and six strains of *Phaeodothis winteri*, identified as potential parasites on liverworts in year 1, were obtained for pathogenicity testing. Four further fungal species, isolated from dying or decaying liverworts, were established on agar media and identified as *Fusarium equiseti, Penicillium velutinum* and *Trichoderma harzianum*.

Preliminary pathogenicity tests and inoculation methods were developed to identify fungal species capable of infecting liverworts. The tests to date have enabled the exclusion of weak-growing strains that do not appear to have any effect on liverwort. *Bryoscyphus atromarginatus* is slow to establish, producing little response. However, as it is reported to attack *Marchantia polymorpha* specifically, efforts are being made to reinvigorate it. *Trichoderma* and Penicillium are soil saprophytes that are secondary colonisers of decaying plant material. The most promising strains are *Fusarium equiseti* (Plate 1), *Phaeodothis winteri* CBS 551.63 and *Phaeodothis winteri.* CBS 102466 and they will be included in glasshouse trials at a commercial nursery during year 3.

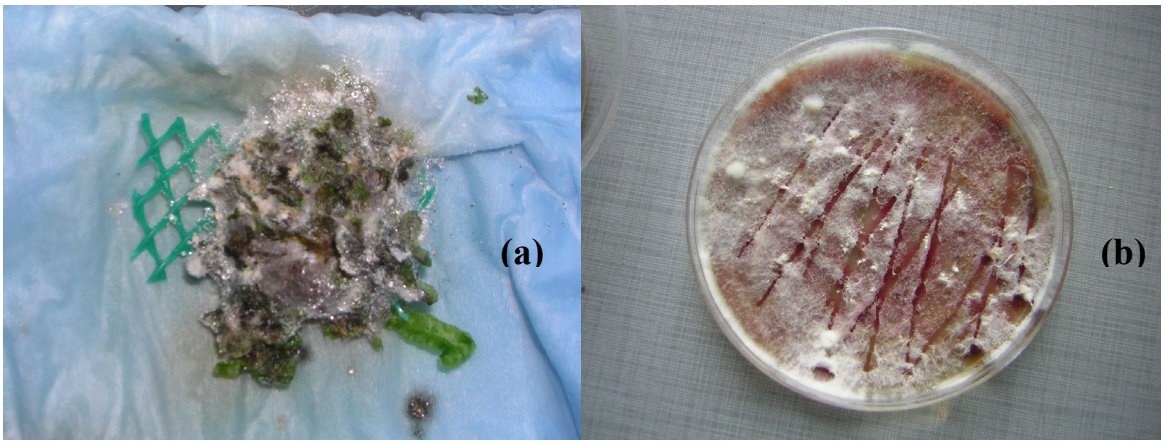


Plate 1(a) Liverwort infected with *Fusarium equiseti* (b) F. equiseti reisolated from inoculated liverwort

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, with growth inhibition causing dormancy in unfavourable environmental.

Methods are being developed to extract and quantify levels of LNA present in liverwort tissue. Further work is required to improve LNA recovery and optimise extraction efficiency. This will be followed by two pieces of work: quantification of lunularic acid present in liverwort at different lifecycle stages and an investigation of levels present during the growth of liverwort in different light, temperature and humidity conditions, using different combinations previously shown to promote or reduce growth.

Financial benefits

This study aims to provide information and develop integrated weed management strategies to reduce the cost of liverwort removal. Results to date indicate that by reducing overhead irrigation and shading of plant containers, liverwort spread and establishment could be reduced; implicit in this is a reduction in water and shading equipment costs.

Action points for growers

An integrated weed management strategy could include adjustments to growing conditions to reduce shade for container plants, and the introduction of irrigation regimes to limit gemmae dispersal. The liverwort gemmae dispersal and gemma cup replenishment experiments both suggest that to reduce the spread of liverwort overhead irrigation should be avoided. If overhead irrigation is used, water applications should be made as infrequently as possible.

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. 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. 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). More chemical options are available following the release of report HNS 93c (Atwood, 2006)

The overall aim of this project is 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. The identity of the liverwort species infesting nurseries will be established; it is commonly considered to be *Marchantia polymorpha.* There will be an investigation of non-chemical means of controlling liverwort: fungal pathogens, glucosinolate hydrolysis products obtained from seed material and cultural methods. Glasshouse trials based on the results of these investigations will consider the use of individual and integrated methods of control.

During year 1, experiments were carried out to investigate the growth and development of liverwort and the dispersal of liverwort gemmae by overhead sprinkler irrigation systems. The statistical analyses of these experiments were completed during year 2 and are presented within the results and discussion sections.

Work on the growth and development of liverwort was expanded with a second experiment using Fitotron growth cabinets at 10 C and 15 C (in year 1, 25 C and 15 C temperature settings were used); all other experimental protocols remained as in year 1.

Continuing from the investigation into the dispersal of liverwort gemmae by overhead irrigation systems, an experiment was carried out to determine the replenishment rate of gemmae dispersed from gemma cups. Gemmae are dispersed from cups by water droplets falling onto them. However, it is not known how quickly more gemmae are produced to replenish cups or if the rate of removal of gemmae affects the rate of replenishment. Does continual emptying of gemma cups result in increased production of gemmae? Does an increased time interval between water applications provide more or less gemmae for

dispersal? This information could be used as a basis for optimising overhead watering regimes to minimise liverwort dispersal.

The ‘clumping’ together of gemmae in large numbers was observed in year 1 during both the growth and development and the gemma dispersal experiments. This phenomenon raised the question of whether there was any biological advantage in clumping for the liverwort life cycle. Perhaps it is a strategy to aid establishment and subsequent growth of new colonies. A laboratory experiment compared the growth and establishment of liverwort colonies initiated with clumps of liverwort gemmae or with freely dispersed groups of individual gemmae.

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 together after dispersal, keeping them in an environment known to be safe until conditions are adequate to produce sexual spores, facilitating long distance dispersal (Equihua, 2005).

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* in year 1. Four further fungal species, isolated from dying liverworts provided by John Atwood (ADAS), were established on PDA and identified by CABI Bioscience, Egham as *Fusarium equiseti, Penicillium velutinum* and *Trichoderma harzianum* (2 samples). Each of these species belongs to Ascomycetes. Species may reproduce by sexual spores (ascospores), although in many species these are seldom found in nature, and asexual spores (conidia) (Agrios, 1997).

Phaeodothis winteri (Niessl) Aptroot syn. *Didymosphaeria marchantiae* is a cosmopolitan, saprophytic species occurring on a wide range of hosts in many countries (Aptroot, 1995a). It has been found on *Marchantia polymorpha* in various locations, including Austria, Poland, Germany, and Sweden (Aptroot, 1995b). It is an endophyte, with ascomata found immersed within the thallus, on archegoniophores and antheridiophores. Black spots on the thallus surface indicate the presence of fruiting bodies (Döbbeler, 2002).

Bryoscyphus is an obligate bryophilous genus only found on mosses and hepatics and one of the only ascomycetes known to parasitize thalloid hepatics (Döbbeler, 1997). *Bryoscyphus atromarginatus* Verkley Aa & G.W. De Cock is a rare ascomycete first collected in the Netherlands in 1995 that appears to parasitize *Marchantia polymorpha*, reportedly killing the liverwort rapidly. (Verkley et al., 1997). Reinfection of *Marchantia* with this species has not previously been tested and the pathogenicity remains unproven (Verkley, 2004).

Penicillium velutinum J.F.H. Beyma is a wide spread fungus, commonly found in soil, decaying vegetation, grain and the air. Some are beneficial to humans, others produce dangerous toxins, and cause post harvest diseases in fruit.

Fusarium equiseti (Corda) Saccardo is a cosmopolitan species found in soils worldwide and isolated from many different plant materials. Although not regarded as an aggressive pathogen, it does infect numerous crops and causes root and post harvest fruit rots (Booth, 1971). It has proven an effective biocontrol agent against water hyacinth (*Eichhornia cassipes*) (Naseema et al., 2001), reduces germination of *Striga hermonthica* (a parasitic plant) seed and affects seedling emergence (Kroschel et al., 1996). There is a concern that *Fusarium* spp. can cause mycotoxicosis or invasive disease in humans by the production of the toxin fumonisin (Kroschel et al., 1996). However, Thiel indicates that strains of *F. equiseti* tested do not produce the cancer promoting mycotoxins Fumonisin B_1 or B_2 (Thiel et al., 1991).

Trichoderma harzianum Rifai is a saprophytic fungus antagonistic to plant pathogens, widely distributed in soil, plant material, decaying vegetation and wood. The antagonist nature of *Trichoderma harzianum* towards plant pathogenic fungi has led it to be widely harnessed as a biocontrol agent against fungal pathogens, such as *Rhizoctonia solani, Sclerotina sclerotiorum* and *Botrytis cinerea* (Batta, 2004)

Pathogenicity tests of fungi cultured during year 1 were developed further, identifying which strains infected or produced a response when applied to liverwort, and which were ineffective.

Lunularic acid (LNA) (Figure 4) 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.

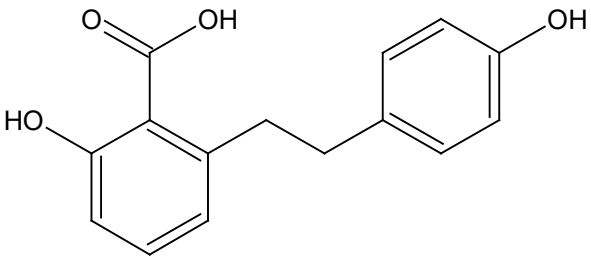


Figure 4. Structure of lunularic acid

LNA was first extracted from the liverwort *Lunularia cruciata* (L.) Dum., and characterised by Valio (1969) (Table 2). LNA causes the Israel strain of *Lunularia cruciata* to become dormant in high temperature, long day conditions; temperatures of 24 C and continuous light induce dormancy after 6 days. Growth recommences within 3-4 days of reinstatement of short day conditions (Valio, 1969; Valio et al., 1969). LNA is a leachable inhibitory factor that accumulates within liverworts, becoming effective above a critical level; increased levels of the inhibitor are found in liverwort species grown in continuous light, and high light intensities (Gorham, 1975).

Table 2. Description of lunularic acid by Valio (1969)

Appearance	Pale yellow needles (2mg)
Melting point	192 C
Molecular weight	258
Suggested molecular formula	$C_{15}H_{14}O_4$

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). A change of LNA content dependent on the cell growth cycle has also been recorded. Low LNA content during the initial exponential cell growth phase increases rapidly, coincidental with the reduced cell growth rate as the stationary growth phase begins (Abe and Ohta, 1983).

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* gemmalings), 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 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. Methods were developed to extract lunularic acid from tissue with minimum losses, in preparation for HPLC analysis to quantify the amount of LNA in plant material at different lifecycle and growth stages.

Materials and Methods

Identification of liverwort species infesting nurseries

HDC members and ADAS consultants were again invited to advise of any liverworts found that appear to be different to the commonly reported *Marchantia polymorpha* in the February 2005 edition of HDC News.

Plate 2. *Lunularia cruciata* found at Hadlow College, Kent (a) growing on a wall (b) detail of crescent-shaped gemma cups (Carey, 2005) (c) round gemma cups of *Marchantia polymorpha*

The liverwort, *Lunularia cruciata*, was found growing on a wall in the nursery at Hadlow College, Kent (Plate 1) (Carey, 2005). It is easily distinguished by the crescent shaped gemma cups found on the thallus surface compared with the round gemma cups of *Marchantia polymorpha* . Although growing within the nursery it has not been found invading container plant pots.

Growth and Development

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

An experiment using four Fitotron growth cabinets (Plate 3) compared the effect of two different temperatures (15 C and 10 C) and two light levels (800 µ mol m^{-2} S^{-1} and 400 µ mol m^{-2} S^{-1}) on the growth (radial expansion), fresh and dry weight accumulation and development of male and female liverworts, with data recorded after 4 and 6 weeks growth.

This experiment expands on work carried out during year 1 where cabinet temperatures used were 15 C and 25 C.

The two different temperatures were replicated at cabinet level, i.e. two cabinets were set at 10 C and two at 15 C. Within each cabinet the shelf heights were adjusted to give two light levels (upper shelf: 800 µ mol m^{-2} S^{-1}, lower shelf: 400 µ mol m^{-2} S^{-1}). This arrangement did not allow for random allocation of light treatments. For each treatment, equal numbers of male and female gemmae were placed in separate blocks. Humidity was set at 65% and a photoperiod of 12 hours.

In total, 288 *Marchantia polymorpha* gemmae (72 per cabinet) were placed individually onto M51C media; in lidded 10 cm diameter Bellaplast pots (one gemma per pot). The cabinets, pot positions and separate male and female areas were all randomly allocated, an example of the layout is shown in Table 3.

Nine pots were removed from each treatment (144 in total) after 4 weeks for data collection. The order of sample removal was determined randomly in advance. On each occasion liverworts were photographed using a Nikon Coolpix 995 digital camera and their areas calculated using ImageJ software. (Web location: http://rsb.info.nih.gov/ij/). Fresh and dry weights of all replicates were also recorded. The number of gemma cups present on each gemma was counted to provide information on liverwort development.

Plate 3. External view of Fitotron growth cabinet

High Light Treatment	M	M	M	M	M	M		F	F	F	F	F	F
	M	M	M	M	M	M		F	F	F	F	F	F
	M	M	M	M	M	M		F	F	F	F	F	F
Low Light Treatment	F	F	F	F	F	F		M	M	M	M	M	M
	F	F	F	F	F	F		M	M	M	M	M	M
	F	F	F	F	F	F		M	M	M	M	M	M

Table 3. Example of the layout within each Fitotron cabinet. Shelves containing high light treatment (800 µ mol m^{-2} S^{-1}) were positioned higher (nearer to the fluorescent tubes) than those containing the low light treatment levels (400 µ mol m^{-2} S^{-1}). M and F refer to male and female liverwort gemmae respectively.

Gemma dispersal

Gemma cup replenishment

In this glasshouse experiment all the gemmae were removed from pre-identified cups using a 100-1000µl pipette and counted. Three treatments were applied whereby gemmae were removed from cups and counted either every 3 days, weekly or at the end of the experiment (control).

The gemma retrieval method used was to apply water to each gemma cup with a pipette, and then remove the gemmae, continuing to add water and remove gemmae until no more could be removed.

Three healthy, active gemma cups per pot of liverwort were identified using coloured pins (red, blue, green); 10 pots of liverwort per treatment (30 pots in total). Identification of gemma cups allowed the tracking of gemma numbers produced by specific cups. The duration of the experiment was four weeks.

Treatments were identified using coloured labels: 3-day, red (R); weekly, blue (B); Control, green (C). The pots of liverwort were positioned in a completely randomised design on capillary matting on a shaded glasshouse bench, providing damp, shady conditions (Table 4).

R1	C1	R2	R3	R4	R5
C2	B1	R6	R7	B2	B3
B4	R8	B5	C3	C4	C5
C6	B6	B7	R8	C7	B8
R10	C8	C9	C10	B9	B10

Table 4. Position of replicates. R = red, B = blue, C = control. Numbers refer to replicates of each treatment.

Clumping of gemmae

This experiment was designed to test whether clumping of gemmae is an aid to establishment and growth of gemmae. The weight of liverworts established by clumps and freely dispersed groups of three different weight classes of gemmae were compared after four weeks growth. 'Clumps' are groups of gemmae found naturally in tightly packed masses and dispersed intact. 'Groups' are clumps of gemmae that have been separated in water and then freely dispersed.

A bioassay was used to devise a means of estimating the number of gemmae in a clump: clumps of gemmae were weighed, separated in water, and the number of gemmae counted and used to produce a linear regression curve. The regression line equation was then used to estimate the number of gemmae within a clump of known weight.

Pots of mature liverwort were grown in shaded conditions and watered from below only, to promote clumping of gemmae by discouraging dispersal. Clumps were removed from gemma cups, weighed on a Sartorius 1712 balance and placed into eppendorfs containing water, to separate the gemmae. They were then tipped onto filter paper and counted.

The linear regression constructed using this data gave the relationship between number of gemmae vs. clump weight with an adjusted R^2 value of 76.8 and F prob. <0.001 (Figure 6). The balance used was not sensitive enough to weigh individual gemmae, and clumps of less than 30 were not found in gemmae cups, providing no reliable weights for small numbers of gemmae. To obtain such data groups of 10 gemmae were weighed on a more sensitive balance (Sartorius 4503 micro balance) and added to the original data. The second regression equation obtained had an adjusted R^2 of 84.6 and F prob. <0.001, indicating an improved fit.

95% confidence intervals were plotted on the regression curve, indicating a 95% confidence that the population mean fell within the interval limits (Figure 5). The relationship between the number of gemmae and clump weight was close enough to use the data to predict the number of gemma within a clump of a specific weight, and this formed the basis of size class definitions. From these bioassays the value for individual gemmae was calculated using the equation: $y = \alpha + \beta x$, where x = no. of gemmae, y = weight of x no. of gemmae □□□□□intercept and □□□□ slope.

The bioassay regression curve data was used to predict the weight of different sized groups of gemmae. From these, three discrete size classes were used as the basis of the

experiment: small (26-50 gemmae), medium (101-125 gemmae) and large (176-200 gemmae) (Table 5).

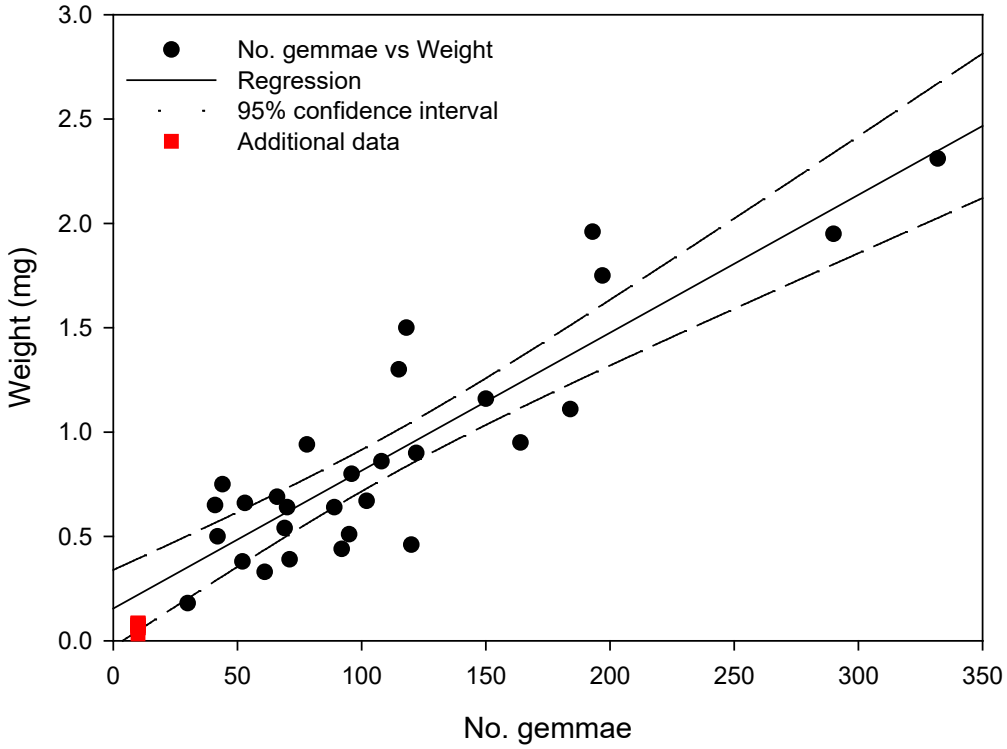


Figure 5. Linear regression of number of gemmae vs gemmae weight. 95% confidence interval indicates a 95% certainty that the population mean will fall between the limits. R^2 = 76.8. F probability = <0.001. 'Additional data' refers to groups of 10 gemma weighed on a more sensitive balance and not included in regression calculations.

