The effects of supplementary UV-B radiation on *Allium vineale var. vineale,* Ecological, Mutualistic and Reproductive Perspectives.

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Abstract.

The plant species *Allium vineale* commonly includes populations with disparate allocation patterns to three modes of reproduction: aerially produced bulbils, belowground asexual offsets and sexual flowers producing viable seed. Adaptive explanations for the persistence of this diversity suggest trade-offs among these alternate components of fitness. Several previous studies of the ecological characteristics between seeds and bulbils produced by *A. vineale* have proffered explanations on the continued prevalence of sexual reproduction over its inherent cost but although this reproductive plasticity is well documented, the role that sexual reproduction plays in *A. vineale* remains contentious. In this study the effects of UV -B lighting regimes, (together with nutrient availability and mycorrhysal colonisation), and the resulting pattern of phenolic secondary metabolite production was examined in order to test the concept of trade-off between growth, photodamage and allocation to sexual / asexual reproduction ratios in this species.

A glasshouse experiment was conducted to examine the effects of UV-B exposure (at a fluence rate of 3.2 μmol m^{-2} s^{-1} of biologically active UV-B, 280 320 nm) on leaf growth and secondary metabolite production in the species *Allium vineale*. Specimen plants were taken from a natural population and subjected to enhanced UV-B over a successive period of growing seasons. Leaves of plants exposed to enhanced UV-B radiation showed changes in secondary metabolites, (specifically flavonol production). Changes in the balance of the production of quercetin, kaempferol and isorhamnetin were identified

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using high performance liquid chromatographic (H.P.L.C.) separation methods and morphological changes were observed in epidermal tissue.

Nutrient treatments in conjunction with supplementary UV-B radiation did not induce any changes in sexual/asexual balance with all plants maintaining asexual bulbil production for the duration of the study. Leaf growth was reduced in the long-term by UV-B exposure but reproductive output as defined by inflorescence viability and dry weight was unaffected suggesting the presence of a dedicated UV-B photoreceptor present in leaf tissue and not oxidative damage *per se.* The plants were able to mitigate effectively any UV-B damage over the period of the study. Phenotypic plasticity was not found for sexual / asexual reproduction modes and in response to nutrient availability. Plants produced bulbils whose average size and weight was unaffected in both UV-B treated and untreated groups. This limited degree of plasticity suggests that the plants in this study lack the capacity to change their allocation patterns between different reproductive modes as nutrient levels or environmental UV-B radiation varies.

I suggest that an important role of plant phenolics in this species may be to protect leaves from photodamage and their levels in leaves may vary depending on environmental levels of UV-B radiation. There is evidence for the presence of a dedicated UV-B photoreceptor in this species underpinned by the shortening of leaves in treated samples.

General Introduction.

Protection from solar UV radiation is provided by a layer of ozone in the stratosphere but this layer has recently suffered depletion. Whether from natural or anthropomorphic factors the predicted result is that more UV radiation will reach the earth's surface. This UV radiation is in the region of the spectrum that has a shorter wavelength than blue light (between 400nm and 250nm) and is subdivided into UV-A (400-320nm), UV-B (320-290nm) and UV-C (290-250nm). UV-A radiation does not interact with ozone as its photonic energy is insufficient to disrupt the necessary photochemical reaction but UV-B carries sufficient energy resulting in its own effective absorption by the ozone layer. UV-C is absorbed by both ozone and oxygen and will be absorbed even under high levels of depleted ozone (Caldwell. M.M. 1971).Well documented analysis shows that ozone has declined globally by 4-5% since 1979 (SORG, (1996)) and this has led to a general assumption that there will be a depletion of the ozone layer of about 15% leading to a possible increase of effective UV-B of 30% over present levels within fifty years depending on global temperature (SORG, Stratospheric Ozone 1996. Within this range there are significant latitudinal variations in UV-B. Equatorial regions experience the highest levels due to the angle of solar incidence but it is also noteworthy that in regions of high latitude, where UV-B levels are relatively low, the thinning of the ozone layer is most marked, especially during winter/spring, and the potential relative increase in UV-B is expected to be of the greatest magnitude. This corresponds with *Allium vineale's* growing season (winter/spring). This species is perennial and therefore renders itself suitable for long-term experimental treatments including UV-B lighting regimes. Variation in

allocation to different modes of reproduction is known to occur in this species providing

scope for the examination of phenotypic plasticity within this study.

Allium vineale L. (liliaceae) is a bulbous perennial plant and is distributed over most of

western and central Europe. In continental Europe it grows in dry, open habitats and

along fields and roadsides. In this country it can be found growing in almost any

environment including moderately shaded conditions in deciduous forests (personal obs).

It is found growing in agricultural settings in north America (Ronsheim, 1994) and is

considered a pernicious weed due to its ability to taint cow's milk when consumed by

dairy cattle. It is a tetraploid ($2n$ = 32), (Rabinowitch and Currah, 2002). It flowers

between May and June, when mature plants produce a scape with a single inflorescence.

A mixture of bulbils and flowers, bulbils only or, flowers only are produced (Ronsheim,

1995). Bulbils mature in August to September with seeds maturing after approximately

eight weeks. The seeds require stratification in cold and moist conditions prior to

germination (Ronsheim, 1995). After flowering, small underground offset bulbs develop

from the parent bulb. These underground bulbs are prone to fungal infection and are of

reproductively less importance than the numerically superior bulbils (Ronsheim, 1995).

Work has previously been carried out in growth chambers, including some

monocotyledonous species (but not A*lliums),* where plants have been grown under

artificial white light to which different levels of supplementary UV-B is added or outdoors,

using artificial UV-B to augment natural sunlight. The results have led to the

acknowledgement that there are both direct and indirect effects of UV-B on plants.

Damage to cellular DNA has long been recognised and the products formed as a

consequence of damage and the role of the photolase enzymes involved in its repair is understood (Tyrell. R. M., 1973). Direct responses also include adaptive responses including the activation of defence mechanisms that afford protection against UV radiation. The most common is the production of pigments, especially flavenoids which act to screen out UV light. These polyphenol compounds are concentrated in epidermal leaf tissue and reduce the amount of UV light reaching the mesophyll cells of the leaf (Middleton *et al*, (1993). This type of response involves the stimulation of expression of specific genes, indicating specific light detection and signal transduction processes (Tevini and Teramura, 1989).

The effect of UV-B enhancements on plants can include decrease in photosynthetic activity, susceptibility to disease, changes in species competition and modifications to plant morphology and pigmentation. Some species are sensitive to existing levels of UV-B radiation while others are effectively unaffected by massive U.V. enhancements. This problem is compounded by studies of equally large response differences among cultivars of a species. Approximately 66% of some 300 species and cultivars tested appear to be susceptible to damage from increased UV-B radiation. Soybean, cotton, winter wheat, and maize were amongst the crops found to be susceptible to damage from increased UV-B radiation (Tevini, M. Braun, J and Fiesser, G. 1991).

There are a small number of experiments that have jointly examined the effects of UV-B and other stress factors on plant responses. The effect of UV-B on plant growth and productivity is difficult to quantify as it varies seasonally and is affected by microclimate and soil fertility. Some studies appear counter intuitive. For example, soybeans are less susceptible to UV-B radiation under mineral deficiency or water stress but sensitivity

increases under low levels of visible range radiation (Teramura *et al*, 1983). Studies over successive growing seasons are necessary in any UV-B impact assessment of agricultural productivity.

The majority of published material indicates that UV-B irradiation is usually detrimental (Caldwell, 1971). 30% of all cultivars tested showed a significant decrease in total biomass in studies using enhanced levels of UV-B radiation. In a six year field study of a UV-B sensitive soybean, a 19%-25% reduction in seed yield in five of the six years under a 25% equivalent ozone reduction level was reported (Tevini and Teramura, 1989). For sensitive cultivars, tiller number and leaf area can also be significantly reduced (Rosema *et al*. 1995).

Effects of UV-B on plant-microbial interactions are complex. Changes in plant chemical composition can reduce microbial activity while a direct effect of UV-B during decomposition can lead to decreased colonisation by fungal decomposers. Increased UV-B may therefore lead to a slowing down of nutrient recycling. Where impacts on forest ecosystems are thoroughly reviewed, (McLeod, 1997), direct effects on growth and physiology of plants relating to UV radiation through forest canopies are considered to have provided evidence for a stimulation of pigment accumulation rather than reductions in growth. The majority of the effects of UV-B radiation promote morphogenetic changes in plants, rather than damage *per se*. Plant morphogenetic parameters may change under UV-B. These include plant height, leaf area, leaf thickness, branching and plant phenology.

There is evidence that increases in leaf thickness is primarily correlated to UV-B. (Rozema *et al,* 1997). Altered plant morphogenesis can lead to changes in ecosystem structure and processes (Caldwell, M. M. *et al* 1995).

The physiological basis for morphogenetic change is, as yet, not fully understood. UV-B effects on phytochrome may be involved in hypocotyl growth reduction (Stapleton, (1992) or photo-oxidation of indole acetic acid to 3-methol oxidol (Ballere *et al,* 1995). Indirect effects can be seen at the whole plant level. Examples include the possible effects of UV-B stress on competitive balance or reproductive allocation.

Symbiotic relationships may also be influenced and this possibility will form part of this study. *Allium vineale* commonly forms associations with Arbuscular Mycorrhisal Fungi (AMF). Arbuscular mycorrhizas are mutual symbionts and commonly form associations with roots of vascular plants. Structural variation exists this phylum (Glomeromycota) but most arbuscular mycorrhizas are characterised by the presence of intra-radical hyphae which may be located either intracellular or extracellular, arbuscules (finely branched hyphae acting as hubs for nutrient exchange), extra-radical mycelium which connect the root to the soil and spores. Associations occur in terrestrial ecosystems globally and have a major impact on plant phosphorus nutrition. The arbuscular mycorrhiza is an endosymbiosis in which the fungus inhabits the root cortical cells and obtains carbon provided by the plant while it transfers mineral nutrients from the soil to the cortical cells. Two major types of Arbuscular mycorrhizal fungi (A.M.F.) have been described: the *Arum* type and the P*aris* type. No attempt at classification was made in this study. Structures are observed in fresh roots using bright field microscopy in some *Allium* species (personal

obs) but usually roots must be processed in order to detect internal fungal structures such as arbuscules and hyphal coiling.

Cytoplasmic contents are normally cleared from cells and stains applied prior to using light microscopy. Roots of some host species react to inoculation by synthesising a yellow pigment. However, this is inconsistent and cannot be used as a diagnostic feature for determining colonization.

There is evidence that UV-B affects other symbiotic relationships between plants and microorganisms. Changes in total arbuscular mycorrhizal colonisation have been demonstrated and decreases in the numbers of arbuscules in inoculated plants have been reported (Van de Staaij *et al,* 2001)**.** Bacteria and non-mycorrhizal fungi increases have been reported in the rhizosphere of UV-B treated *A. saccarum* (Klinonomos *et al,* 1995).These results indicate that the functioning of below-ground microorganisms can affect nutrient availability (particularly phosphorus and nitrogen) and this can influence primary production of ecosystems.

Flavonoids are phenolic compounds commonly found in plant tissues. They play pivotal roles in plant development and the interaction of the plant with pathogenic and mutualistic organisms. Flavonoid production is influenced by UV-B radiation in that they are recruited for the production of protective sun screening compounds in addition to the many signalling roles they are known to have. These compounds and their derived pigments now provide tools to investigate a number of central plant mechanisms including the biology of transposons, the regulation of gene expression, gene silencing and the organisation of metabolic pathways. (Akagi et al, 1995).The flavonoids are a large family of over 4000 secondary metabolites. Many flavonoids are found in plant tissue in

high concentrations as glycocides or other sugar conjugates. Most flavonoids, however, have restricted distributions within the plant kingdom, with many only occurring in only one genus or even species (Markham, 1988). Quantification of flavonoid compounds frequently use methods of separation in conjunction with UV detection (Harbourne, 1984, Romani, A. 2000). In this study, paper chromatography was initially used to separate flavonoid compounds. This technique, although useful in identifying potential qualitative differences between samples, is not able to reliably identify or quantify these phenolic compounds. The technique itself has been largely superseded by High Performance Liquid Chromatography (HPLC) which has routinely become an important tool in the identification and separation of phenolic compounds (including flavonoids) from raw plant extracts.

The flavonoids examined in this study belong to the group known as the flavonols and the specific flavonols under examination namely Quercetin, Isorhamnetin and Kaempherol have been shown to be present in this and other members of the *Allium* family in varying proportions (Bilyk *et al* 1984, Leighton 1992).

Flavonols have been found to contain significant antioxidants and perform free radical scavenging activity (Shahidi and Wanasundara, 1992) and epidemiological studies have suggested that their consumption is associated with a lower risk of contracting some cancers and cardio-vascular disease (Hertog *et al,* 1994).

Induction of UV-B absorbing flavonoid synthesis by UV-B is thought to be a means of plant protection against cellular damage. Flavonoids absorb UV-B radiation and epidermal flavonoids screen internal tissues and stems. Epidermal structures such as leaf hairs in *Olea europaea* not only increase reflectance and scattering of UV-B, but often contain UV-

B absorbing compounds which act to reduce the transmittance of UV-B through the epidermis (Karabourniotis, Kyparissis, and Manetas,1993). Further evidence is provided by mutant flavonoid deficient varieties of *Arabidopsis thaliana* that are shown to be hypersensitive to UV-B (Stapilton *et al* 1992).

Flavonoids act as scavengers of active oxygen and prevent peroxidation of lipids (Takeuchi *et al,* 1996). Flavonols, induced by UV-B are secreted into the soil by legume roots. These compounds can regulate gene expression in nodulating nitrogen-fixing bacteria (Phillips, *et al,* 1995). These results indicate that the functioning of below-ground micro-organisms can affect nutrient availability (particularly phosphorus and nitrogen) and this can influence primary production of ecosystems.

Potential changes in flavenoid production induced by changes in lighting regimes will have far reaching consequences for these mechanisms.

The majority of the research dealing with UV-B radiation influences on growth and flowering in the field have not been over successive growing seasons and has involved annual plants or preliminary growth stages of perennials (Caldwell & Flint, 1994). It is recognised that UV-B radiation can alter the timing of flowering (Ziska *et al,* 1992) as well as the ratio and colour of flowers in certain species (Musil, 1995),and that such effects are due to regulatory effects in the plant and not to cellular damage *per se* (Caldwell & Flint 1994; Ballare *et al* 1995). Treatment with supplementary levels of UV-B radiation in an experiment with *Arabidopsis thaliana* reported decreases in expression in both the photosynthetic genes L*hcb* and *psb*A and increases in jasmonic acid levels and ethylene production (Mackerness, 1999). UV-B induction of specific flavonoids was reported in a

growth chamber study using *Brassica napus* (Wilson *et al* 2001). A robust set of early low-level UV-B responsive genes, independent of known photoreceptors at UV-B wavelength has been identified in *Arabidopsis thaliana* (Ulm *et al* 2004). Rau *et al* (1995) found substantial decreases in flowering from UV-B radiation when *Calamagrostis purpurea* were grown outdoors at levels of UV-B radiation representing 25% ozone depletion.

Temporal changes in the timing of flowering will have obvious implications for pollinators. Any factor that affects the timing or morphology of flowering in any clonal plant will have consequences for the availability of pollinators and out-breeding ratios. Responses to UV-B radiation in *Trifolium repens l.* were investigated in conjunction with water stress under controlled climate room conditions. Levels of flavonoids were elevated under exposure to increased UV-B radiation and this response was synergistically enhanced by water stress (Hofmann *et al,* 2003).

In contrast to growth chamber/room experiments, there have been outdoor experiments conducted using screening foils to manipulate light transmittances. 2000).

The expected evolutionary outcome for a species polymorphic for reproductive mode is that when fitness values are constant, either mode will go into fixation (Joshi & Moody, 1995). Given the supplementary costs of sexual reproduction, (Maynard Smith, 1975) mechanisms have been proposed for its maintenance.

M.L. Ronsheim (1994), proposed that differences in predation rates or dispersal distances between seeds and asexual bulbils might provide a suitable explanation for the maintenance of sexual reproduction while later research (Ronsheim, 1996) examined frequency dependant interactions (sibling competition) as a possible driving

mechanism for sexual reproduction. The 1994 study did not provide such evidence and the 1996 study, whilst providing evidence against negative frequency-dependent interactions, indicated that mutualistic interactions with mycorrhizal fungi amongst similar genotypes of *A. vineale* may influence such interactions.

M.L. Ronsheim, (2000), proposed allocation shifts in response to nutrient availability and reported broad-sense heritabilities for flower / bulbil ratios. Further related work (A. Ceplitis, 2001) used RAPD fingerprinting and concluded that a stable balance between reproductive modes was driven by annual (environmental) fluctuations in fecundity. Symbiotic associations were, however in this study, not considered. Whilst highlighting the complexity of mutualistic associations, and the obvious need to include them in field experiments, these studies do not provide support to the evolutionary theory underpinning them.

The effects of increasing solar UV-B involve changes in secondary plant chemistry and, specifically, in the shikimic acid pathway. Pigments including flavonoids and phytoalexins are commonly produced in response to UV-B radiation. These secondary chemicals, part regulated by UV-B, can influence interactions between vegetation, microbes and herbivores. Some flavonoids are regulatory compounds, such as in *Rhizobium* infection of roots (Cooper, 2004). Mycorrhizae have been shown to be attracted to root exudates (Xie *et al,* 1995) and to affect asexual ramet development (Miller *et al,* 1987). Thus, if increased solar UV-B elicits changes in these secondary products, plant / mycorrhizal relationship changes might be expected. Such change may act to re-define the parameters of the

reproductive mode of this species. To date there is no published work that explores

either the direct effects of UV-B radiation on the genus *Allium,* its effects on

mycorrhysal infection or the balance of flavonols produced which not only act as anti-

oxidants for the plants themselves but have health implications as being constituents in

the quality of crop production.

Chapter 1.

1.1. The Genus *Allium*.

The taxonomic position of *Allium* and related genera has been a matter of debate for some time. In early classifications of the angiosperms, they were placed in the *Liliaceae.* Later, they were placed in the *Amaryllidaceae* (on the basis of inflorescence structure), but recently, molecular data has favoured division into a larger number of small mono-phyletic families. In the most recent taxonomic treatment of the monocotyledons, *Allium* and its close relatives were recognised as a distinct family, the *Alliaceae* close to the *Amaryllidaceae.* The following hierarchy has been adopted (Takhtajan, 1997):

1. **Class *Liliopsida.***

2. **Subclass *Liliidae.***

3. **Superorder *Liliiiianae.***

4. **Order   *Amaryllidales.***

5. **Family   *Allioideae.***

6. **Subfamily *Allioideae.***

7. **Tribe   *Allieae.***

8. **Genus   *Allium.***

Some classifications still have their proponents and are still used in some literature e.g. Brewster, (1994) and Stace (1997).

Allium vineale (Crow Garlic) is a perennial bulbflower in the genus *Allium*, native to Europe, north Africa and western Asia. The species was introduced in Australia and North America where it has become an invasive species (plate 1).

Plate 1. *A vineale.* Inflorescence.

1.1. General Characteristics of Alliums. (Rabinowitch and Currah, 2002)

- Underground storage organs: bulbs, rhizomes or swollen roots.

- Bulbs: often on rhizomes; true bulbs (one or two extremely thickened prophylls) or false bulbs (thickened basal sheaths plus thickened prophylls (bladeless 'true

- scales')); several tunics, membraneous, fibrous or coriaceous; annual or perennial roots.

- Rhizomes: condensed or elongated; rarely runner-like; with very diverse branching patterns.

- Leaves : basally arranged, frequently covering the flower scape and thus appearing cauline.

- Bracts: two to several, often fused into an involucre (spathe).

- Inflorescence: faciculate to umbelor head-like, (one) few-to-many-flowered, loose to dense, (plate one).

- Flowers: pedicelled, actinomorphic, hypogynous, trimerous.

- Tepals: in two slightly differentiated whorls, free.

- Stamens: in two whorls, sometimes basally connected, the inner ones often widened and/or toothed.

- Ovary: triocular three septal nectaries, two or more curved ovules per loctule, developing into a loculicidal capsule dehiscing along the midrib of the carpels.

- Style: single, with slender capitate or, more rarely, trilobate stigma.

- Seeds: angular to globular, black, ornamentation of the cells highly variable.

- Chemical characters: reserve compounds consist of sugars, no starch; enzymatic decomposition products of several cysteine sulphoxides, characteristic odour.

- Karyology: predominant basic chromosome numbers x = 8 and x = 7 with polyploids in both series (Rabinowitch and Brewster, 1990).

Basal bulblets and bulbils are important in vegetative propagation. Most *Allium* are allogamous. Spontaneous interspecific hybridisation is not as rare as formerly believed but strong crossing barriers exist in some groups, even between morphologically similar species.

1.2. Distribution, ecology and domestication.

The genus *Allium* is widely distributed over the holoarctic region from the dry subtropics to the boreal zone. Some species even occur in the subarctic belt e.g. *A. schoenoprasm* and a few alliums are scattered in mountains within the subtropics and tropics. Only *A. dregeanum* Kth. has been described from the southern hemisphere (de Sarker *et al* .1997). A region of especially high species diversification stretches from the Mediterranean basin to Central Asia and Pakistan. A second such region (but less pronounced) occurs in western North America. These centres of diversity possess differing percentages of the several subgroups of the genus and are therefore clearly distinguishable in taxonomic terms (Rabinowitch & Currah, 2002).

Evolution of the genus has been accompanied by ecological diversification. The majority of species, however, grow in open, sunny, rather dry sites in arid and moderately humid climates. *Allium* species have also adapted to other ecological niches. Different types of forests, European sub-alpine pastures and moist sub-alpine and alpine grasslands of the Himalayan and Central Asian mountains all contain some *Allium* species. Even saline and alkaline environments are tolerated by some taxa.

Spring, summer and autumn flowering taxa exist within A*llium* species. There are short and long living perennials, species with one or several annual cycles of leaf formation and

continuously leafing ones. Species may show winter or summer dormancy. For many

species (ephemeroids) annual growth is limited to a very short period in spring and early

summer and the cycle in this case from leaf sprouting to seed maturation is as short as 2

or 3 months (Bosch, Serra and Currah, 2002).

Conditions for seed germination vary between species. For most species the germinability

of the seeds is limited to a few years unless the seed is stored under dry and cold

conditions where the seed life can be greatly extended (Rabinowitch and Currah, 2002).

The genus is of great economic importance because it includes several important

vegetable crops and ornamental species. In contrast, some *Allium* species, including the

species detailed in this study, are considered as noxious weeds of cultivated ground.

1.3. The significance of *Alliums* as a food group.

The genus *Allium* is commonly associated with agriculturally important crops such as

onions, garlic, chives and leeks. World onion production is increasing with production

being around 44 million tonnes in 2002, (Griffiths, 2002), making it the second most

important horticultural crop after tomatoes. Most horticultural A*lliums* are versatile and

durable. They are easily stored and traded and are often used as an ingredient in many

dishes in most cultures. Onion consumption is significantly increasing in the USA owing to

health promotion activities. *Alliums* contain two chemical groups with accepted health

benefits comprising of flavenoids and the alkenyl cysteine sulphoxides (ACSO). Two

flavonoid subgroups are found in most species, the anthocyanins, from which a

red/purple colour is seen in some varieties of onion and flavonols such as quercetin,

responsible for the yellow and brown skins of many other varieties. The ACSOs are the

flavour precursors, which when cleaved by the enzyme alliinase, produce the odour characteristic of onion. The downstream products are compounds which include thiosulphinonates, thiosulphonates, mono, di- and tri-sulphides (Rabinowitch and Currah, 2002).

Compounds from onion and garlic species of A*llium* are reported to have a range of health benefits which include anti-carcinogenic properties, anti-platelet activity, anti-thrombotic activity, anti-asthmatic and antibiotic effects, (Griffiths, 2002).

1.4. Sexual reproduction.

Many, if not the majority, of *Allium* spp. reproduce sexually by the production of flowers and seed. Flowering of various taxa within the genus *Allium* is extremely diverse with regard to morphology.

1.4.1. Vegetative reproduction.

The terminology between various authors describing vegetative reproduction in *Alliums* varies widely, leading to the potential confusion of the description of plant tissues. The term "bulblet" has been used for example to describe both ground-level or aerially derived bulbs in the genus. These bulbs have been described as increase bulbs, sister bulbs, daughter bulbs, offset bulbs, sets, cloves and bulbils. Some terms are applied according to the size of the structure produced with larger bulblets being referred to as daughter bulbs, cloves or offsets and smaller structures being referred to as sets and bulbils. The term bulbil is usually used to describe the bulbs that develop in the inflorescence. They can be referred to as pips,

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apomictic bulbils/bulblets, aerial bulbils/bulblets, topsets or aerial sets (Rabinowitch *et al*, 1990).

For the purpose of clarification of terms in this report, it is necessary to clarify the meanings of the terms used. The term bulbil will be used to describe the propagules that form within the inflorescence.

1.4.2. Forms of vegetative reproduction.

Five distinct forms of vegetative reproduction have been identified in some *Allium* species (Kamenetsky, 1993). They are:

- Vegetative replacement.
- Vegetative increase.
- Bulblet production.
- Vegetative production in rhizomatous species.
- Bulbil production.

1.4.3. Replacement or renewal.

In *A. vineale* two bulbs are regularly produced from the parent bulb after flowering or bulbil production. The largest of these is known as the renewal bulb, (also referred to as the main, principal or terminal bulb), and serves to continue the parent plant. The renewal bulb arises from the condensed stem or growing point of the parent bulb. Sister bulbs, however, arise from buds which form in the axils of the foliar leaves. Due to this developmental difference, and because renewal may occur independently of all other forms of vegetative reproduction, this is considered to be a separate process to the production of sister bulbs.

1.4.4. Vegetative increase.

Increase bulblets are formed from buds in the axils of the foliage leaves (Kamenetsky, 1993). The number and size vary. Many species produce a large single sister bulb as well as the larger renewal bulb (e.g. *A. giganteum)* whereas others produce numerous smaller bulblets. *A. ampeloprasum* can produce up to 350 bulblets per plant (personal obs). This form of reproduction is typical throughout the genus.

1.4.5. Production of bulblets.

Bulblets are small increase bulbs that often have a tough outer sclerified layer and often an accompanying inner layer. They are produced by the subterranean main bulb and therefore their potential for dispersal would appear limited. In some species (e.g. *A. caeruleum and A. truncatum)* bulblets are formed at the end of a stolon arising from the mother bulb. In *A. ampeloprasum* they may be dispersed up to 250mm away from the bulb by stolons (Galil, 1965). In this species, agricultural cultivation methods increase dispersal and the double layer coat serves to limit water ingress, promoting dormancy and the resulting temporal dispersal of the bulblet. These bulblets are confusingly sometimes referred to as bulbils.

1.5. Vegetative reproduction in rhizomatous species.

The rhizomatous group includes members of the subgenera *Rhizirideum* and *Amerallium.* The fleshy rhizomes are built up through successive concrescence of the basal plates over several generations and function primarily as underground storage organs. Despite the

presence of a rhizome, vegetative reproduction in some species of *Allium* e.g. *A. tricollum* can differ from that in non-rhizomatous species. After flowering, two or three bulbs are formed, one of which will be the renewal bulb and the remaining bulbs will serve as increase bulbs. In some rhizomatous species, e.g. *A. mutans,* this process is distinct from bulblet production making it difficult to distinguish between vegetative reproduction in rhizomatous and non-rhizomatous species due to the difficulty in distinguishing bulbs from other subterranean storage organs (Rabinowitch and Currah, 2002).

1.5.1. Bulbil production.

Bulbils are small bulbs that develop in the inflorescences of many alliums (plate 2.). They have no significant protective layers and may appear mixed in with flowers, or they may replace the flowers completely as in *A. cepa var. Viviparum*. They are morphologically similar to bulbs although commonly much smaller. They vary in number according to species and environmental conditions and individual bulbils are capable of growing into a new plant (plate 1.).

1.6. Reproductive plasticity in *Allium vineale.*

The contentious nature of flowering / bulbil production in *A. vineale* has provoked adaptive explanations for the persistence of this diversity citing trade-offs among alternate components of fitness. Advantages exist for producing sexual progeny in the presence of pathogens or unstable environments whilst asexual progeny may be favoured where environments are relatively stable. Genotypes are known to vary their allocation to these different reproductive modes with respect to plant density in several species (Ogden, 1974). Selection should favour such phenotypic plasticity if different propagule

types were deployed when environmental conditions change (loehle, 1987). For natural selection to currently maintain diverse competitive strategies and phenotypic plasticity in allocation patterns, the variation must have a genetic basis. Negative genetic correlations between life history traits suggest trade-offs in reproductive strategies, (Stearns, 1991), as selection for a favoured allocation for one trait will necessarily cause a reduction in allocation to the other. There is considerable variation in the ratio of bulbils/seed produced even within species of *Allium.* This is demonstrated by the subject of this study- *A. vineale.* The occurrence of seed and bulbils in the same umbel can range from 100% bulbils to 100% seed set with all intervening states. Research suggests that the proportion of each respective propagule may be genetically determined (Ronsheim, 1997, 2000). This matter forms the basic tenet upon which the evolutionary theory surrounding asexuality will be examined in subsequent chapters of this study.

Chapter 2. The collection and UV-B treatment of *Allium vineale*.

2.1. Collection of *Allium vineale*.

A population of *Allium vineale* was identified in May 2004 at map reference SS 270420

near to Carmarthen south Wales. The plants were established near two partially managed

municipal reservoirs (upper and lower Cwmoernant) used by local residents and anglers.

Permissions were granted (see appendix 5) for their removal with the proviso that they

were to be taken almost immediately as the area was being cleared to provide more car

parking.

The plants were growing in the partially managed banks of the lower reservoir and the

upper reservoir. The soil texture was coarse and sandy and the plants were growing in

partial shade. Both areas of reservoir were divided into ten sections of two metre^2 areas

and up to eight mature plants per section were selected by apportioning the number 1 to

the first plant in the Northwest corner of the grid and plants selected by removing every

other plant followed by every third plant from each section, working alternatively

clockwise and then anticlockwise between areas. In excess of one hundred and fifty

plants were removed in total using a tulip planter in order to remove as much original soil

as possible and also to minimise root damage. The plants were then initially transported,

to the author's home glasshouse and potted up individually into three-inch pots (courtesy

of Bransford Nurseries, Bransford, Worcester) in an equal mixture of their original soil,

sterilised John Innes no 1 © compost and washed sharp sand (Tudors Building Supplies,

Hereford). This medium was an approximation in texture to the original soil conditions

and pH 6.8. The plants were left in this medium for two months to allow them to complete their flower, bulb and /or bulbil production. The plants were transported to the University glasshouse the following spring.

The plants were all collected from one area and being partially clonal are unlikely to represent the population as a whole. However, for the purpose of this experiment, homozygosity is beneficial and indeed subsequent clonal generations used in experiments reduced non-treatment variability.

2.2. Planting arrays

The arrays were constructed from 200mm lengths of 50mm diameter drainage pipe. Six pipes were glued to a centre pipe that remained unplanted but served as a guide to watering (Plate 3). Each of the six pipes was filled with a mixture comprising 50% John Innes no 1 ©loam based compost and washed sharp sand. No original soil was used in this final experimental mixture. Each filled pipe was a discrete unit in order to negate any mycorrhizal hyphal sharing.

Plate 3. Experimental lighting arrays showing arrays made from a total of seven short lengths of drainage pipe glued together.

Two months after collecting the *Allium vineale* the plants died back as would be expected. Some of the original plants (genets) had produced additional small sister bulbs (section 1.4.4.). All the bulbs, including the attached sister bulbs, were washed in a fungicidal solution (5ml / litre Benlate©). From each original genet, the main bulb was then selected. These were planted into each of the tubes of an array at a depth of 3cm. A total of 24 experimental arrays were prepared, each array contained six plants. The pH of the experimental growing medium was found to be 6.8. using a pH meter (Mettler Toledo, Leicester, UK).

To ensure that no mycorrhizae remained to re-inoculate the plants from the new soil the arrays containing the bulbs received a drench of fungicide (5ml / litre Benlate©) before transportation to their final position at the University greenhouse.

2.3. Additional plants

Several main bulbs from additional genets which were not included in the experimental arrays were treated in a similar manner. The bulbs were planted in additional arrays in the same compost and treated in a similar manner in relation to the fungicide treatment. These spare plants were also then kept in the greenhouse along with the experimental plants on an adjacent staging but were not placed under the light banks (2.5.) unless used as replacements for experimental plants that had died.

2.4. Test for mycorrhizal infection.

To confirm the absence from typical mycorrhizal colonisation 20 mm of root were excised from each of six of the additional plants during the late spring of the initial growing season. These roots were carefully rinsed with tap water and boiled for 3 minutes in 10% potassium hydroxide (w/v), rinsed again with tap water and re-boiled in tryphan blue stain (0.05% / acetic acid (5%)) (Phillips, J.M. and Hayman, D.S., 1970). Slides were simply prepared by placing the root material on a slide with a coverslip and examined using a Leica DMLB digital microscope. No arbuscular mycorrhizal infection was observed in the excised root sections under bright field conditions.

2.5. Experimental lighting regime.

Two softwood lighting boxes were made, vented with plastic louvres and lined internally with aluminium foil. A total of ten 18" fluorescent batten lights were modified with higher capacitance 40 watt chokes (Newlec NLKC40STV2) in order to reliably start, eight Phillips TL 12 Blacklight U.V. tubes (four per box) and two warm white tubes (one per box) in order to provide additional photosynthetically active radiation (PAR). Ambient light levels were reduced by the shading of the lighting boxes themselves and the boxes were equally spaced to provide equal shading in the greenhouse itself. Overall ambient light levels were equal for both groups, as was temperature and ventilation. The control box was run with energised (but filtered) lamps to ensure equal radiant heat output. Skirting Bekaert Armorgard© WWHC foil was fixed between the light boxes to prevent UVB radiation

becoming incident on the control rig. The tubes themselves were renewed every 400

hours to ensure spectral consistency (plate 3).

2.6. Spectral output of Phillips TL 12 Blacklights.

The TL 12 tubes were used exclusively in the experiment due to their emission ratio of

UV-A/UV-B light. When used with a cellulose diacetate filter it has a ratio of 0.99. It has

been suggested that the UV-A contribution from these lamps may be considered

negligible (e.g. Petropoulou *et al* 1995). Previous studies have removed UV-A radiation by

the use of Mylar© film (Sission & Caldwell 1975). There is also evidence that UV-A

mitigates against the accumulation of UV-B inducible flavenoids (Wilson, K.E., 2001).

However in this case a decision was made to include these frequencies (315-400nm) as

incident UV-A would be screened by the glasshouse itself and therefore render the

experiment less realistic. For the pilot study the prospect of PAR+UV-B+ low UV-A against

PAR was under investigation only. Four U.V. tubes were fitted to each light box in addition

to two standard Phillips (Croydon, UK) warm white fluorescent tubes. The spectral output

of UV-B lamps includes wavelengths in the UV-C range (≤280nm) which do not occur at

the earth's surface and must be eliminated for the purpose of this experiment. Previous

research has shown that cellulose diacetate film (McLeod .1997, Mackerness, 1997)

excludes these wavelengths. A roll of this film was sourced from Clarifoil and a sample

was tested using a Shimadzu spectrophotometer. (see appendix 2). The sample

successfully filtered out all wavelengths below 280nm and was subsequently used in the

treatment array. UV-B wavelengths degrade cellulose acetate over time, (Mackerness,

1997), and so the film was replaced on a weekly basis. (The ageing and changing spectral

characteristics of cellulose diacetate filters has been well described by Adamse & Britz (1992). For the control light box Bekaert Armorgard© WWHC foil was used to eliminate all types of UV light.

The fluence rate for the incident photosynthetically active radiation (400-700nm) provided by the white tubes was in the region of 150 µmol m^{-2} s^{-1}(Mackerness, 1999) in addition to the incident light (-UVA) inside the glasshouse. The UV-B fluence rate (between 280 and 320nm –UV-B) was 3.2 µmol m^{-2} s^{-1}. This fluence rate is equivalent to a 15% increase in transmitted UV-B radiation at this latitude (Mackerness, 1999). Previous studies have used a "square wave" design where supplementary light is simply switched on during a given period of time. A "stepped design" was used in this experiment.

2.7. Stepped design and photoperiod.

The six tubes in each lighting box were switched independently through three independent timers. This enabled the UV light to be stepped in intensity around midday where solar UV-B radiation would naturally occur and also to avoid the plants receiving UV-B radiation in the absence of normal light intensity. The plants received a photoperiod that approximated natural day length throughout the treatment period.

Chapter 3.

Morphological and flavenoid changes in *Allium vineale* following UV-B irradiation for a ten week period.

3.1. Introduction

The majority of the research dealing with UV-B radiation influences on growth and flowering in the field have not been over successive growing seasons and has involved annual plants or preliminary growth stages of perennials (Caldwell & Flint, 1994). In contrast to growth chamber/room experiments, there have been outdoor experiments conducted using screening foils to manipulate light transmittances. Flavonoid concentrations in grape (*Vitis vinifera cv Silvaner*) increased tenfold in response to elevated UV-B radiation (Kolb *et al,* 2001). Phenolic sunscreens in field-grown soybean crops were shown to be affected by solar radiation and their phenylpropanoid levels specifically induced by the UV-B component within it (Mazza *et al*, 2000). The primary aim of the following pilot experiment was to determine potential changes in flavonoid output in this species induced by short-term UV-B radiation between treated groups of plants by qualitatively comparing separated phenolic compounds using paper chromatography and also comparing leaf length between groups of UV-B treated and untreated plants as a measure of somatic output.

The particular species (*Allium vineale*) was chosen as the subject of the experiment as no other research has considered the effect of winter/spring growing species when UV-B exposure can be proportionally higher than at other times of the year. Allium vineale produces above ground growth from late October and therefore the effect of UV-B

exposure during this growing period (winter/spring) on plant growth as defined by summed plant leaf length and flavonoid production was examined.

3.2. Chemistry and Distribution of flavonoid pigments.

The flavonoids are all structurally derived from the parent substance flavone and are mainly water soluble compounds. They can be extracted from plant tissue with 70% ethanol and remain stable in the aqueous layer, following partition with petroleum ether. Flavonoids are phenolic and hence change colour when exposed to ammonia (Harborne,1973). They contain conjugated aromatic systems and display intense absorption bands in both the visible and UV spectral range.

Almost every higher plant contains a characteristic pattern of flavonol glycosides in leaf tissue and these substances are ideal taxonomic markers for use in studying problems of plant classification, hybridization or phytogeography.

3.3. Method

At the beginning of the rapid growing season (March) each of the 24 arrays was randomly assigned to a numbered grid positioned beneath one of the two light boxes (plate 3.). The arrays were randomly relocated and rotated through ninety degrees weekly during the treatment period under each rig. The light boxes were themselves interchanged monthly in order to negate pseudoreplication. An automatic watering system was used and the plants were watered twice daily until their free draining planting tubes were overflowing. The arrays generally remained dry between watering. The plants were subjected to the lighting

regime (section 2.6., 2.7.)for a total of 10 weeks between March and May (1 year on

from collection) up until flowering / bulbil production.

3.3.1. Leaf length.

The length of each leaf produced by a genet was measured from the soil surface to leaf

tip. The total of all leaf lengths produced by a genet was taken as an indication of somatic

growth during the 10 week growing period. A total of 71 genets were examined, one

plant from the UV B irradiated and one from the untreated group having died.

3.3.2. Extraction.

Having completed the leaf length measurements (3.2.1.) 20cms of fresh leaf tip tissue was

excised from each leaf from all UV B irradiated plants and placed together into one bag to

provide one sample of material. This was repeated for the untreated plants. Using the

method outlined in Harbourne, (1973) for the specific extraction of phenolic compounds

and the subsequent identification of flavenoid compounds from plant material. 100gm of

plant material was ground with washed sand and 25ml 95% ethanol to form a paste. All

the ground plant material from each treatment group was then boiled with an additional

100 ml of 95% ethanol in a Leibeg reflux apparatus for 10 minutes and left for 90 minutes

to allow the chlorophyll and other impurities to deposit and for the extract to become

more concentrated. At this point both extracts were volumetrically equivalent. 10ml of

the aqueous concentrate was then directly collected in a capillary for spotting on the

chromatography paper. 3ml of the concentrate from both samples was also transferred

to a cuvette (pathway length 10mm) and the absorbance spectrum recorded using a

scanning Shimadzu UV IR spectrophotometer (fig 3.1 , 3.2).

3.2.2.a. The detection of flavonoids from UV B irradiated and untreated leaf extracts in paper chromatography.

Following the indicated absorption differences in the crude extract the aqueous concentrate from the UV B irradiated and non-irradiated plants was collected in a capillary and two drops applied directly to a chromatography paper. In addition a 1 molar solution of a standard flavonoid marker, : quercetin-3-rutinoside (rutin) was prepared (Sigma Labs code R5143) and three drops added to the right hand margin of the paper. This flavonol was selected as a marker as it occupies a position approximately in the middle of the chromatogram itself (Harbourne,1973) and any separated flavonol 3-glycosides will migrate to a corresponding distance along the solvent front.

Two chromatograms were prepared for the UV B treated material and two for the untreated material (plate 4). These chromatograms were for comparative purposes and were therefore prepared under identical conditions and in parallel.

The papers were processed as two-dimensional chromatographs by placing into a tank containing the solvents BAW (n-butanol–acetic acid-water, 4:1:5, top layer) and 5% HOAC. (total vol 500ml).

Results.

3.2.2.a. Absorbance of crude extracts

The UV absorption spectra from the original extracts (3.2.2.a) are shown in Figs 3.1 and 3. 2.The peaks between the red lines (fig 3.3) correspond to individual flavonol compounds with absorption spectra between 250 and 350nm. Inspection of this region indicates that there appears to be a difference within the region of flavonoids such that there appear to

be more peaks at a higher absorbance in the UV irradiated plant extract compared to the

extract from the untreated plants.
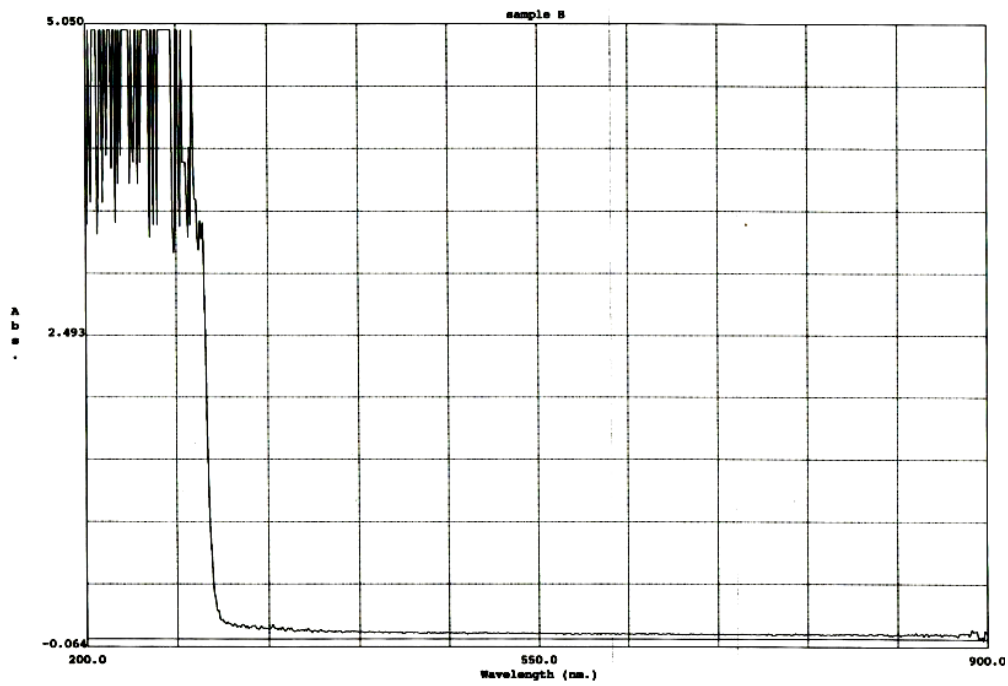
Figure 3.1. Absorbance spectra of untreated plants.
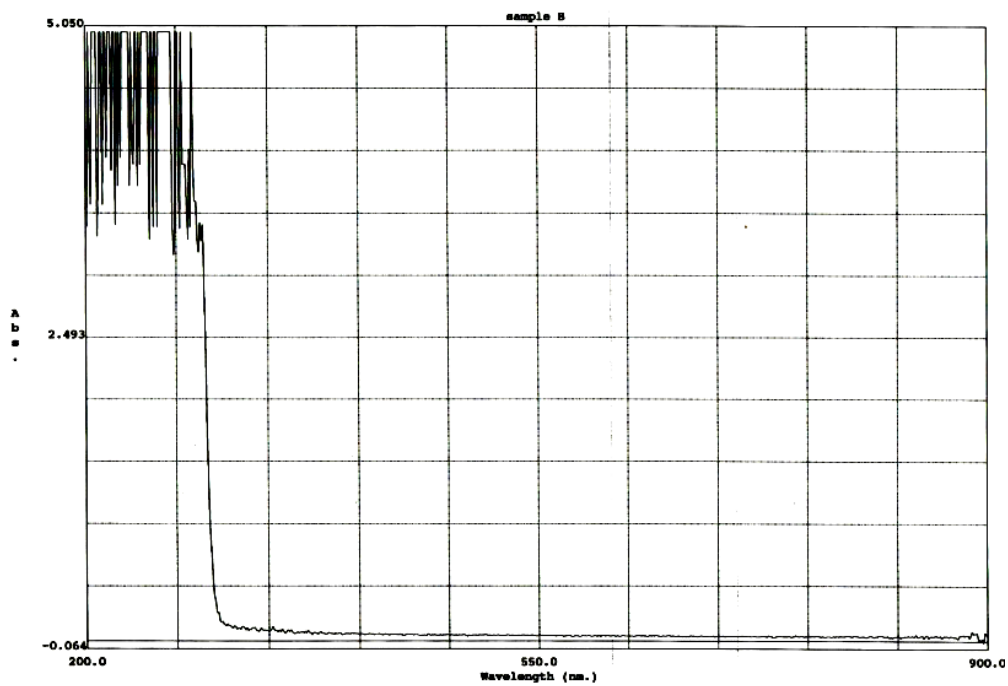
Figure 3.2 Absorbance spectra of treated plants.

Expanded view of individual flavonoid absorption peaks in treated plants.

Expanded view of individual flavonoid absorption peaks in untreated plants.

200 nm   250nm   350nm
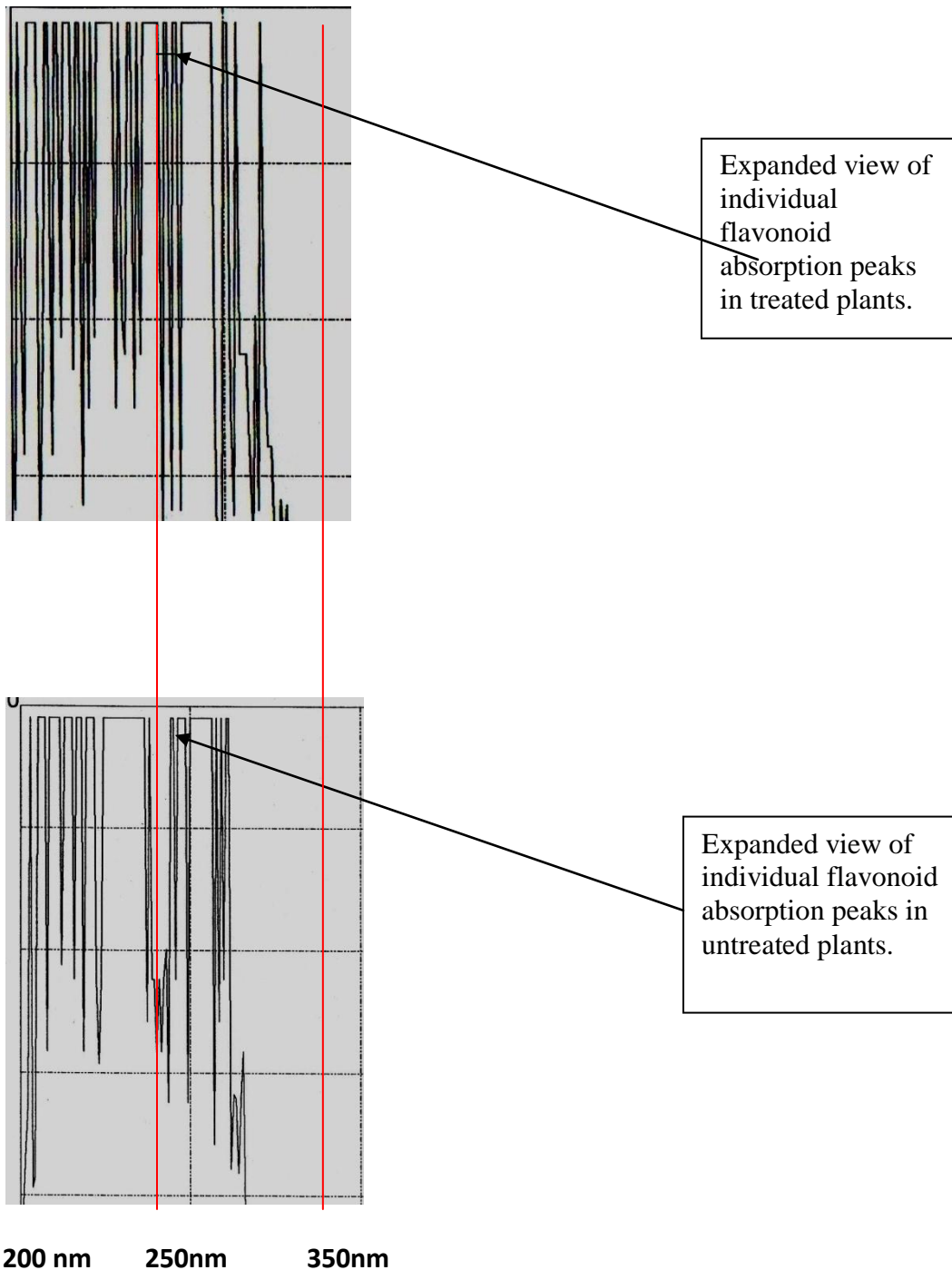
Figure 3.3. Expanded view of UV spectra of crude extracts.

Peaks correspond to individual flavonoid compounds with absorption spectra between

200 and 350 nm.

Plate 3. UV B irradiated and untreated samples being chromatographed using paper chromatography.

There are three key indicators of flavonoids in paper chromatography (Harbourne 1974).

- the sample fluoresces under UV light

- the sample turns yellow when exposed to ammonia fumes

- the sample shows a bathochromatic shift when exposed to sodium hydroxide (appendix 8).

The paper chromatographs containing the extracts from the UV B irradiated plants, the untreated plants and the flavonoid marker Rutin were exposed to UV light on a UV transilluminator (spectral output at 250-370nm). (plates 5., 6. And 7.).

Results.
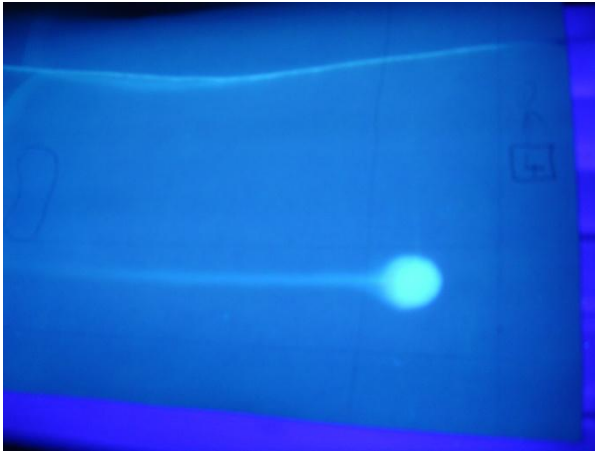
Plate 4. Paper chromatograph of flavonoid marker Rutin.

Plate 5. Paper chromatograph of separated flavonoid compounds in untreated plant extract.

Plate 6. Paper chromatograph of separated flavonoid compounds in UV-B treated plant extract.

On each paper a large spot was seen to fluoresce when exposed to UV light. The spots for both light treatments which had migrated in line with the rutin were then exposed to UV light where marked differences were observed using a transilluminator. The separations provided visible differences in the chromatograms with the untreated plant chromatograms showing a distinct three-pronged pattern (Plate 6). In contrast the treated plant chromatographs showed one larger and brighter spot within the overall pattern of separation (Plate 7).

The sample turns yellow when exposed to ammonia fumes:

Two chromatographed papers were transferred to a second chromatography tank which contained 100ml of 0.5 molar aqueous ammonia. The papers were exposed to the fumes

for 10 mins. The areas on the paper chromatographs that turned yellow were compared with

the areas that fluoresced on the transilluminator (plates 6, 7.) as a cross reference. The

two chromatographs not receiving the ammonia treatment then had the fluorescing

areas outlined in pencil for future incision. As a further confirmation the section of

chromatogram containing a Rutin spot (plate 5) was cut out and the rutin was re-eluted

(3.2.2.). 3ml of the elute was inserted in a cuvette (pathway length 10mm) and the

absorbance spectrum recorded. One drop of 1 molar sodium hydroxide was then added

to the cuvette and a bathochromic shift was observed in the spectrum characteristic of a

flavonol 3 – glucoside (Harbourne,1974). (see appendix 8 for results).

3.2.2.c. Re-chromatography of the separated flavonoid sections following excision.

The (fluorescing) spots on the remaining chromatograms were cut out from the papers

for the UV-B irradiated, the untreated sample and the Rutin marker. Sample material was

eluted using 70% ethanol. The elute was concentrated in a laminar flow cabinet to

volumes of 10ml per sample.

The same chromatographic procedure was repeated as before(3.3.2.b) and the final dry

chromatographs examined on a transilluminator in the UV spectral range (plates 9 and

10).The UV absorption spectrum of the final elution was measured using a Shimadzu UV

IR spectrophotometer.

Results.
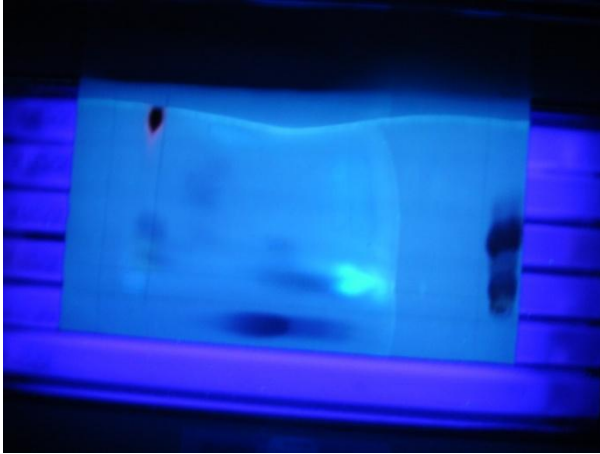
Plate 7. Re-eluted flavonol chromatogram (untreated plants).
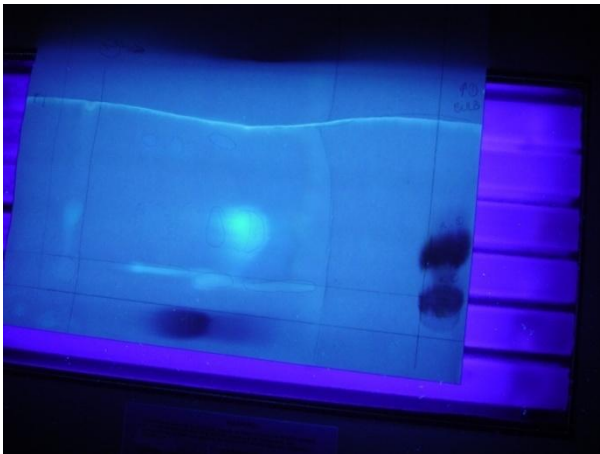
Plate 8. Re-eluted flavonol chromatogram (treated plants)

The above chromatograms again show differences in patterns in the fluorescing

flavonoid areas with the treated chromatograph indicating a minor flavonoid streak

below the main pattern. The Untreated chromatogram shows a more concentrated and

well defined spot.

The action spectra from spectrometric data as examined in the range of 200-300 nm in

re-eluted re-chromatographed material proved unreliable. It is probable that the

concentration

of final eluted solution was at or near the detection limit of the spectrophotometer. It is

also possible that impurities from the paper used may have interfered with the UV

absorption in the range of 260-270nm (Harbourne, 1974), at these concentrations.