Size class	No. gemmae	Weight prediction (mg)
	1	0.0788
	25	0.2497
Small (S)	26	0.2569
	50	0.4278
	51	0.4350
	75	0.6059
	76	0.6131
	100	0.7840
Medium (M)	101	0.7911
	125	0.9621
	126	0.9692
	150	1.1402
	151	1.1473
	175	1.3183
Large (L)	176	1.3254
	200	1.4964

Table 5. Weights and predicted number of gemmae in small, medium and large size classes

Liverworts were grown in glasshouse conditions, watering from below only to encourage gemmae clumping. Naturally formed clumps (C) and groups (G) of separated gemmae of each size class (Table 6) were placed on damp compost in 10 cm pots (five pots per treatment). 'Groups' of gemmae were obtained by weighing clumps, placing them into eppendorfs with water and shaking vigorously to separate the gemmae. These were emptied onto the compost, distributing the gemmae randomly, then rinsed and emptied twice more.

The experiment was replicated three times, giving a total of 90 pots. Pots were arranged in a completely randomised design on capillary matting on a shaded glasshouse bench, providing damp, shady conditions (Table 7). Fresh and dry weights of liverworts were recorded after four weeks and an establishment rate calculated.

	Small (S)						**Medium (M)**						**Large (L)**					
Replications	1		2		3		1		2		3		1		2		3	
Treatment	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G
No. pots	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Table 6. Experimental design showing size classes and treatment structure. Cl = clumps, G = groups. Size classes: small (S) = 26-50 gemmae, medium (M) = 101-125 gemmae, large (L) = 176-200 gemmae

M-3-C-1	L-1-G-1	M-3-G-1	S-1-C-1	L-1-C-1	S-1-G-1	L-2-G-1	L-2-G-2	S-1-G-2
S-2-G-1	L-1-C-2	L-2-G-3	L-3-C-1	M-3-C-2	S-2-G-2	L-2-C-1	L-2-G-4	S-2-G-3
M-1-C-1	M-2-G-1	L-3-G-1	M-2-G-2	M-3-C-3	S-3-C-1	M-2-C-1	M-1-G-1	S-3-G-1
L-3-G-2	L-1-G-2	S-1-G-3	S-3-C-2	L-2-C-2	M-2-C-2	S-1-C-2	L-1-G-3	M-3-C-4
S-2-C-1	M-2-G-3	L-1-C-3	M-2-C-3	S-3-G-2	L-1-C-4	S-1-C-3	L-2-G-5	M-3-C-5
M-2-C-4	M-2-C-5	M-1-G-2	L-2-C-3	M-1-G-3	L-1-G-4	M-1-C-2	M-1-G-4	L-3-C-3
M-1-G-5	S-1-G-4	L-3-G-3	S-2-G-4	L-1-C-5	L-3-C-2	M-1-C-3	M-3-G-3	S-2-G-5

S-2-C-2	M-3-G-2	S-3-C-3	L-3-C-4	M-1-C-4	S-3-G-3	M-1-C-5	M-3-G-4	S-2-C-3
S-1-C-4	M-3-G-5	M-2-G-4	L-3-G-5	S-3-C-4	L-1-G-5	S-1-G-5	L-3-C-5	S-2-C-4
S-1-C-5	L-3-G-4	S-3-C-5	L-2-C-5	M-2-G-5	L-2-C-4	S-3-G-4	S-2-C-5	S-3-G-5

Table 7. Completely random design of pots on glasshouse bench. Cl = clumps, G = groups. Size classes: small (S) = 26-50mg, medium (M) = 101-125mg, large (L) = 176-200mg

Fungal antagonism

Isolation of fungal pathogens from dying liverwort samples

Pathogens were removed from dying liverwort samples (Plate 4) using a heat sterilised wire loop and spread onto potato dextrose agar (PDA) media in petri dishes, sealed with parafilm and incubated at 20 C. Once colonies had developed enough to distinguish between different species, each was reisolated onto new media as before, this time 'streaking' them to separate colonies and produce plates containing individual species (Plate 5).

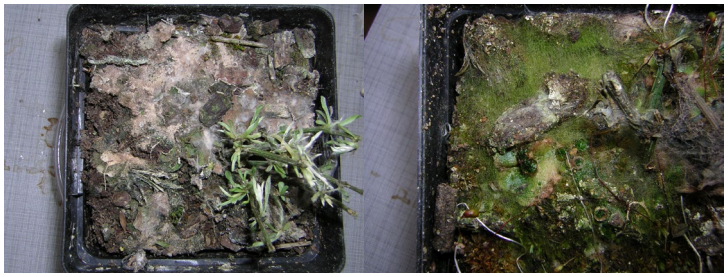


Plate 4. Samples of dying liverwort provided by John Atwood (ADAS).

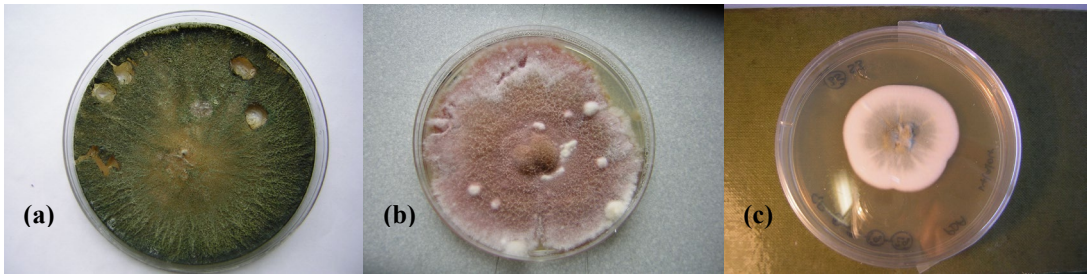


Plate 5. Fungal cultures isolated from dying liverwort samples. (a) *Trichoderma harzianum* (b) *Fusarium equiseti* (c) *Penicillium velutinum*.

Specimens sourced from Centraalbureau voor Schimmelcultures (Plate 6) were subcultured onto PDA and malt extract agar (MEA) according to the information provided.

Each of the fungal species has been established in culture on slopes as a long-term maintenance procedure and in petri dishes for current experimental use.

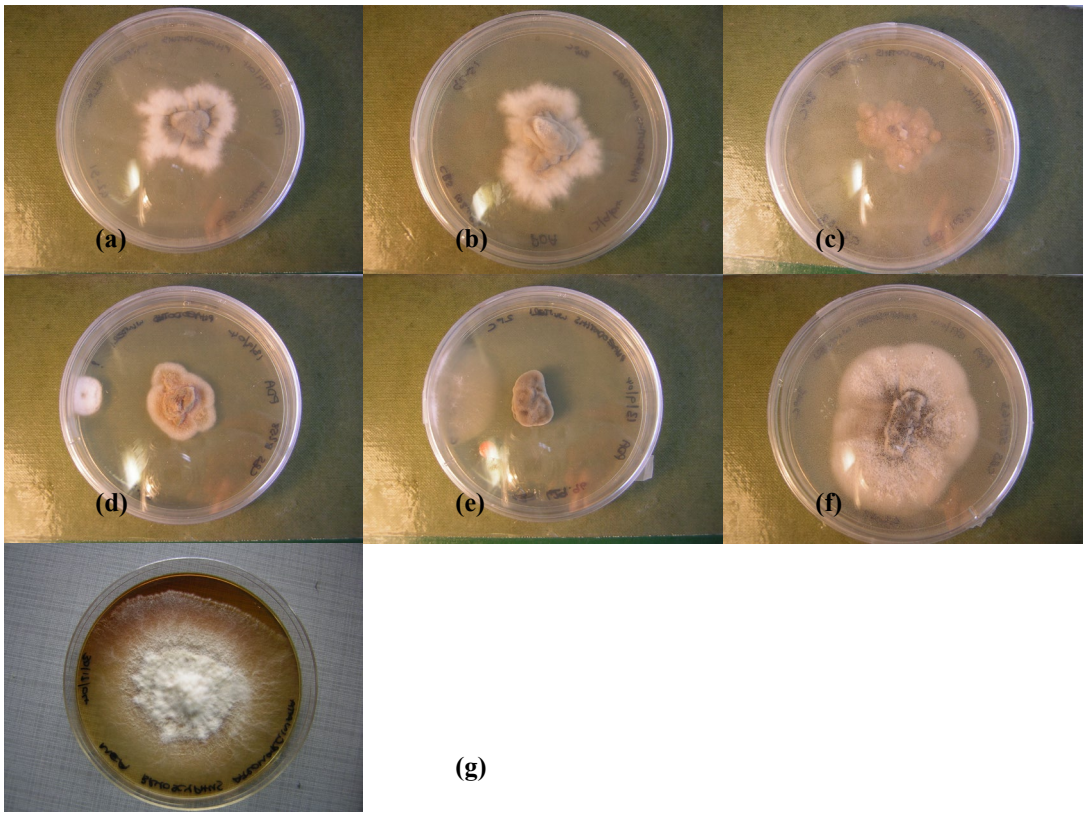


Plate 6. Fungal specimens sourced from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands: *Phaeodothis winteri* (a) *Phaeodothis winteri* CBS 102466 (b) *Phaeodothis winteri* CBS 102483 (c) *Phaeodothis winteri* CBS 162.31 (d*) Phaeodothis winteri* CBS 182.58 (e) *Phaeodothis winteri* CBS 429.96 (f*) Phaeodothis winteri* CBS 551.63 (g*) Bryoscyphus atromarginatus.*

Inoculation of liverwort with fungal species

Preliminary pathogenicity tests were developed, designing inoculation methods, aiming to identify fungal species capable of infecting or eliciting a response from liverworts.

Spore suspension preparation

A small amount of sterile distilled water was poured over a petri dish of sporulating fungi. An inoculating loop was flame sterilised and used to scrape the surface of the fungi. The fluid was filtered via a double layer of muslin, to remove mycelium, into a centrifuge tube. It was centrifuged for 2.5 minutes at 3000 rpm, the water poured off and the spores resuspended in

sterile distilled water. The spore concentration was calculated using a haemocytometer, aiming for around 5 x 10^5 spores cm^{-2}.

Preliminary spore germination bioassay

A preliminary bioassay was carried out using *Trichoderma harzianum*, to confirm viability of spores. Three 20 µl drops of spore suspension in water were placed individually on a microscope slide, incubated and examined microscopically for germination after 24 hours. Incubation chambers used for the bioassay and inoculated liverwort were non-airtight plastic boxes with the lid lined with tissue moistened with sterile water, to create a humid atmosphere (Plate 7). The *Trichoderma harzianum* spores did not germinate therefore the process was repeated, suspending spores in water (i.e. no added nutrients), and 50%, 25%, 12.5% and 6.25% nutrient solution (potato dextrose broth (PDB)). The spores did germinate within 24 hours when suspended in PDB (Table 18).

Plate 7. Incubation box containing liverworts inoculated with *Penicilium velutinum*, suspended in water and 0.625% PD broth.

Liverwort inoculation 13/12/04

Phaeodothis winteri CBS 551.61, *Fusarium equiseti,* and *Penicillium velutinum* also produced spores and were used for the first liverwort inoculations (13/12/04) together with *Trichoderma harzianum* (Table 8). Spore germination bioassays were carried out as before, at the same time. Young liverworts of approximately 2cm diameter were used for inoculations; they were removed from their growing media (M51C, as used in the growth and development experiments), rinsed and placed on sections of plastic mesh in an incubation box (four per box). The surface of the liverwort was allowed to dry then inoculated with three 20 µl drops of spore suspension in serial dilutions of PD broth as before. Two liverworts were used for each broth dilution treatment.

Fungal growth from the thallus surface of liverworts that had been inoculated was reisolated using the initial isolation method. A portion of fungus was scraped from the liverwort, 'streaked' onto media in a petri dish, and then incubated as before.

Table 8. Fungal species used in liverwort inoculation 13/12/04

	Spore Density
Trichoderma harzianum	2.4×10^7 cm^{-3}
Phaeodothis winteri CBS 551.63	9.05×10^5 cm^{-3}
Fusarium equiseti	9.0×10^5 cm^{-3}
Penicillium velutinum	6.48×10^6 cm^{-3}

Liverwort inoculation 23/2/05

A further set of inoculations were carried out to include fungal strains that had subsequently started to produce spores (Table 9). Although *Bryoscyphus* was not sporulating it was applied as a suspension of mycelium. 2cm^2 liverwort sections were inoculated using spores in 50% PDB and water. Two liverworts thalli were used per treatment, each inoculated with five 20 µl drops of inoculant. The control was non-inoculated liverwort sections. Spore germination bioassays were also carried out, as before, using three 20 µl drops of inoculant per microscope slide.

Table 9. Fungal species used in liverwort inoculation 23/2/04

	Spore Density
Phaeodothis winteri CBS 551.63	1.12×10^6 cm^{-3}
Phaeodothis winteri CBS 102466	7.0×10^6 cm^{-3}
Phaeodothis winteri CBS 429.96	5.32×10^6 cm^{-3}
Bryoscyphus atromarginatus CBS 211.96	mycelium
Fusarium equiseti	5.2×10^6 cm^{-3}
Control	

Liverwort inoculation 15/3/05

An alternative inoculation method was developed as some fungal strains still had not produced spores. Plugs of established mycelium on agar media were taken using a No. 2 cork borer and inverted onto the liverwort thallus so that contact was made between the mycelium and liverwort tissue. For each fungal strain, four liverwort samples were inoculated with five plugs each; of these, half were wounded with a needle to investigate whether the response would be greater in wounded or unwounded liverworts.

Observations were characterised to denote the amount of fungal growth:

- Growth visible on plug
- Growth extending beyond plug by less than 1 plug diameter
- Growth extending beyond plug, greater than 1 plug diameter

Liverwort response was graded as 'slight', or 'collapsed'

Liverwort inoculation 31/3/05

Plugs of mycelium were again used for this set of inoculations. For each fungal strain used, six liverwort samples were inoculated with 2 plugs each; of which three were wounded and 3 remained unwounded. Uninoculated plugs were applied as a control. The *Bryoscyphus* and *Fusarium* cultures were not at a suitable stage to provide enough plugs.

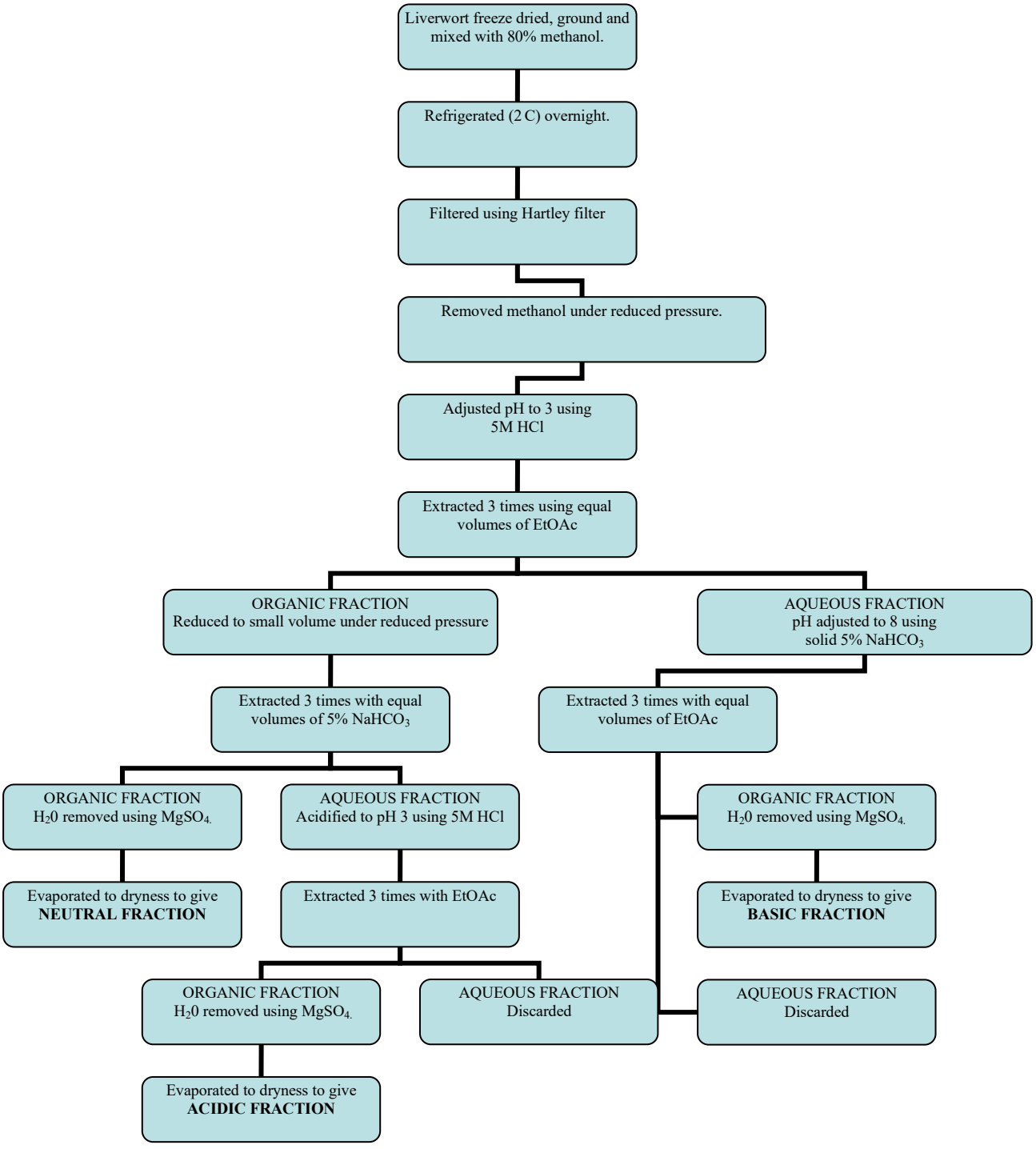
Lunularic acid

Methods are being developed to extract and quantify levels of LNA present in liverwort tissue. A full description of the method development to date is given in Appendix (I).

LNA is not available commercially, therefore it was initially necessary to extract and purify a sample of liverwort tissue to use as a standard during HPLC analysis. The extraction and purification method used was adapted from Valio (1969) and is summarised in Figure 6.

A sample of LNA was subsequently provided by Professor Asakawa of Tokushima Bunri University, Japan. Mass spectroscopy confirmed its molecular weight as 258, authenticating the sample. It was therefore no longer necessary to extract and purify a sample from liverwort tissue.

Figure 6. Summary of lunularic acid extraction method



HPLC analysis techniques were developed to identify optimum procedures for detecting LNA. Both fluorescence and UV detectors were used. As most molecules do not exhibit fluorescence this can be an extremely sensitive tool. The structure of lunularic acid (Figure 4), incorporating phenyl, carboxyl and hydroxide groups suggested that fluorescence would elicit a good response. Fluorescence excitations and emission curves were produced to identify optimum detector settings so that LNA would produce the maximum peak size.