3.2.1. Leaf length.

The total leaf lengths (m) within the two treatments showed little variation between

genets. This is also the case when comparing the genets exposed to the two lighting

regimes (Table 3.1.) which are not significantly different (z = 1.64, p >0.05).

Table 3.1. Summary statistics for total length of leaf (m) in UV B treated and untreated *Allium vineale* after 10 weeks of supplemental irradiation.

	treated	*control*
Std error	0.031	0.029
Mean	1.352183	1.383183
Known Variance	0.067	0.06
Observations	71	71
Hypothesized Mean Difference	0	
Z	-0.73297	
z Critical one-tail	1.644854	
P(Z<=z) two-tail	0.463574	
z Critical two-tail	1.959964	

3.3. Discussion.

The avoidance and tolerance of UV-B induced stress is displayed in a number of plant strategies, the most common being the increase of UV screening compounds. The different phenolic compounds found in epidermal and mesophillic cells of leaves reflect UV response with epidermal flavonoids playing a major role (Cen *et al, 1993., *Bornman, *1991).* Two main approaches have been used to examine the relationship between elevated UV-B levels of radiation and pigment accumulation. The first is the examination of the induction of phenolic compounds (as in this study) and secondly, the performance of mutants deficient in the capacity to synthesise flavonoid compounds.

In this study, the paper chromatographic separation process itself was limited to a qualitative comparison between samples. Absorption spectra of unseparated extracts displayed differences between treated and untreated samples albeit from a cluttered spectrograph. The separated extracts shared retention parity with the known flavonoid marker and displayed a characteristic colour change when exposed to ammonia fumes. Re-chromatographed sections of separated paper chromatographs also displayed visible differences in the final distribution of flavonoid spots. Previous studies have reported marked increases in the ratios of major flavonoids (Quercetin-glycoside and Kaempherol-glycoside) induced in other species (Cen *et al,* 1993 and *Lui* et al, 1995). The experimental technique employed in this study, using paper chromatography, whilst capable of identifying visible differences between samples was not capable of quantification or reliable identification of individual flavonoid compounds. Attempts to obtain action spectra from spectrometric data in re-eluted re-chromatographed material proved unreliable. The paper chromatograms and the UV absorption spectra of their eluted

separated segments indicated that the UV-B treated plants had modified their production of secondary metabolites in qualitative terms (plates 6 and 7) following short-term irradiation. Previous studies have reported similar results using other species and lighting regimes. High performance liquid chromatographic (HPLC) profiles obtained following analysis of crude extracts of *Brassica napus* after exposure to 16 days of enhanced UV-B radiation (8.9KJ m^{-2} day^{-1}) demonstrated changes in levels of both kaempherol and quercetin induced by the UV-B lighting regime (Cen *et al*, (1993).

From the above data there is no evidence in this study that enhanced UV-B radiation will influence plant leaf length in this species in the short term. Under these growing conditions and time frame, these plants were able to either to acclimate or mitigate effectively supplementary UV-B radiation.

There are surprisingly few available reported findings regarding the short-term effects of UV-B radiation on plants. Considerable attention has been devoted to season-long effects of UV-B radiation in crop species where morphological effects vary between cultivars. The majority of studies carried out on the molecular impact of UV-B on plants have used fully expanded mature leaves. However, UV-B sensitivity of *Cah* genes in pea seedlings is found to be dependent on the specific age and maturity of the tissue studied (Jordan *et al.,* 1994).

Some evidence is contradictory citing decreases in plant height and increases in branch dry weight in some cultivars of the same species (Mepsted *et* al, 1996). Sullivan and Teramura (1988) reported stunting and reduced seedling height in three out of ten species of P*inus* following a twenty two-week treatment with supplementary levels of UV-B radiation. Short-term effects of increasing UV-B radiation on biomass parameters

for 18 days was examined under acclimatised greenhouse conditions by Rozema *et al* (1991). Ariel plant architecture was affected in the short term and growth rate was reported to have been reduced by 23% during this period.

Teramura (1980) reported reductions in photosynthesis, leading to biomass reduction and anatomical changes including the development of shorter, thicker leaves following long-term exposure to UV-B. It is now recognised that longer wavelength radiation (P.A.R.) can minimise UV-B induced damage and that this conferred protection is effective at a physiological and molecular level (Cen and Bornman, 1993, Flint *et* al, 1985). Pre-treatment with high levels of P.A.R. can ameliorate UV-B induced damage (Teramura, 1980). These findings contribute to the relevance of long-term experiments using biologically realistic levels of supplementary UV-B radiation in parallel with supplementary levels of P.A.R. The possibility remains that photosynthetic reductions are not necessarily a causal factor in reduced leaf length and/or structure in these studies but that UV-B receptors in some experimental species are influencing observed changes in plant architecture. In this case, however, changes may not have been observed in this species due to the short exposure time or that the exposure was too late in this species growing season to be registered.

Differences in plant architecture between species, dosages in UV-B radiation and lengths of exposure together with differences in the developmental stages of the species within individual studies involved make direct comparisons between studies difficult. The stepped and P.A.R. augmented experimental design in this case may have facilitated the successful acclimation of the plants to the experimental lighting regime in the short term.

As a bulbous perennial, growth is not solely dependent on light or nutrient regimes and any measurable differences in metabolic output may be partly masked by differences in storage organ mass between individual plants. The possibility also exists that potential effects are solely through changes in genetic expression which came too late to affect leaf length and that longer-term exposure to UV-B radiation would therefore be required in order for any potential differences to manifest themselves.   There is no previous literature relating to short-term UV-B exposure in a bulbous perennial.

Chapter 4.

Season-long investigation into potential morphological and reproductive changes in UV B treated and untreated plants.

4.1. Introduction.

The short-term exposure to supplementary UV-B radiation used in the pilot experiment in chapter 3 induced qualitative differences in phenolic flavonoid metabolites but no effect on plant architecture *per. se.* Most available literature has examined effects over complete growing cycles examining various species and using various UV-B lighting regimes rendering direct comparison or speculation in this species tenuous. No literature, exists for a long-term study on a bulbous perennial.

 Epidermal structures are the first barriers to the penetration of UV-B radiation entering the leaf. The leaf epidermis is very effective in transmitting PAR whilst at the same time screening the ingress of UV-B light. Such effectiveness is reliant on such factors as leaf thickness, UV screening compounds and leaf surface properties. Transmittance of UV-B varies between species and is, for example, less than 10% in *Peperomia obtisifolia* and *Yukka treculeana* but can be in excess of 90% in the epidermis of onion, *Allium cepa (*Gausman *et al* 1975). A study by Day *et al, (*1993) examined the leaves of 22 plant species and found UV-B penetrated deepest into the leaves of herbaceous dicotyledons. Intermediate penetration into monocotyledons and grasses was reported and almost no penetration was found when conifer needles were examined.

Leaf thickening has been shown as a typical structural response of some terrestrial plants to UV-B in other studies (Bornman and Teramura 1993, Sullivan *et al,* 1989) but epidermal tissue thickening as a direct result of UV-B in this species has remained hitherto unexamined.

Leaf length and plant architecture is only one factor important in assessing the impact on plants of UV-B radiation. Phenological changes may reflect somatic output and also reproductive output and/or allocation. The reproductive phenotypic plasticity of *Allium vineale* is outlined in section 1.6.

The following experiment measures inflorescence fresh weight, leaf length and epidermal cuticle thickness following season-long exposure to UV-B in order to examine possible changes in photomorphology and reproductive timing and output. Most previous studies have used elevated levels CO_2 in conjunction with supplementary UV-B. (Garbutt & Bazzas, 1984; Slack *et al* 1988; Krupa & Kickert, 1989). Earlier flowering is reported in these studies. In contrast, where no supplementary CO_2 is used, the findings are contradictory (Johanson *et al* (1996) and no effect on phenology was reported.

Clearly gross morphological changes such as in leaf length and production of bulbils reflect underlying changes in cellular structure, changes to gene expression and therefore molecular components in plants. The mechanisms by which plants perceive UV-B wavelengths (in particular the regulation of gene expression) is not fully understood. There are several possible mechanisms which will be considered in section 4.6.

Structural and biochemical changes induced by lighting regimes can modify the potential penetration of UV-B radiation into the plant. The production of U.V. screening pigments , (typically flavonoids in this case), may reduce the penetration of UV-B radiation to underlying tissue. Increased wax deposits on leaf surfaces can also lead to reduced penetration caused by incident light reflecting from leaf surfaces. Increased length of inner leaf cells or increases in cell number, (both palisade and spongy mesophyll) generally determine the penetration of UV radiation across a leaf at the cellular level (Caldwell *et al,* 1998).

 In particular, leaf damage and leaf thickening has been shown to be a typical structural response of terrestrial plants to UV-B radiation (Bornman and Teramura 1993, Sullivan *et al.* 1996).

4.2. Method

4.2.1. Plant arrays and UV B treatment

Plants growing in arrays that had been used in the experiment outlined in Chapter 3 were left in the greenhouse with no supplementary lighting from the end of the previous experiment (May) until late September of the same year. During this time the plants continued to be watered and rotated as before. In late September when the plants were starting to show above ground growth the supplementary lighting regime was reinitiated (see Chapter 2 for details). No other alterations or additions were made to the plants in their arrays. Therefore a total of 24 arrays were again re-used (12 per treatment). Plants

were irradiated for a growing season starting from the end of dormancy (late September) and ending when all the plants had produced bulbils in July the following year.

4.2.2. Leaf length

All leaves within each individual 'pot' were measured as before (Chapter 3) and the leaf length totals for each genet recorded prior to statistical analysis using commercial software (Microsoft © Excel).

4.2.3. Inflorescence weight

At the end of the full growing season each genet had produced just one inflorescence. These were deemed mature when all the main foliage had died back. At this point all the inflorescences were cut just at the base of the inflorescence and weighed (table4.3).

4.2.4. Cellular structure: epidermal cuticle thickness

Possible changes in leaf tissue morphology between UV B treated / untreated plants were examined by preparing stained sections of fixed material. The method by Steeves and Sussex (1989)had been adapted for a study on *Allium babintonii* (Herbert 1991; Cottrell 1999).

A total of twelve individual plants from both treatment groups were randomly selected and 10mm sections of leaf tissue were excised from a point 100mm from the leaf tip. The

remainder of the plants were similarly treated but the tissue was collected and subjected

to the extraction method outlined in 3.2.2. and analysed using High Performance Liquid

Chromatography (section 7.2.1.). The tissue was collected and fixed as rapidly as possible

in order to minimise decay and dehydration. There are various fixatives available and

Clarke's Fluid (100% ethanol : glacial acetic acid (3 : 1, v/v)) was appropriate as it

minimises tissue shrinkage (Peacock, 1966). The tissue was placed into plastic cassettes

and fixed until pigmentation was absent from the samples.

Each 10mm length of leaf was then divided into two 5 mm lengths before being fixed in

freshly melted wax. One 5mm length from each genet (i.e. twelve for each treatment)

were positioned for transverse sectioning (TS) and the remaining 24 leaf lengths were

positioned for longitudinal sectioning (LS). They were cooled rapidly to -20°C then stored

at 4°C.

The wax blocks were sectioned using a Spencer Microtome set at 5 μm. The ribbons of

sections were floated on the surface of a water bath at 42°C containing 4 ml/l subbing

solution (1% potassium dichromate and 1% gelatine). Slides were previously ethanol

washed (70 % aq.) and air-dried at room temperature. Samples were positioned on the

slides and air-dried horizontally at room temperature.

The dry slides were stained by transferring them sequentially along a series of Coplin jars

containing the appropriate solutions (Table 4.1.) The initial treatment contained

histoclear which dissolved the wax around the plant tissue samples on the slide. This

process took approximately 20 minutes. The slides then undertook a sequential hydration

process before immersion in distilled water. The slides were hydrolysed in 1%

hydrochloric acid for 8 minutes at 60^0C, transferred to Schiffs reagent for 1 hour and

transferred to 45% acetic acid for 1 minute. This was followed by 1 part ethanoic acid: 3

parts ethanol for 1 minute and two rinses (for 1 minute each) of sodium dioxide water

(1g of potassium metabisulphate (disulphate (VI)) dissolved in 200ml distilled water

followed by a rinse (for 1 minute) in 10ml of 1M hydrogen chloride.

Table 4.1.: Staining procedure for *Allium* leaf sections (adapted from Herbert 1991;

Cottrell 1999).

Treatment	Time (min.)
Histo-Clear	20
100% ethanol	5
70% ethanol	5
50% ethanol	5
30% ethanol	5
Distilled water	5
1M Hydrochloric acid at 60°C	8
Schiffs Reagent	60
45% Ethanoic acid	1
Sulphur dioxide water	1
Sulphur dioxide water	1
30% ethanol	5
50% ethanol	5
70% ethanol	5
100% ethanol	5
Light green stain	20 seconds
100% ethanol	1
100% ethanol	1
Histo-Clear	1

A dehydration sequence followed in solutions containing 30 to 100% ethanol for 5

minutes each. The slides were dipped in fast green stain (0.2% w/v dissolved in 98%

ethanol) for 20 seconds and rinsed in 100% ethanol twice for 1 minute. The slides were then finally rinsed in histo-clear®(Flogen Bioscience) for 1minute.

Slides were made permanent by dipping them in xylene twice for 1 min each (Hopkin & Williams Ltd GPR Grade A) in a fume cupboard, then placing a drop of DPX mountant (Fluka 44581) over the samples before pressing down the cover slip for a few seconds. They were kept level and allowed to dry for 48 hours in a fume cupboard, before examination.

A total of twelve 'treated' and twelve 'untreated' transverse section slides were examined using a Leica DMLB digital microscope. The twelve radial measurements were taken of the cuticle per sample using an eyepiece graticule resulting in an adjusted scale of 1 eye piece unit equalling 2 µm. The 12 radial measures were taken at approximately 30^0 intervals around one cell on each slide. The 12 measures taken from each cell from a slide were totalled.

4.3. Results.

The results indicate that in genets given the different irradiation treatments the UV B irradiated *Allium vineale* produced a shorter total leaf length per genet than the untreated plants. An F_{max} test confirms that the data are parametric and a Z-test confirms that this difference is significant (Z = 2.146, P = 0.03186). In contrast however there is no significant difference between the fresh weights (g) of the inflorescence in UV B treated and untreated plants Z = 0.37826. P = 0.707237.

Table 4.2. External and cellular changes to morphology in *Allium vineale* irradiated with UV B for 10 months compared to untreated plants.

		UV B irradiated genets	Untreated genets	Significance
Total leaf lengths per genet (cm)	Mean	1.355	1.445	0.031857
	Standard Error	0.0311	0.031	
Weight of inflorescence (gm)	Mean	1.641	1.668	0.705237 N/S
	Standard Error	0.058	0.056	
epidermal cuticle thickness (μm)Total per slide (12 obs).	Mean	71.23333	52.85	
	Standard Error	2.447958	2.062006	8.89×10^{-6}
Mean epidermal cuticle thickness per leaf		5.935 μm	4.40 μm	

Plates 9 - 12 illustrate the changes observed in epidermal thickness.

Analysis of the total thickness for the epidermal layer cells indicates a 35% increase in the UV B treated plants.

Plate 9. Mag 40x / 0.65. Untreated c/s A. vineale leaf showing cutical.
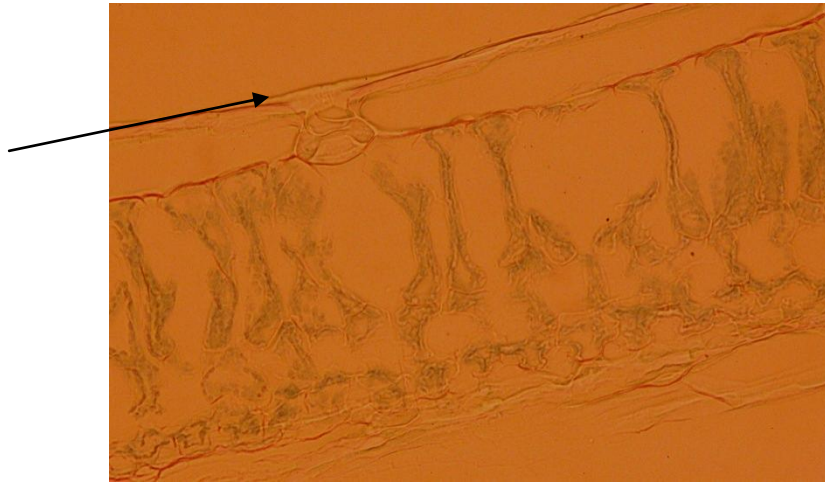
Plate 10. Mag 40x / .065. Treated c/s A vineale leaf showing cuticle thickening.
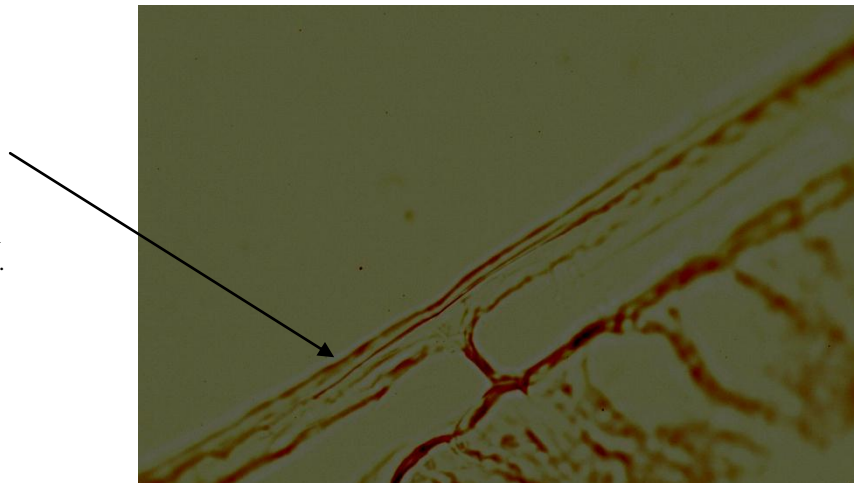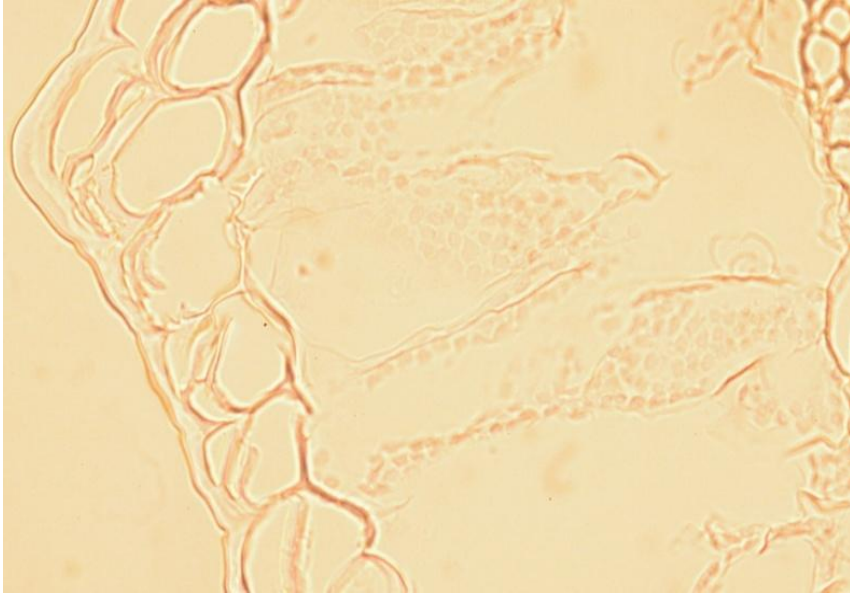
Plate 11. Mag 200x / 0.65.
Unreated c/s A. vineale leaf .
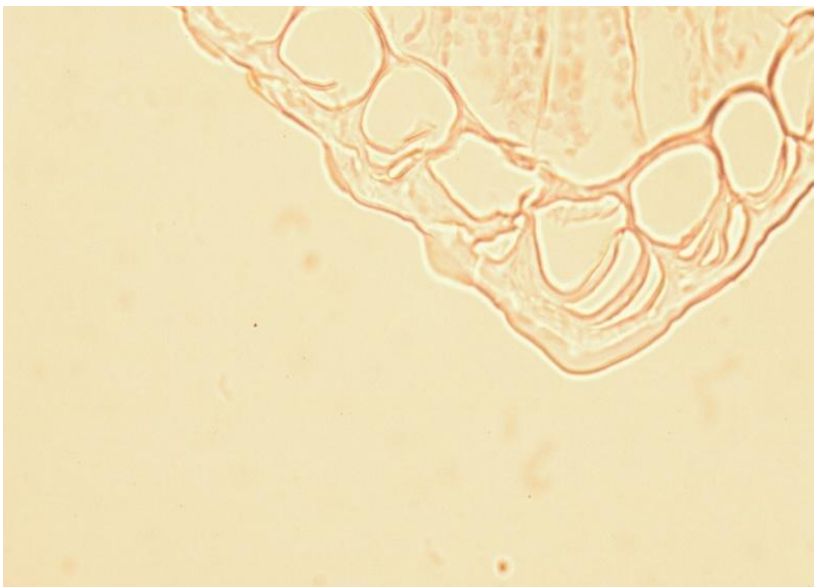
Plate 12. Mag 200x / .065. Treated
c/s A vineale leaf showing cutical
thickening.

4.6 Discussion.

The above results demonstrate a reduction in overall leaf growth length in plants treated with UV-B over an entire growing season but no significant change in inflorescence weight.

Photosynthesis and photosynthetic productivity can be inhibited in many plant species by increased exposure to UV-B radiation (Caldwell, Teramura & tevini; 1989; Musil, 1995; Middleton & Teramura, 1993). Reduction in rubisco activity and also changes in stomata conductance have been identified as factors limiting CO_2 assimilation in leaves exposed to elevated UV-B levels. Season-long exposure to elevated levels of UV-B in leaves of species of pea has been shown to lead to decreases in both rubisco activity and content (Jordan *et al.*, 1992; Vu, Allen & Gerrard, 1984). Such decreases can be correlated with empirical decreases in leaf carboxylation efficiency as determined from the response of carbon dioxide assimilation to increasing carbon dioxide concentration when leaves are given supplemental UV-B radiation (Ziska & Teramura, 1992).

Exposure to UV-B can also provoke changes in the rates of stomatal opening and closing and cause reductions in the rate of leaf transpiration (Tevini & Teramura, 1989; Middleton & Teramura, 1993).

Several studies have shown that the photosystem II (PSII) is the most susceptible part of the thylakoid membrane photosynthetic apparatus to elevated levels of UV-B radiation (Middleton & Teramura, 1993). Consequently PSII damage has often been cited as the

major potential limitation to photosynthesis in UV-B treated leaf tissue (Bornman, 1989; Teramura & Sullivan, 1994) as it is in the photoinhibition of photosynthesis by PAR (380-700nm) (Middleton & Teramura, 1993).

In this case there were observable differences between treated and untreated plants in the shortening of leaves and the thickening of the epidermal cuticle in UV-B treated plants. In contrast, the reproductive output between treated and untreated groups as defined by changes in inflorescence dry weights remained non-significant suggesting that any decreases in photosynthesis were mitigated in UVB treated plants.

Whilst responses to external signals, such as UV-B radiation, may be studied at the whole plant level, the signals are perceived and responded to at the cellular level. Specific cellular components, termed receptors, allow signals to be detected and acted upon. Reception is then relayed to the terminal response by transduction mechanisms. This signal transduction process can amplify the initial signal and can also store it for periods of time (Knight & Knight 1995), Maathuis & Sanders, 1995).

There are several possible mechanisms for the specific detection of UV-B. The first is direct absorption of UV-B by DNA in the nucleus which could, at least theoretically, result in a type of signal that mediated the rate of transcription of individual genes. There is no experimental evidence currently to support this conjecture but it would be premature to dismiss it completely. Secondly, UV-B could be detected via its ability to generate reactive oxygen species. If this was the case then the increases in transcription following UV-B exposure would be oxidative stress responses rather than photo-responses *per se*. There is some evidence to support this hypothesis. The accumulation of a pathogenesis-related protein, PR-1 was mediated, in response to UV-B in tobacco leaves, by the production of

reactive oxygen species (Green and Fluhr, 1995). UV-B response was greatly diminished in the presence of antioxidants and the generation of a singlet oxygen could substitute for UV-B radiation in inducing PR-1 accumulation. This somatic response, were it to apply in *A. vineale*, may provide some explanation as to the observed decreased leaf length in treated plants but as no significant changes in inflorescence weights were observed in this experiment, this explanation is unlikely.

 UV-B could be detected by photoreceptor molecules similar to those photoreceptor molecules found in higher plants. Likely predictions regarding the nature of a specific UV-B photoreceptor can be made from other well known UV/ blue light absorbing proteins in plants. Studies in the action spectra of various UV/ blue photo-responses indicate that plant UV/blue photoreceptors are likely to be proteins with a bound flavin chromophore (Gallard and Senger, 1988, Short and Briggs. 1994). The existence of specific UV/blue photoreceptors and of chromophores that could absorb UV-B wavelengths supports this hypothesis.

Exposure of plants to UV-B stimulates expression of genes encoding PAL, (phenylalanine ammonia-lyase), CHS (chalcone synthase) and several other phenyl-propanoid and flavonoid biosynthesis enzymes (Hahlbrock & Schell, 1989; Beggs & Wellmann, 1994) in a range of species. In *Petroselinum crispum* (parsley cell) cultures, UV-B induces genes encoding PAL and CHS (Hahlbrock & Schell, 1989). Action spectra of PAL and CHS transcript accumulation in *Daucus pastinaca* (carrot) cultures indicates the involvement of a UV-B receptor (Takeda, Obi & Yoshida, 1994). In common with other aspects of their regulation, the stimulation of PAL and CHS genes by UV-B is likely to involve signal perception and transduction processes resulting in the stimulation of transcription within

these systems. It is also possible that the three above mechanisms are not mutually

exclusive. It is perfectly possible that UV-B regulates gene expression by photoreceptor

mediated and oxidative stress mediated signalling processes working in parallel.

Chapter 5

Investigation into potential morphological changes and floral initiation induced by the interaction of UV-B radiation and an enhanced nutrient regime.

5.1. Introduction.

Following the result of the previous study (section 4.3),an experiment was set up to investigate the observed changes in gross morphology in *A. vineale* exposed to supplementary UV B. In order to determine whether these effects were simply due to oxidative stress and UV-B damage *per se* or due to the potential effect of a UV-B / blue light photoreceptor. Studies, Teramura 1980) have demonstrated that morphological changes provoked by UV-B radiation can be moderated by such factors as drought or by a lack of available phosphorus. It is possible that the results of this study (sections 3.3 and 4.2.) thus far, demonstrated morphological changes brought on by UV-B exposure only in the presence of a secondary stressor. Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* has been demonstrated under excess light and drought stress (Tattini *et al* 2008). It was therefore necessary to examine any changes induced by UV-B radiation in the presence of an enhanced nutrient regime.
 As the plants were watered to excess in the second year and the lighting regime output remained constant, the possibility of a mineral or general nutrient deficiency exists. To eliminate this possibility it was decided to augment half of the plants with an enhanced generic nutrient regime and take terminal measurements of inflorescence weight and leaf length at the end of the growing season in order to investigate any interaction

between the effects of UV-B exposure and reproductive/somatic output. Initiation of scape initiation per treatment was also recorded.

Examples of earlier flowering of plants grown at elevated levels of CO_2 are well documented (Garbutt & Bazzas, 1984; Slack, 1986; Krupa & Kickert, 1989). Studies using UV-B but not involving CO_2 are less well documented. Johanson *et al* (1995a), reported that a UV-B treatment simulating a 15% ozone reduction over a subarctic heath had no effect on the phenology of leaf-bud break, flowering, or ripening in several heathland species.

Interactions between storage and growth temperatures play an important role in normal scape elongation and flowering of *Allium* species although light conditions and photoperiodism can affect this process (Rabinowich and Currah 2002). In the case of a bulbous perennial environmental pressures from previous seasons may determine floral initiation.

5.2. Method.

5.2.1 Supplementary lighting

The procedure remained consistent with previous years. The UV B treated and untreated plants remained undisturbed in their respective arrays. This resulted in the possibility of any persisting bulbs having been irradiated for more than a single treatment period in comparison with any renewal or sister bulbs (section 3.3.1.) leading to the possibility of magnification of results due to additive effects. The plants were again irradiated (Chapter 2) for a complete growing season (late September – July).