Salicylic acid (SA) was selected as an internal standard (Figure 7) as it has a similar structure to LNA and therefore also detectable by fluorescence. Using an internal standard (spiking) helps to quantify the amount of LNA in a sample and it also helps to detect losses during sample preparation.

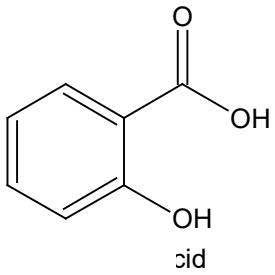


Figure 7. Structure of salicylic acid

Sample preparation methods were then developed for the extraction of LNA from plant material in methanol over a steam bath (Figure 8). The extract was extremely green and was extracted with diethyl ether to remove the chlorophyll (Figure 9), and then magnesium sulphate to remove any water present. An amino-propyl solid phase extraction (SPE) column was used for final preparation before HPLC analysis (Figure 10). Three extracts were prepared in this manner to produce three samples:

- extract 'spiked' with 2 ml SA
- extract without SA
- 2 ml salicylic acid (SA) standard containing no liverwort extract.

Figure 8. Summary of steam bath extraction method

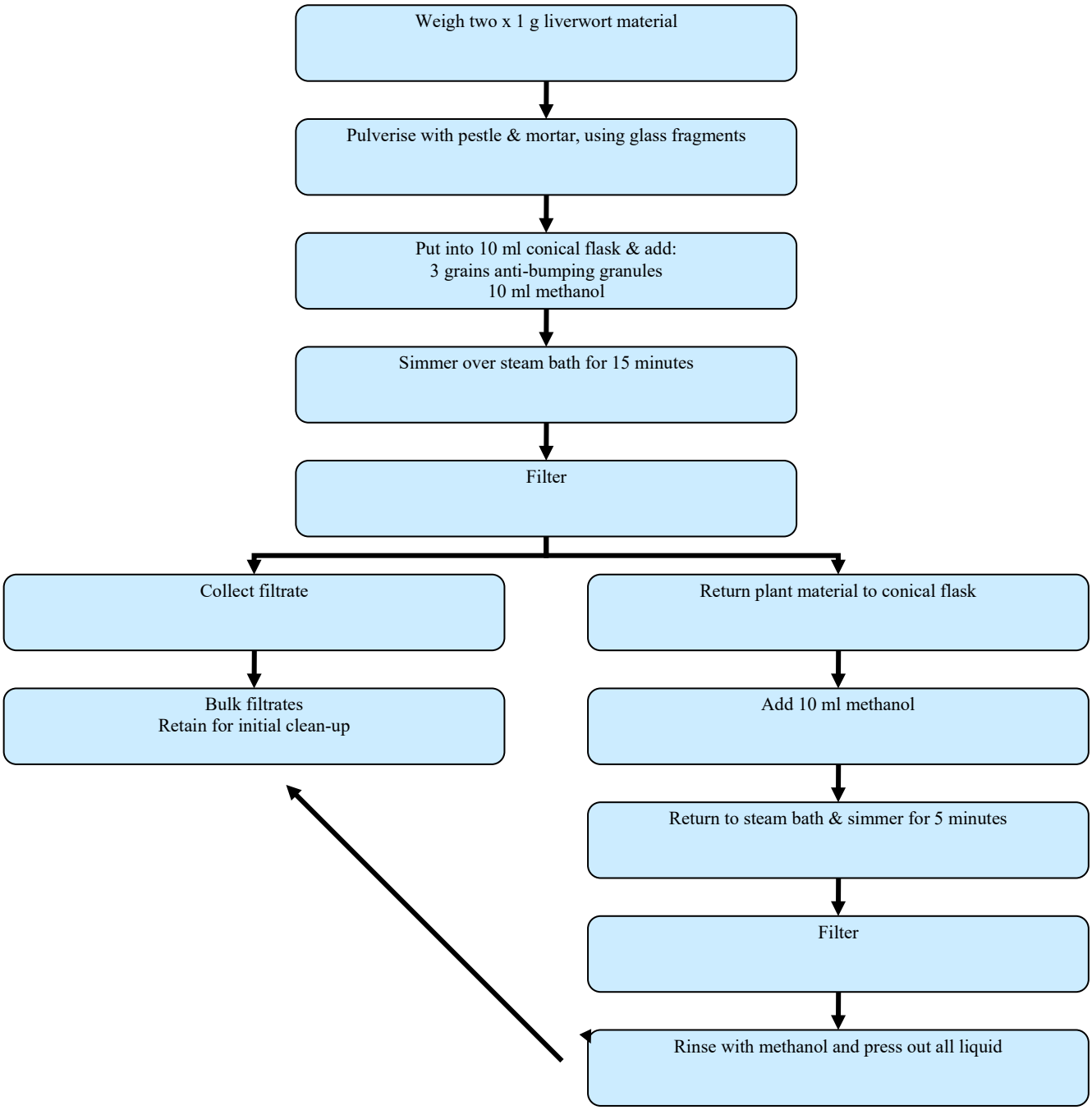


Figure10. Summary of initial sample preparation method

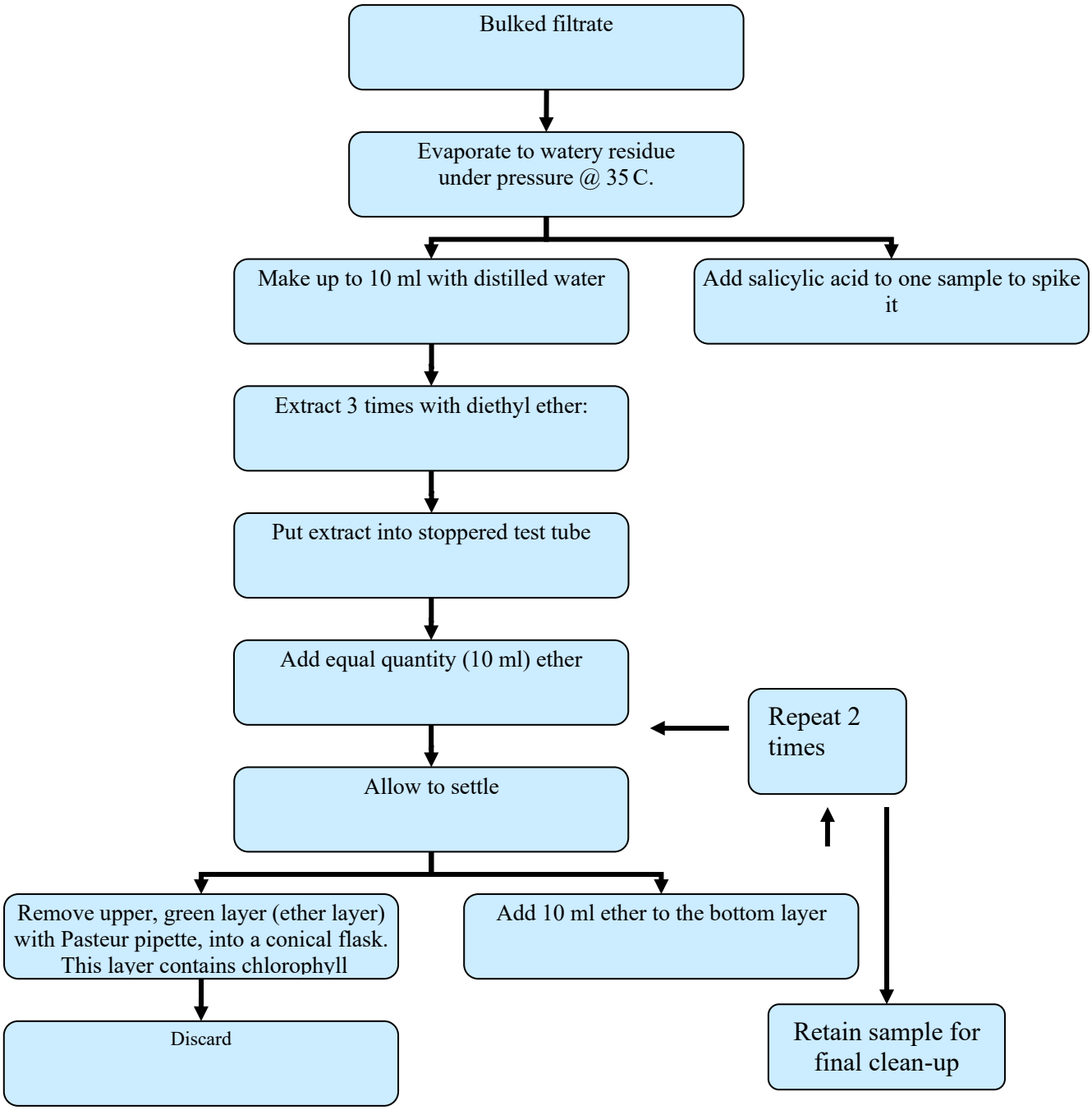
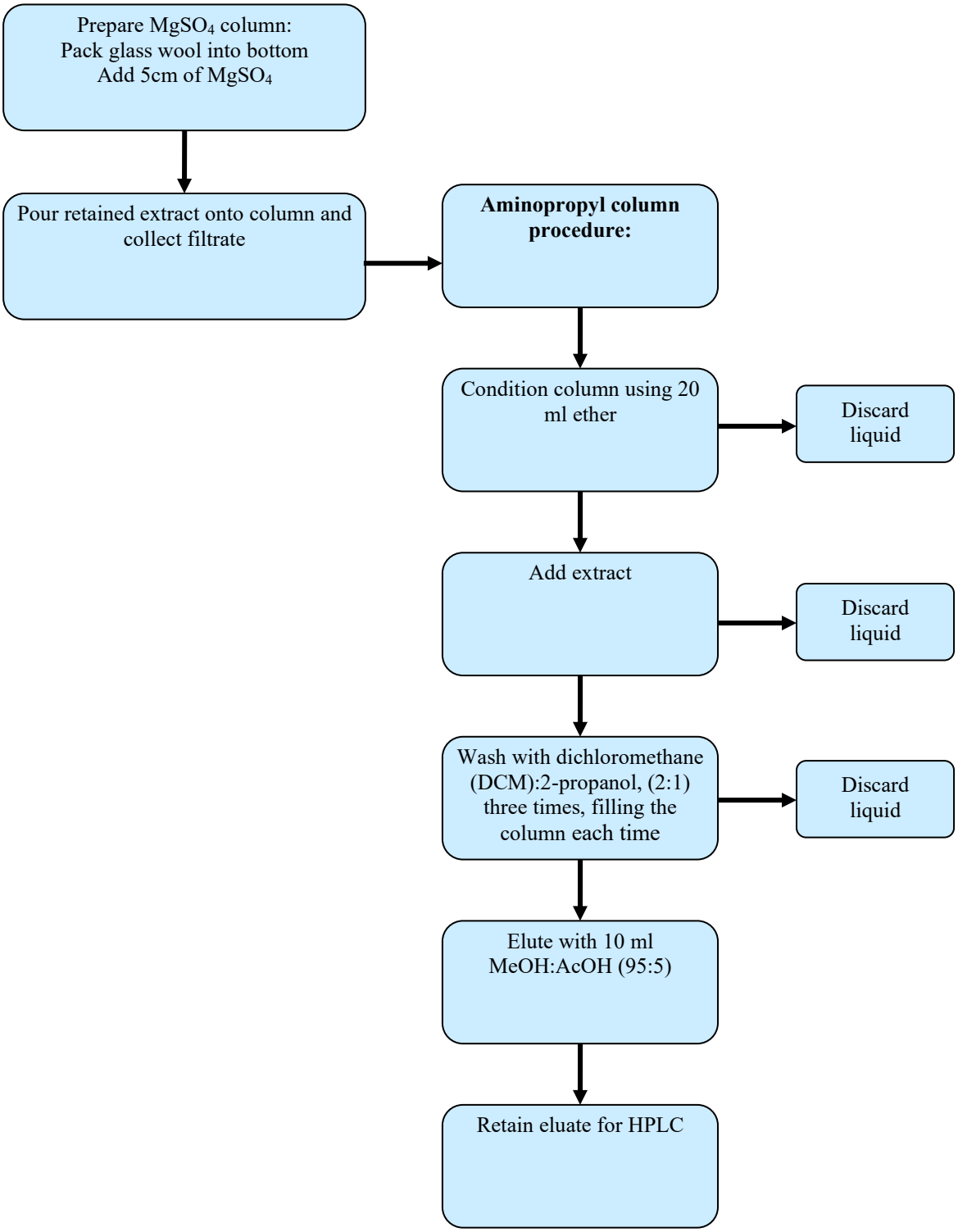


Figure 9. Summary of final sample preparation method



Each extract was dissolved in 1 ml of acetonitrile ready for HPLC analysis. Salicylic acid was identified in the unspiked liverwort extract, rendering it unsuitable for use as an internal standard. 3-hydroxy-2-nahthoic acid (HNA) was therefore investigated as an alternative (Figure 11). A UV spectrophotometer was used to produce the characteristic UV absorbance spectrum for HNA and identify UC setting that produced the largest peaks, indicating maximum absorbance (Table 20).

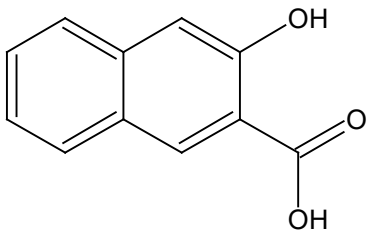


Figure 11. Structure of 3-hydroxy-2-naphthoic acid (HNA)

HNA standards were analysed by HPLC using the same solvent solution as for LNA and were found unsuitable as an internal standard. The peaks produced were broad and tailing, and was not detectable by fluorescence with the detector at the correct settings for LNA.

A simplified extraction and detection process was then 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.

Experimental results

Statistical analysis of the gemma cup replacement, growth and development and clumping experiments is still to be completed.

Growth and development

Year 1: The effect of light level and temperature on growth and development of liverwort

This experiment explored the effect of two different temperatures (25 C and 15 C) and two light levels (800 µ mol m^{-2} S^{-1} and 400 µ mol m^{-2} S^{-1}) on the growth (radial expansion), fresh and dry weight accumulation and development of male and female liverworts, with weekly data collection. The results were present in year one, the statistical analysis is now completed.

Using natural logarithms, statistical analysis indicates that the effect on growth (radial growth) due to temperature, light and gender are significant at the 0.1% level, as were the interactions between temperature and light and temperature and gender. The graphs (Figure 12) suggest an interaction as the trend lines are not parallel, intersecting at weeks 1 and 2.

The results show an effect on growth (radial expansion) due to light level for both temperature treatments. At both temperatures gemmaling (a young liverwort developing from a gemma) growth (radial expansion), and fresh and dry weights after six weeks were greater when grown in low light levels compared to high light. Overall, dry weights and gemmaling growth were greater for liverworts grown at 25 C. Statistically there was a significant effect of temperature and light at the 0.1% level on dry weight.

The effect of light on fresh weight was significant at the 0.1% level; the interactions between temperature and light and temperature and gender were significant at the 1% and 5% levels, respectively. Male and female gemmalings had greater fresh weight in low light levels at both temperature treatments. Female gemmalings, in low light fresh weight was greater at high temperature than low. Conversely, in high light growth was greater in high temperatures than low. For male replicates fresh weight was greater in low light than high, but there was little effect due to temperature.

The growing conditions had a different effect on male and female gemmaling growth. In the high temperature treatments female gemmalings grew larger than male in low light, however in the high light conditions the reverse is seen. Although there is no intersection shown in the graph for 15 C the lines are still not parallel, indicating an interaction. The interactions between gender, temperature and light are significant at the 0.1% level.

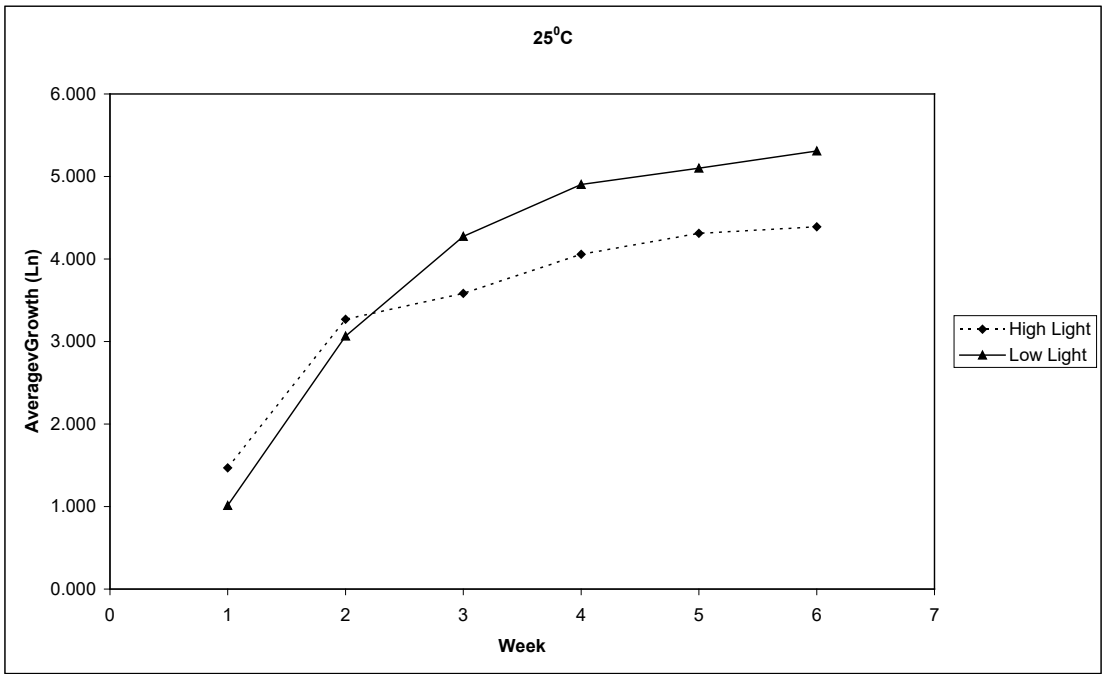


Figure 12a. Gemmaling growth (Ln) at temperatures of 25C and 15C.

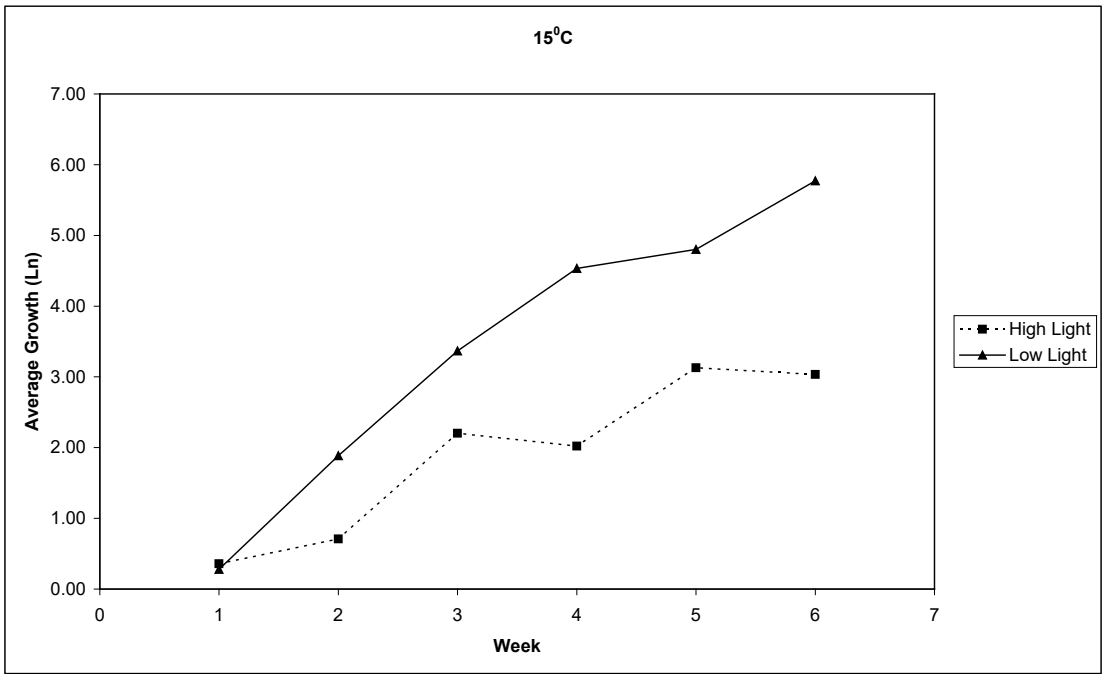


Figure 12b. Gemmaling growth (Ln) at temperatures of 25C and 15C.

Liverwort development was also monitored, with the presence and number of gemma cups on each gemmaling used as a measurement. Gemmaling 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. During week six there was a large increase in the number of gemma cups present in the 15 C treatment, where more gemma cups were produced overall. Statistical analysis indicates these results are not significant due to temperature, however there is a significant interaction between temperature and light at the 0.1% level ($P<0.001$)

There was an effect on the number of gemma cups produced depending on liverwort gender, with the results significant at the 0.1% level. More gemma cups were produced on male plants than female at both light levels.

Year 2: The effect of light level and temperature on the growth and development liverwort

Dry weight, fresh weight, area, and no. of gemma cups were all greater in high temperature than low; greater in low light than high light after both four and six weeks growth (Figures 12 to 17). Statistical analysis, fitting the same statistical models as in year 1, will establish the significance of these results.

The effects of temperature were the same for the number of gemma cups mm^{-2}, with the exception of the high temperature treatment where more gemma cups mm^{-2} were produced in high light than low after both four and six weeks.

After four weeks there were more gemma cups on male than female gemmalings in high light than low light treatments. However, in low temperature the gemmalings were particularly small and gemma cups were only produced in the female, low light treatment. Overall there appeared to be no discernable trend relating to gemmaling sex.

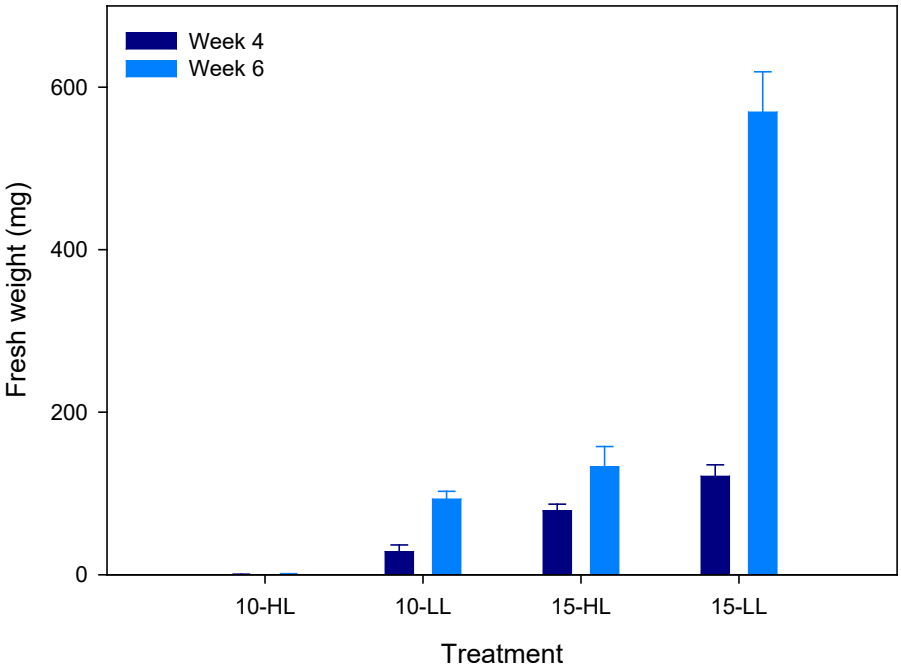


Figure 12. Fresh weight of gemmalings after 4 and 6 weeks of growth. HL=high light (800 µ mol m^{-2} S^{-1}), LL = low light (400 µ mol m^{-2} S^{-1}) Temperature = 15 C & 10 C

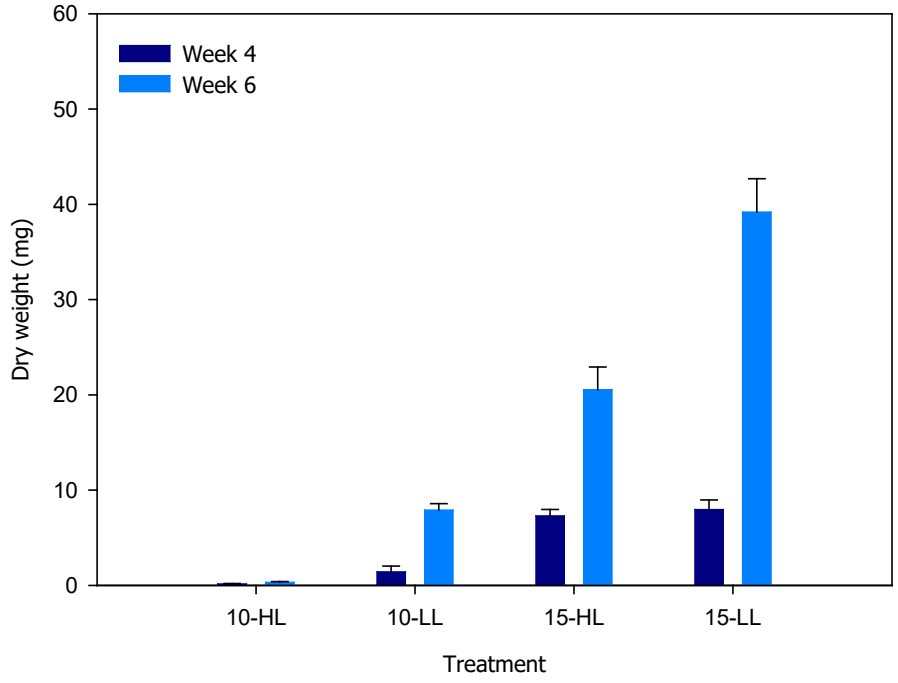


Figure 13. Dry weight of gemmalings after 4 and 6 weeks of growth. HL=high light (800 µ mol m^{-2} S^{-1}), LL = low light (400 µ mol m^{-2} S^{-1}) Temperature = 15 C & 10 C

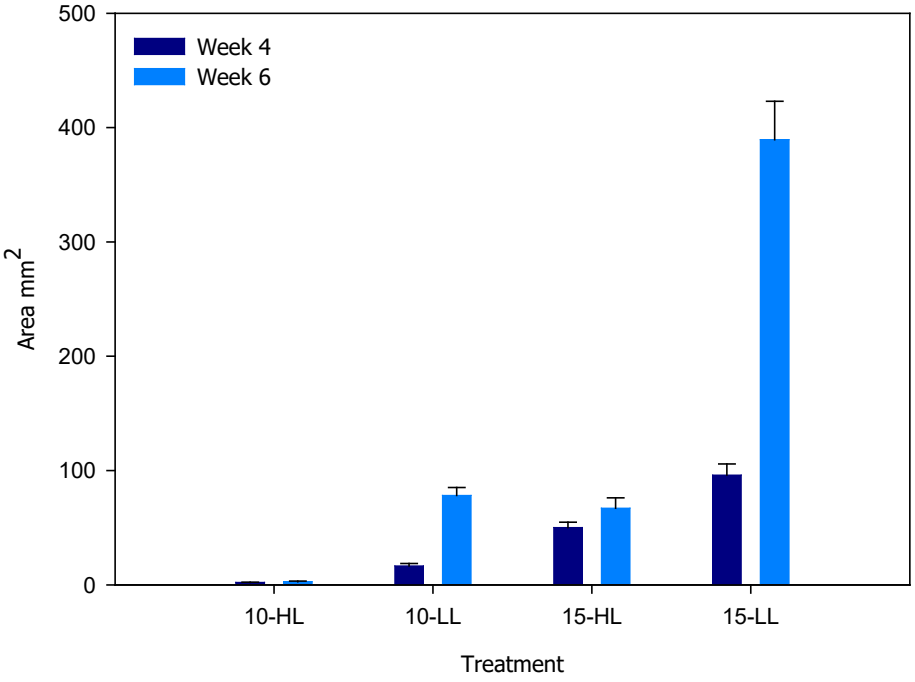


Figure 14. Areas of gemmalings after 4 and 6 weeks of growth. HL=high light (800 µ mol m^{-2} S^{-1}), LL = low light (400 µ mol m^{-2} S^{-1}) Temperature = 15 C & 10 C

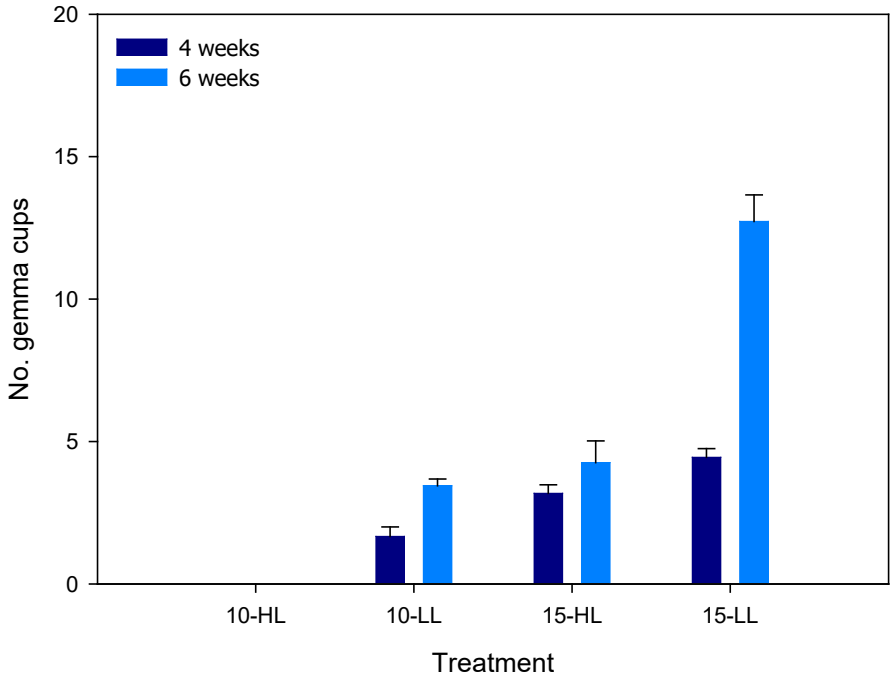


Figure 15. No. of gemma cups present on gemmalings after 4 and 6 weeks of growth. HL=high light (800 µ mol m^{-2} S^{-1}), LL = low light (400 µ mol m^{-2} S^{-1}) Temperature = 15 C & 10 C

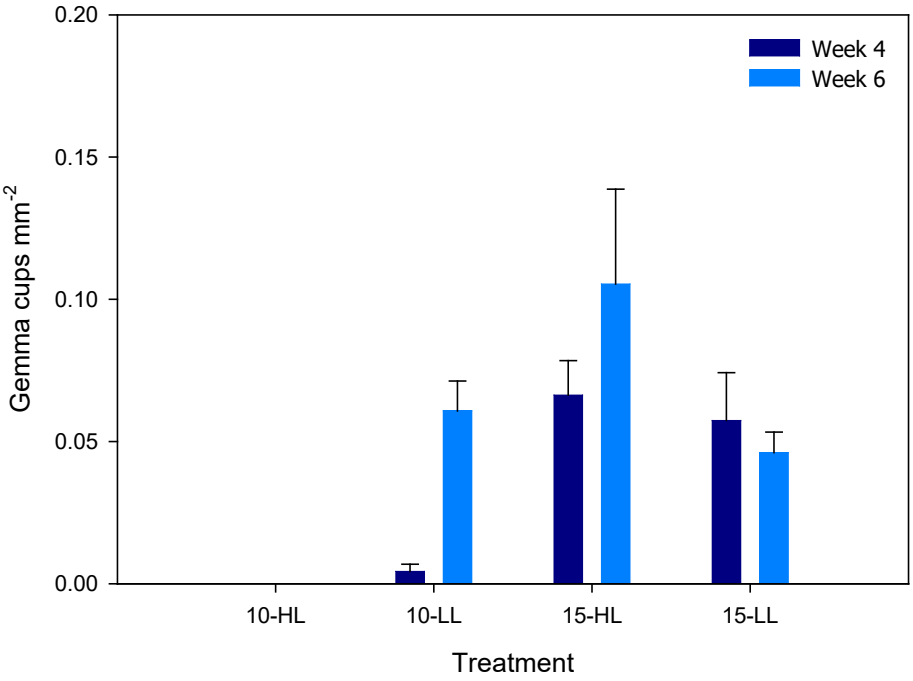


Figure 16. No. of gemma cups mm^{-2} after 4 and 6 weeks of growth. HL=high light (800 µ mol m^{-2} S^{-1}), LL = low light (400 µ mol m^{-2} S^{-1}) Temperature = 15 C & 10 C

Gemma dispersal

Year 1: The effect of nozzle size, water pressure and nozzle height on gemmae dispersal using an overhead sprinkler system.

This experiment investigated the dispersal distance of liverwort gemmae by a glasshouse overhead sprinkler system, using three different sprinkler nozzles, four different water pressures (1.5, 2, 2.5 and 3 bar) and two different nozzle heights (1 and 2 metres). The nozzles used were Ein Dor Agridor 700 Dynamic Sprayers, colour coded by the manufacturer to denote the flow rate: brown – 160 lh^{-1}, blue – 105 lh^{-1} and grey, 60 lh^{-1}. A half tray containing mature liverwort thallus bearing gemma cups was placed on the ground beneath the nozzle. 10 cm collection pots were arranged in 3 lines, at right angles to each other and starting at the half tray. For each treatment water was applied to the liverwort via the sprinkler system for 15 minutes and the number of gemmae that had fallen into each collection pot counted.

Using a log-linear model, regression curves fitted to gemma dispersal data were highly significant, with F-probability <0.001 for all treatments (Figure 18). 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.

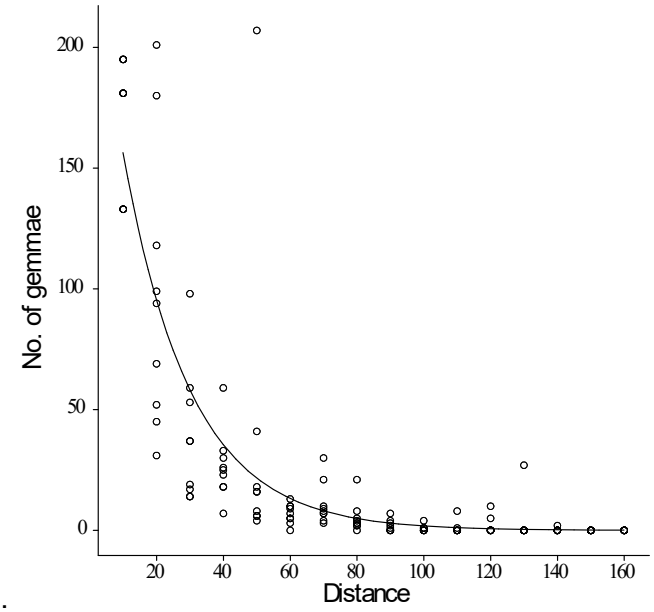


Figure 18. Regression curve for blue nozzle at 2 m height and 2 bar water pressure treatment. Adjusted $R^2 = 0.7708$

This graph represents the number of gemmae collected in each 10 cm collection pot for the treatment described. Regression analysis was used to give a dispersal gradient for each treatment, showing the relationship between treatments and dispersal distances obtained. I've added more method description which should help when reading the results – the experiment was carried out in year 1, and analysis in year 2

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 (Table 10). Maximum dispersal distances obtained were 160 cm (Table 10); 60 cm and 121.4 cm were previously recorded by Brodie (1951) and Equihua (1987) respectively.

Nozzle height (m)	Pressure (bar)	Grey 60 lh^{-1}	Blue 105 lh^{-1}	Brown 160 lh^{-1}
1	**1.5**	110	130	-
	2	90	120	100
	2.5	80	150	160
	3	110	150	90
2	**1.5**	150	110	-
	2	130	140	110
	2.5	90	140	90
	3	140	140	110

Table 10. Distance travelled by gemmae (cm).
The brown nozzle did not operate at 1.5 bar.

Generally more gemmae were dispersed at 2 and 2.5 bar water pressure. The brown (160 lh^{-1}) nozzle did not operate at 1.5 bar. More gemmae were dispersed at the 2m height for the brown and grey (60 lh^{-1}) nozzles, and at 1 m height for the blue nozzle (105 lh^{-1})

A measure of relative droplet size for each nozzle was obtained using water sensitive paper (Plate 8). The droplet pattern produced was photographed digitally and analysed using Image J software. Distribution charts constructed using this data identified the range of droplets sizes produced during each treatment (Figure 19). 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).