5.2.2. Fertiliser.

The selection of plants to receive the fertilizer treatment was randomised. Six arrays within each light treatment were selected at random and the plants within these arrays had fertiliser added during the growing season. Each 'fertiliser' group received fortnightly liquid fertilizer treatment of Scotts miracle Gro©,(15 : 30 : 15 by ratio)of nitrogen, phosphorus and potassium. (Full details are in the appendix)

Spare plants (Chapter 2) that had not been irradiated before were added to the arrays to make up the full complement of 36 plants per 4 treatments.

5.2.3. Leaf length and inflorescence weights. 63

The leaf lengths from each individual plant and the inflorescence weights were measured as before (Chapter 3 and 4 respectively). The data was subjected to an F_{max} test and subsequently to a two- way ANOVA statistical analysis (Microsoft Excel ©).

5.3. Results.

Analysis of the data indicates that the total leaf length per genet is very highly significantly longer in the plants not irradiated with UV B compared to those that were irradiated (Table 5.1., F = 5.550, p = 0.02), and between the plants treated with fertiliser compared to those not treated with fertiliser (F = 20.4, p =1.31x 10^{-05}). However there is also a very highly significant interaction between the effect of the fertiliser and the UV B

treatment (F =19.87, p < 0.001) such that the fertiliser had the greatest effect on leaf

length in the absence of UV B treatment.

Table 5.1. Effect of fertiliser treatment and UV -B exposure on total leaf length (cm) per genet in *Allium vineale.*

	Total leaf length (cm) per genet in *Allium vineale*			
	Supplementary UV B treatment		No supplementary UV B treatment	
	Fertiliser	No fertiliser	Fertiliser	No fertiliser
Mean	135.64^{ab}	127.93^{acd}	159.90^{bce}	135.41^{de}
Std error	0.0340	0.0369	0.0395	0.0316
n	36	36	36	36

The results from a Tukey's test are shown in superscripts (Holmes, Moody and Dine, 2006).

The only insignificant pairing of means (Table 5.1.) is between the 'UV treated + fertilized'

group versus the 'untreated + no fertilizer' group with the greatest variation accounted for in

the 'UV untreated + fertilizer' group compared to the 'UV treated + no fertilizer' group. This

result demonstrates that the additional growth promoted by the enhanced nutrient regime

in the non-irradiated plants is absent in the irradiated groups. The UV treatment effectively

reduces leaf length in nutrient enhanced plants to the values found in the non-irradiated + no

fertilizer group.

Table 5.2. Effect of fertiliser treatment and UV- B irradiation on weight (g) of inflorescence in *Allium vineale.*

	Supplementary UV B treatment		No Supplementary UV B treatment	
	Fertiliser	No fertiliser	Fertiliser	No fertiliser
Mean	1.581	1.564	1.641	1.554
Std error	0.0342	0.0303	0.0262	0.0309
n	36	36	36	36

There is very little noticeable effect of either supplementary UV B treatment or fertiliser on the weight of the inflorescence (Table 5.2.) and this difference has been confirmed as being non significant (F = 0.49. NS (fertiliser), F = 0.12, NS (UV B irradiation), F = 0.23, NS (interaction)

5.3 Discussion.

The resulting data from this experiment demonstrates that the fertilized plants exhibited highly significant (p= 1.31 x $10^{-3)}$ shortening of leaf length under UV-B radiation. Should such morphological change arise solely from oxidative stress, then it is reasonable to assume that a similar trend would be found in the fresh inflorescence weight of corresponding plants. From table 5.1. it is clear that the supplemented nutritional regime offers no degree of mitigation from the effect of UV-B in this case. It is possible that changes are due to the involvement of phytochrome or a dedicated UV-B photoreceptor.

Flavonoids can modify hormonal activity in plants. Indole-3 acetic acid (IAA) is a growth regulator and cofactors of the enzyme IAA oxydase are monohydroxy B-ring flavonoids such as kaempherol. This enzyme cleaves IAA. Dihydroxy B-rings (e.g. quercetin) are inhibitors of IAA oxydase (Harbourne, 1980). Mono and dihydroxy B-rings both inhibit the transport of auxin through the plasma membrane (Markham, 1975, Stafford, 1991). It follows that in this case a UV-B induced alteration in relative concentrations in keampherol and quercetin can lead to modified IAA concentration and activity. IAA can also be broken down by UV-B into photo-oxydation products (Tevini and Teramura, 1989) leading again to morphological changes. Auxin has an important function in the regulation of apical dominance, cell division and cell elongation (Singh, 1996). Alterations such as leaf size and shoot length may be caused by lowered cell elongation and/or cell division can be indirectly then regulated via flavonoids and auxins (Jansen *et al,* 1998).

Plant development and the timing of flowering can be modified by numerous environmental factors including stressors. In this case there is sufficient overlap in floral initiation between both groups of plants receiving an enhanced nutrient regime and both groups not receiving it to postulate that the UV-B treatments did not induce any temporal changes in the timing of flowering in these plants indicating that the genetic control of flowering in this species is independent of the above morphogenical modifications and hormonal changes induced by UV-B (Appendix 8).

Chapter 6.

Investigation into morphological and phenological changes induced by supplementary UV-B radiation following inoculation of plants with Arbuscular Mycorrhisal Fungi.

6.1. Introduction.

So far, in this study, lighting and nutrient regimes have been manipulated in the absence of AMF for experimental reasons. The following experiment and observations were performed with the confirmed presence of AVF structures. *Allium vineale* commonly forms associations with AV fungi (Ronsheim, 1994).

6.2. Establishing VA- mycorrhiza symbiosis.

Root exudates of the host plant are implicated in establishing AVF interactions. Excreted flavonoids have marked effects on the fungus (Siqueira *et al,* 1991). This interaction is, however, complicated and controversial. Quercetrin, for example, has the effect, depending on concentration, to both stimulate and inhibit hyphal growth. Flavonoids serve as plant signal molecules in the role of establishing AVF associations for the majority of plant species but they are not essential in all cases. Maize plants, deficient in the essential enzyme chalcone synthase show normal mycorrhizal development (Shirley, 1996).

6.3.The effects of enhanced UV/B radiation on VA-mycorrhriza.

There is little research in this area but a study by Klironomos and Allen (1995) reported altered VA-mycorrhiza associations under controlled environmental conditions. Numbers

of arbuscules decreased under elevated UV/B radiation and vesicle numbers increased in roots of *Acer saccharum.* Given that arbuscules organise active exchange of nutrients and that vesicles are essentially storage organs it was postulated that this result indicated a lower rate of activity of VA-mycorrhiza associations under high UV/B fluxes. Changes in flavonoid concentrations in root exudates may influence spore germination in addition to hyphal length.

UV/B can also affect AMF associations in natural ecosystems. Work on elevated UV/B effects on a coastal-dunes grassland ecosystem showed significant decreases in VAM infection rates in *Calamagrostis epigeios* and *Carex arenaria* (van de Staaij 1996). These two species are non nitrogen fixing monocots and show high infection percentages. In plots that had been irradiated continuously for five years, infection percentages dropped by 18% and 20% respectively when root tissue was examined (as measured by reduced arbuscular numbers).

6.4. Method.

6.4.1. AVF inoculation of plants.

In late September following the investigation into the effects of fertiliser, when the plants were about to break dormancy, the bulbs in the arrays treated previously were prepared for the next experiment. Each tube was emptied one at a time. The main bulb and soil was retained but the weeds and any bulblets were discarded. The tube was refilled to a depth of 700mm with the original soil. A proprietary preparation of mycorrhizal fungi (Rootgrowplus ™) was made up according to the manufacturer's guidelines and 25mls was added to the tube and covered by a further layer of the original soil. The bulb was

placed into this media so that the bulb was 3cm down from the soil surface and the roots were coiled into the Rootgrowplus. A further dressing of 5mls of Rootgrowplus was sprinkled onto the top of the soil to allow the AVF inoculate to be watered into the soil. To ensure consistency of the degree of disturbance this treatment was repeated for each plant in all of the 24 arrays.

 The 12 arrays previously exposed to supplementary UV B and the 12 arrays not so irradiated were placed back under their same light banks to continue to receive the same light treatment. Six arrays in each light treatment were selected at random and given a fungicidal drench at monthly intervals during the growing season and were the 'no AVF' treatment. The pH of the soils was measured throughout the growing period using a Mettler-Toledo, Leicester, UK.   The pH of the growing medium remained at 6.8 throughout the treatments. The plants received no nutrient supplements during this time and were watered to excess through their free -draining tubes. The plants were irradiated as before from late September until June.

6.4.2. Confirmation of AVF infection in treated bulbs.

At the end of the following growing season the bulbs were tested to check the level of AVF inoculation. Six plants were selected at random from each of the two no AVF treatments (i.e. + / - UVB). 10mm of root tissue was excised from each of the growing tubes and examined for evidence or absence of mycorrhizal structures within the excised root tissue using a Leica DMLB digital microscope and the staining protocol described before (2.4.). No AVF infection was seen.

All the bulbs from the two + AVF treatments were examined. Here 33 bulbs in the + AVF + UVB treatment and 34 bulbs in the + AVF - UVB treatment were infected (e.g. Plates 6.1. and 6.2.). To simplify analysis of the data relating to morphological changes in the plants, 33 plants were therefore studied from all treatments. In the –AVF treatments and the +AVF / - UVB treatment these were sampled at random.

6.4.3. Leaf length and inflorescence fresh weight.

In June after the growing season the leaf length and inflorescence fresh weight was recorded as outlined in 3.2.1. and 4.2.3. respectively.

6.4.5. Phenology.

During the investigation into the effect of fertiliser on plant morphology (Chapter 5) the timing of the early development of the inflorescence was recorded and an effect noted (Appendix 4).   Therefore this characteristic was included in this final experiment.
 The number of plants demonstrating spathe eruption was recorded twice weekly during May, approximately 8 months after the start of the growing season. Spathe eruption is a clear stage in this species when the leaf displays a characteristic light green discolouration over the erupting spathe.
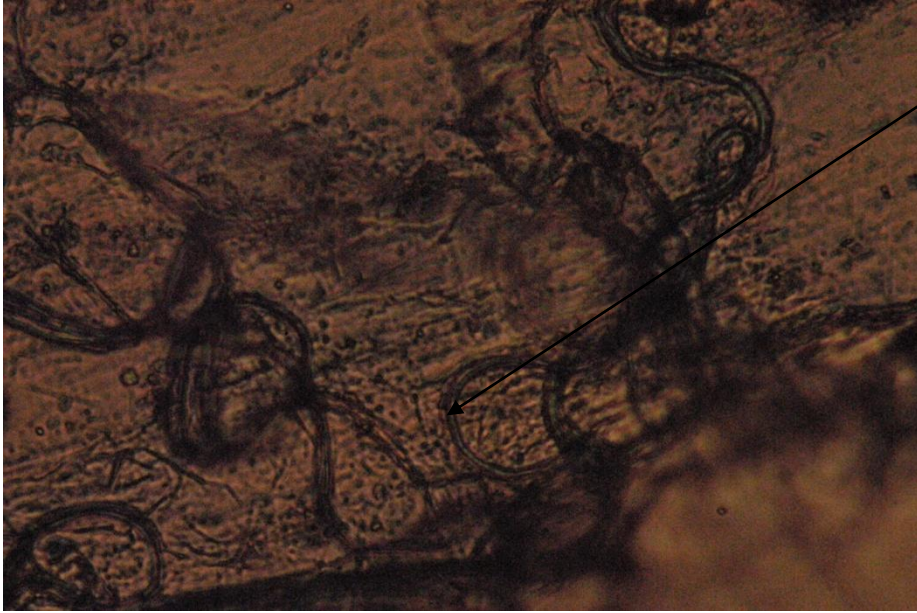
Hyphal
Coiling

Plate 13. AVF treated root of *Allium vineale* with *Paris*-type arbuscular mycorrhisal

association showing extensive hyphal coiling. Mag 20x / 0.4 . 72

Arbuscules.

Hyphae

Plate 14. AVF treated root of *Allium vineale* confirming AVF presence. Mag 20x / 0.4 72

6.5. Results.

As in previous experiments leaf length showed a highly significant leaf shortening as a result of supplementary UV- B treatment (F = 17.71, p < 0.0001, Table 6.1.) however, there was no significant effect on total leaf length per genet of the AVF treatment. In addition there was also a significant interaction between treatments such that the AVF infection appears to enhance the difference in total leaf length in the presence of U -B compared to no UV-B (Fig 6.1).

Table 6.1. Effect of AVF treatment and UV B exposure on total leaf length (m) per genet in *Allium vineale*

	Total leaf length (m) per genet in *Allium vineale*			
	Supplementary UV B treatment		No supplementary UV B treatment	
	+ AVF[a]	No AVF[bc]	+ AVF[ac]	No AVF[b]
Mean	1.415	1.313	1.595	1.485
SE	0.047	0.037	0.0385	0.043
n	33	33	33	33

The results from a Tukey's test are shown in superscripts (Holmes, Moody and Dine, 2006).

When the fresh weight of the inflorescence is examined (Table 6.2.) the reverse pattern is seen. The effect of the supplementary irradiation with UVB is not significant. However,

the effect of inoculation with AVF is highly significant (F = 55. 14, p < 0.0001). There is

again a significant interaction (F = 6.49, p = 0.012). The successful AVF infection has

resulted in additional reproductive effort especially in the non irradiated plants.

Table 6.2. Effect of AVF treatment and UV B exposure on inflorescence fresh weight (g) in *Allium vineale*

	Inflorescence fresh weight (g) in *Allium vineale*			
	Supplementary UV B treatment		No supplementary UV B treatment	
	+ AVF[ab]	No AVF[ac]	+ AVF[cd]	No AVF[bd]
Mean	1.746	1.530	1.760	1.522
SE	0.031	0.026	0.030	0.034
n	33	33	33	33

The significant results from a Tukey's test (p = 0.05) are shown in superscripts (Holmes,

Moody and Dine, 2006).

Fig 6.1. shows that those plants inoculated with mycorrhiza were substantially earlier in

showing spathe eruption than the non-inoculated plants.
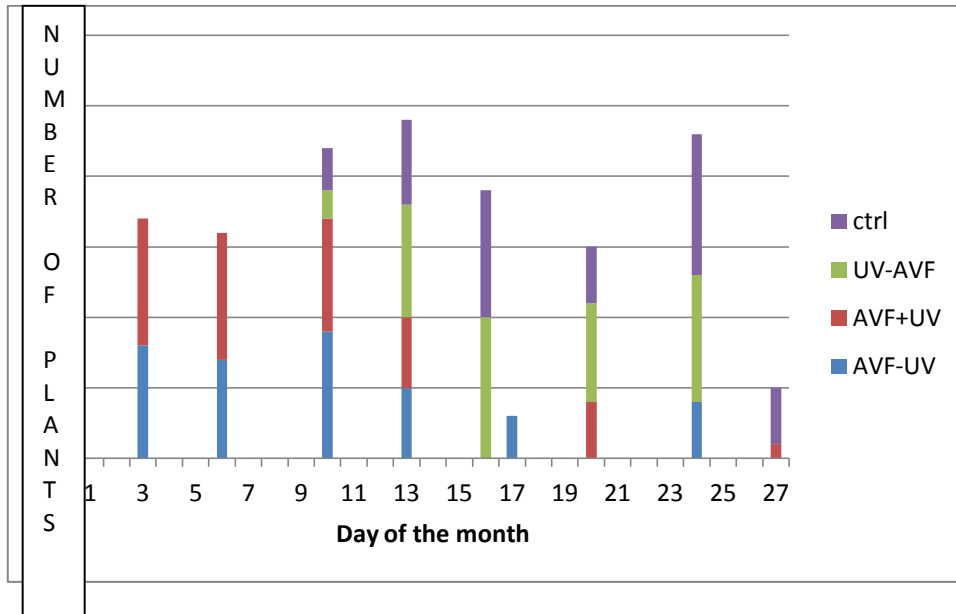
Fig 6.1. Spathe eruption in *Allium vineale* during May plants treated with AMF and supplementary UV-B irradiation.

The results of a modified Kruskal-Wallace test, ranking values for days of the month (and corrected for tied ranks) show χ^2_{crit} at p = 0.0001 = 16.27. (sig difference p = < 0.001). The result of a multiple comparison Q test are; Q initial = 2.635, p = 0.01, Q = 3.144. p= 0.05, Q = 3.765.

Sig at p = 0.05*. P = 0.01** p = 0.001***All highly significant differences except − UVB+AVF , CF +UVB-AVF and +UVB-AVF CF-UVB-AVF.

Interaction between UV-B treatment and flowering time is shown in figure 6.2.
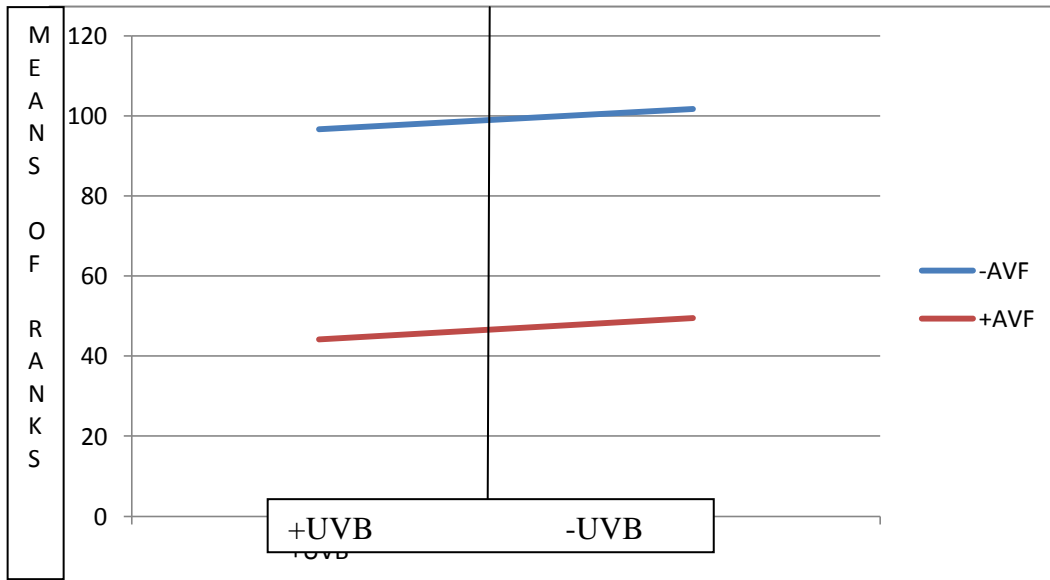
Figure 6.2. Interaction of UV-B and AMF.

6.6. Discussion.

The results again show an overall decrease in leaf length with increased reproductive

output coupled with earlier floral initiation in AMF inoculated plants. In parallel with this

finding is the interaction between the UV-B treatment and AMF inoculation.

Reproductive output determines the ultimate success of a plant species and despite the

obvious importance of vegetative growth it is only necessary to provide the structure and

resources needed for reproduction. Any additional effort expended in reproduction by

the parental generation will determine the abundance and success of the next generation. For most annual plant species vegetative growth is often correlated with reproductive output and therefore if mycorrhizal colonisation increases vegetative growth then similar increases in reproduction should follow (Lu and Koide, 1994, Bryla and Koide, 1990a). Evolutionary theory reasons that selection for such a trait as being mycorrhizal can occur if that trait increases fitness (increasing capacity to leave viable offspring relative to other conspecifics). The fact that so many mycorrhizal plant species exist suggests that this is the case.

Possible explanations for the increase in reproductive output might include supplementary somatic growth induced by the mutualistic AMF infection and/or the benefits of not having to synthesize the photoscreening phenolics needed during UV/B exposure. Alternately there may be below ground interaction involving UV/B induced flavonoid compounds and AMF function.

Mycorrhizal infection can influence host plant populations by exerting an influence on the extent of variation between individuals. Micro-site environmental variation and genetic differences amongst individual plants will be magnified during the process of competition. Increased size inequality of infloescences induced by mycorrhizal inoculation may lead to disproportionate distribution of offspring by the dominant individuals. In this experiment sample variance for the irradiated groups indicate greater variation in reproductive output in inoculated plants. In this case, where AVF were not present the UV-B radiation efectively removed the size inequalities seen in the other treatment groups. It is likely that heavier and nore numerous propagules will have impacts on fecundity and dispersal

mechanisms. Propagule germination and seed quality are also likely to be affected. Further implications of this will be discussed in section 7.8.

Whilst the additional reproductive output is unsurprising it is interesting that when viewed in conjunction with the net loss of leaf length in UV/B treated plants, it indicates that these particular somatic photomorphogenic changes may again be the result of a photochromatic UV/B receptor and not photo-damage *per se.* The results from chapter 5 demonstrated the species ability to maintain reproductive consistency under UV/B exposure and varying nutrient regimes. This result demonstrates that inoculation with AVF actively promoted a net increase in reproductive output that was not observed previously in an experiment using an enhanced nutrient regime. Moreover, AVF inoculation generated variation of reproductive output between individual plants not seen in other treatments. A possible explanation may be that this species is highly mycorrhizal and that the variance in reproductive output is simply a reflection of the individual plant's level of inoculation/genetic susceptability. Whatever the explanation, a mechanism for selection remains. In most cases for any increase in reproductive output induced by AVF inoculation the benefit must be conferred before or during the reproductive phase. This is not necessarily the case in this species (as as bulbous perennial) but any mechanism that allows for the capacity to reproduce earlier in the growing season will exert selection pressure on non-AVF individuals.

Other explanations include the possibility that the species is inefficient at metabolising phosphorus in commercial fertilizer form at low spring temperatures that do not permit adequate phosphorus uptake.

There is evidence that under stressful conditions the AVF fungus may act as a parasite, draining plant resources (Johnson *et al* 1997), but there is no evidence of this from these results possibly indicating that the UV/B treatments did not induce observable stress in the plants.

Preliminary examination of the excised root tissue showed that the number of plants in both the treated and untreated groups displaying AMF colonisation was similar and that any perturbation or flavonoid root exudates induced by UV-B radiation had little or no effect in this case on the overall number of infected plants. This technique, however was not sufficient to measure the relative extent of root colonisation. It was possible that the extent of root colonisation varied between groups. Further investigation would be necessary using image analysis software to establish any potential differences in gross colonisation.

Chapter 7.

High-performance liquid chromatographic determination of selected flavonols.

Introduction.

Allium vineale, (together with other edible *Alliums*) contains flavonoids and many other

phenolic compounds which exist in a multiplicity of complex conjugates with sugars and

organic acids including quercetin, isorhamnetin and keampherol conjugates in varying

proportions (Bilyk, Cooper, Sapers, 1984). These flavonoids are physiologically active as

potent antioxydants and metal chelators and screening pigments in plant epidermal

tissues (Jordan *et al*., 1992). Epimediological evidence has been presented setting out

implications for health, specifically cancer and heart disease (chapter 2). Quantification of

flavonoid compounds frequently use methods of separation in conjunction with UV

detection (Harbourne, 1984, Romani, A. 2000). Previously in this study, paper

chromatography was used to separate flavenoid compounds but this technique, although

useful in identifying potential qualitative differences between samples, is not able to

reliably identify or quantify these phenolic compounds. The technique itself has been

largely superseded by High Performance Liquid Chromatography (HPLC) which has

routinely become an important tool in the identification and separation of phenolic

compounds (including flavonoids) from raw plant extracts.

High Performance Liquid Chromatography is a separation technique in which components

of a mixture can be separated by allowing the sample (*analyte)* to be transported through

a packed bed of material (*the stationary phase)* by a fluid *(mobile phase)*. The individual

components move through the packed bed at different rates and separation occurs, the rate depending on the rates of migration. In HPLC the stationary phase is chemically bonded to an inert support material which is packed into a narrow (commonly 4-6mm) column. The mobile phase in then pumped through at rates of $1\text{-}5cm^3 min^{-1}$.The final separated components are then scanned using a photo-diode detector which compares the finally separated eluted compounds with known previously prepared standards, often in conjunction with a UV spectral detector calibrated to the known absorption spectra of the target compounds. Mass spectroscopy is then sometimes used to identify compounds by atomic weight. The flavenoid molecules are prone to decomposition on heating and therefore cannot be separated by gas chromatography making HPLC a suitable tool for this procedure.

Flavonoids have been extracted and quantified in 28 vegetables and 9 fruits commonly consumed in the Netherlands (Hertog *et a* 1992).HPLC has been used for the quantification of the flavonol quercetin itself in several cases (Bilyk *e tal, Hertog et al,* Crozier *et al, and* Price and Rhodes, 1997). High concentrations of quercetin occur in onion (Bilyk *e tal, Hertog et al,* and Leighton *et al)* and the amounts vary with bulb colour and type. The quercetin content of commercially grown onion (*Allium cepa*) is of interest to nutritionists, food technologists and plant breeders in order to assess breeding lines for genetic selection HPLC was performed as early as 1988 (Daigle, 1988). A HPLC gradient system was developed (Pietta, 1991) and applied to raw extractions of *Ginkgo bilboa* for separation of naturally occurring flavonoids. Hasler *et al* separated 33 flavonol glycocides found in Ginkgo leaves and Kressmann *et al* (20020 determined flavonol content from hydrolysed extracts in order to determine the pharmaceutical quality of several brands of

Ginco biloba products available on the U.S. market. Reported concentrations of quercetin found in HPLC analysis of onions vary. Lombard *et al*. (2002) reported total values ranging from 253.6 to 515.3 mg.kg^{-1}fwt whilst Price and Rhodes (1997) reported values of 1369-1788mg.kg^{-1}fwt. Other research reports intermediate values (Hertog *et al.* 1992, Patil *et al* 1995). Discrepancies in concentrations may indicate differences in sample techniques or simply varietal differences in onions grown in differing geographical locations. No data is available for likely concentrations of quercetin in the species *A. vineale.*

Aim

To evaluate any potential changes in target metabolites induced by UV/B treatment.

Objectives.

1. To identify the specific flavenol metabolites.

2. To highlight any changes in the ratio of these metabolites produced between the treated and untreated plants.

Qualitative analysis performed using paper chromatography (section 3.4) had highlighted major observable differences in individual flavenol distributions in previous extractions. in flavonol output it was necessary to use High Performance Liquid Chromatography techniques on freshly extracted leaf tissue.

7.1 Method: extraction and characterisation of flavonoids.

7.1.1 Extraction.

Following season long exposure to the UV-B lighting regime (as detailed in section 4.2.1.) extractions were carried out on plants.

10cm lengths of fresh leaf tip tissue was excised from all treated and untreated plants and extraction was carried out as before (section. 3.3.2.).

7.1.2. Standards.

The standards were purchased from Sigma Aldrich Co. Ltd. Poole (rutin & kaempferol) and MP Biomedicals UK London (isorhamnetin & quercetin).

Stock solutions were prepared at 200ugcm3 w/v in HPLC grade methanol purchased from Fisher Scientific. A range of standards were prepared by further dilution in 1:1 HPLC grade methanol: uhp water.

These were prepared for injection onto the column at the following concentrations: 5, 10, 15, 20 and 25 µg cm^{-3}.

7.1.3. HPLC method.

A suitable HPLC protocol has been developed to identify and quantify flavonol glycocides in *Ginkgo biloba* as a commercially available solid oral dosage (Dubber and Kanter , 2004). This paper describes a precise, simple and reproducible method for quantification of five relevant flavonol marker compounds using HPLC. This protocol was adopted in an attempt to replicate the analysis in *Allium vineale* following extraction in fresh plant material (Harbourne, 1973).

7.1.3.1. **HPLC Protocol.** This method is based on that given by: Dubber and Kanter (2004).

The equipment used was the Thermo Separation Products P4000 HPLC pump/degasser , a AS300 autosampler fitted with a UV 6000LP Photodiode array. The column used was supplied by   Phenomemex 4 um Synergi Max-RP 250x2mm.

The Solvent System comprised, A : Acetonitrile 0.3% Formic Acid and B: 0.3% Formic acid. The following gradient was used to elute the column:

Gradient: 0 mins 15% A : 85% B

                      12mins 25% A : 75%B

                      40mins 25%A : 75% B.

Other conditions were set at:

Flow rate: 0.4cm^3/min.

Column temperature: 45°C

All measurements were made at 350nm. (Dubber and Kanter , 2004). (Maximum absorbance of flavonols at this wavelength).

The software used to process the results was: Thermo Xcalibur 1.2.