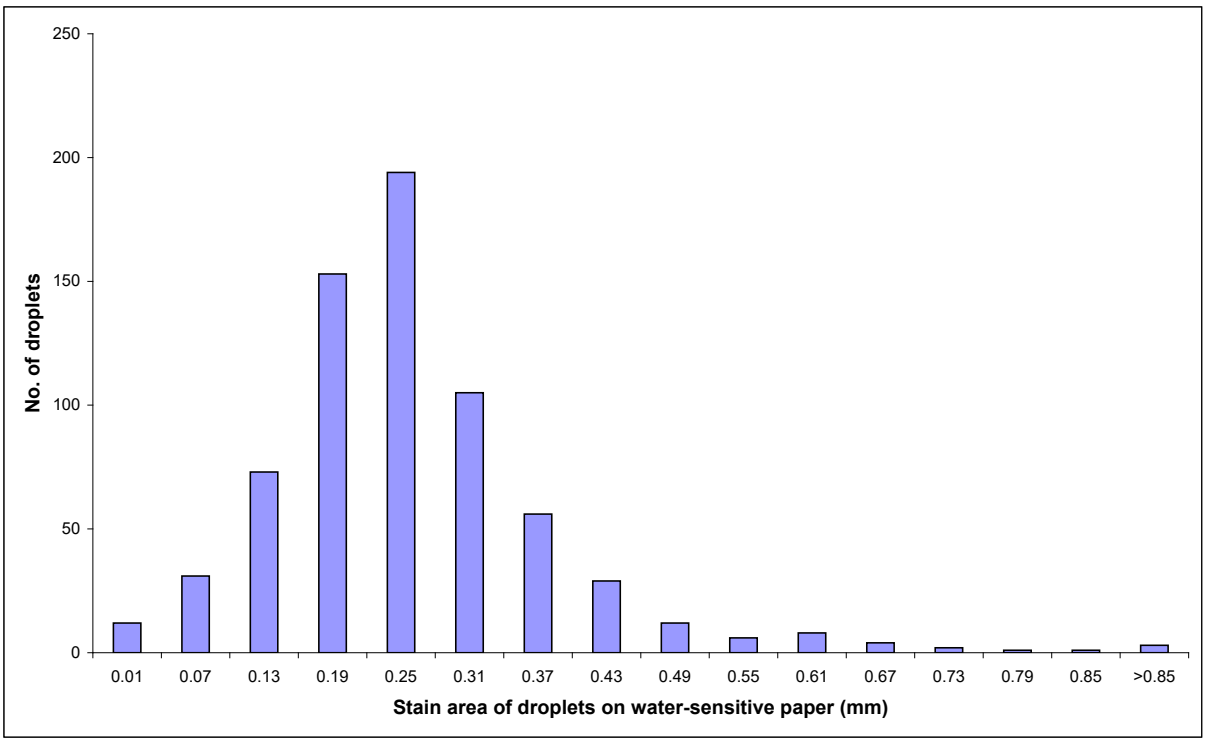


Figure 19. Droplet size distribution chart for blue nozzle at 2 m height and 2 bar water pressure treatment

Plate 8. Water sensitive paper showing droplet stains for the blue nozzle, 2 m height and 2 bar water pressure treatment.

Gemma cup replenishment

The number of gemmae collected during the initial removal of gemmae was fairly constant across all treatments (Table 11). For subsequent collections, however, markedly more gemmae were collected during the 3-day treatment than either the weekly or control treatments (Table 12, Figure 20).

Table 11. Initial gemma collection

	No. Gemmae Collected	Average for treatment	Standard error
3-day	3131	104	21.28
Weekly	3180	106	21.02
Control	3646	122	25.74

Table (12) Total no gemma collected, excluding initial gemma collection

Treatment	Average no. Gemmae Collected
3-day	6543
Weekly	4884
Control	4200

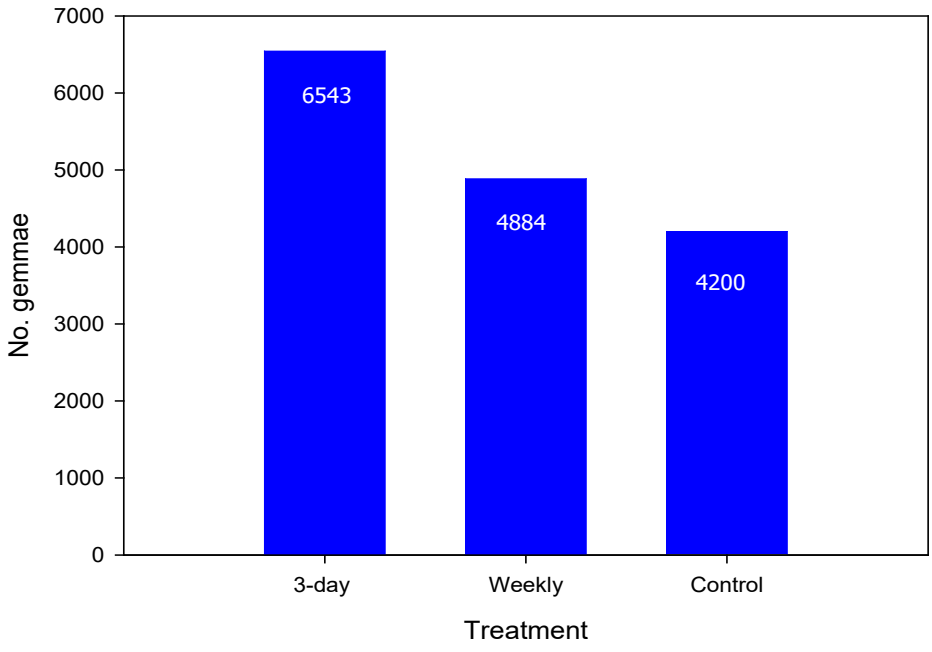


Figure 20. Total number of gemmae collected, excluding initial gemmae count

For the 3-day and weekly treatments the average number of gemmae collected each week declined overall during the course of the experiment. This effect was more pronounced for

the first three data collections of the 3-day treatment (Figure 21), becoming more or less constant for the final four collections. For the control, more gemmae were counted in the second (final) collection.

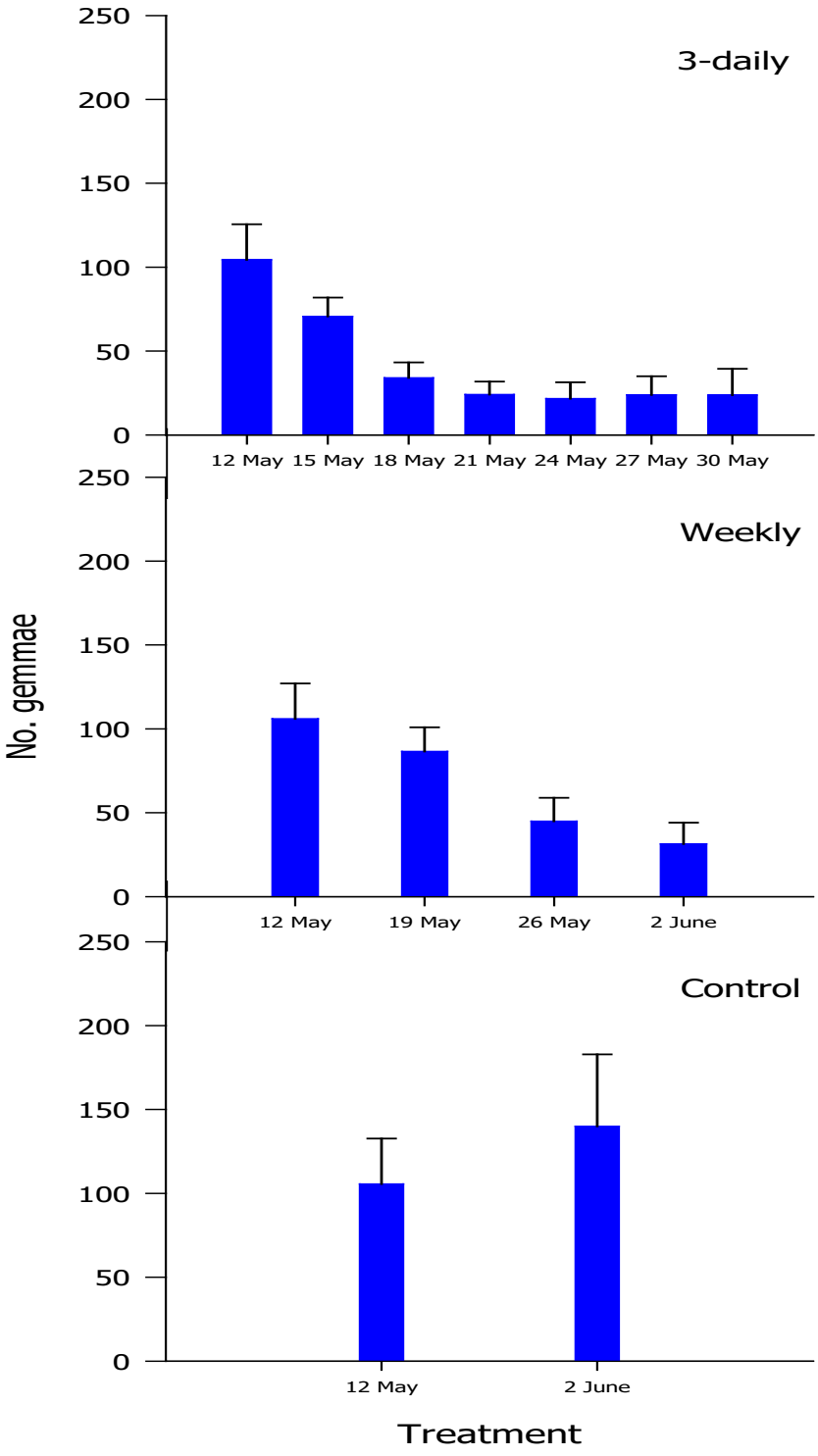


Figure 21. Gemma cup replenishment. 3-day and weekly treatments and control.

During the experiment some cups began to degenerate, becoming brown, and producing few, if any, gemmae. Some previously healthy gemma cups became discoloured and surrounded by areas of dieback, possibly due to scorching during a period of hot, sunny weather; other gemma cups may have been adversely affected by gemma removal. 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 control, for the final data collection.

The high growth rate of thallus caused some gemma cups to be overgrown by new thallus. These were re-exposed and gemmae counted as planned.

Clumping of gemmae

The aim of this experiment was to investigate whether there was any biological rationale for gemmae to disperse in intact, tightly packed clumps rather than individually that would aid their growth and establishment. However, both the fresh and dry weights were greater for groups of individual gemmae than clumps in all three size classes (Table 13, Figures 22 and 23). For groups of gemmae this effect increased with increased class size.

Groups of gemmae also established more successfully, with 100 % establishment in the small and medium size classes (Figure 24).

Table 13. Fresh and dry weights and percentage establishment of small (S) = 26-50 gemmae, medium (M) = 101-125 gemmae, large (L) = 176-200 gemmae

	Clumps			Groups		
	S-C	**M-C**	**L-C**	**S-C**	**M-C**	**L-C**
Fresh weight (mg)	151.456	96.070	110.973	287.866	382.310	533.307
Dry weight (mg)	8.753	6.532	9.107	22.459	31.089	41.773
% Establishment (mg)	67	60	73	100	100	87

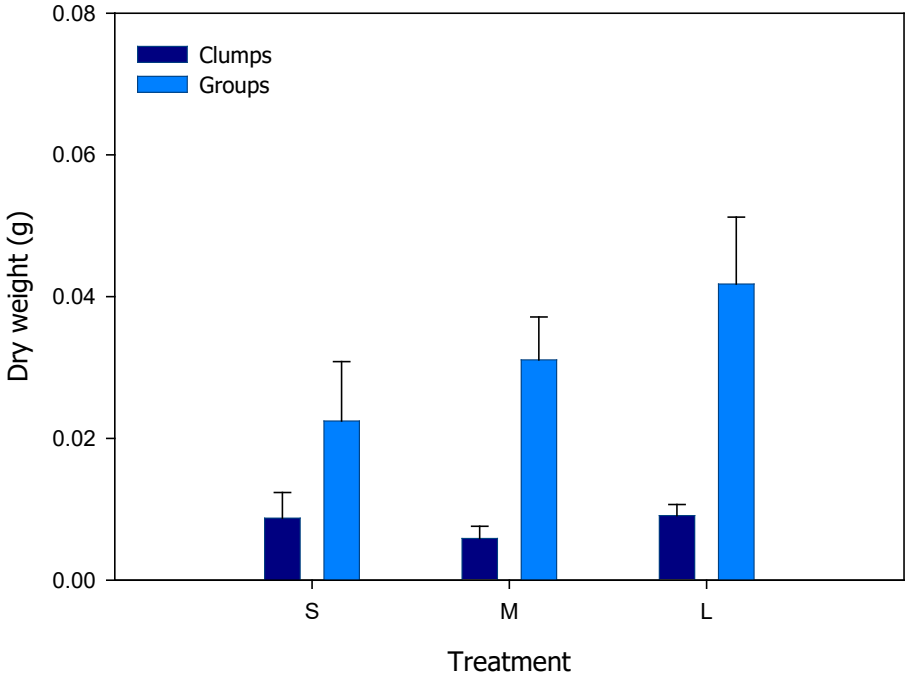


Figure 22. Dry weights of small (S) = 26-50 gemmae, medium (M) = 101-125 gemmae, large (L) = 176-200 gemmae. 'Clumps' = groups of gemmae found in a tightly packed mass and dispersed intact. 'Groups' = clumps of gemmae that have been separated and then freely dispersed.

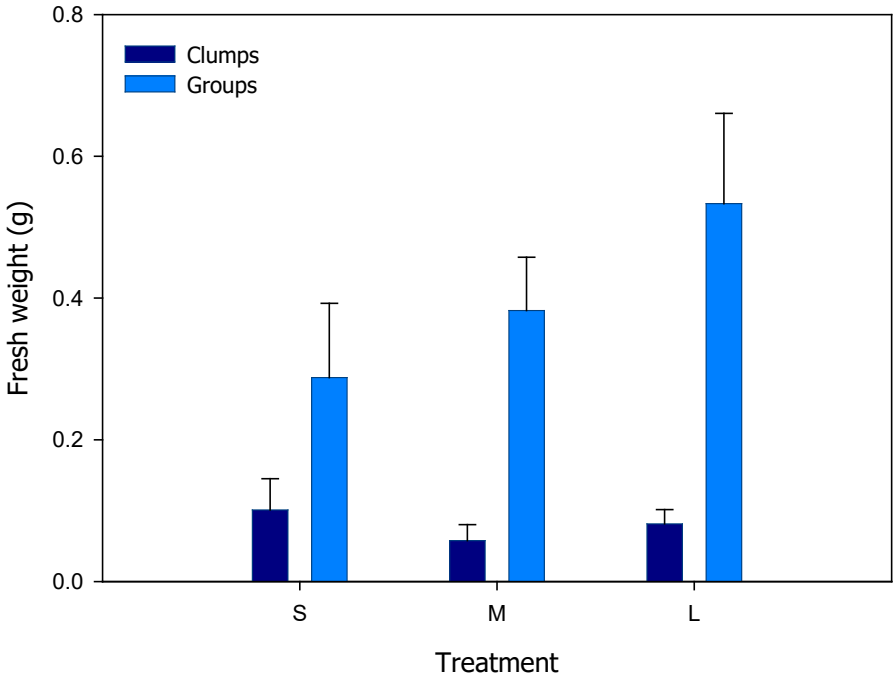


Figure 23. Fresh weights of small (S) = 26-50 gemmae, medium (M) = 101-125 gemmae, large (L) = 176-200 gemmae. 'Clumps' = groups of gemmae found in a tightly packed mass and dispersed intact. 'Groups' = clumps of gemmae that have been separated and then freely dispersed.

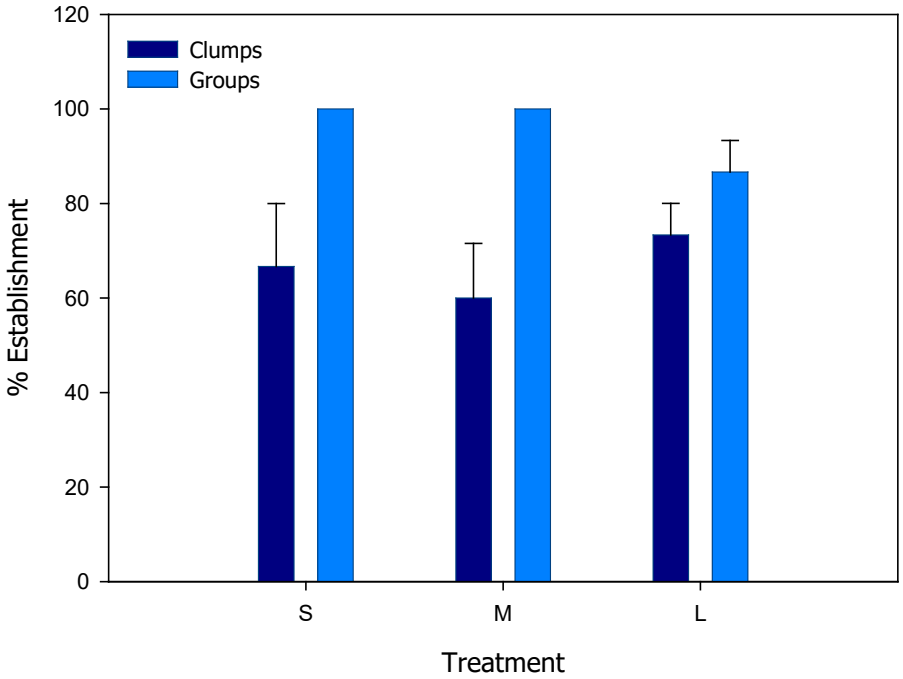


Figure 24. Percentage establishment of small (S) = 26-50 gemmae, medium (M) = 101-125 gemmae, large (L) = 176-200 gemmae. 'Clumps' = groups of gemmae found in a tightly packed mass and dispersed intact. 'Groups' = clumps of gemmae that have been separated and then freely dispersed.

Fungal antagonism

Liverwort inoculation 13/12/04

The *Trichoderma harzianum* and *Penicillium velutinum* did not germinate in water (Table 14); however spores of all species did germinate within 24 hours when suspended in above 25% Potato Dextrose Broth (PDB). Future inoculations were therefore limited to comparing spores suspended in water and 50% PDB.

Table 14. Germination bioassay of spores suspended in water and PD broth (13/12/04)

Fungal species	Water	PDB concentration			
		50%	25%	12.50%	6.25%
Trichoderma harzianum	x	□	□	x	x
Phaeodothis winteri CBS 551.63	□	□	□	□	□
Fusarium equiseti	□	□	□	□	□
Penicillium velutinum	x	□	□	□	□

Semi-permanent slides were made of inoculated liverwort tissue – those inoculated with *Penicillium velutinum* showed mycelium attached to the liverwort, although it was not clear if the tissue had been penetrated, as the hyphae appear to be growing over the surface of thallus and rhizoid tissue (Plate 9). The liverworts inoculated with 50% and 25% PDB collapsed: one treatment using 50% PDB had died completely and rotted after 2 months.

Others that had almost completely died away showed signs of new growth at the outer edges.

The *Trichoderma harzianum* did not appear to infect the liverwort. The spores germinated and appeared to begin to invade the tissue, but then stopped. The majority of liverworts inoculated with *Phaeodothis winteri* CBS 551.63 were growing strongly after 2 months, with no real trend relating to PDB dilution.

Fusarium equiseti was the most successful of the species tested, causing severe collapse with all dilutions of PDB and water, although three 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 (Plate 10).