7.1.3.2. Standards.

Retention times for each standard on the column was determined by injection of the standard on the column and monitoring its emergence at the detector. Linear calibration curves for Kaempferol and Isorohamnetin Quercetin were constructed by analysis of a mixture containing each of the individual flavonols at five concentration levels and then plotting peak areas against the concentration of each reference standard. Calibration curves were then constructed at the relevant wavelength of maximum absorption of each

reference compound. Specificity was determined by calculation of peak purity facilitated

by the photodiode-array detector.

Prior to running the extracts, the previously prepared standards of rutin, quercetin,

kaempferol and isorohamnetin were run through the column as follows :

The calibration standards at each concentration:
5 µg cm^{-3} standard
10 µg cm^{-3} standard
15 µg cm^{-3} standard
20 µg cm^{-3} standard
25 µg cm^{-3} standard
Following each of these a methanol blank was eluted to wash the column.

7.1.3.3. Limits of detection (LOD) and quantification (LOQ)82

The limits of detection were determined by the means of serial dilution based on a signal

to noise ratio of 3:1 for the limit of detection and 10:1 for the limit of quantification

(Dubber and Kanter (2004).

7.1.3.4.1. Plant tissue preparation.

At the end of the growing season 20 cm lengths ^{of} fresh leaf tip tissue was excised from all

33 of the plants non UV-B treated group found to be inoculated with AVF and 33

fungicidally treated plants from the non UV-B treated group and boiled separately with

95% ethanol in a Leibig reflux apparatus for 10 minutes. Both extracts were

volumetrically equivalent. The samples were refluxed in 50 cm^3 ethanol: 3M HCl (70:30)

for 2 ½ hours. The cooled sample

was adjusted to 100 cm^3 and 0.3 cm^3 of ethanolic extract was diluted 1:10 with distilled

water.

7.2. Plant sources.

7.2.1. Types of extracts: with and without UV-B full season.

HPLC analysis was performed on extracts from plants described in chapter 4 section 4.2.3. and the extraction method as described in chapter 3 section 3.2.2.

7.2.2. Plants plus mycorrhiza.

HPL analysis performed on extracts from plants using the collection method described in chapter 6 section 6.2.4.,the extraction method as described in section 7.1., and the SPE method 7.1.3.4.

7.2.3. Plants plus mycorrhiza plus UV-B.

Plants were grown on and irradiated as before for a second year following the SPE analysis of the inoculated plants in chapter 6. There were no further inoculations and plant tissue was collected at the end of the growing season in July and subjected to HPLC analysis performed on extracts from plants using the collection method described in chapter 6 section 6.2.4.,the extraction method as described in section 7.1., and the SPE method (section 8.1).

SPE tubes failed so unconcentrated samples were sent for HPLC analysis and only plants treated with UVB (+/-) mycorrhriza were examined by HPLC.

The standards served two purposes. They formed a calibration curve for quantifying the amount of flavonoids in the samples, and also were used qualitatively, as retention time markers. This enabled identification of where the peaks of interest would occur in the

samples, and also as a quality control check. They showed whether the retention times remained constant throughout the run. The retention times for the standard materials were determined by the HPLC method described above. Figures 7.1, 7.2, 7.3 and 7.4. show the chromatograms obtained for the individual runs.
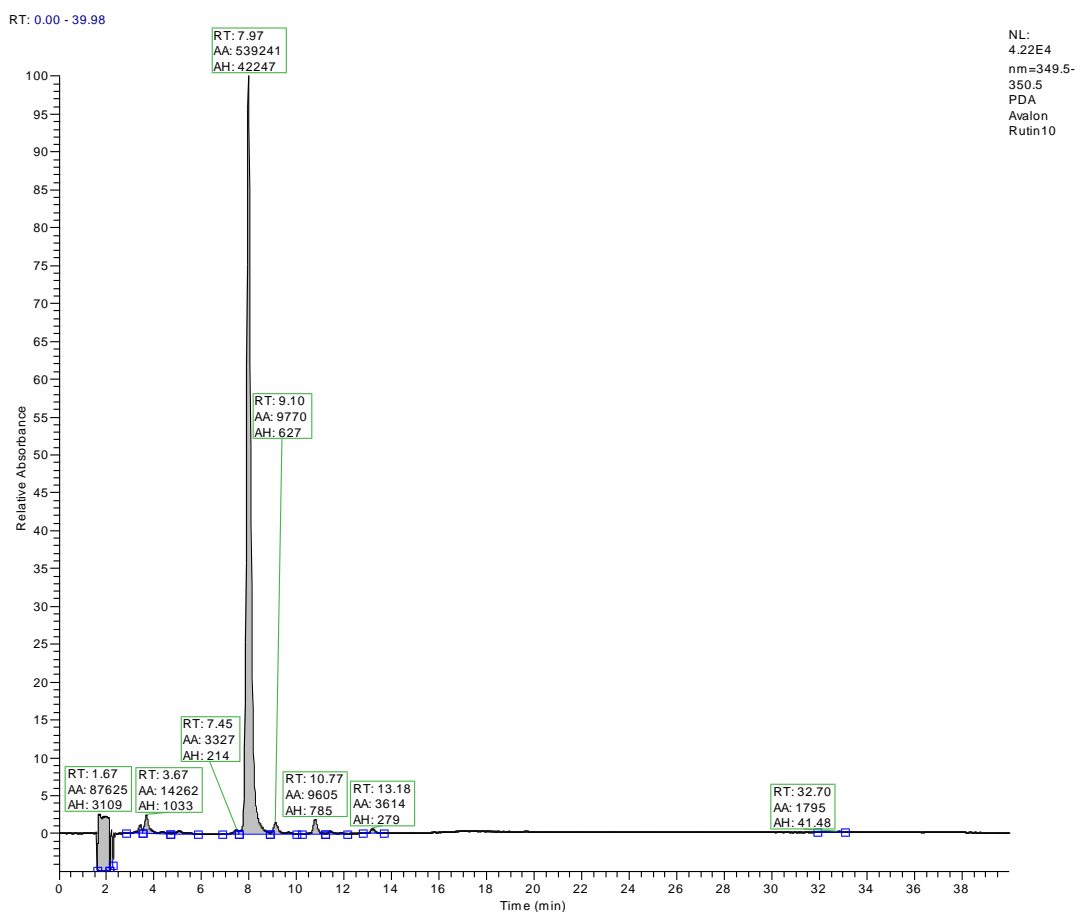
Figure 7.1. Chromatogram for Rutin.

The major peak found on the chromatogram represents rutin and has a retention time 7.97. minutes. Other minor peaks are considered to be impurities as degradation products during HPLC analysis have not been reported.
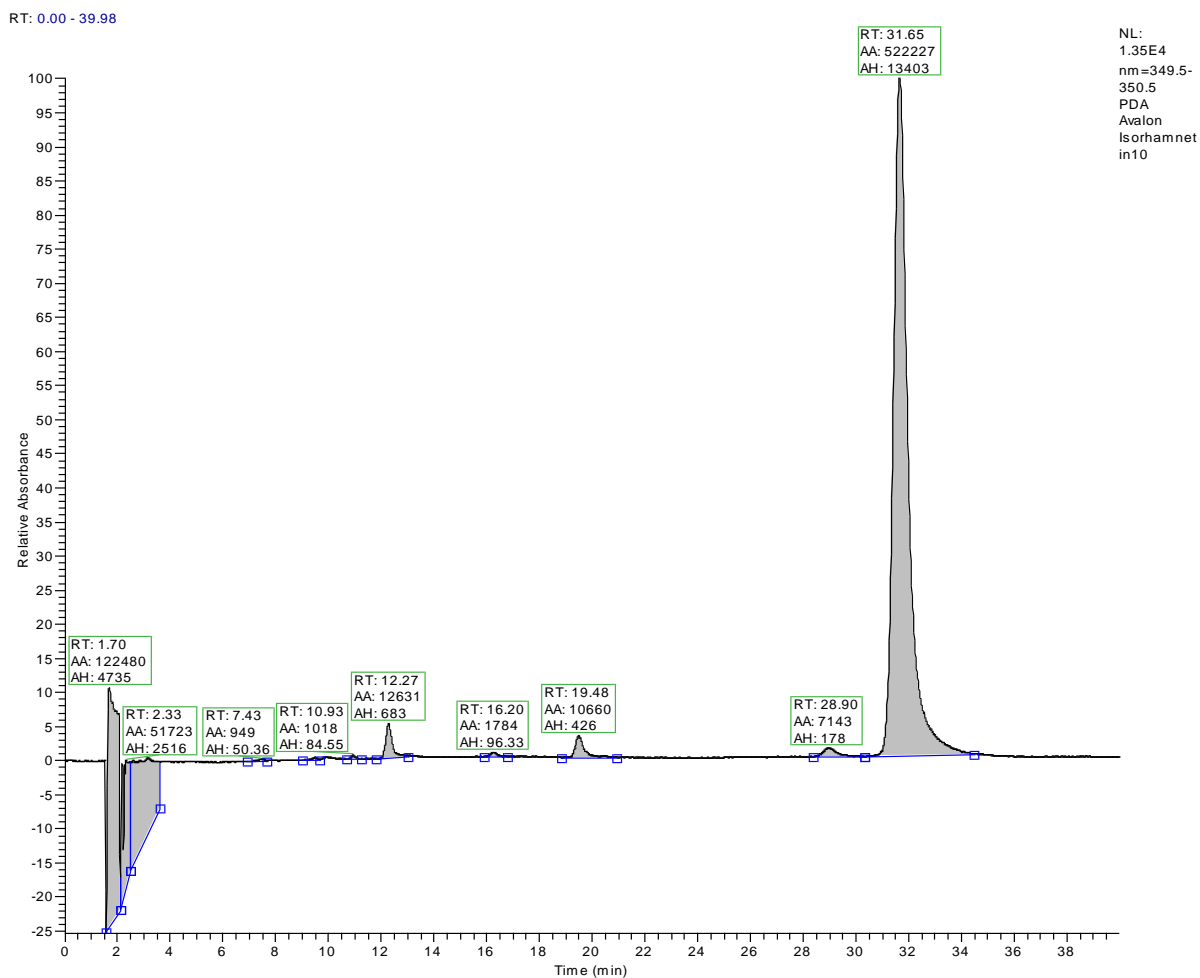
Figure 7.2. Chromatogram for isorhamnetin.

The major peak found on the chromatogram represents isorhamnetin and has a retention time 31.65 minutes. Other minor peaks are considered to be impurities as degradation products during HPLC analysis have not been reported.
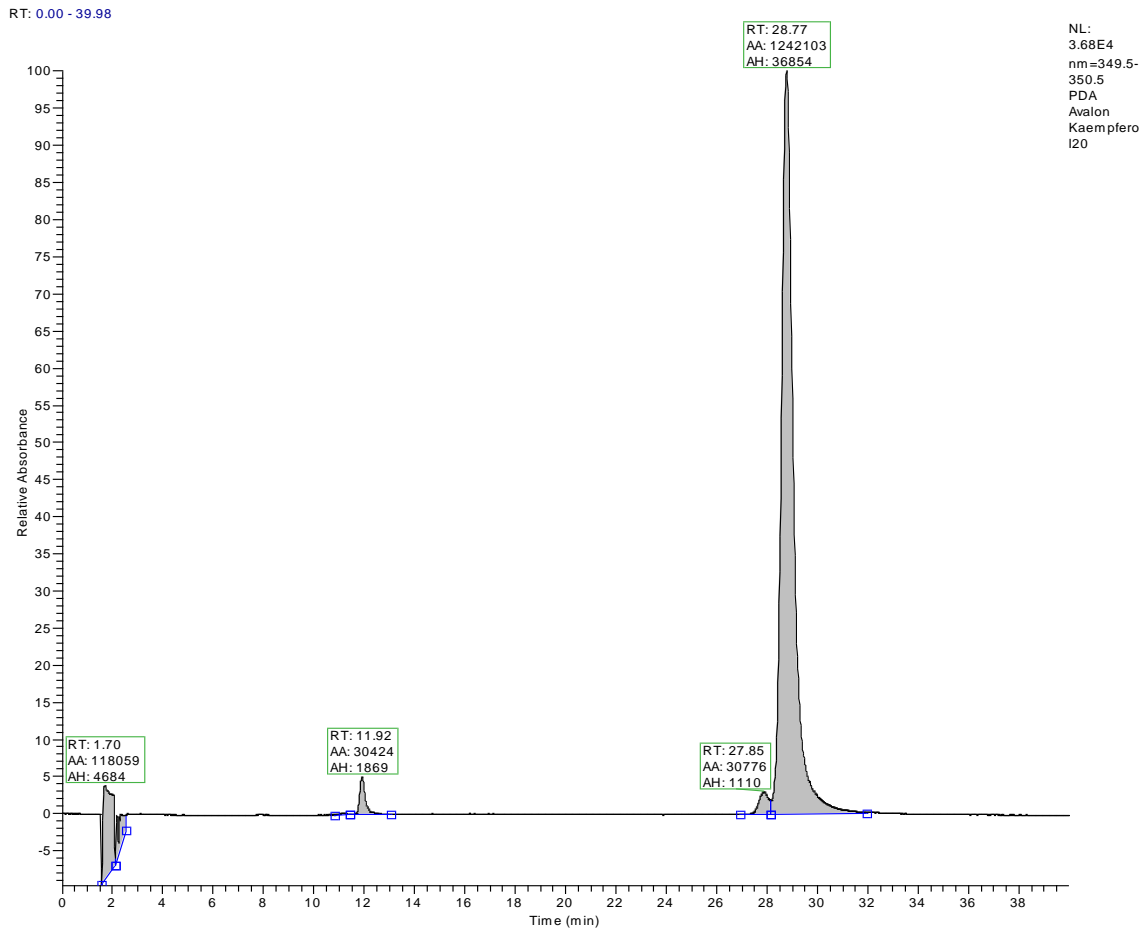
Figure 7.3. Chromatogram for kaempherol.

The major peak found on the chromatogram represents **kaempherol** and has a retention

time 28.77 minutes. This chromatogram shows fewer minor peaks. These are also

considered to be impurities.
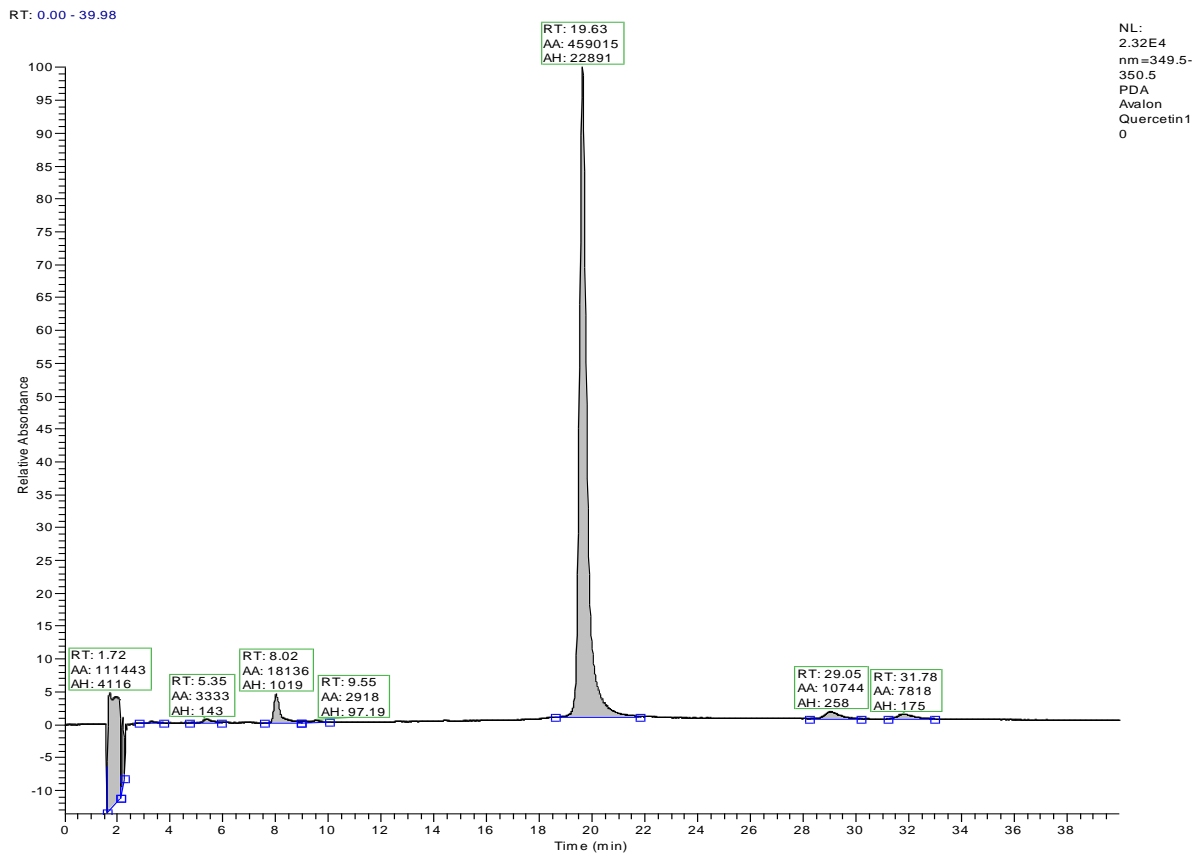
Figure 7.4.Chromatogram for quercetin.

The major peak found on the chromatogram represents quercetin and has a retention time 19.02 minutes. Other minor peaks appearing both with slower and faster retention times are considered to be impurities.

Observed minor peaks may have been a result of degradation products within the pure compounds or column impurities. Flavonoid glycocides are, however, relatively stable and hydrolyse only under rigorous extraction conditions and/or incorrect storage conditions. They therefore serve as excellent quality control indicators (Dubber and Canter, 2002). Therefore, minor peaks observed represent likely column impurities .

Figures 7.1, 7.2., 7.3 and 7.4 show the HPLC-UV (350nm) chromatogram of the four target flavonols, rutin, isorhamnetin, kaempherol and quercetin with retention times of 7.97, 31.65, 28.77 and 19.02 minutes respectively. This result is supported by the retention times found by Dubber and Canter (2002) where corresponding retention times for these flavonols were 8.7, 31.5, 28.1 and 19.8 minutes respectively. Assessment of peak purity showed homogeneity, therefore excluding the possibility of the presence of interfering components and rendering the method specific.

7.3.1.2. Calibration curves for the standards.

The various concentrations of each of the standards were run through the column as described in the method (7.1.3.2.) the area under the peak was determined by the software and these values plotted against concentration in order to be able to obtain quantitative values for the subsequent peaks when running the extracts. These standard curves are given in figures 7.4.1., 7.4.2.,7.4.3. and 7.4.4. and can be found in appendix 13.

7.3.1.3. Calibration curve non-linearity.

In the cases of kaempherol and quercetin the regression line has been forced through zero. There are several methods available to use a calibration curve to quantify samples. Most methods use UV detection in conjunction with HPLC. Where the UV detector response versus peak area is linear over five or more orders of magnitude it is assumed that linear response is possible when using a method calibration that covers a wide range of concentrations. This is the expectation and the retention times support this

assumption. However, the regression line in the case of isorhamnetin shows non-linearity. This analysis primarily compares relative differences between treated and untreated samples in the context of the study rendering absolute values of flavenols of secondary importance.

Linearity in this case is defined as; $y = mx$ where y is the response (area), x is the concentration and m is the slope of the curve. In the case of kaempherol a regression (Appendix 7.) table plotted from the values in figures 7.4.1. shows that the y intercept value at 0.717205 is less than the standard error value for the entire curve (0.83779). This allows the equation $y = mx$ to be applied (b = 0) and the curve to again be forced through zero.
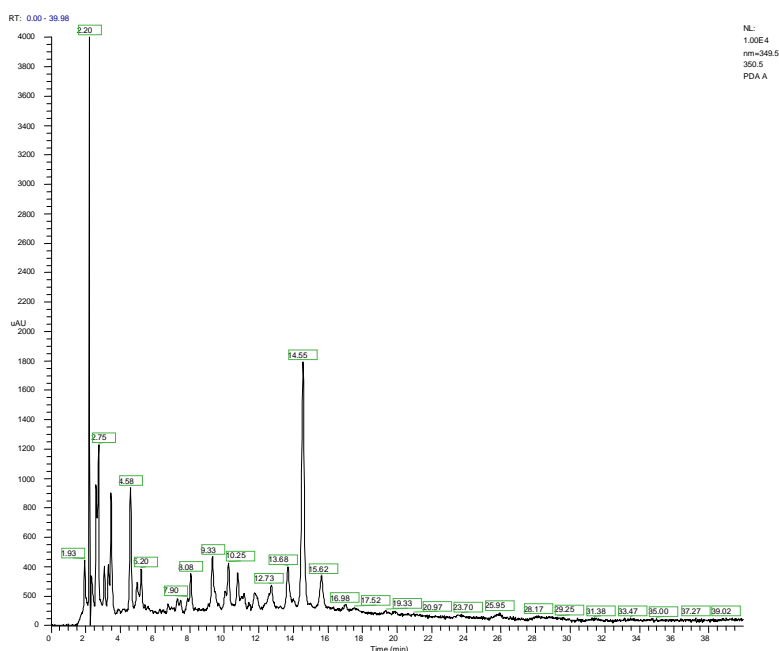
Figure 7.4.5. HPLC chromatograph of untreated plant extract.
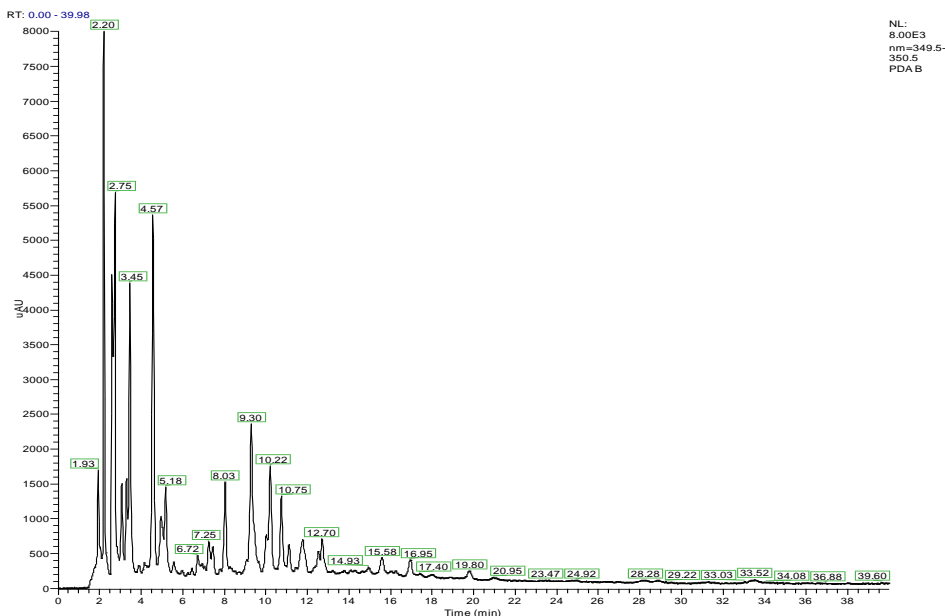
Figure 7.4.6. HPLC chromatograph of UV-B treated plant extract.

7.3.1.3. Results – UV-B treated / Untreated plants, full season (crude extract). The

chromatograms for the untreated and treated plants were compared and it is evident

that the traces are different with a number of major peaks being identified for one

sample and not the other. A number of the other peaks are however present in both

chromatograms as would be expected. Table 7.1 below gives the retention times for the

peaks from both samples for comparison.

UV/B Treated	Untreated	Comparison
2.2	2.2	In common
2.75	2.75	In common
4.58	3.45	
5.2	4.57	
7.91	5.18	
	6.72	
	7.25	
8.08	8.08	rutin
9.33	9.33	In common
	10.22	
10.25	10.25	In common
12.73	12.70	
13.68		
14.55	14.9	
15.62	15.5	
16.9	16.9	In common
17.52	17.4	
19.8	19.8	quercetin
20.9	20.9	In common
23.7	23.4	
25.9	24.9	
28.1	28.2	In common
29.2	29.2	In common
31.38	31.37	isorhamnetin
33.47	33.3	
	33.5	
	34.0	
35.0	36.6	
37.2		
39.2		
	39.6	

Table 7.1 : retention times (minutes) for major peaks for both the UV-B treated and untreated plants.

The peaks of interest corresponding to the comparative retention times of the target metabolites in table 7.1 are highlighted in yellow. The UV absorption spectra of the peaks

of interest were then examined and compared with reference absorbance data in order to confirm the identify and quantify the target flavonol metabolites by measurement of areas under peak values using Xcalibur software(Quantginko09b).(Examples of UV quantum targets in Appendix 9). Concentrations for quantitative analysis were then calculated from measured areas using said software (figure 7.3.2.2.) using the data from calibration curves performed on standards. Results indicate that there are indeed differences in the retention times of peaks for each sample. Unidentified compounds with corresponding In particular the peak values are highlighted in green. Peaks at 8.08, 19.8, and 31.38 mins were identified as: rutin, quercetin, and isorhamnetin respectively using UV absorption data. Kaempherol could not be reliably identified. There is a peak seen (e.g. at 16.38mins) in the treated sample that has no corresponding value in the untreated sample. Several other peaks have no corresponding values.

Figure 7.4.7. Concentrations of target flavonol metabolites in treated and untreated plants (µgcm^3).
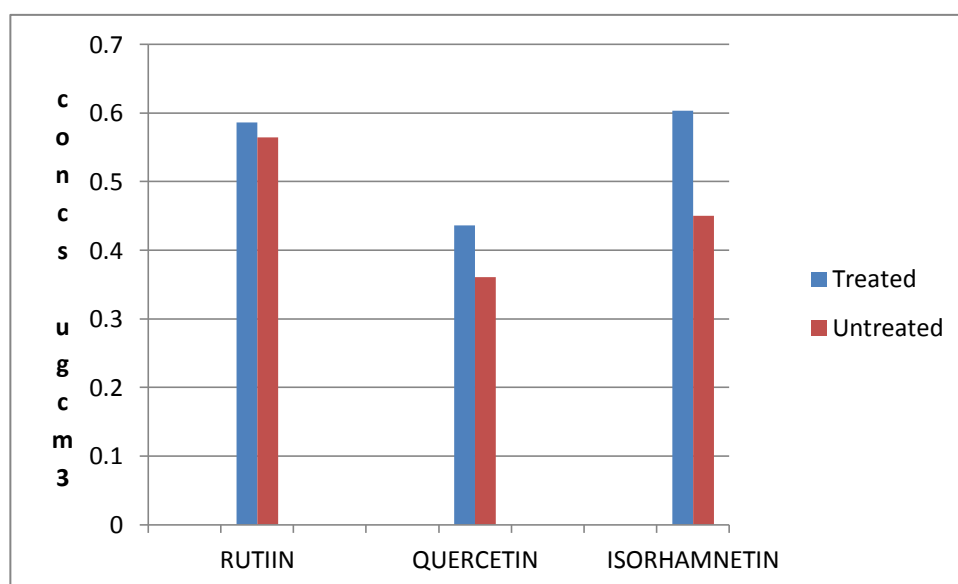
Relative concentrations of three target flavonols: rutin, quercetin and isorhamnetin have

been modified and are higher in UV-B irradiated samples.

Chapter 8.

Examination of plant material treated with mycorrhizae using solid phase extraction methods.

8.1. Plant treatment with mycorrhorizae (no UV-B).

All plants were inoculated with AVF (section 6.4.1). Leaf material was collected as in section 4.2.3. and extractions carried out as in section 3.2.2. and 7.1.1. Only non-irradiated plants (+ AVF / -AVF) were used in this analysis in order to establish a baseline value of target flavonols between inoculated and non-inoculated samples. Four standards were initially chromatographed (figure 7.9) and standards run at five concentrations as before (7.3.3.2).

8.2. Use of SPE tubes.

Solid Phase Extraction is an efficient method for isolating and concentrating solutes from relatively large volumes of liquid. This technique is effective, even when the solutes are present at extremely dilute concentrations (e.g. ppb). Materials extracted in this way can be used for subsequent chromatographic separation, spectroscopic examination, or biological assessment. The apparatus consists of a simple tube, which, in this case was 4 mm in diameter and 4 cm long and made from suitably inert polymer. The extraction tube was packed with an appropriate bonded phase (Phenomenex Uk). All the solutes accumulate at the front of the packing. The solutes can then be displaced from the adsorbent by elution with methanol or acetonitrile (usually by reverse flow techniques to minimize extract dilution).The results can lead to a concentration factor of 2000-4000.