Inoculated liverworts show signs of infection, with tissue collapsing (Plate 11). Fungal growth has been observed on the surface of liverwort tissue inoculated with *Fusarium equiseti, Trichoderma harzianum* and *Penicillium velutinum.* Attempts made to reisolate the fungi were successful with *F. equiseti* (Plate 10) and *P. velutinum.*

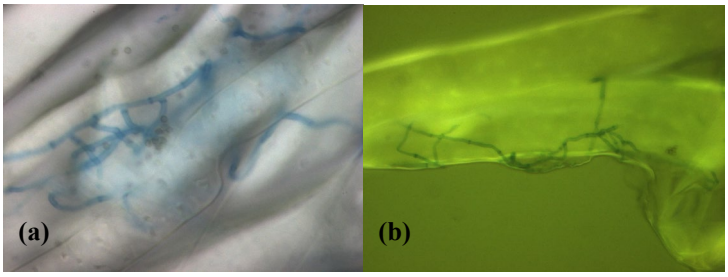


Plate 9 Liverwort infected with *Penicillium velutinum* (a) thallus (b) rhizoid. Magnification X400

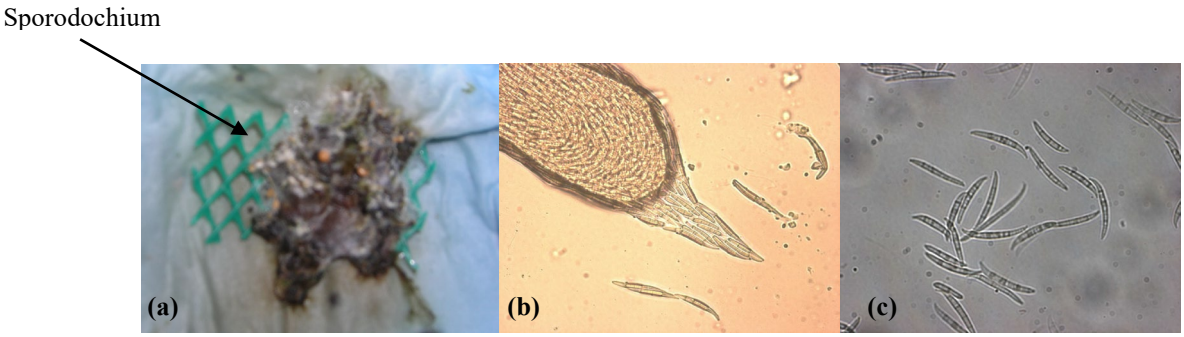


Plate 10 (a) Liverwort infected with *Fusarium equiseti* with orange sporodochia. (b) Sporodochium releasing macroconidia, found on liverwort inoculated with *F. equiseti*. (c) *F. equiseti* macroconidia. Magnification x 400.

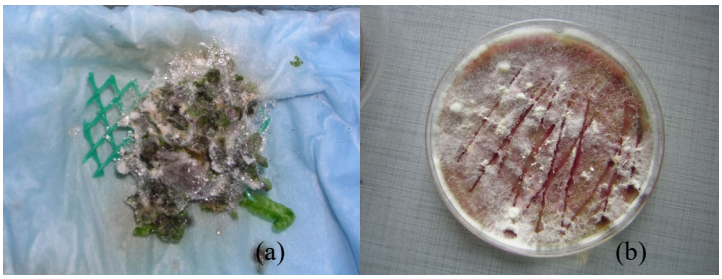


Plate 11 (a) Liverwort infected with Fusarium equiseti (b) F. equiseti reisolated from inoculated liverwort

The outcome of the spore germination bioassays proves both the viability of the fungal spores tested and suggests the addition of nutrients improves spore germination (Table 14).

Liverwort inoculation 23/2/05

The spores and mycelium in 50% PDB all germinated within 24 hours. *Phaeodothis winteri* CBS 102466 spores did germinate in water only, but more sparsely than when suspended in nutrient solution (Table 15).

Table 15. Spore germination bioassay for 23/2/05 inoculation

	Water	50% PDB
Phaeodothis winteri CBS 551.63	□	□
Phaeodothis winteri CBS 102466	□	□
Phaeodothis winteri CBS 429.96	x	□
Bryoscyphus atromarginatus CBS 211.96	□	□
Fusarium equiseti	□	□

The results of this inoculation were varied (Table 16), but again *Fusarium equiseti* produced the greatest response from the liverwort, with 95% collapse of one sample by 29th March. Subsequently, some weak liverwort regrowth was observed around the edge of the dying liverwort, but this did not develop further (Plate 12).

Table 16. Results for 23/2/05 inoculation indicating levels of collapse of liverwort

Data collection: 16/03/05	**Spores + Water**		**Spores + 50% PDB**	
	Sample 1	**Sample 2**	**Sample 1**	**Sample 2**
Phaeodothis winteri CBS 551.63	x	x	25%	slight
Phaeodothis winteri CBS 102466	x	x	x	x
Phaeodothis winteri CBS 429.96	x	50%	slight	x
Bryoscyphus atromarginatus CBS 211.96	x	x	slight	slight
Fusarium equiseti	50%	25%	25%	25%
Control (no spores applied)	slight	slight	-	-

Plate 12. Liverwort inoculated with *Fusarium equiseti* showing signs of regrowth.

Liverwort inoculation 15/3/05

All fungal species were well established on the agar plugs, covering the upper plug surface by 23rd March (Table 21). However, *P. winteri* CBS 102483 and CBS 162.31 had not extended beyond the plug by this time; *P. winteri* CBS 182.58 also grew weakly. *Fusarium equiseti* again proved vigorous, as did *P. winteri* CBS 551.63. The greatest response by the liverwort was induced by *F. equiseti*, *P. winteri* CBS 551.63, *Penicillium velutinum* and *P. winteri* CBS 182.58, although the latter exhibited weak growth. The control liverworts were all healthy on 23rd March except for one sample that was soft and beginning to collapse in the centre; by the 30th March they were all pale green and two were collapsing.

Wounding the liverworts did seem to produce an effect, with more samples collapsing in four strains, an equal number in 3 strains and less effect observed in two strains (Table 17).

Small, black structures were found growing on the surface of liverworts inoculated with *P. winteri* CBS 551.63. These were examined microscopically and found to be pycnidia which when crushed released thousands of spores (Plates 13);

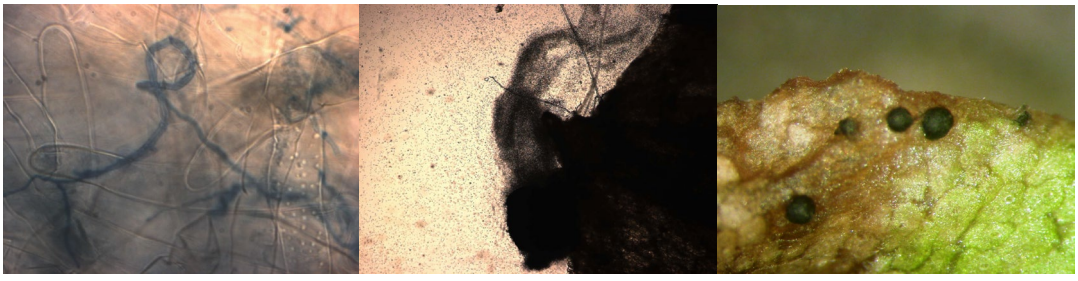


Plate 13. *Phaeodothis winteri* CBS 551.63 (a) Mycelium infecting liverwort thallus. (b) Pycnidia releasing thousands of conidia, (c) Pycnidia on the thallus surface. (a) and (b) Stain = lactophenol tryphan blue. Magnification x 400

It was decided not to continue with pathogenicity testing on fungal species that were slow to grow and elicited little response from the liverwort: *P. winteri* CBS 182.58, *P.w.* CBS 429.96,

Penicillium velutinum and *Trichoderma harzianum.* Although *P. velutinum* did attack liverwort tissue and cause some collapse, the liverwort was not overcome and continue to develop new growth.

Liverwort inoculation 31/3/05

All fungal strains used for this inoculation established well with growth extending beyond the agar plugs, except for *Phaeodothis winteri* CBS 162.31 and *Bryoscyphus atromarginatus*. *P. winteri* CBS 551.63 and *Fusarium* elicited the most vigorous response from the liverwort (Table 18).

By May all replicates inoculated with *Phaeodothis winteri* CBS 102483 were completely covered with fungal mycelium; liverworts inoculated with *P.winteri* CBS 551.63 were completely collapsed, black and slimy. *P. winteri* CBS 102466 and CBS 162.31 were dull green and collapsing, but so too was the control, so this was unlikely to be caused by the inoculant.

Work to reisolate fungal strains of liverwort continued, being successful with *Fusarium equiseti, Bryoscyphus atromarginatus* and*, Phaeodothis winteri* CBS 162.31 and 102466*.* These isolates will be used to reinoculate and reisolate, as passaging through the host reinvigorates the fungi. The *Bryoscyphus* is proving slow to establish and grow, therefore this process should prove beneficial.

Lunularic acid

Extraction and purification of lunularic acid

The aim of the extraction and purification was to provide enough lunularic acid to use as a standard during HPLC analysis; however the final TLC plate did not contain enough LNA on this occasion. A sample of lunularic acid was subsequently obtained and the process no longer required

Table 17. Results for 15/3/05 inoculation

Data collection 23/3/05	Growth visible on plug		Growth extended beyond plug but within 1 plug diameter		Growth extended beyond plug > 1 plug diameter		Liverwort response			
							Wounded		Unwounded	
	Wounded	Unwounded	Wounded	Unwounded	Wounded	Unwounded	Slight	Collapsed	Slight	Collapsed
Phaeodothis winteri CBS 551.63	*10	10	10	10	6	4	-	10	2	4
Phaeodothis winteri CBS 182.58	10	10	2	2	-	-	2	8	3	7
Phaeodothis winteri CBS 102466	10	10	10	9	-	-	3	5	4	6
Phaeodothis winteri CBS 102483	10	10	10	10	-	1	5	-	6	-
Phaeodothis winteri CBS 162.31	5	3	-	-	-	-	5	-	3	-
Phaeodothis winteri CBS 429.96	10	10	-	3	-	-	4	1	3	2
Bryoscyphus atromarginatus CBS 211.96	9	10	10	8	-	-	1	5	4	3
Fusarium equiseti	10	10	10	10	9	7	-	10	-	10
Penicillium velutinum	10	10	10	10	-	-	-	7	-	6

* number denotes how many plugs affected

Table 18. Results for 31/3/05 inoculation

Data collection 11/4/05	Growth visible on plug		Growth extended beyond plug but within 1 plug diameter		Growth extended beyond plug > 1 plug diameter		Liverwort response			
							Wounded		Unwounded	
	Wounded	Unwounded	Wounded	Unwounded	Wounded	Unwounded	Slight	Collapsed	Slight	Collapsed
Phaeodothis winteri CBS 551.63	*6	6	5	6	-	-	-	6	2	4
Phaeodothis winteri CBS 102466	6	6	6	6	-	-	2	2	3	3
Phaeodothis winteri CBS 102483	6	6	6	6	2	-	2	2	3	3
Phaeodothis winteri CBS 162.31	1	-	-	-	-	-	-	-	-	-
Bryoscyphus atromarginatus CBS 211.96	-	-	-	-	-	-	-	-	-	-
Fusarium equiseti	6	6	6	6	6	6	-	6	-	6
Control	-	-	**	**	-	-	-	-	-	-

* number denotes how many plugs affected ** denotes bacterial contamination

LNA produced peaks using both UV and fluorescent detectors. With fluorescence a single large peak was produced, indicating that LNA could be detected in tiny quantities in plant samples by fluorescence, compared to the UV. The configuration of solvents and detector settings were determined and used for all subsequent HPLC analysis (Table 19). The optimum setting for the UV detector was 285 nm (Abe and Ohta, 1983).

Detector Settings:	**Wavelength λ**
Fluorescence	excitation 300 nm emission 405 nm
Ultra violet	285 nm
Solvent ratios:	
H_2O + 0.1% acetic acid	60
95% acetonitrile	40

Table 19. Optimum settings for fluorescence and ultraviolet detectors

Salicylic acid (SA) produced a peak at 4.47 minutes retention time. A larger peak was produced by fluorescence than UV. Analysis of the plant sample produced a second peak at 8.68 min retention time, confirmed as LNA when compared with the LNA standard. However, there was also a peak at the 4.47 produced by the plant sample without SA added, indicating that liverwort probably contains some SA, and therefore it is not ideal as an internal standard.

The UV absorbance spectrum for HNA produced peaks at 283.60 and 295.20 nm, close to that of LNA (285 nm) (Table 20). One produced at 360.40 nm was at the end of the spectrum where fewer response peaks are found for plant tissue samples are used.

No.	**Wavelength (nm)**	**Absorbance**
1	360.40	0.172
2	295.20	0.227
3	283.60	0.391
4	272.80	0.339

Table 20. UV absorbance spectra for 3-hydroxy-2-naphthoic acid (HNA)

The initial HPLC analysis of HNA produced small peaks (Table 25) that would be difficult to identify using the UV detector when analysing plant tissue samples; the

more concentrated standard (1 mg ml^{-1} HNA) did produce a larger peak, however it was too broad and tailed. Fluorescence produced a number of small peaks that would not be easily identifiable, as opposed to the single large peak produced by salicylic acid and lunularic acid; the setting was correct for LNA, not HNA. This substance was therefore unsuitable for use as a standard.

Using the simplified extraction process, peaks detected in the fluorescence during HPLC of gemmae extract, SA and LNA were an acceptable shape for both SA and LNA standards. A peak was produced by the gemmae extract with a retention time of 8.08 minutes that could have been LNA. To confirm this LNA standard was added to the liverwort extract and analysed. A single large peak was produced by fluorescence, suggesting the two peaks found previously co-migrated to form one, confirming the peak produced by the gemmae extract as LNA.

The recovery of LNA was calculated using serial extractions: the average peak area for each five extractions, across three samples, and their percentage of the total amount recovered was calculated (Table 33. Figure 28). The peaks indicated that the majority of the LNA was recovered during the first four extractions. The presence of LNA in the fifth extraction indicated that further extractions were necessary for 100% recovery. The results can be used to determine an acceptable recovery rate, which can then be incorporated into the extraction protocol.

Discussion

Growth and development

Years 1 and 2. The effect of light level and temperature on the growth and development of male and female liverwort

In years 1 and 2 growth room experiments compared liverwort growth and development in varying temperature conditions (25 C, 15 C and 10 C), while light levels, humidity and day length remained constant. The results from year 2 are yet to be analysed statistically.

Results from both experiments show that dry weight, fresh weight and number of gemma cups are all greater in high temperature than low temperature; and in low light than high light.

In year 2, dry weights, fresh weights and growth (radial expansion) were all greater for female gemmalings than male, except for the high light, low temperature treatment where male gemmalings were larger. These gemmalings were the smallest of all the treatments, showing very little growth.

In year 1 there was a significant effect at the 0.1% level depending on gender, with more gemma cups were produced on male plants than female at both light levels.In year 2, after four weeks there were more gemma cups on male than female gemmalings in high light than low light treatments. However, in low temperature the gemmalings were particularly small and gemma cups were only produced in the female, low light treatment, with no discernable trend relating to gemmaling sex overall.

It was observed that growing liverworts in high light at 25^{o}C had an effect on their colour and morphology. By week 5 they were a dull green colour, and by week 6, 8 of the 12 replicates were developing dark brown colouration and reduced relative growth rate, showing signs of tissue damage due adverse growing conditions. The thallus had a thicker, dome-shaped appearance. Liverworts grown in lower temperature and light conditions were flatter and were brighter green colour.

In year 2, where there was no 25°C treatment, none of the replicates developed the dark brown colouration. Some replicates grown at 15C and high light level became domed shape, with little radial growth. Liverworts grown in low light levels were, again, flatter and brighter green in colour.
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 temperature and humidity levels, for example, would promote liverwort growth. The implications are that a reduction in shading and humidity could reduce liverwort infestations. Experiments during year 3 will further investigate the effects of shading in at Palmstead Nursery, Wye.

This seems to indicate 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. This may be linked to LNA accumulation, which is known to increase in high temperatures and continuous light, causing liverworts to become dormant in these adverse growing conditions. This effect will be investigated in a further fitotron experiment in year three, where LNA levels will be measured in liverwort gemmalings and linked to their growth at different temperature and light conditions. The aim will be to identify levels where LNA becomes toxic to liverwort and investigate using environmental conditions and LNA levels to inhibit liverwort growth.

Gemma dispersal

Year 1: The effect of nozzle size, water pressure and nozzle height on gemmae dispersal using an overhead sprinkler system.

More gemmae were generally dispersed using 2 and 2.5 bar water pressure, and these were dispersed closer to the source, coinciding with the manufacturers recommendation that 2 bar is the optimum operating pressure for this particular nozzle range.

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.

In each treatment a number of gemmae travelled further than the majority, reaffirming the ability of liverwort to invade and colonise new ground, clearly demonstrating its ability to disperse and colonise new areas in nursery situations.

Possible solutions as part of an integrated pest management system could include altering the irrigation regime to reduce or eliminate use of overhead sprinklers.

Gemma cup replenishment

The aim of this experiment was to investigate how gemma cups replace dispersed gemmae, whether this is affected by irrigation regime. 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.

These results and those of the gemma dispersal experiment complement the conclusions of another HDC study (HNS 107) that compares the cost and use of capillary matting and overhead irrigation practices. Sub irrigation can provide costs savings of 25-35% over well designed overhead irrigation, improving water use efficiency, uniformity of water distribution, and plant quality (Burgess, 2003).

Clumping of gemmae

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, with 100% establishment rates in the small and medium size classes.

Fresh and dry weights were also greater for groups of gemmae than clumps, increasing with class size for ‘groups’ of gemmae, but remaining more constant for clumps. Statistical analysis will determine the significance of these results.

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; there may be other factors that influence their formation and that may affect growth and establishment.

Fungal antagonism

The inoculations to date have enabled the exclusion of weak growing, non aggressive strains that do not appear to elicit a strong response from liverwort. *Bryoscyphus atromarginatus* is slow to establish, producing little response, however, as it is reported to attack *Marchantia polymorpha* specifically efforts are being made to reinvigorate it by passing it through liverworts. The most promising strains are *Fusarium equiseti, Phaeodothis winteri* CBS 551.63 and *P.winteri* CBS 102466.

Lunularic acid

The literature indicates that there is evidence of the inhibitory effect of lunularic acid, however this has not been characterised either in terms of liverwort lifecycle or in relation to environmental conditions.

Method development is almost complete. Further work on improving LNA recovery and optimising extraction efficiency include use of a TissueLyser to pulverise liverwort tissue, maximising the extraction of LNA whilst reducing the number of extractions necessary is in the final stages.

Research Plan

Fungal antagonism:

Pathogenicity testing of fungi already in culture is almost complete. During year 3 experiments will be designed to test selected fungal species under nursery conditions.

Lunularic acid

Method development for lunularic acid is almost complete. This will be followed by two pieces of work: quantification of lunularic acid present in liverwort at different stages of the lifecycle and an investigation of LNA levels present during the growth of liverwort in different light, temperature and humidity conditions, particularly where liverworts show signs of stress due to adverse growing conditions.

The effect of glucosinolate bioactive products on liverworts:

Glucosides are present in members of Cruciferae and related families. They break down to form secondary metabolites that are volatile defence substances - glucosinolates, (Taiz and Zeiger, 1998). Biologically active isothiocyanates, highly toxic to pests and pathogens, are one of the products of glucosinolate degradation, released from *Brassica* tissues following cell damage (Bones and Rossiter, 1996). The structure of glucosinolates and toxicity of isothiocyanates varies depending on plant species, resulting in variable biofumigation potentials of brassicas (Matthiessen et al., 2001).

A product, 'AlbaGro' is being tested in America using seed meal from *Limnanthes alba,* a waste product following oil extraction. Studies indicate that short term (30 days) control of liverwort is possible using this product (Svenson and Deuel, 2000). It is proposed to extract glucosinolates from *Limnanthes alba* seeds. Seed sourcing is proving difficult as the seed in America is protected by patents belonging to seed oil companies. Other promising species are currently being selected.

A selection of other glucosinolate bioactive hydrolysis products with potential for liverwort control will be isolated and tested against optimal and sub-optimal growth conditions identified in year 1, under laboratory conditions, and the growth stage when application would produce most effective results will be determined.

Final year scaling up experiments

Larger scale trials will be designed to investigate further the results of growth and development and dispersal experiments and to test the effects of applications of fungal species and glucosinolate products as liverwort controls. This will transfer the main tenets of the research from the artificial environment of controlled laboratory experiments to nursery conditions. Combinations of treatments will be applied, linking environmental conditions to irrigation methods using a variety of irrigation systems such as those developed at East Malling Research.

Technology Transfer

An article in the February 2005 issue of HDC News gave a project update and offered members another opportunity to advise us of any unusual liverwort plants or incidents of unexplained decline or dieback of established liverworts.

A poster was presented at the XIII European Weed Research Society Symposium at Bari, Italy in June 2005 (Appendix II).

A poster was presented at the annual Post Graduate Symposium held at the Department of Agricultural Sciences, Imperial College London, Wye Campus on 6th July 2005.

A further poster presentation is planned for January 2006 at the Northeastern Weed Science Society meeting in Providence, Rhode Island, New York. A number of weed scientists currently researching liverwort control have been contacted and will be attending.

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Appendices

Appendix (I) Lunularic acid method development and results

Extraction and purification of lunularic acid

The following extraction and purification method was adapted from (Valio, 1969) and is summarised in Figure 6. 13 g of freeze dried liverwort were ground down in a Moulinex coffee grinder, placed into a conical flask with 500ml 80% methanol and then refrigerated at 2 C on a stirrer overnight. It was then filtered to remove plant debris using a Hartley funnel, filter paper and Hyflo Supercel 'Filter Aid' (from BDH). The methanol was extracted from the filtrate under reduced pressure using a Büchi Rotavapor RE120 rotary film extractor at 35-40 C. The volume was made up to 15 ml with distilled water and the pH lowered to 3 using 5M HCl. Acid is not soluble in ethyl acetate (EtOAc) and is therefore separable as a fraction using ethyl acetate.

To obtain the neutral fraction the organic fraction was extracted from the solution by washing it with an equal volume of EtOAc: The filtrate and ethyl acetate were poured into a separating funnel, the flask was shaken and volatiles released three times then left for a few minutes for the fractions to separate. The upper green organic layer was put into a conical flask. The lower aqueous layer was replaced in the separating funnel and extracted again with an equal volume of EtOAc. The extraction was repeated three times, bulking the organic fractions in the conical flask. The aqueous fraction was used to produce the basic fraction.

The organic fraction was reduced to a small volume (50ml), using the rotavapor, and extracted 3 times with equal volumes of 5% sodium bicarbonate ($NaHCO_3$) as before. The upper organic layers were bulked, the lower aqueous layer returned to the funnel each time for the next extraction. The aqueous fraction from this extraction was used to produce the acidic fraction; the organic fraction formed the neutral fraction.

To obtain the acidic fraction the aqueous fraction was acidified to pH 3 using 5M HCl. It was then extracted 3 times using equal volumes of EtOAc as before. The organic fraction was used to form the acidic fraction; the aqueous fraction was discarded.

To obtain the basic fraction solid $NaHCO_3$ was added to the aqueous fraction from the first extraction to increase the pH to 8. It was then extracted three times with

EtOAc as before. The final aqueous fraction was discarded; the organic fraction was used to form the basic fraction.

The three organic fractions (acidic, basic and neutral) were then dried. Each was placed into a separating funnel, allowed to settle and the layer of water that formed at the bottom drawn off and discarded. Magnesium sulphate ($MgSO_4$) was used to remove the remaining water from the organic fraction, and then removed by filtration.

The filtrate was reduced to dryness using the rotavapor. The dry filtrate was rinsed with EtOAc, transferred to a smaller round bottom flask and re-dried. The filtrate was again rinsed with ethyl acetate and transferred to a small vial. The extract was then dried using a Techne Dri-Block DB-3A sample concentrator.

Purification of lunularic acid

A small amount of the neutral (6mg) and acid (5mg) fractions were used. The acid fraction was discarded as there was very little of it and Valio (1969) found it contained no lunularic acid. 150µl of ethyl acetate was put into each vial to dissolve the compounds. The start line was drawn onto the TLC plate approximately 2.5 cm from the bottom. Even streaks of each sample were applied to the plate in layers along the start line, 20µl at a time, producing a narrow line. Each layer was allowed to dry before more was added.

Two solvent systems were used during purification (Table 21). The choice of solvent system depends on the substance being analysed. The aim is to separate the components of the fraction. Enough of solvent system 1 was poured into the chromatography tank to cover the bottom of the TLC plate. The plate was placed vertically into the solvent with the start line at the bottom. After approximately 1 hour the solvent front had almost reached the top of the plate. Its position was marked with pencil, the plate removed from the tank and placed vertically within the fume cupboard to dry. The plate was viewed with ultraviolet light to see the positions of separated compounds.

Table 21. Solvent systems

Components	Component Ratio

1	Chloroform	Ethyl acetate	Acetic Acid	60:40:5
2	Toluene	Ethyl acetate	Acetic Acid	50:5:2

The area between the start line and solvent front line was divided into 16 sections, 2cm apart, with pencil lines (Figure 25). The silica gel containing the separated compounds between each line was scraped off the plate and transferred to small, labelled conical flasks. 20ml methanol was put into each flask to extract the eluate from the silica gel and refrigerated overnight. It was filtered to remove the silica gel, and methanol was extracted from the filtrate using the rotavapor. The round bottom flasks were rinsed with a small amount of methanol to dissolve the dried filtrate and transferred to small, pre-weighed vials. The Dri-block was then used to remove the remaining methanol.

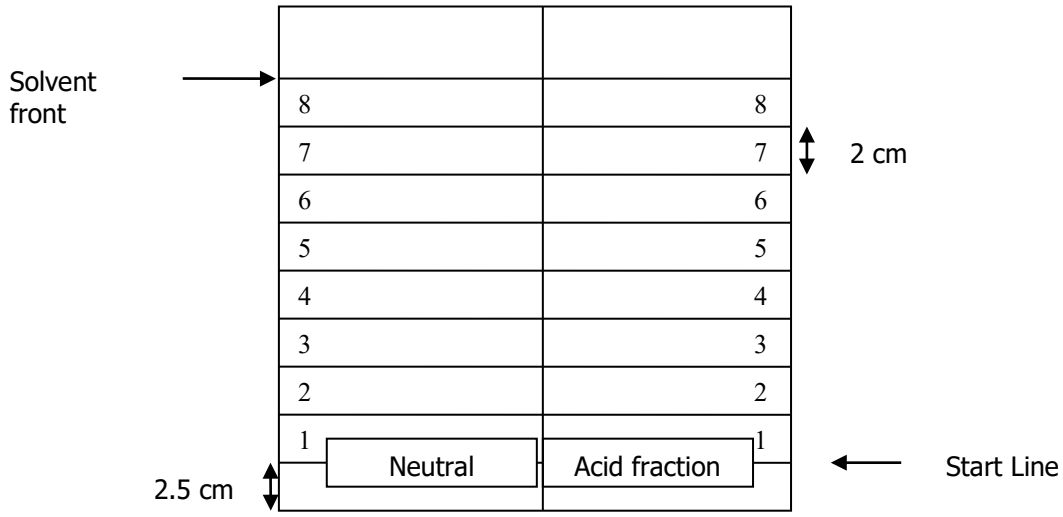

Figure 25. Chromatography plate layout

A second TLC plate was run to further separate the compounds. A plate was prepared with 16 points marked along the spotting line, approximately 1cm apart. Two 2.5µl spots of each of the 16 compounds was placed at each point on the spotting line and allowed to dry. The plate was placed in the chromatography tank and developed as before, using solvent system 2. As the eluate fronts had not moved very far, once the plate had dried it was redeveloped.

Two compounds were visible under the ultraviolet light. The remainder of the compounds used to produce these spots were used on a third plate to obtain a larger

quantity of this substance. Each of the two substances was streaked separately along the spotting line, 20µl at a time, in the same manner as the first plate. The compounds did not move very far, therefore the plate was developed twice more.

HPLC analysis

A sample of lunularic acid was obtained to use as a standard for comparison during analysis of liverwort tissue. Mass spectroscopy confirmed its molecular weight as 258, authenticating the sample. It was therefore no longer necessary to extract and purify a sample from liverwort tissue.

HPLC equipment was arranged as in Figure 9. A Waters spherisorb ® S5 ODS 2, reverse phase column was used, at 35 C. Solvent flow rate was 1 ml min^{-1}. Both fluorescence and UV detectors were used. As most molecules do not exhibit fluorescence this can be an extremely sensitive tool. The structure of lunularic acid (Figure 26), incorporating phenyl, carboxyl and hydroxide groups suggests that fluorescence would elicit a good response.

A stock solution was made up of 0.19 mg LNA in 1 ml ethanol (C_2H_6O), then diluted with the HPLC solvent to give 19 µg ml^{-1} LNA.

Table 22. Solvent system I

Solvent A	milli-Q water + 0.1% acetic acid
Solvent B	95% acetonitrile (CH_5CN),

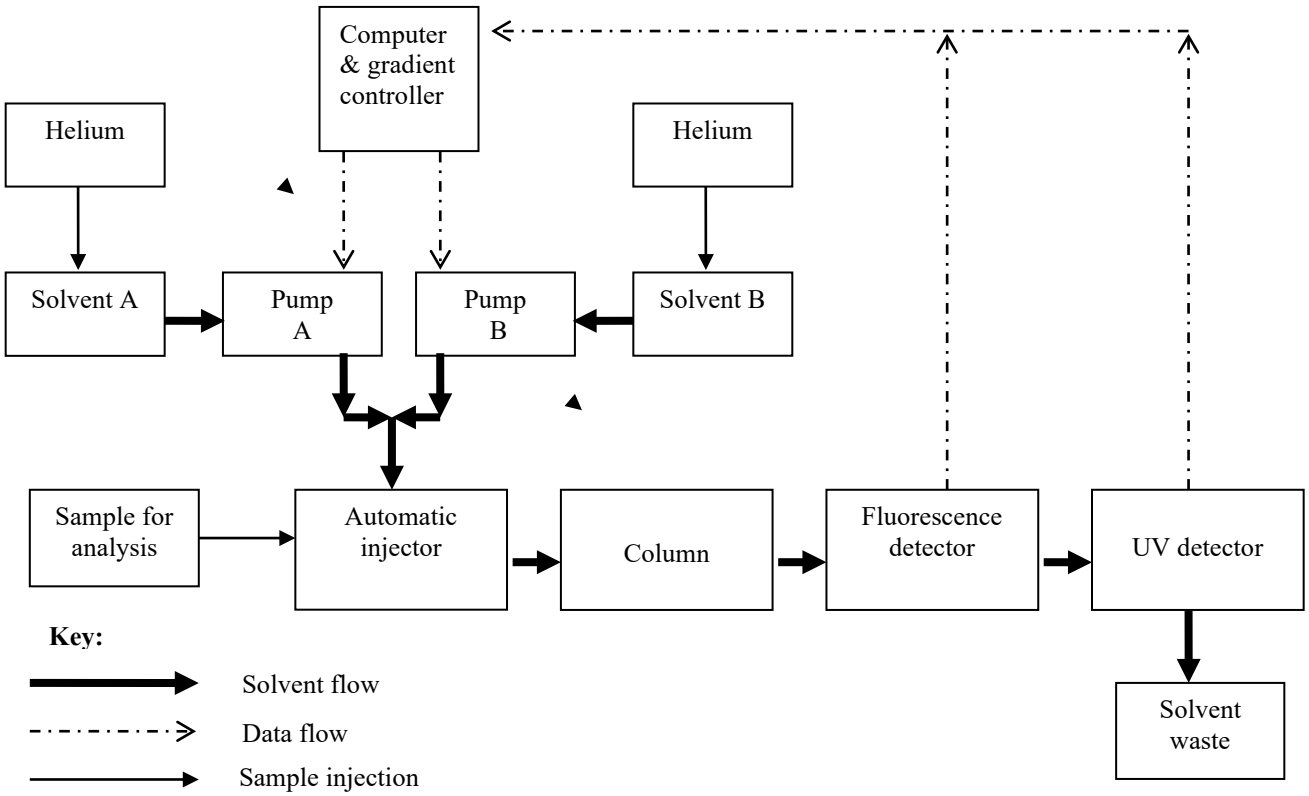

Figure 26. Layout of equipment used for HPLC analysis. Solvents A and B were degassed with helium, then passed through pumps A and B, where they were combined and through the automatic injector. The sample to be analysed was injected into the system and was carried by the solvent flow through the chromatography column where it was separated before passing through the detectors.

The solvents (Table 22) were introduced via two separate pumps (A and B), operating isocratically. Four solution ratios were tested (30:70, 40:60, 50:50, 60:40) to determine which produced a peak corresponding with LNA at a suitable retention time. A 60:40 ratio (solvents A:B) produced a peak after 8 minutes, with the fluorescence detector (Merck/Hitachi F-1050 fluorescence spectrophotometer) set at 300 nm excitation wavelength and 400 nm emission wavelength. The optimum setting for the UV detector (Waters 486 tuneable absorbance detector) was 285 nm (Abe and Ohta, 1983).

Fluorescence excitation/emission curves:

A fluorescence excitation/emission curve was produced to optimise the detector settings so that LNA would produce the maximum peak size.

Excitation □ (nm)	Energy (mV)	Emission □□(nm)	Energy (mV)
250	10	355	72.3
255	11.8	360	74.4
260	18.4	365	82.3
265	30.4	370	96.1
270	57.9	375	113
275	102.6	380	141.3
280	136.8	385	168
285	167.9	390	197.6
290	194.2	395	217
295	212.3	400	225
300	232.8	405	225.8
305	225	410	218.5
310	184.3	415	209
315	128.1	420	196
320	87	425	180.7
325	57.4	430	161
330	41.1	435	142.9
335	28	440	123.3
340	21.9	445	107
345	17.3	450	90.8
350	13.7	455	75.3
355	11.1	460	64.8
360	8	465	49.5
365	7.6	470	42.2
		475	36.7

Table 23. Data for excitation and emission spectra

Each sample was injected directly into an injection point on the fluorometer. With the emission wavelength set at 400 nm, energy level readings were taken at an initial excitation wavelength of □250 nm, increasing by 20 nm increments to 365 nm for subsequent injections. This was repeated with the excitation wavelength set at 200 nm, beginning at 355 nm, again increasing in increments of 20 nm to 475 nm. Once plotted graphically (Table 23, Figure 27) these peaks indicate emission and excitation settings required to produce maximum peaks for LNA.

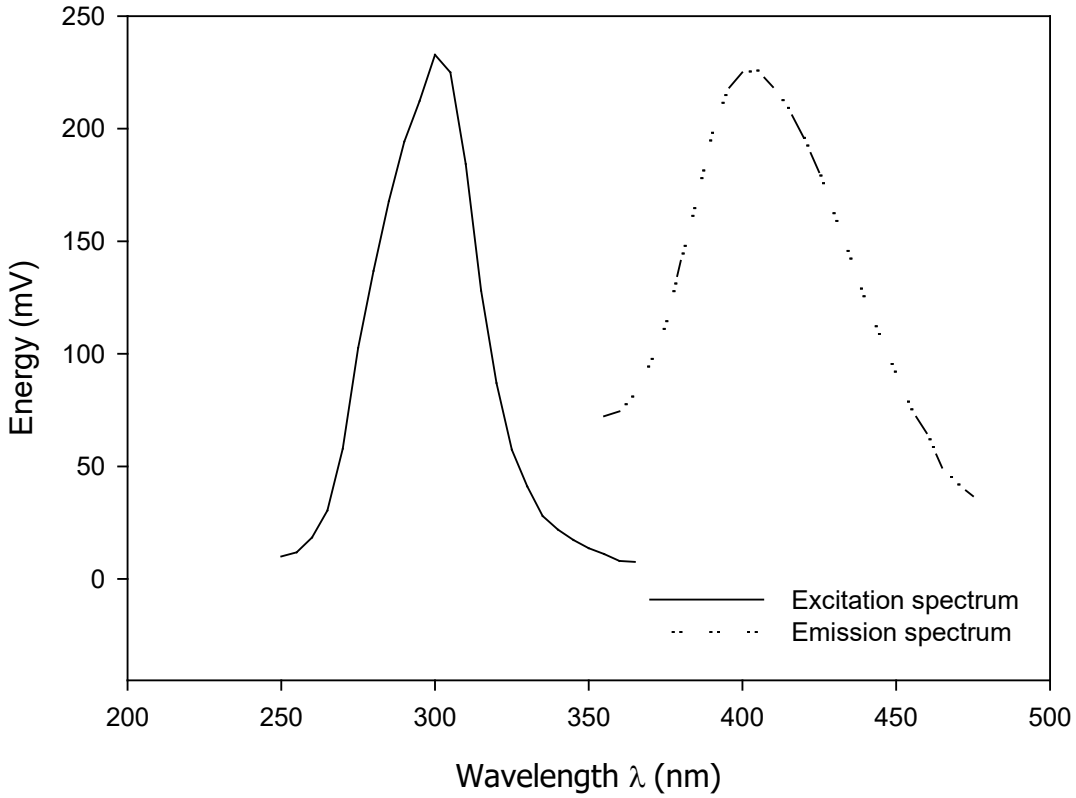

Figure 27. HPLC analysis of lunularic acid using fluorescence detection. Excitation spectrum produced at 405 nm wavelength emission. Emission spectrum produced at 300 nm wavelength excitation.

Sample preparation for HPLC analysis

1 g of liverwort thallus was placed into a mortar with 10 ml methanol (MeOH) and some glass fragments, then pulverised using a pestle. This was transferred to a 100 ml conical flask with 3 grains anti-bumping granules and another 10 ml solvent. This was extracted over a steam bath for 15 minutes then filtered. The liverwort residue was returned to the conical flask, with 10 ml methanol and extracted for a further 5 minutes. It was then re-filtered and the two filtrates bulked. This was repeated to produce two samples (Figure 8).

The filtrate was dried to a watery residue under reduced pressure using a Büchi Rotavapor RE120 rotary film extractor at 35 C. Salicylic acid (0.01 mg ml^{-1} ether) was used as an internal standard; 2 ml were added to one plant extract sample then made up to approx 10 ml with distilled water. To remove the chlorophyll in the sample it was extracted three times with 10 ml diethyl ether. The green upper layer was

removed into a conical flask each time (Figure 9). The samples were finally passed through a magnesium sulphate ($MgSO_4$) column to remove any water.

Amino-propyl solid phase extraction (SPE) column

Salicylic acid (SA) was selected as an internal standard (Figure 7); SA and LNA have similar structures (phenyl, carboxyl and hydroxide groups), therefore both are detectable using fluorescence. Using an internal standard (spiking) helps to quantify the amount of LNA in a sample. It would also help to detect losses during sample preparation.

The column was conditioned with 20 ml ether and the solvent discarded. The column was then filled with six volumes of the sample and the liquid collected discarded. The column was then washed with three volumes of Dichloromethane:2-propanol (2:1) and the liquid again discarded. The lunularic acid was then eluted with 10ml MeOH:AcOH (95:5) and the eluate collected (Figure 10).