The extraction process can be very efficient, e.g.>99% and higher concentrations of target metabolites can be obtained using this method (Dr D. Hodgeson, Phenomenex UK, personal communication).

The solutes or usually desorbed with acetonitrile or an acetonitrile-water mixture. A solid phase extraction (SPE) method was used in an attempt to purify and concentrate the extracted samples prior to injecting them on to the column.

8.3. Solid-phase extraction procedure.

Solid Phase Extraction tubes (Strata X reversed phase polymeric sorbent) were obtained pre-loaded from Phenomenex UK Ltd for the identification of quercetin, kaemphferol and isorhamnetin. The Strata (SPE) tubes were equilibrated with 2 cm^3 of a mix of 1:1 methanol:distilled water and then loaded with 3 cm^3 of the 1/10th diluted sample. The extraction tubes were then subjected to a wash of 2ml 5% aqueous methanol. A second wash was carried out with 2 cm^3 of methanol: 50mM NH$_4$OAc (ammonium acetate) (30:70) adjusted to pH5. The extraction cartridge was dried for 15 minutes with an air vacuum and then the flavonoids were eluted from the tube with 3 cm^3 MTBE/methanol (80:20). (The protocol for this was supplied by Dr. R. Hodgeson, Phenomenex UK Ltd).

8.4. HPLC of materials following SPE extraction.

The concentrated samples were injected on to the column using the same conditions as stated in 7.1.3.1. but with some minor alterations as below:

Figure 8.1. HPLC chromatogram of the standard mixture of the 4 flavonoids at concentration of at 15ug cm^{-3}
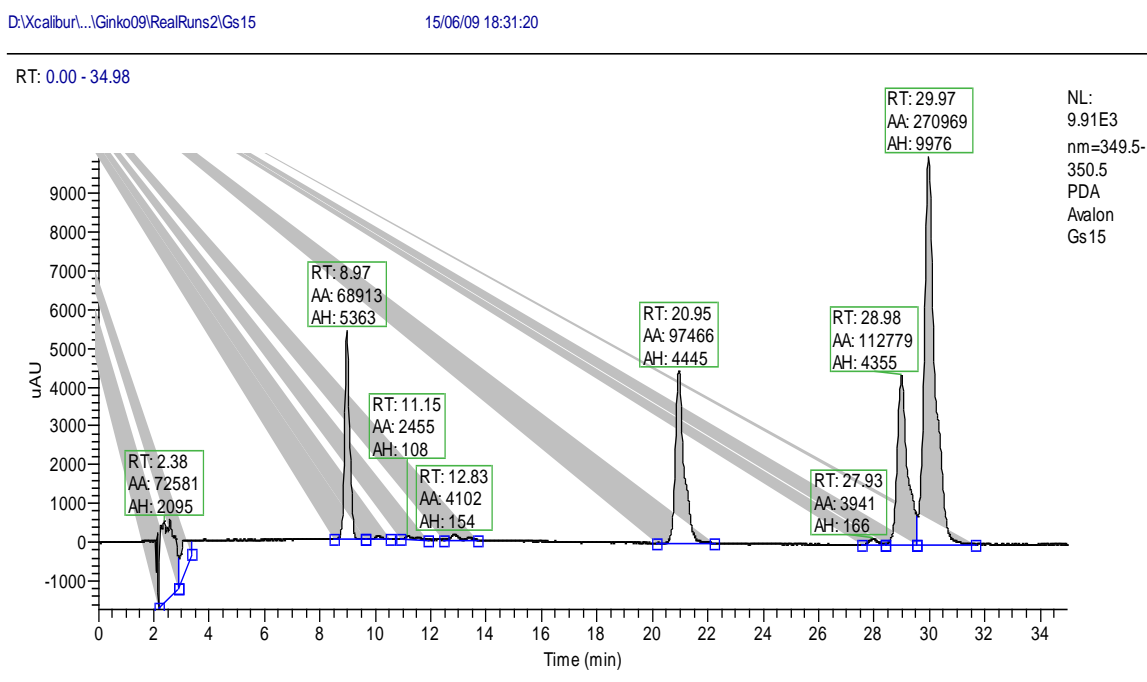
This chromatogram is shows 4 major peaks for the standards at 8.97 (rutin), (20.95 quercetin), 28.98 (kaempherol) and 29.97 (isohaemetin) minutes. These retention times are very similar to those previously recorded and discrepancies in the values would be due to small changes in conditions experienced. There are also 3 minor peaks within this region plus the solvent front eluting at 2.38 minutes. The 3 minor peaks could be impurities eluting form the column, the possibility of breakdown products are unlikely as

mentioned previously. These results provided a standard against which extracts from irradiated plants could be compared.

8.5. Calibration curve.

Calibration curve data is presented as produced by the quantification module of the Xcalibur 1.2. software(Quantginko09b). The four tables below give numerical values to the four standard marker flavonols injected at five concentrations and the resulting regression equation for calibration. Data tables for regression equations are to be found in appendix 11.

8.6. SPE – HPLC.

SPE extracted plant samples were subjected to HPLC analysis (section 7.1.3.1.) at 10μ and 20 μl volumes and two resolutions. Resultant chromatographs are below.
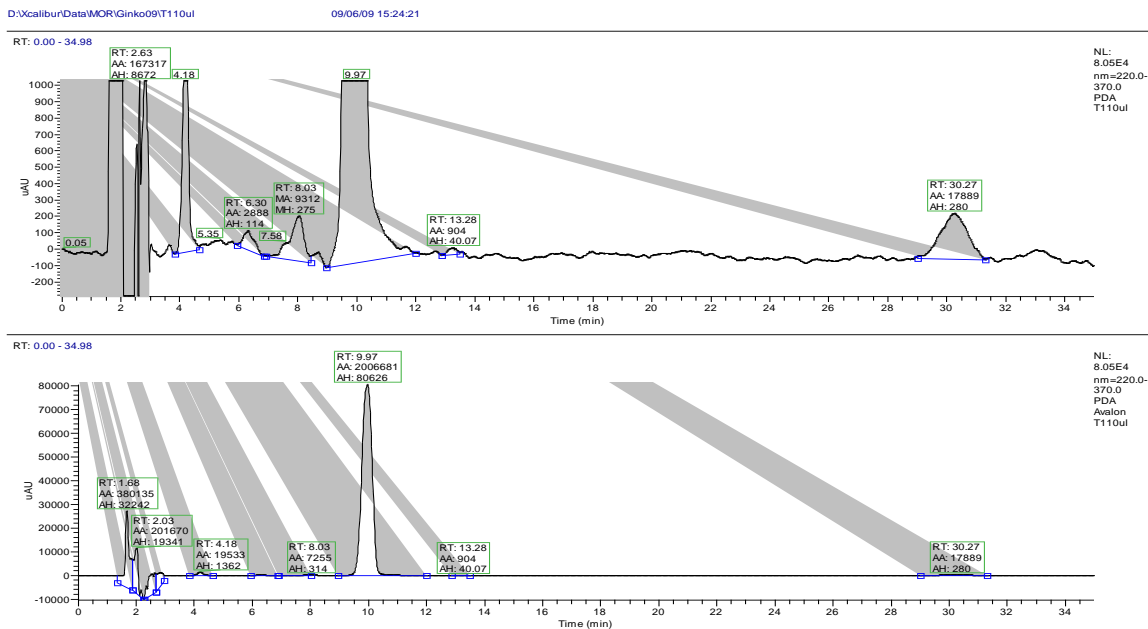
8.7. Results.

Figure 8.2. Chromatograph of AVF treated 10 ul sample at 2 resolutions in order to get

area for major peaks.

For this HPLC analysis the extract was concentrated using SPE tubes prior to injection on

the column. A 10μL volume was injected on this occasion and the results have been

reproduced

at two resolutions so that major peak at 9.97 minutes could be resolved and the area

under the peak calculated.
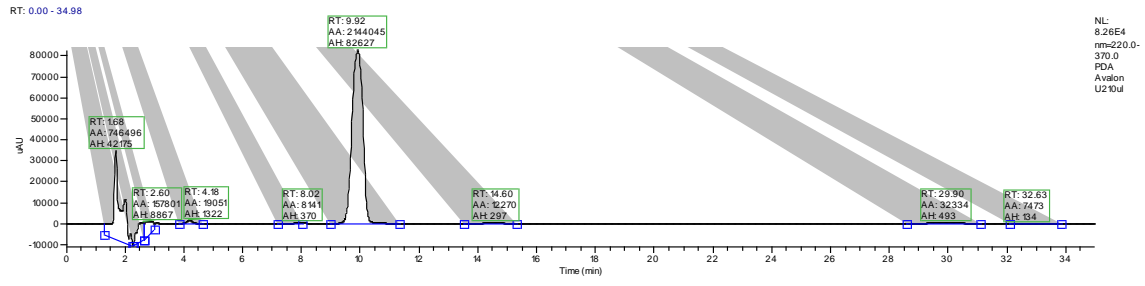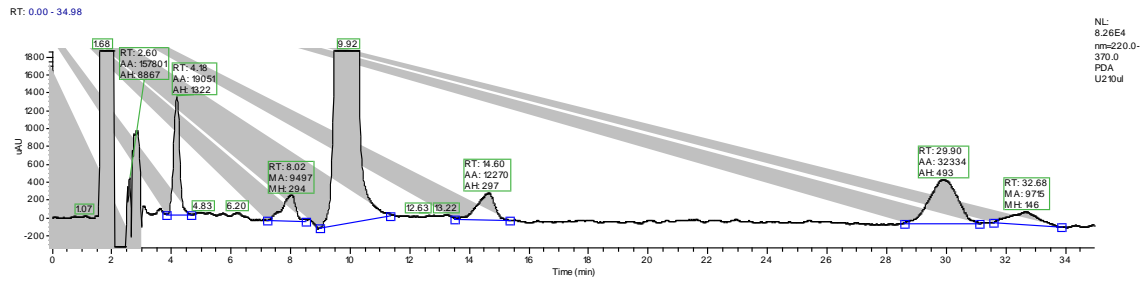
Figure 8.3. Chromatograph of untreated 10 ul sample at 2 resolutions in order to get area for major peaks.

AVF	1.68	260	4.18	4.83	5.20	8.02	9.92	12.63	13.22	14.60	29.9	32.68
Non-AVF	1.68	2.60	4.18			8.02	9.92			14.60	29.9	32.63

Table 8.1. Comparative retention times (mins) of major peaks.

The above retention times demonstrate qualitative differences between the AVF inoculated and non-inoculated plant extracts. Peaks are seen at 4.83, 5.2, 12.63 and 13.22 minutes in the AVF inoculated plant group retention times that do not have corresponding values in the non-inoculated group. Areas of similar retention time values are highlighted in green.

Values highlighted in red represent indicate the synthesis of novel compounds induced by AVF infection. Retention times varied with previous values found in section 7.3.1.1.(non-S.P.E. extraction) and thus rendered the identification of the target flavonols unreliable. As the S.P.E. protocol is designed in this case to absorb and concentrate flavonol glycosides (phenomenex, uk) it is likely that the major peaks represent these substances but further method development is required in order to confirm their precise identities. Three flavonoid compounds were identified under peaks at 9.92, 29.9 and 32.68 mins using comparative action spectra UV data. Quantification the target flavonol metabolites was achieved by measurement of areas under peak values using Xcalibur software(Quantginko09b).(figure 7.9.2.). Concentrations for quantitative analysis were then calculated from measured areas using said software (figure 7.9.4) using the data from calibration curves performed on standards. Discussion of the results can be found in section 7.7.
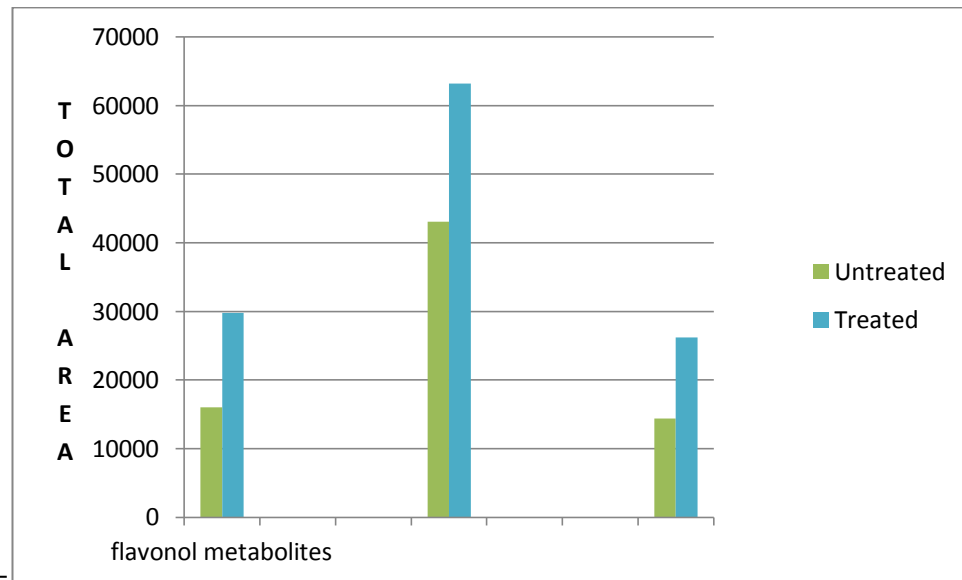
Figure 8.4. Total areas of target flavonol metabolites – Xcalibur software(Quantginko09b).
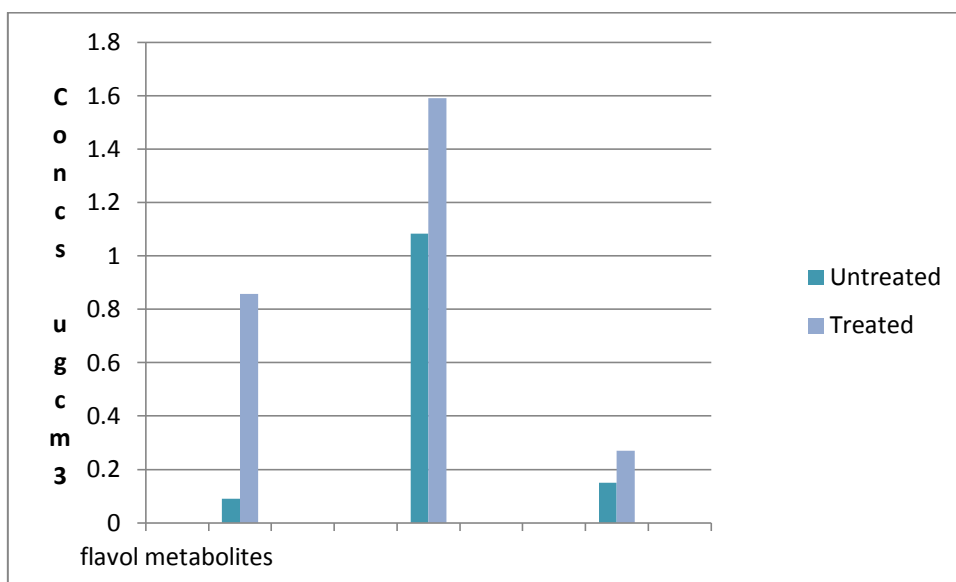
Figure 8.5. Relative concentrations of target flavonols (ugcm^3).

Chapter 9.

Investigation into the effect of UV-B on treated/untreated plants following successful inoculation with AVF.

9.1. method.

Plants were subjected to a further year's treatment during the growing period of October

09 to May 2010. Successfully inoculated plants were positioned as before into arrays

consisting of four groups of 33 plants. (UV-B, AVF. Non UV-B AVF. UV-B, Non AVF and

Non UV-B Non AVF).

 Aims.

To determine relative flavonoid output.

To establish any changes in extent of AVF colonisation (uncompleted).

Objectives.

To compare flavonol output with baseline SPE / HPLC analysis values from the previous year
(uncompleted).

To develop a protocol for the quantification of AVF inoculation using Image Analysis
Software (uncompleted).

9.2. Extraction.

Following season long exposure to the UV-B lighting regime (as detailed in section 4.2.1.)

extractions were carried out as in section 3.3.2. on irradiated plants only (UV-B + AVF, UV-

B – AVF).

9.3. Use of SPE tubes.

Strata X. S.P.E. tubes (Phenomenex, uk) were used as before (section 7.1.3.4.).

9.4. Plant tissue preparation.

Plant tissue was prepared as in section 7.1.3.4.1.

9.5. HPLC Protocol.

HPLC protocol was performed as in section 7.1.3.1.

9.6. HPLC method.

HPLC method was performed as in section 7.1.3.

9.7. Standards.

Standards were prepared as in section 7.1.3.2.
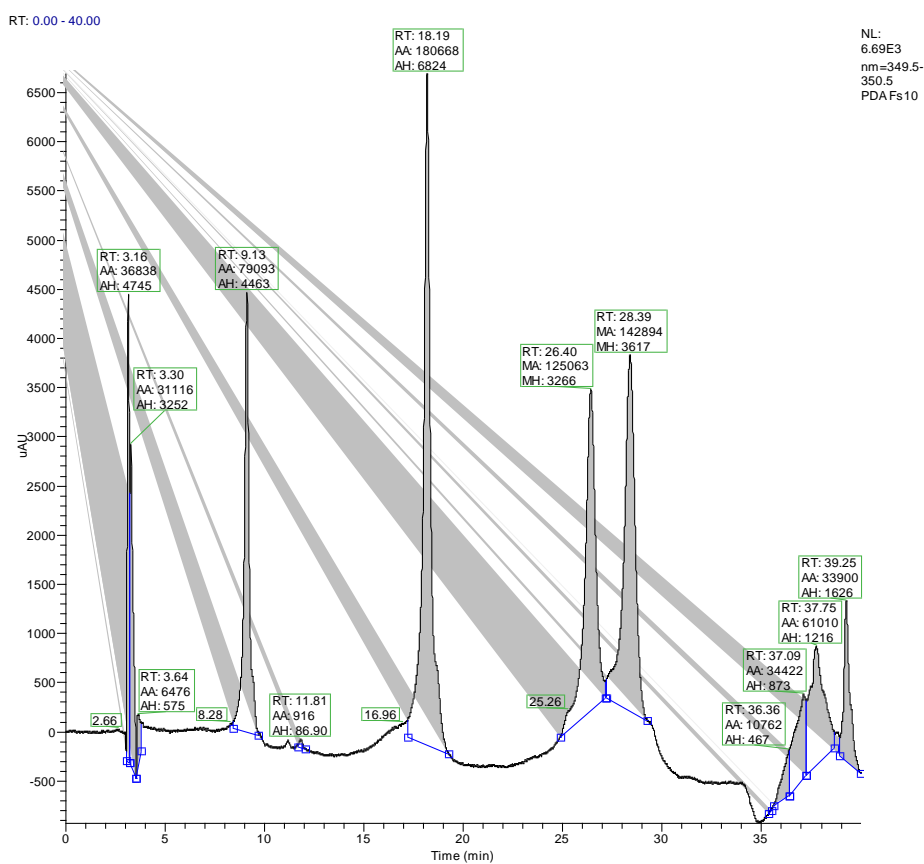
Figure 9.7. Chromatogram of standard mixture of 4 flavonoids at 10ugcm3.

The standard marker flavonols rutin, quercetin, kaempherol and isorhamnetin are showing peaks at 9.13, 18.19, 26.40 and 28.39 minutes respectively. (All shown at 350nm. Maximum absorbency for flavonol compounds).

9.7.1. Calibration curves for the standards.

Calibration curves were prepared as in section 7.3.1.2.(figures 9.7.1., 9.7.2., 9.7.3. and 9.7.4.). Calibration curves can be seen in appendix 14.

9.8. Results.

9.8.1. S.P.E. Extractions.

When samples were chromatographed no flavonol compounds could be detected. HPLC analysis of non- S.P.E. extractions was then performed. Possible explanations for this are contained in section 9.9.

9.8.2. Spare crude extract as in section 7.1 was then used in the following HPLC analysis.

9.9. HPLC Full season results. AVF + UV-B / AVF –UV-B.
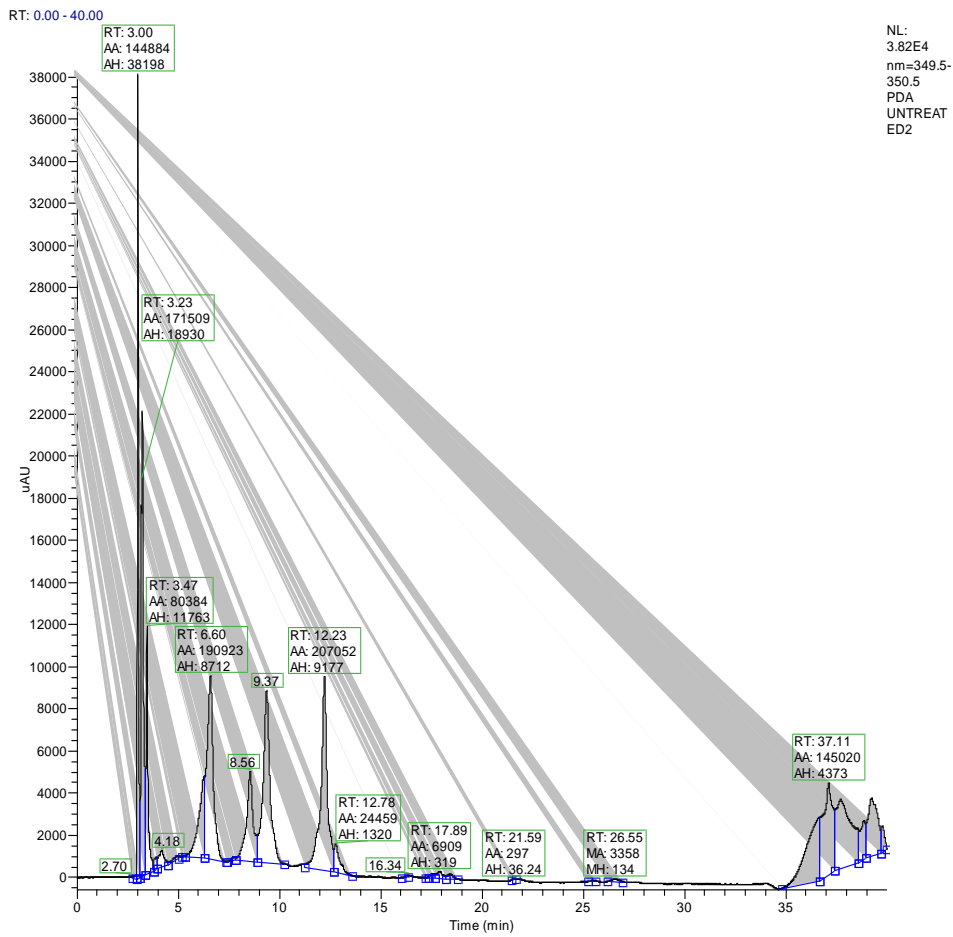
Figure 9.7.5. Chromatogram of AVF – UV-B crude extract (non S.P.E.).

Figure 9.7.4. shows the chromatogram of non-irradiated AVF inoculated plants. Peaks areas

of interest are tabulated (table 9.3.).
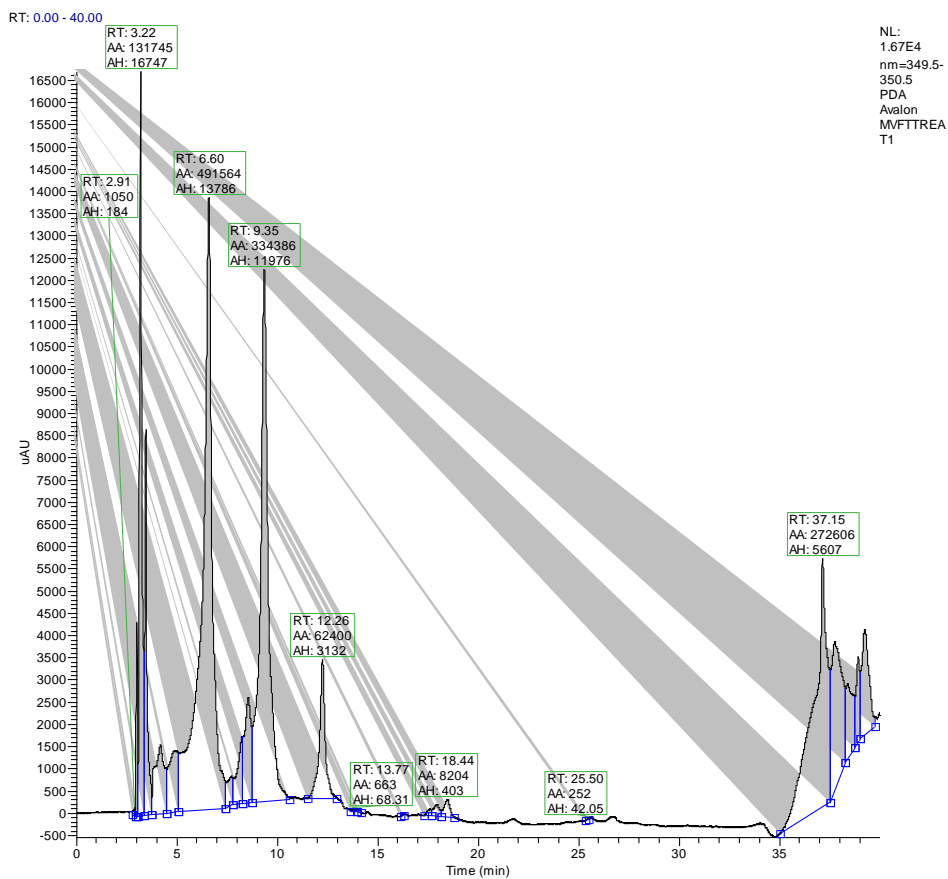
Figure 9.7.6. Chromatogram of AVF + UV-B treated crude extract (non S.P.E.)

Figure 9.7.5. shows the chromatogram of irradiated AVF inoculated plants. Peaks areas of interest are tabulated (table 9.3).

Table 9.3. Comparative retention times (mins) of major peaks.

UVB + AVF	3.23	3.47	6.6	8.56	9.37	12.23	12.78	16.34	17.89	21.59	26.5	
UVB − AVF	3.22		6.6		9.35	12.28	13.77		18.44			28.6

Retention times varied with previous values found in section 7.3.1.1.(non-S.P.E. extraction) and thus rendered the precise identification of the target flavonols problematic. The UV absorption spectra of the peaks of interest were then examined and compared with reference absorbance data in order to confirm the presence of flavonoids at peak values 9.37, 12.33/8 and 17.89/44 mins. Isorrhamnetin was not see in either sample. Peaks at 8.56, 16.34, 21.59, and 26.5 mins displayed in the UV-B + AVF table of peak values (figure 9.3.) are not present in the non-irradiated sample indicating induced synthesis of novel metabolites. Similarly, a peak at 28.6 mins is seen in the non- irradiated sample. Three HPLC runs were performed on each sample.

Quantification three target flavonoid metabolites was achieved by measurement of areas under peak values (section 7.3.2.3.1.) using Xcalibur software version 1.2.).

Concentrations for quantitative analysis were then calculated from measured areas using said software using the data given below from calibration curves performed on standards (table 793.).
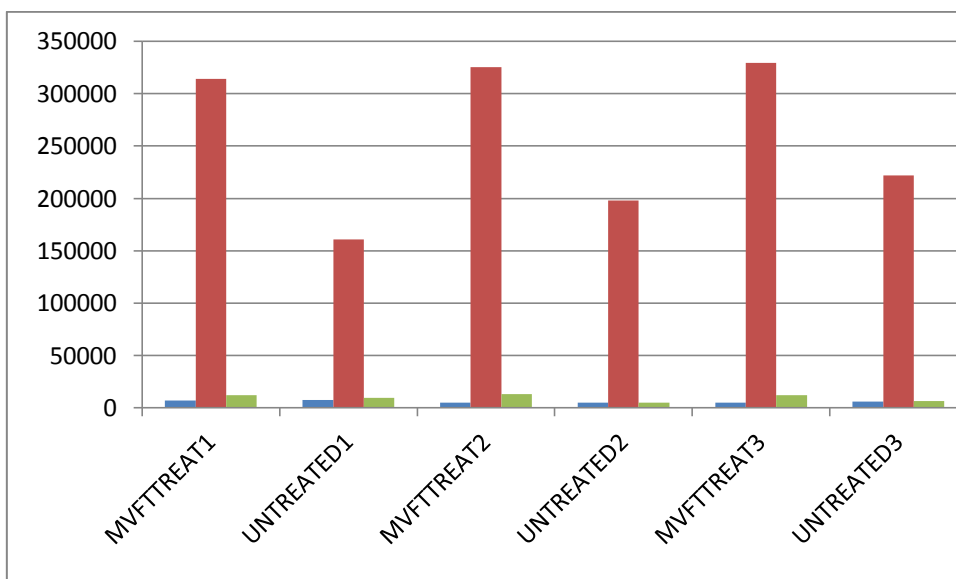
Figure 9.8. Total areas of target flavonoid metabolites – Xcalibur software(Quantginko09b).

Rutin AVF + UV-B	Quercetin AVF + UV-B	Kaempherol AVF + UV-B	Rutin AVF − UV-B	Quercetin AVF −UV-B	Kaempherol AVF − UV-B
32.062	0.569	0.391	16.397	0.452	0.431
33.186	0.628	0.274	20.2	0.241	0.284
32.640	0.570	0.272	22.64	0.319	0.356

Table 9.4. Relative concentrations of target flavonols (ugcm^3).

9.9.1. Discussion.

The extraction technique (Harbourne, 1973) and the following HPLC separation highlighted a number of differences between peak values on chromatographs of the UV-B treated and untreated plant samples and provide evidence that treated plants have modified their photo-screening pigment output in response to UV-B irradiation. Table 7.1. shows the relative concentrations of the target flavonols found in the samples. Whilst this is a useful indication of the differences between samples, the concentrations were derived from the measurement of peak areas which were themselves small (section 7.3.1.4.). The resulting levels of flavonols found were at low concentrations rendering absolute concentration calculations, (figure 7.9.1.) subject to a high margin of error. For comparative purposes this is not critical. A revised extraction protocol using Solid Phase Extraction methods was adopted in an attempt to pre-filter and concentrate the extraction solute prior to HPLC (section 7.9.).

The S.P.E. protocol resulted in less cluttered chromatographs. Retention times varied with previous values found in section 7.3.1.1.(non-S.P.E. extraction). Peaks are seen at 4.83, 5.2, 12.63 and 13.22 minutes in the AVF inoculated plant group retention times that do not have corresponding values in the non-inoculated group. This would indicate novel flavonoid metabolites being synthesised in the leaves of the inoculated plants or, alternatively, flavonoids missing from plants devoid of AVF infection (table 7.2.). Results from the UV-B only analysis (table 7.1.) also demonstrate changes in flavonoid output between treated samples.

Table 6.1. demonstrates a statistical interaction between groups of plants receiving UV-B irradiation and AVF inoculation with greater leaf shortening in UV-B/AVF groups than non UV-B/AVF groups suggesting that some commonality may exist in the flavonoid metabolites induced by both UV-B and AVF treatments and that some requisitioning of resources in response to UV-B perturbation is occurring.

Flavonoid compounds are often present in plants bound to sugar as glycosides and any single glycoside aglycone may occur in a single plant in several glycosidic combinations. These closely related compounds have similar absorption spectra and corresponding retention times (Harbourne, 1973). The S.P.E. protocol and conditioning process may also have been a factor but as the study is of a comparative nature, the precise flavonol glycosides being synthesised remains a topic for further investigation.

Where S.P.E. tubes were used (section 7.3.2.3.) retention times varied with previous values found in section 7.3.1.1.(non-S.P.E. extraction) and no exact correlation was found between corresponding retention times and measurable peaks thus rendered the identification of the target flavonols unreliable. As the S.P.E. protocol is designed in this case to absorb and concentrate flavonol glycosides (phenomenex, uk) it is likely that the major peaks represent these substances but further method development is required in order to confirm their precise identities. One objective was to establish a baseline separation and resulting chromatograph showing retention times in non-irradiated AVF inoculated / non-inoculated samples from which future comparisons could be made with samples of UV-B + and –UV-B / AVF inoculated plants. The S.P.E. technique failed to show any of the flavonol compounds when samples were processed through HPLC. Possible explanations include delays in between extraction and HPLC analysis inducing breakdown

of the flavonol compounds within the SPE tubes themselves. Alternatively, the SPE tube sorbent may have been incorrectly loaded by the supplier or a revised protocol not provided with the tubes.

HPLC was performed on the crude extract that accompanied the SPE tubes and the results, although highlighting differences between samples, were disappointing in that a comparative table of retention times for the samples AVF/non-AVF / UV-B AVF / UV-B non-AVF would have explored the possible above-ground interaction between AVF inoculation and flavonoid output and UV-B exposure and flavonoid output.

The results demonstrate that it is evident that the traces are different with a number of major peaks being identified for one sample and not the other. A number of the other peaks are however present in both chromatograms as would be expected. Three target flavonols were reliably identified and quantified abeit at low concentrations.(section 7.3.1.4.). SPE procedure allowed generic flavonoids to be identified in higher concentrations with evidence of four novel flavonoids being synthesised by the presence of AVF inoculation. It was also demonstrated that the relative concentrations of likely target flavonols had been modified in response to UV-B radiation. Results from the irradiated AVF / non-AVF separations (tables 7.3 and 7.4.) again demonstrated differences in peak values and novel flavonoid metabolites induced in samples.

9.9.2. Conclusions.

Plant growth and reproductive output is regulated by environmental factors throughout its life cycle. Particular responses are elicited in response to a range of environmental factors which act as cues to initiate developmental transitions. Some environmental signals are potentially harmful and in this case the plant's response is to minimise potential damage. Examples of abiotic stresses include drought, extremes in temperature and UV-B radiation.

Response to UV-B radiation have been examined at the whole plant level in this study and evidence of leaf shortening and epidermal thickening were seen. For this to occur signals must be perceived and responded to a cellular level. Changes in flavonoid metabolites (both qualitative and quantitative) were seen in UV-B treated plants in this study. Such responses therefore rely on signals being detected by specific cellular components termed receptors. Reception is linked to the terminal response by transduction mechanisms. This signal transduction process normally serves to amplify the initial signal but this signal may not have immediate effects.

UV-B irradiation has well documented effects which are outlined in chapter 2 and elsewhere throughout this report many of which are known to be damaging to cellular components such as DNA. Such damaging effects are likely to be caused by direct absorption of UV-B by the molecules themselves (Mitchell & Karentz, 1993) and further damage may result indirectly from the generation of reactive oxygen species (Green & Fluhr, 1995). It is clear from this study that not all the effects of UV-B on plants cause macromolecular damage. The synthesis of UV absorbing pigments such as the flavonoids examined in this study demonstrate that rather than a damage response in this species

the UV-B radiation is stimulating the expression of particular genes. In this case it is likely

that a specific set of UV-B photoreceptors is involved. The possible mechanisms of UV-B

perception are given in sections 3.3. and 4.2. Photo-morphogenical aspects of UV-B

exposure were seen in the form of leaf shortening and epidermal thickening. There is

evidence of a UV/A/blue photoreceptor named CRY1 (cryptochrome) that mediates

several growth responses to UV-A/ blue light in *Arabidopsis* (Ahmad & Cashmore).

Interestingly, further research (Fuglevand, Jackson and Jenkins, 1996) provided evidence

that separate UV-B receptors regulate the expression of the *Arabidopsis* CHS gene. (Blue

light within the visible range was produced in addition to the UV-B by the Phillips TL20

lighting regime). The nature of a dedicated UV-B receptor would probably be a protein

with flavin and/or pterin chromaphores. Pterins are likely candidates for the

chromophore of a UV-B receptor as their absorption spectra are dependent on their

redox state and a reduced pterin would be able to absorb in the UV-B spectral region.

(Gallard & Senger, 1988a).

Many classes of flavonoids are implicated in biological activity and most are notorious for

their antioxidant properties. Flavenoids also appear to be complicit in directly interacting

with transport and signal transduction pathways. One example is the importance of

flavonoids in fertility. Although a minority of flavonoid-deficient plants are able to

germinate, grow and set fertile seed, the majority of plants require flavonoids to ensure

fertility and normal pollen development (Napoli *et al* 1999). Flavonoid modulation of

auxin transport in addition to local auxin accumulations are observed during nodulation

(Novak, *et al* 2002). Flavenoid signalling is seen as playing a role in the mediation of

interactions between the plants themselves and also with other organisms in the

environment. It is seen at competitive and cooperative levels where mycorrhizal association is involved. (Garcia-Garrido, 2002).

Induction of increased secondary metabolites by enhanced UV-B radiation acts not only as UV filters but also in other ecological relationships. UV-B radiation has been demonstrated as having the potential not only to change litter decomposition, carbon cycling and herbivory in terrestrial ecosystems, but also to change symbiotic relationships between higher plants and micro-organisms (Stacey, 1997). Isoflavenoid phytoalexins are both expressed and suppressed by fungal plant symbionts (Harrison, 1993). It is possible that plant compounds, specifically flavonoids, are implicated in the level of microbial communities in the rhizosphere as the symbiotic relationship between plants and mycorrhizal fungi become less efficient under an elevated UV-B environment. It has been demonstrated that UV-B radiation can induce significant reductions in the number and size of nodules produced in rhizibium- plant symbiosis and a reduction in the number of arbuscules in mycorrhysal fungal associations (Rozema, 1999). Quercetin was seen to accumulate in roots of clover inoculated with mycorrhizae but was not seen in non-inoculated plants (Ponce *et al,* 2004). In a five year study it was found that increased solar UV-B radiation reduced infection by mycorrhizal fungi in an area dominated by the species *Calamagrostis epigeios* and *Carex arenaria* by 18% and 20% respectively (van de Staaaij *et al,* 2001).

Predictions interactions of species in ecosystems based on studies of plants in isolation is impossible. It is possible to postulate changes in competitive balance brought about by one species being detrimentally affected by UV-B radiation more than another. If this occurs, then even a small degree of perturbation would magnify into significance over

time. This study has highlighted morphological changes and alterations in plant secondary chemistry. Differences in the ratios and concentrations of flavonol metabolites found between treatment groups (tables, 7.6.2., 7.4.) may have health and oncological implications in human populations in closely related edible *Allium* species. If the morphological changes found were to apply growing in a mixed-species stand even subtle changes in plant height may affect inter-specific competition by reducing the ability to compete for light. Seed and/or bulbil dispersal may also be affected by the limiting effects on the variance values of inflorescence weights by the effect of UV-B on AVF inoculated plants therefore influencing the development of reproductive inequality. (section 6.6).

 Mycorrhizal inoculation may influence plant fitness by having effects that carry over to the next generation by increasing seed/bulbil quality and resultant offspring fecundity. Germination trials between AVF and non-AVF derived seeds/propagules might provide interesting results in further experiments. This experiment has examined the effects of AMF inoculation on female reproductive output even though the potential influence on male function is large. Half of the genetic sporophytic material is inherited through pollen. The effects of AVF on pollen quality or production is largely unknown in this species. One conclusion arising from this study and related literature is that the effect of supplementary UV-B radiation in this species of Allium is idiosyncratic due to epigenetic modifications (permanent methylation of genes following histone modification) that have led to a decrease in phenotypic plasticity in this sampled population and will be further discussed in the following chapter.

Chapter 10

Overview and further investigations.

10.1. Evidence for a UV-B photo-receptor.

The physiological pathways and genes controlling flowering in *Arabidopsis thaliana* have been partly characterised. Studies have identified pathways that either promote or repress the transition of vegetative meristem to floral meristem leading to inflorescence development. The inputs from the various pathways are integrated and subsequently regulated. Inflorescence meristem identity genes are eventually activated (figure 10.1., adapted from Levy and Dean, 1998a). The species *A. vineale* may contain a dedicated UV/B receptor. Molecular information on this photo-receptor named CRY1* (Ahmad and Cashmore, 1993) mediates several responses to UV-A / blue light in *Arabidopsis hy4* mutant alleles, (deficient in CRY1) have provided evidence of its function from phenotypic characterisation. The original *hy4* mutant, *hy4-2,23N* (Koornneef *et al* 1980) appears to be a null mutant and has longer hypocotyls than the wild-type in blue, UV-A and green light. In addition, it has modified extension growth responses and has reduced blue light induction of CHS,CHI and DFR transcripts (Jackson and Jenkins,1995). Anthocyanin induction by blue light is also greatly reduced. Interestingly, *hy4-2.23N* is not altered in the UV/B induction of CHS transcripts (Fuglevand, Jackson & Jenkins,1996) providing genetic evidence that separate UV-B and UV-A/ blue photoreceptors regulate expression of the *Arabidopsis* CHS gene.

Figure 10.1. (overleaf). Proposed representation of the relationship between UV/B radiation and somatic growth / flowering in *A. vineale.*

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Legend box (top right):
Increase in somatic / flowering competence.
Decrease in somatic/ lowering competence.
UV/B light.   Ambient light

Diagram labels:

VISIBLE LIGHT   UVB

UV/A

UVB

uvb

PHOTORECEPTION

SIGNAL TRANSDUCTION

TRANSCRIPTION FACTORS.

BZIP myb

PAL   4- COUMARYL –COA

PHENYLALANNE

SINAPTIC ACID ESTERS

CHALCONE SYNTHASE

CHI   NIAGRIN CLALCONE

5'   3'

LRU2   LRU1

DISTURBANCE OF PHOTOSYSTEM 11.THYLKALOID MEMBRANE FUNCTION.

DAMAGE TO MEMBRAME PPPROTEINS .PEROXIDAT ION OF FATTY ACIDS.

NIAGRIN

FLAVONES

3-OH FLAVENONES

DEWER PRODUCT

GLYCLOBUTANE DIMER

PHOTO-DAMAGE

IAA AND FLAVENOID SCREENING PRODUCTS INTERACT. SOMATIC GROWTH

PHOTO-PRODUCT

FLAVO NOIDS

PHOTO-HORMONES

AUTONOMOUS PROMOTION

VRN
VERNALISATION

COMPETANCE

FPA FVE

FAR- RED

+ +

AVF MYCORRHIZAL INFECTION. PHOSPHORUS INCREASE. PATHOGEN RESISTANCE.. PHOTOMORPHISM.

VEGETATIVE MERISTEM

FLF

PHY ELF3 CRY1* BLUE LIGHT RECEPTOR / UV/B RECEPTOR.

LFY   AP1
AG   TFL1

UVB DECREASES ARBUSCULAR NUMBERS IN AVF. RESTRICTS EXUDATES AFFECTS SIGNALLING AND HOST RANGE SPECIFICITY. STOMATAL OPENING

PHOTOPERIOD
CO FWA FT

REPRESSION
EMFI FRI FLC TFL1 CLF

EPIGENETIC MRTHYLATION MODULATED BY LONG STRAND RNA FOLLOWING HISTONE ACYLETATION * SEE TEXT

CAPITALS IN GREEN DENOTE KNOWN GENES IN *A. THALIANA*..

FLORAL MERISTEM

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As described in previous chapters, levels of screening flavonols together with photomorphogenic changes were shown to be modified by supplementary UV/B light in this study. Floral initiation was delayed by an enhanced nutrient regime but promoted by inoculation of AVF in this population of *A. vineale.* This is in itself a surprising result. Research into aspects of floral initiation in *A. thaliana* shows that vernalization looks largely epigenetic. The processes underling this have been confirmed at the chromatin modification level. Figure 10. 1. shows the overlying effects of UV-B light on terrestrial plants and the key gene involved in flowering is known as flowering locus C (FLC).

10.2 Epigenetic mediation of flowering.

Epigenetics is the study of heritable changes in gene expression or cellular phenotype without change in DNA. Epigenetic regulation of gene expression is accomplished by DNA methylation, histone modifications, histone variants, chromatin remodelling, and may involve small RNAs. DNA methylation at cytosine is carried out by enzymes called DNA Methyltransferases and is involved in many cellular processes, such as silencing of transposable elements and pericentromeric repeats (Ahmada 2010). X-chromosome inactivation and genomic imprinting, etc. Histone modifications refer to posttranslational covalent attachment of chemical groups onto histones such as phosphorylation, acetylation, and methylation, etc. Histone variants, the non-

canonical histones with amino acid sequences divergent from canonical histones, can have different epigenetic impacts on the genome from canonical histones. Higher-order chromatin structures maintained or modified by chromatin remodelling proteins also play important roles in regulating gene expression. Small non-coding RNAs play various roles in the regulation of gene expression at pre- as well as posttranscriptional levels (Heo & Sung 2010).

10.3. Epigenetic mechanism for vernalisation.

FLC FLOWERING LOCUS C (FLC) has a key role in the timing of the initiation of flowering in Arabidopsis and encodes a transcriptional repressor that binds to FT, SOC1 and FD.(Deng *et al* 2011). Before a cold period (winter) the FLC gene promoter carries multiple histone modifications that promote gene expression. Consequently, the FLC gene is highly expressed and represses target genes by binding them with the produced protein it is coded to produce. Vegetative growth follows as normal. Following winter, however, the histone modifications seen on the FLC gene switch from promoter to repressive states. Increased sunlight levels during spring initiate the expression of the FT gene. Crucially now, FLC levels have decreased. If FLC levels have not decreased, the FT gene cannot respond to the stimulus of sunlight (Dennis and Peacock, 2009).

Experiments with epigenetic enzymes in mutated versions of *A. thaliana* show histone modifications at FLC gene sites are integral in flowering response. A gene SDG27 ads methyl groupings to lysine at position 4 on histone H3 is a known epigenetic writer known to promote gene expression (Ahmad et al 2010). If SDG27 is mutated

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experimentally to disable its active protein, plants have less of active histone modification at the FLC gene promotor and subsequently less active FLC protein and do not then suppress the genes that suppress flowering. These mutants flower prematurely (Pien *et al* 2008). This demonstrates that epigenetic modifications can both alter expression and activity level of a gene. Cold weather induces the VIN3 protein in plants (Sung and Amasino 2004). This may bind to the FLC promoter and acts as a chromatin re-modeller (opening up the local structure of the chromatin and leading to an increase in gene expression). In this case VIN3 can add methyl groups to histone proteins by attracting an enzyme. Lysine is thus methylated at position 27 on H3. This represses gene expression and is the primary mechanism for switching off FLC (De luca *et al* 2008).

10.3.1. Targeting mechanism.

The precise targeting mechanism has not been fully described. However, one of the stages has been described. Following exposure to cold periods, cells in A. thaliana. Produce long non-coding RNA. This RNA chain has been called COLDAIR and is localized specifically at the FLC gene where it binds to the enzyme complex. This creates the significant repressive mark at position 27 on histone H3. COLDAIR therefore acts as a target for the enzyme complex (Heo and sung 2011).

When A. thaliana sets seed the repressive histone marks on the FLC gene are removed and replaced with activating chromatin modifications thereby ensuring that when germination occurs, the FLC gene is switched on and repress flowering until a cold period re-initiates the process.

Sample plants of A . vineale in this study did not flower and no seed was set. Floral initiation however was modified by experimental manipulation as previously described. Autonomous promotion due to bulb size remains an unsatisfactory explanation due to the fact that plants treated with an enhanced nutrient regime flowered displayed a temporal delay in floral initiation.

Bulbil derived plants do not have the luxury of transposable elements found in seed endospermic tissue to remove dangerous genomic elements or any way of removing methylation from genes using paternally derived DNA.

There is also the question of the resetting of the repressed activity level of the FLC gene, following vernalisation, to the default high activity level and whether it occurs during both male and female gametogenesis.

Regulation of expression of seed transcriptome, involvement of direct tandem repeat elements in the PHE1 imprinting in addition to PcG proteins activity, paramutation, and epigenetic barriers in species hybridization currently areas of research (Ahmed *et al* 2010).

The complex multigenic system which is responsible for the patterns of gene activity which bring about hybrid vigour in crosses between genetically similar but epigenetically distinct parents is reviewed by Groszman *et al,* where epigenetic systems have been identified as contributing to the heterotic phenotype. They are identified as the 24nt siRNAs and their effects on RNA dependent DNA methylation (RdDM) at the target loci is leading to changed expression levels. If this is correct then it should provide a fitness benefit to sexually derived plants leading to fixation within a population.

What are the proposed mechanisms of the reduced transcription and the memory of

the vernalisation treatment through vegetative development seen in successive bulbil

derived generations of *A. vineale?* It is still possible that all previous research into the

subject of sexual allotment in this species failed to note correctly whether individual

plants ever fully reverted to flowering after previously <u>exclusively</u> producing bulbils.

However, a further experiment using successions of both clonal bulbil derived

generations of plants grown together with seed derived plants followed with

molecular investigations might resolve some of the epigenetic questions regarding

vernalisation and other molecular memory issues.

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Laboratory recipes

Sulphur dioxide water

50ml of lM HCl was added to 1l of distilled water. 5g of potassium metabisulphite was dissolved in this, the solution being made up fresh each time.

Subbing solution

A 1:1 mixture of 1% potassium dichromate and 1% gelatine (w/v) was made up. The solution was used without dilution for pre-subbing slides. 4 ml were added per litre to the water bath subbing solution.

Light green stain

A 0.2% w/v solution was made of Light green stain, in 98% ethanol.

Clarke's Fixative

3:1 (v/v) mixture of absolute ethanol and glacial ethanoic acid.

Feulgen Stain

4g of Basic fuchsin (pararosalanine) was dissolved in 800ml of boiling distilled water. This solution was allowed to cool to 50°C and then filtered. 120ml of 1M HCl and 12g of potassium metabisulphite were added to the filtrate and left overnight in the dark. 2g of de-colourising charcoal was added, and the mixture filtered. The stain was stored in the dark at 4°C
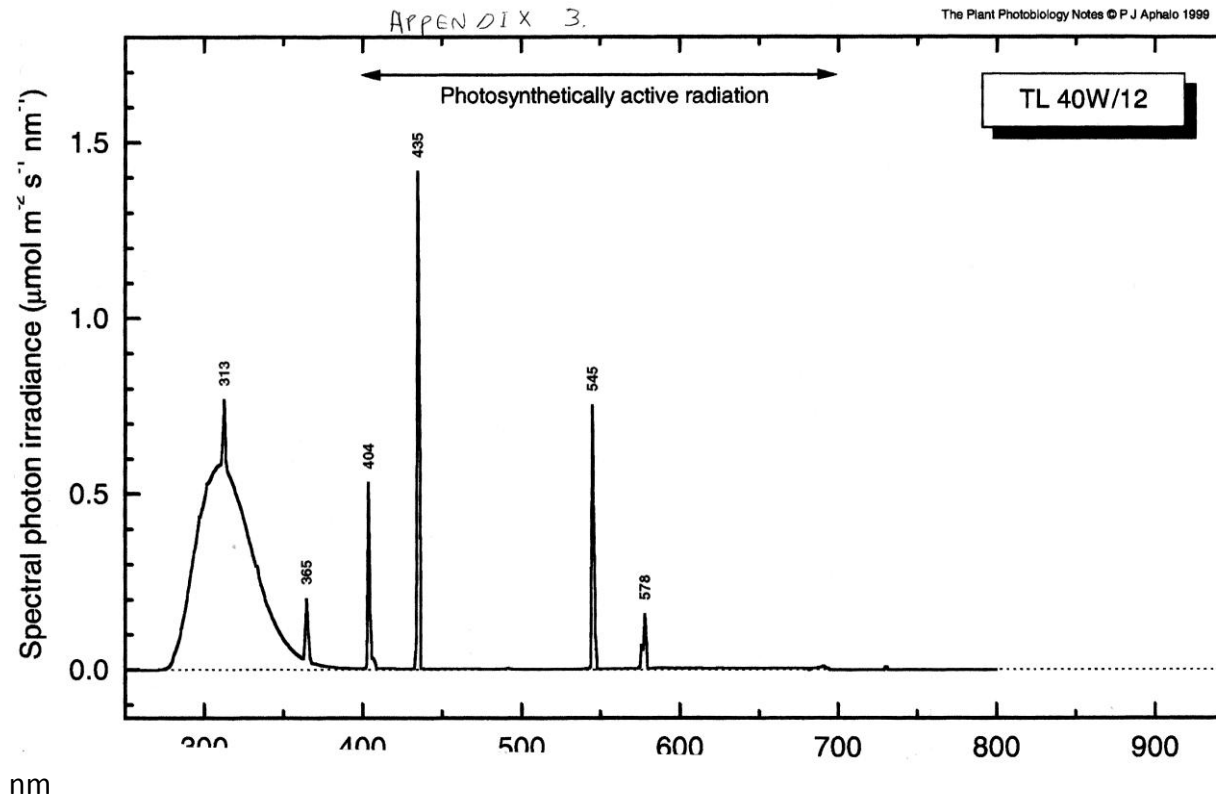
Appendix 2.

Point of collection of *Allium vineale.*

Spectral output of Phillips TL40/12 lamps.
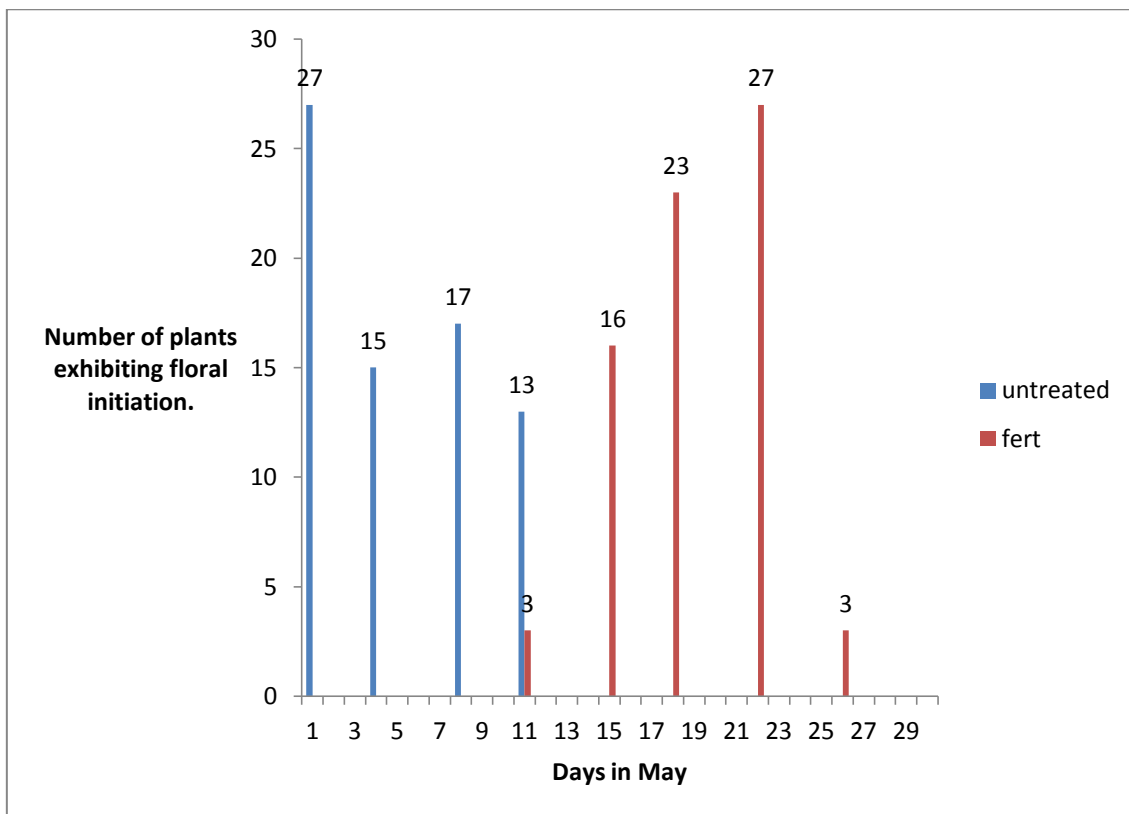
Appendix 3.

APPENDIX 3.

Photosynthetically active radiation

TL 40W/12

Spectral photon irradiance (μmol m^{-2} s^{-1} nm^{-1})

313
365
404
435
545
578

1.5
1.0
0.5
0.0

300   400   500   600   700   800   900

nm

Appendix 4.

Staining procedure for *Allium* leaf sections (modified from Herbert 1991;Cottrell 1999)

treatment	Time (min.)
Histo-Clear	20
100% ethanol	5
70% ethanol	5
50% ethanol	5
30% ethanol	5
Distilled water	5
1M Hydrochloric acid at 60°C	8
Schiffs Reagent	60
45% Ethanoic acid	1
Sulphur dioxide water	1
Sulphur dioxide water	1
30%ethanol	5
50% ethanol	5
70% ethanol	5
100% ethanol	5
Light green stain	20 seconds
100% ethanol	1
100% ethanol	1
Histo-Clear	1

During the investigation into the effect of fertiliser in the UV B irradiated and untreated plants (Chapter 5) the number of plants displaying spathe eruption was recorded twice weekly (Tuesdays and Fridays) during May. This was a clear stage in the plants development when the leaf displayed a characteristic light green discolouration over the erupting spathe.