Three solutions were passed through the column to produce three samples:

- 2 ml salicylic acid (SA) standard
- extract 'spiked' with 2 ml SA
- extract without SA.

To remove the acetic acid each sample was dried under reduced pressure using the rotavapor, then azeotroped twice with 2 ml butan-1-ol at 50 C. The residue was dissolved in 1 ml of acetonitrile ready for high pressure liquid chromatography (HPLC) analysis.

3-hydroxy-2-naphthoic acid (HNA) as an internal standard

An alternative compound, 3-hydroxy-2-naphthoic acid (HNA) was tested as an alternative internal standard to SA (Figure 11).

A solution of 0.01 mg ml^{-1} HNA in ether, injected into the HPLC system, did not produce a peak. A gradient solvent system was then used, but again no peak was detected.

A UV spectrophotometer was used to produce the characteristic UV absorbance spectrum for HNA and UV setting that produced the largest peaks, indicating maximum absorbance (λ_{max}) (Table 20).

Three HNA standards (1 mg ml^{-1}, 0.1 mg ml^{-1}, 0.01 mg ml^{-1}) were prepared using the HPLC solvent system (H_2O + 0.1% acetic acid:95% acetonitrile, 60:40) and analysed. Three HPLC runs were made with the UV detector set at 360.40, 283.60 and 310 nm, with 0.1 mg ml^{-1} HNA, followed by a run with the HNA concentration increased to 1 mg ml^{-1}. 310 nm was used as there was at a dip in the UV spectrum at this point (the HPLC trace should not have a peak there); an aid to confirming the peaks were produced by HNA.

To try to improve the peaks produced purified (99.99% pure) HNA was then used at concentrations of 1 mg ml^{-1} and 0.1 mg ml^{-1} HNA in acetonitrile. The UV detector was set at 284 nm.

For further HPLC analysis and to improve the peaks the solvent system was acidified and the proportion of acetonitrile increased (Table 13). The pumps were operated on a gradient system, using 1 mg ml^{-1}, 0.1 mg ml^{-1} and 0.01 mg ml^{-1} purified HNA in acetonitrile. The UV detector was set at 284 nm.

Table 13. Solvent system II

Solvent A	milli-Q water + 0.5% acetic acid:95% acetonitrile + 0.5% acetic acid (60:40)
Solvent B	95% acetonitrile (CH_5CN) + 0.5% acetic acid

This analysis was repeated using a new column (same type), as this could improve the peaks produced, and solvent system with reduced acetonitrile content (Table 25). Both fluorescence and UV detectors were used.

Table 25. Solvent system III

Solvent A	milli-Q water + 0.5% acetic acid
Solvent B	95% acetonitrile (CH_5CN) + 0.5% acetic acid

Simplified extraction and detection process.

Due to the negative results in identifying a suitable internal standard, a simplified extraction process was designed. By simplifying the preparation process the opportunity for losses to occur is reduced, and although an internal standard would be preferable, it was not essential. SA and HNA were tested again for use as an internal standard before making a final decision (Table 32).

25 mg liverwort gemmae were ground with a pestle and mortar in 150 µl 95% acetonitrile, centrifuged and the supernatant removed. The extraction was repeated twice more and the extracts bulked. This was then analysed by HPLC, along with SA and LNA standards. Solvent system I (Table 11) was used for the HPLC system and to prepare standards of SA and LNA in this and subsequent analyses, to regain the original retention times for LNA (Table 30).

A peak was produced in the plant sample with a retention time of 8.08 minutes that could have been LNA. To confirm this, the LNA standard was diluted 1:20, (the peak size of the standard was approximately 20 times larger than that produced by the gemmae extract) and aliquots of LNA standard and gemmae extract were combined and analysed. An increased peak at 8.08 minutes retention time by the same amount as the standard would confirm its identity as LNA (Table 31).

A further HPLC analysis combining HNA, SA and LNA, directly compared the retention time and peak characteristics of each (Table 32).

LNA recovery

LNA recovery rates were calculated using serial extractions, without bulking the supernatants, then analysing them individually. The area beneath the LNA peak was used to determine how many extractions were needed to retrieve all the LNA from the tissue sample; losses can then be minimised. The final extraction should contain no LNA.

Three 25mg samples of liverwort thallus were weighed into separate epindorfs and extracted with 50 µL 95% acetonitrile + 0.1% acetic acid added to each. They were ground up with a pestle, and then centrifuged for 5 minutes at 13000g per minute. The supernatant was removed into a vial, and the extraction repeated with more solvent, keeping the supernatants separate. Five extractions were completed from

each sample and analysed by HPLC. Areas beneath the peaks produced by fluorescence were recorded and percentage recovery calculated for each (Table 33).

Results

Extraction and purification of lunularic acid

The aim of the extraction and purification was to provide enough lunularic acid to use as a standard during HPLC analysis; however the final TLC plate did not contain enough LNA on this occasion. A sample of lunularic acid was subsequently obtained and the process no longer required.

The configuration of solvents and detector settings were determined and used for all subsequent HPLC analysis (Table 19).

LNA produced peaks using both UV and fluorescent detectors. With fluorescence a single large peak was produced, indicating that LNA could be detected in tiny quantities in plant samples by fluorescence, compared to the UV.

Extraction of lunularic acid from plant material

Amino-propyl solid phase extraction (SPE) column:

The salicylic acid (SA) produced a peak at 4.47 minutes retention time. A larger peak was produced by fluorescence than UV. Analysis of the plant sample produced a second peak at 8.68 min retention time, confirmed as LNA when compared with the LNA standard. However, there was also a peak at the 4.47 produced by the plant sample without SA added, indicating that liverwort probably contains some SA, and therefore it is not ideal as an internal standard.

3-hydroxy-2-naphthoic acid (HNA) as an internal standard:

The UV absorbance spectrum for HNA produced peaks at 283.60 and 295.20 nm, close to that of LNA (285 nm) (Table 26). One produced at 360.40 nm was at the end of the spectrum where fewer response peaks are found for plant tissue samples are used.

No.	Wavelength (nm)	Absorbance
1	360.40	0.172

2	295.20	0.227
3	283.60	0.391
4	272.80	0.339

Table 26. UV absorbance spectra for 3-hydroxy-2-naphthoic acid (HNA)

The initial HPLC analysis of HNA produced small peaks (Table 27) that would be difficult to identify using the UV detector when analysing plant tissue samples; the more concentrated standard (1 mg ml^{-1} HNA) did produce a larger peak, however it was too broad and tailed. Fluorescence produced a number of small peaks that would not be easily identifiable, as opposed to the single large peak produced by salicylic acid and lunularic acid.

UV setting	HNA Conc mg ml^{-1}	≈ Retention time (mins)	Peak size (mV)
360.4	.1	5	14.75
310	.1	5	5
283.6	.1	5	25
283.6	1	5	292.7

Table 27. HPLC using 3-hydroxy-2-naphthoic acid (HNA)

The peak produced using 0.1 mg ml^{-1} purified HNA was very small. A larger peak was produced using 1 mg ml^{-1} HNA, but it was very broad and tailed, which may indicate overloading of the column. The retention time had also increased to 8 and 7.76 minutes (Table 28), coinciding with that of lunularic acid.

UV setting	HNA Conc mg ml^{-1}	≈ Retention time (mins)	Peak size (mV)
284	.1	8	64
284	1	7.75	187.5

Table 28. HPLC using purified 3-hydroxy-2-naphthoic acid (HNA)

The peaks produced using the acidified solvent system were still broad and tailing, but slightly improved. The retention time had moved to 3.7 minutes (Table 29).

UV setting	HNA Conc mg/ml	≈ Retention time (mins)	Peak size (mV)
284	1	-	195.3
284	.1	3.7	49
284	.01	3.7	7

Table 29. HPLC analysis of purified 3-hydroxy-2-naphthoic acid (HNA) with acidified solvent system.

The peaks produced after changing the column were extremely small, broad and tailed. No substantial peak was detected by fluorescence; the setting is correct for LNA, not HNA. This substance appeared unsuitable to use as a standard.

Simplified extraction and detection process.

The peaks detected in the fluorescence following HPLC of gemmae extract, SA and LNA were an acceptable shape for both SA and LNA standards (Table 30). A peak was produced by the gemmae extract with a retention time of 8.08 minutes that could have been LNA. To confirm this, the LNA standard was diluted 1:20, (the peak size of the standard was approximately 20 times larger than that produced by the gemmae extract). Aliquots of LNA standard and gemmae extract were mixed together and analysed. An increase in size of the peak with 8.08 minutes retention time by the same amount as the standard would confirm its identity as LNA.

The peak areas for the LNA standard and the gemmae extract combined was 887726; very similar to the single peak produced using gemmae extract plus LNA (955117) (Table 31). The peak shape was also good. This suggested the two peaks found previously (Table 30) co-migrated to form one peak and confirmed the peak produced by the gemmae extract as LNA.

Table 30. HPLC analysis of liverwort gemmae, LNA and SA standards

	UV		Fluorescence	
	≈Retention time (mins)	Peak size (mV)	≈ Retention time (mins)	Peak area
SA std 0.001 mg ml^{-1}	4.98	5.5	4.88	5217797
LNA std 0.019 mg ml^{-1}	8.74	6.5	8.58	9244885
25 mg gemmae extract	9.25	10	8.08	487064

Table 31. HPLC analysis of liverwort gemmae detected using fluorescence

Sample	≈ Retention time (mins)	Peak area
LNA standard 1:20 dilution	8.68	400662
25 mg gemmae extract	8.08	487064
LNA standard 1:20 dilution + 25 mg gemmae extract	8.25	955117

The results of the analysis using HNA, SA and LNA showed peaks as expected produced by SA and LNA (Table 32). HNA, however, did not produce a peak in the fluorescence and there was a very small, unidentified peak at approximately 7.50 minutes in the UV. If this was produced by HNA it was too small to use as a standard, and its non-appearance in the fluorescence confirmed HNA to be unsuitable as a standard.

Table 32. HPLC analysis of SA, LNA and HNA

		UV			Fluorescence	
Conc (mg ml^{-1})	**Setting**	**≈ Retention time (mins)**	**Peak area**	**Setting**	**≈Retention time (mins)**	**Peak area**
SA std 0.01	285	5.04	75645	300/405	4.87	2547734
LNA std 0.0095	285	8.79	2309	300/405	8.64	1419641
HNA std 0.01	285			300/405		

LNA recovery

The average peak area for each extraction across the three samples and the percentage of the total amount recovered was calculated (Table 33. Figure 28). The peak areas show that the majority of the LNA was recovered during the first four extractions. The presence of LNA in extraction five indicates that further extractions are necessary for 100% recovery. The results can be used to determine an acceptable recovery rate, which can then be incorporated into the extraction protocol.

Extraction no.	Sample 1	Sample 2	Sample 3	Average	% of total	% recovery
1	22073170.00	16841700.00	22388536.00	20434468.67	67.51%	67.51%
2	9861853.00	5361145.00	8235117.00	7819371.67	25.83%	93.34%
3	2478929.00	3526.00	1479737.00	1320730.67	4.36%	97.71%
4	860277.00	254097.00	412643.00	509005.67	1.68%	99.39%
5	298072.00	113246.00	144146.00	185154.67	0.61%	100.00%

Table 33. Recovery of LNA during five serial extractions from 25 mg liverwort thallus. Figures relate to areas beneath LNA peaks

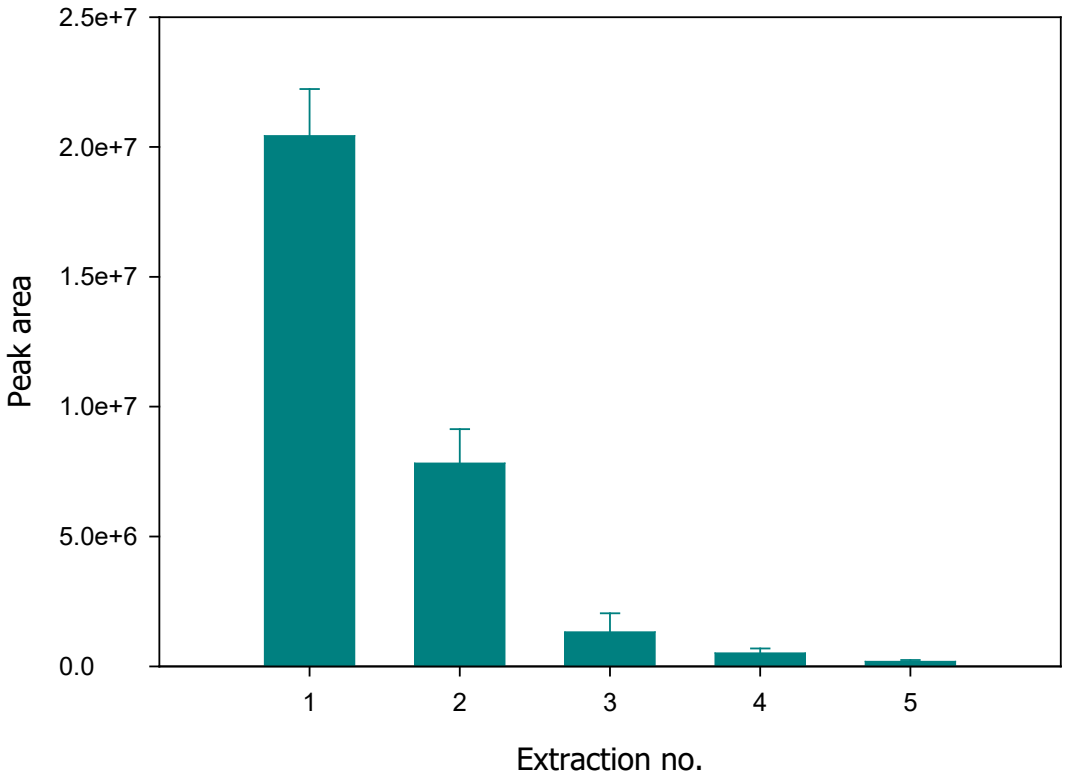

Figure 28. LNA recoveries from serial extractions of 25 mg liverwort thallus

Appendix (II) Poster presented at XIII European Weed Research Society Symposium, Bari and Department of Agricultural Sciences, Imperial College London, Wye Campus

Imperial College London

The effect of nozzle size, water pressure and nozzle height on dispersal of liverwort (*Marchantia polymorpha)* gemmae, using an overhead sprinkler system

Jill England and Mike Jeger. Department of Agricultural Sciences, Imperial College London, Wye Campus.

INTRODUCTION

Liverworts growing on the surface of container plants are a major problem in nurseries, estimated to cost the UK horticultural industry £13 million each year[1].

Marchantia polymorpha has a complex lifecycle with a variety of mechanisms to ensure its continued spread and establishment. It can reproduce asexually by gemmae (Figure 1a), vegetative propagules produced in circular gemma cups (Figure 1b) found on the dorsal surface of the thallus. The aim of this experiment is to provide information on the epidemiology of liverwort infestation.

Gemma cups are constructed with sides at an angle of 60-70^0 to the horizontal which, together with lentil-shaped gemmae, form an efficient splash-cup mechanism. Water droplets fall into the gemma cup and displace water, thrusting gemmae upwards along the cup sides and transporting them away from the parent plant. Dispersal distances of 0.6 metres by small raindrops[2] and 1.21 m by individual water drops of 4.74 mm diameter[3] were previously recorded.

In the artificial environment of the plant nursery liverworts are exposed to overhead irrigation systems designed to provide even distribution of water to the whole bed or tunnel – simultaneously producing ideal conditions for the spread of liverwort by gemmae.

Characterisation of the effect of overhead sprinklers on gemma dispersal could provide an insight into the effective design and use of irrigation regimes as part of an integrated pest management system.

METHOD

This experiment investigates the dispersal distance of liverwort gemmae by a glasshouse overhead sprinkler system, using three nozzle sizes and four water pressures (1.5, 2, 2.5 and 3 bar) at two heights (1 and 2 metres).

The nozzles used were Ein Dor Agridor 700 Dynamic Sprayers, colour coded by the manufacturer to denote the flow rate (Brown = 160 lh^{-1}, Blue = 105 lh^{-1} and Grey = 60 lh^{-1}).

Incident water drops were applied via an adjustable in-line water pressure adapter, anti-drip device and nozzle onto a half tray containing mature liverwort thallus bearing gemma cups. Dispersed gemmae were collected in 10cm diameter pots arranged in 3 lines of 16 at right angles to each other (Figure 2). The number of drops landing in each collection pot were counted.

For each treatment incident drops produced via the nozzle were collected on water-sensitive paper (Figure 4c), photographed digitally and droplet diameters calculated using ImageJ software.

Each combination of nozzle, nozzle height and water pressure produced a characteristic range of drop sizes (Figure 4 c), that can be represented by drop distribution charts (Figure 4 b).

ACKNOWLEDGEMENTS

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Figure 1

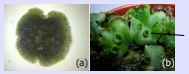

Figure 2

Nozzle Height (m)	Pressure (bar)	Grey 60 lh^{-1}	Blue 105 lh^{-1}	Brown 160 lh^{-1}
1	1.5	110	130	-
	2	90	120	100
	2.5	80	150	160
	3	110	150	90
2	1.5	150	110	-
	2	130	140	110
	2.5	90	140	90
	3	140	140	110

Figure 3. Distance travelled by gemmae (cm). The brown nozzle did not operate at 1.5 bar.

RESULTS

•Using a log-linear model, regression curves fitted to gemma dispersal data were highly significant, with F-probability <0.001 for all treatments (Figure 4a). 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 blue and brown 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 nozzle gemmae travelled further with the nozzle at 2 m (Table 1). Maximum dispersal distances obtained exceed those previously recorded[2,3].

•Generally more gemmae were dispersed at 2 and 2.5 bar water pressure. The brown nozzle did not operate at 1.5 bar.

•More gemmae were dispersed at the 2m height for the brown and grey nozzles, and at 1 m height for the blue nozzle.

•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).

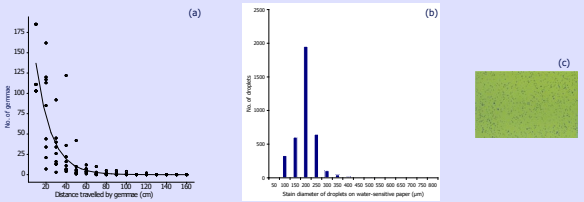

Figure 4. Blue nozzle, 1m, 2 bar treatment (a) regression curve (b) droplet size profile (c) droplet stains on water-sensitive paper

DISCUSSION

More gemmae were generally dispersed using 2 and 2.5 bar water pressure, and these were dispersed closer to the source. This appears to coincide with the manufacturers recommendation that 2 bar is the optimum operating pressure for this particular nozzle range; outside of 2.5 and 1.5 bar water distribution becomes uneven and may cause damage to the emitters.

It is difficult to draw conclusions from these results; 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. There may be an optimum combination for dispersing maximum gemmae numbers.

In each treatment a number of gemma travelled further than the majority, reaffirming the ability of liverwort to invade and colonise new ground, clearly demonstrating its ability to disperse and colonise new areas in nursery situations.

Possible solutions as part of an integrated pest management system could include altering the irrigation regime to reduce or eliminate use of overhead sprinklers.

REFERENCES

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