All plants treated with fertilizer exhibited an average observable ten day delay in floral initiation regardless of their lighting regime.

Miracle-Gro plant food UK fertiliser declaration.

N.P.K. blend 24-8-16

Nitrogen	24%	
Ammoniacal nitrogen	3.5%	
Ureic nitrogen		20.5%
Phosphorus pentoxide soluble in ammonium citrate and in water		8%
Phosphorus pentoxide soluble in water		8%
Potassium chloride soluble in water	16%	
Boron. Soluble in water		0.02%
Coppersoluble in water		0.03
Iron. Soluble in water	0.19	
Manganese. Soluble in water	0.05	
Molybdenum. Soluble in water		0.03

Appendix 7.

Regression table. (for calibration curves- see section 7.3.1.2.).

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.99578
R Square	0.991578
Adjusted R Square	0.98877
Standard Error	0.837779
Observations	5

ANOVA

	df	SS
Regression	1	247.8944
Residual	3	2.105623
Total	4	250

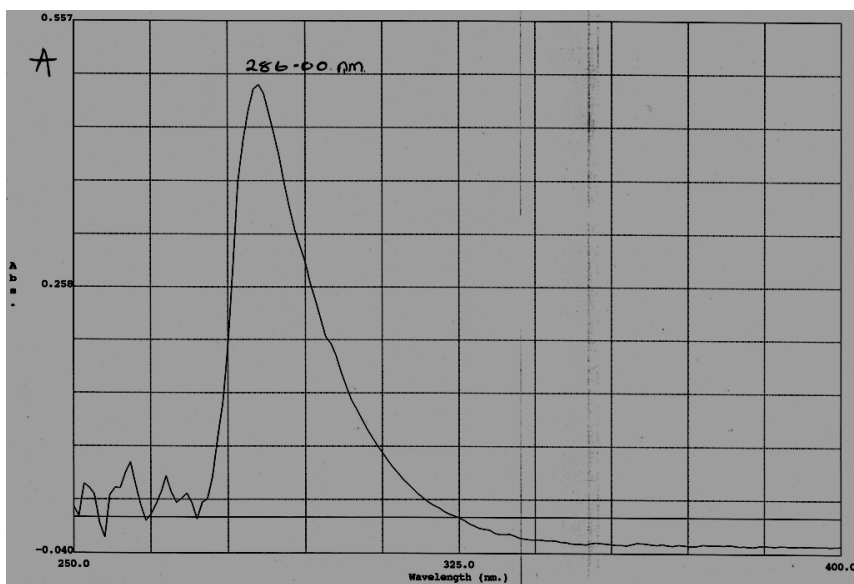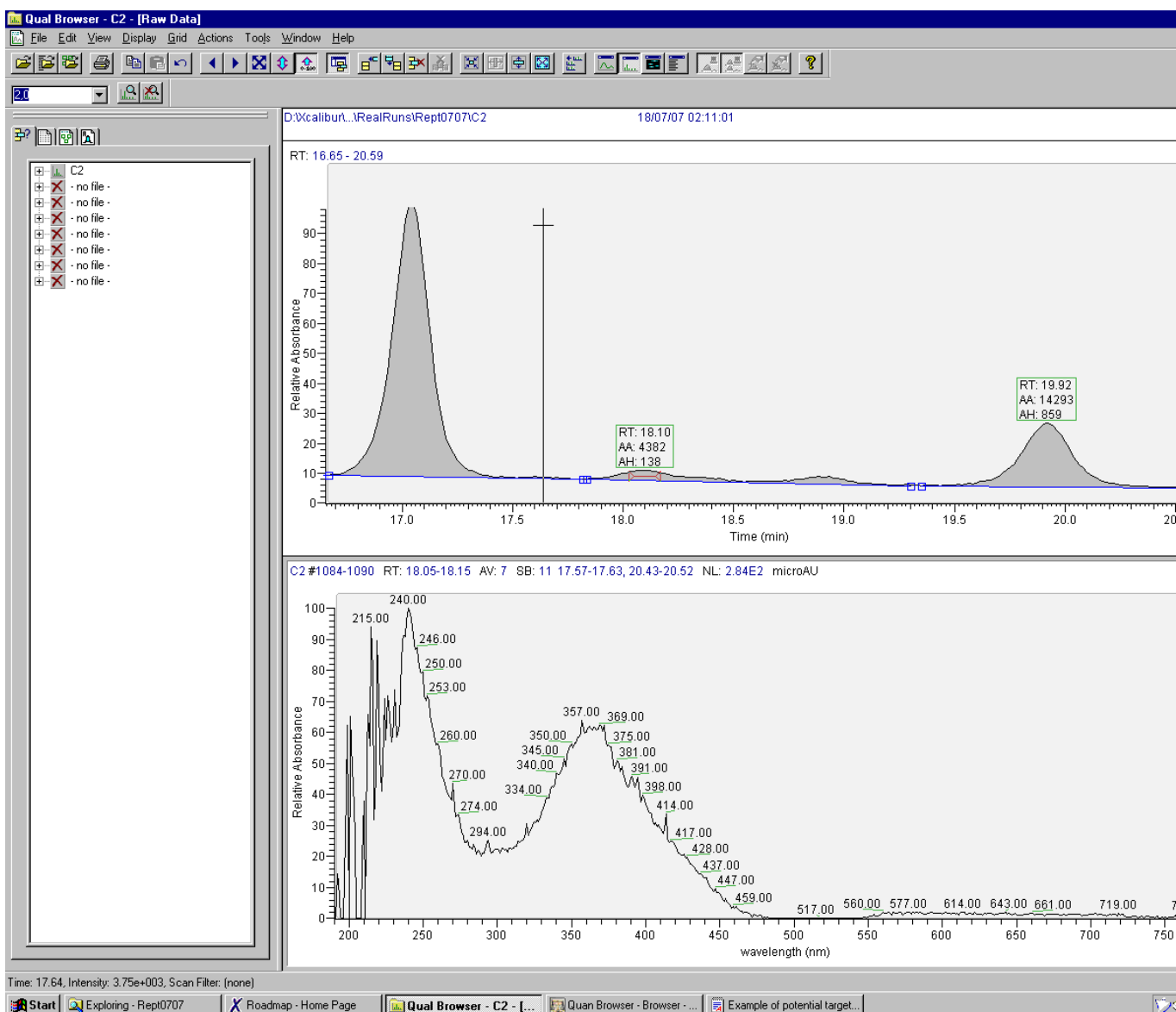|	Coefficients	Standard Error
Intercept	3.506715	0.717205
X Variable 1	0.000188	9.99E-06

Appendix 8.

Absorbance spectra of rutin showing bathochromatic shift characteristic of the flavonoid group of phenolics.
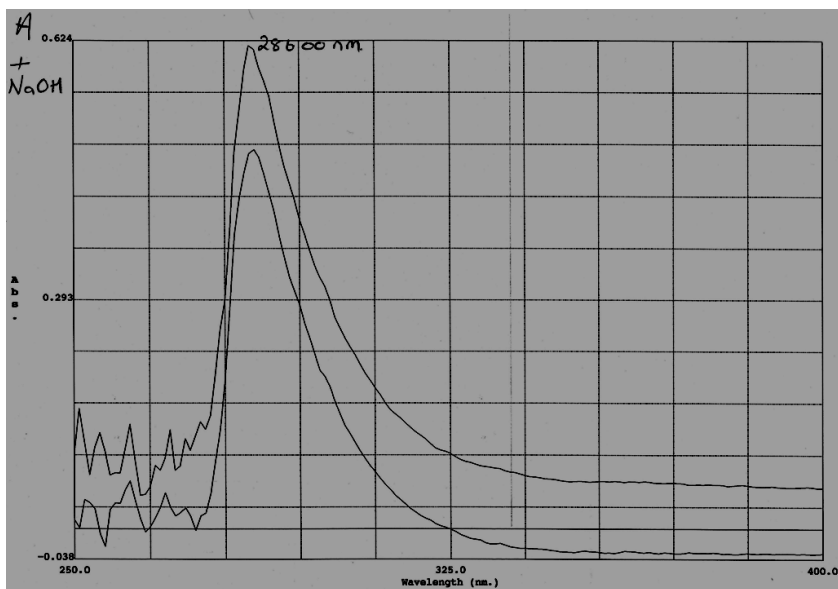
Absorbance spectra of rutin.

Appendix 9.

Appendix 10.

Leaf length measurements.

Descriptive statistics and Z test.

F calc = 1.088 F crit = 1.51

Sed = $\sqrt{\dfrac{.0491 + .0451}{72 + 72}}$ = .03784.   Z calc = $\dfrac{1.57611 - 1.4405}{.03784}$ = 3.5978

Z crit = 2.326 @ P = .005 for a two tailed test.

Cont	
Mean	1.576111
Standard Error	0.026121
Median	1.57
Mode	1.39
Standard Deviation	0.221643
Sample Variance	0.049126
Kurtosis	-1.22828
Skewness	0.14336
Range	0.8
Minimum	1.19
Maximum	1.99
Sum	113.48
Count	72

uvb tr	
Mean	1.440556
Standard Error	0.025041
Median	1.4
Mode	1.4
Standard Deviation	0.212476
Sample Variance	0.045146
Kurtosis	-0.02069
Skewness	0.008683
Range	1
Minimum	0.9
Maximum	1.9
Sum	103.72
Count	72

Appendix 11.

Calibration curve data for solid phase extraction (section 8.5).

Component Name		Curve Index	Weighting Index	Origin Index	Equation
Quercetin		Linear	Equal	Force	Y = 6948.09*X R^2 = 0.98

Filename	Sample Type	Sample Name	Sample ID	Exp Amt	Calc Amt	Units	%I
GS10	Std Bracket Sample		1	10.000	9.536	ugcm3	
GS15	Std Bracket Sample		1	15.000	13.510	ugcm3	
GS20	Std Bracket Sample		1	20.000	20.064	ugcm3	
GS25	Std Bracket Sample		1	25.000	26.091	ugcm3	
GS5	Std Bracket Sample		1	5.000	4.685	ugcm3	

Component Name		Curve Index	Weighting Index	Origin Index	Equation
Rutin		Linear	Equal	Force	Y = 4839.45*X R^2 = 0.9959

Filename	Sample Type	Sample Name	Sample ID	Exp Amt	Calc Amt	Units	%Dif
GS10	Std Bracket Sample		1	10.000	10.333	ugcm3	
GS15	Std Bracket Sample		1	15.000	14.092	ugcm3	
GS20	Std Bracket Sample		1	20.000	20.147	ugcm3	
GS25	Std Bracket Sample		1	25.000	25.310	ugcm3	
GS5	Std Bracket Sample		1	5.000	4.924	ugcm3	
	Linear	Equal	Force		Y = 7937.2*X R^2 = 0.9880		

Kaempferol

	Sample						
Filename	Type	Sample Name	Sample ID	Exp Amt	Calc Amt	Units	%
GS10	Std Bracket Sample		1	10.000	9.950	ugcm3	
GS15	Std Bracket Sample		1	15.000	13.500	ugcm3	-
GS20	Std Bracket Sample		1	20.000	20.037	ugcm3	
GS25	Std Bracket Sample		1	25.000	25.977	ugcm3	
GS5	Std Bracket Sample		1	5.000	4.562	ugcm3	

Y = 19165.5*X+222.667*X

Isorhamnetin   Quadratic   Equal   Force   R^2 = 0.9956

	Sample						
Filename	Type	Sample Name	Sample ID	Exp Amt	Calc Amt	Units	
GS10	Std Bracket Sample		1	10.000	10.705	ugcm3	
GS15	Std Bracket Sample		1	15.000	14.214	ugcm3	
GS20	Std Bracket Sample		1	20.000	20.037	ugcm3	
GS25	Std Bracket Sample		1	25.000	25.124	ugcm3	
GS5	Std Bracket Sample		1	5.000	5.192	ugcm3	

Jeremy Cox

From:	Gerwyn Thomas [GerThomas@carmarthenshire.gov.uk]
Sent:	19 May 2004 14:35
To:	Jeremy Cox
Cc:	Elwyn Hughes
Subject:	FW: Allium vineale

Dear Mr Cox,
Thank you for your enquiry Re: the collection of Allium.
If as I understand it the location your intrerested in is Cwmoernant in Carmarthen then I have no problem with this.
If you need to collect from other locations then I would need to know where.
The only thing I ask in return is if you be so kind as to forward a copy of your findings so that I add this to our files.
If you require any further help, please do not hesitate to get in touch.

Regards

Gerwyn Thomas
Senior Urban Parks Officer
01554 747503

-----Original Message-----
From: Elwyn Hughes
Sent: 19 May 2004 12:18
To: Gerwyn Thomas
Subject: FW: Allium vineale

Can you handle this please asap. Relates to Cwmoernant Ponds. Tie up with CCW?

Elwyn
-----Original Message-----
From: Jeremy Cox [mailto:Jeremy.Cox@herefordpct.nhs.uk]
Sent: 19 May 2004 12:00
To: 'ehughes@carmarthenshire.gov.uk'
Subject: Allium vineale

Dear Mr Hughes, Thank you for your help today. As I outlined on the telephone, I am a part-time PhD student and I need to collect specimens of Allium Vineale currently growing within your local authority. I require written permission. I can confirm that the species is neither rare or protected. My University Tutors contact address is :

Dr Debbie Holmes,
University College Worcester,
Henwick Grove,
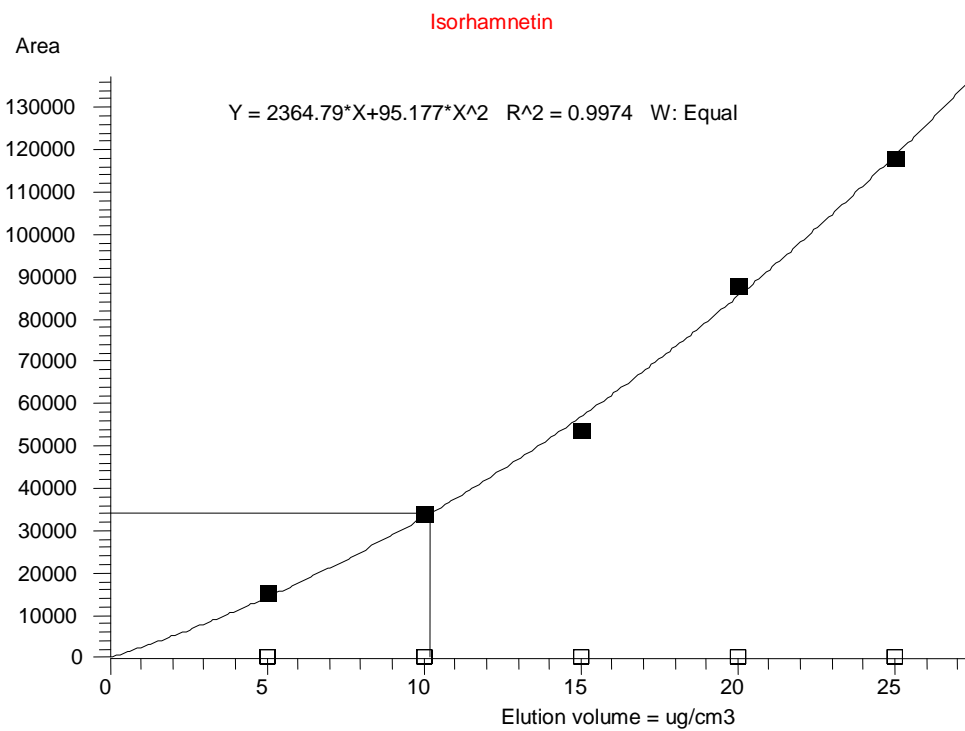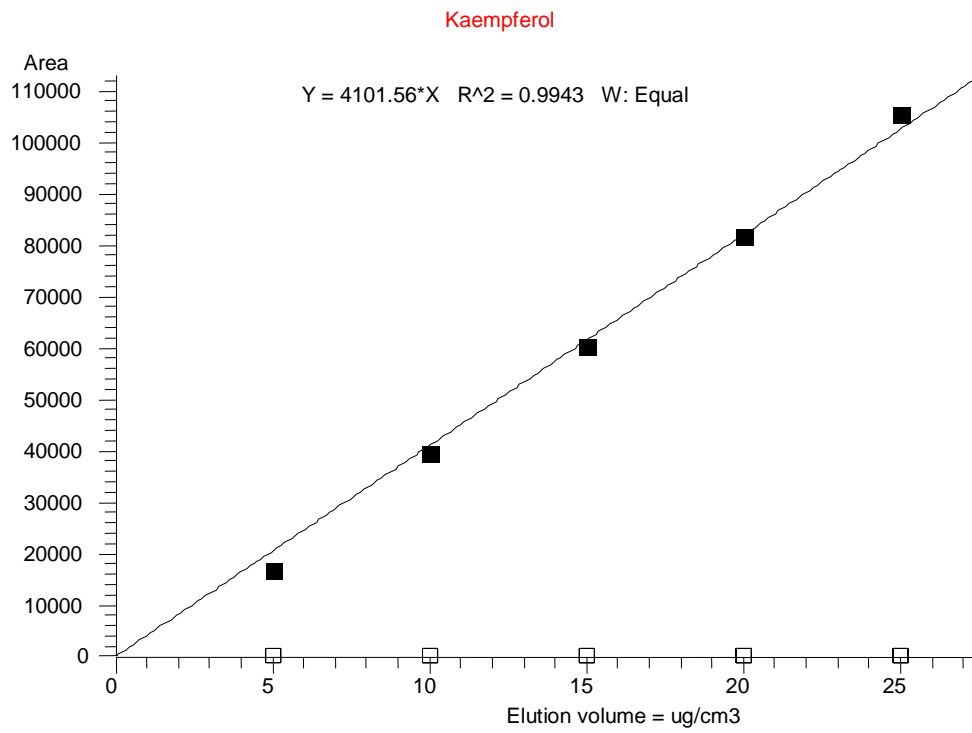Worcester.
Tel 01905855000 .

Please forward any correspondence to my home address - Golden Post House, Allensmore, Hereford HR2 9AA
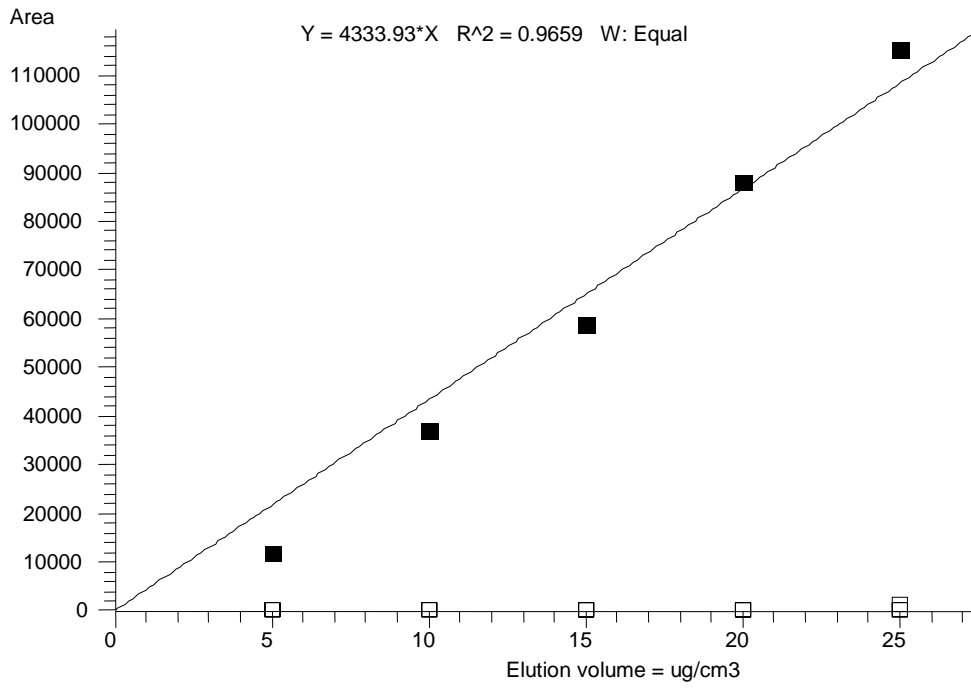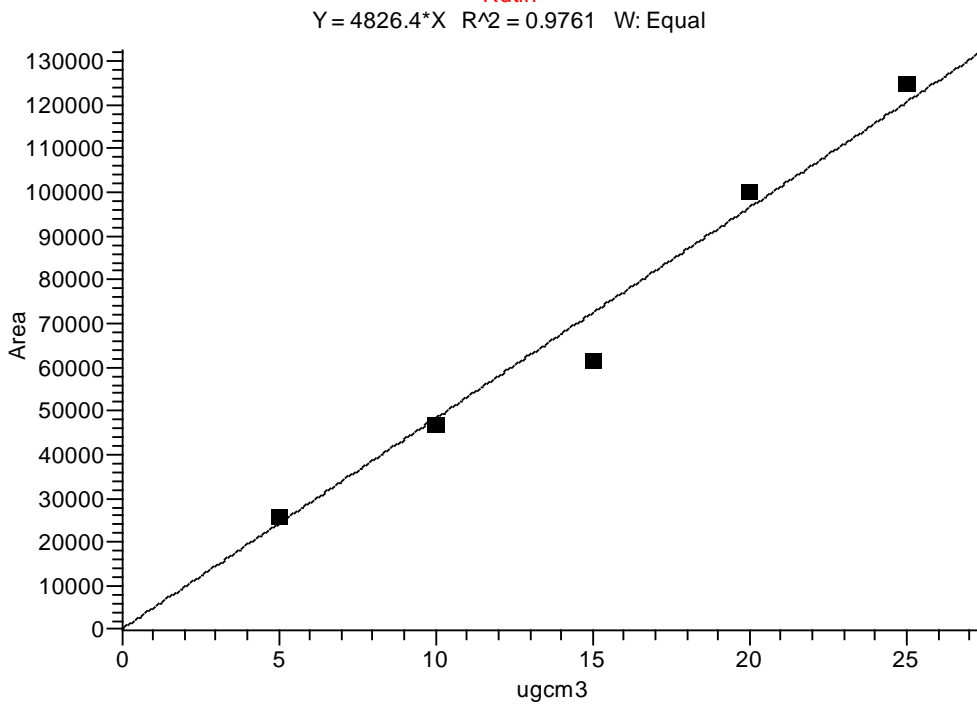
Many thanks, Jeremy Cox.

20/05/2004

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Appendix 13.

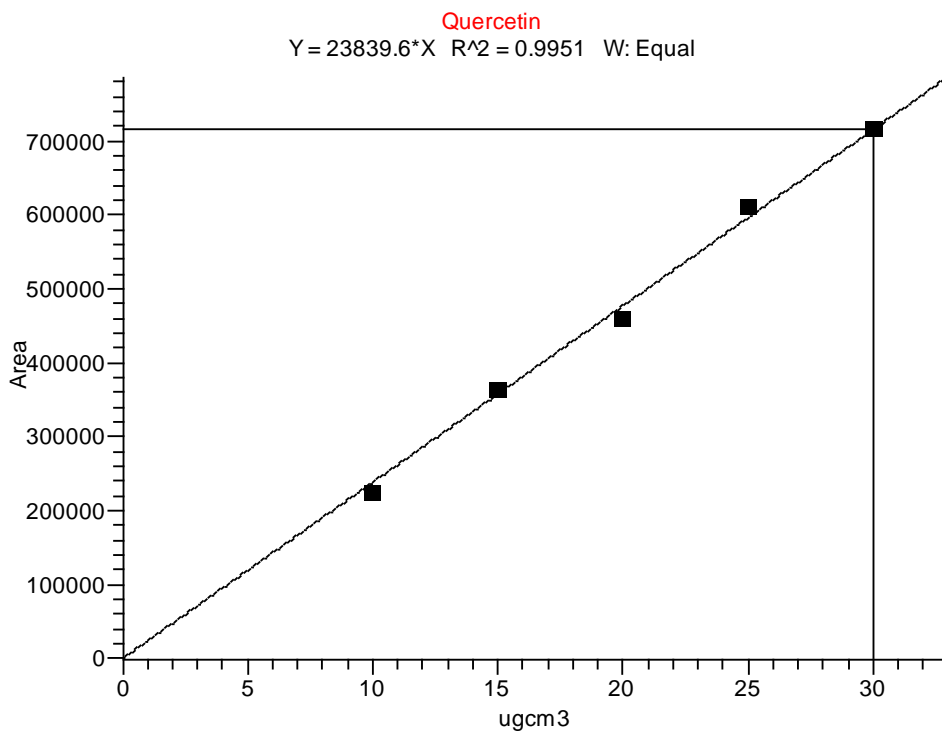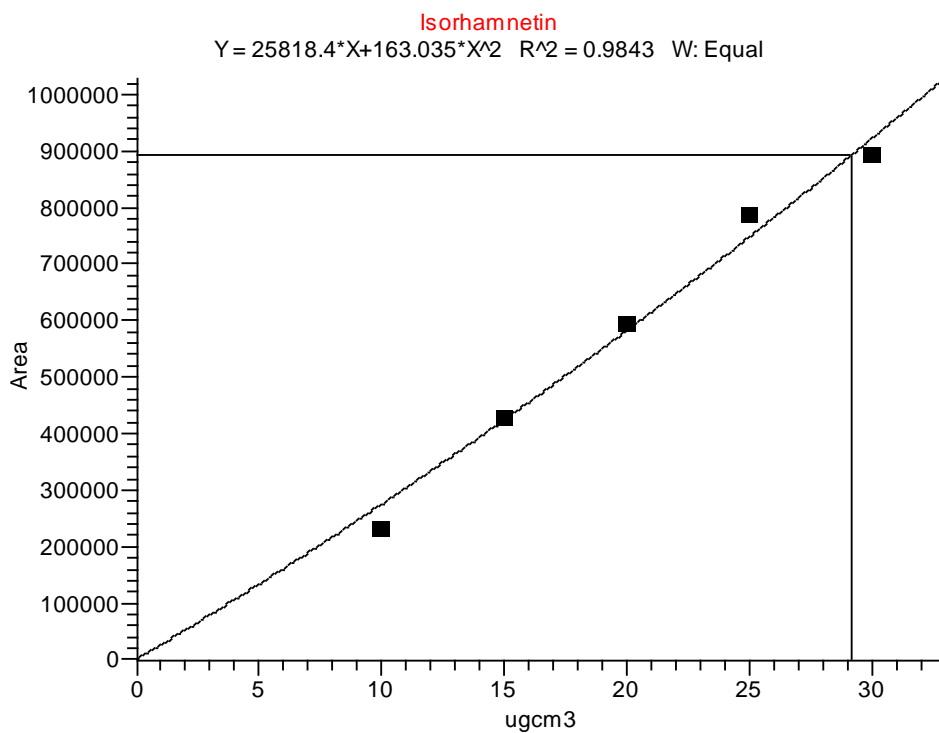## Calibration curves for HPLC section 7.4.5.

Kaempferol

Area

$Y = 4101.56*X \quad R^2 = 0.9943 \quad W: Equal$

Elution volume = ug/cm3

Isorhamnetin

Area

$Y = 2364.79*X+95.177*X^2 \quad R^2 = 0.9974 \quad W: Equal$

Elution volume = ug/cm3

Quercetin

Y = 4333.93*X   R^2 = 0.9659   W: Equal

Area

110000

100000

90000

80000

70000

60000

50000

40000

30000

20000

10000

0

0   5   10   15   20   25

Elution volume = ug/cm3

Rutin

Y = 4826.4*X   R^2 = 0.9761   W: Equal

130000
120000
110000
100000
90000
80000
70000

Area

60000
50000
40000
30000
20000
10000
0

0   5   10   15   20   25

ugcm3

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Appendix 14.

Calibration curves for Chapter 9.

Isorhamnetin
Y = 25818.4*X+163.035*X^2   R^2 = 0.9843   W: Equal

Quercetin
Y = 23839.6*X   R^2 = 0.9951   W: Equal

Kaempferol
Y = 20497.9*X   R^2 = 0.9785   W: Equal
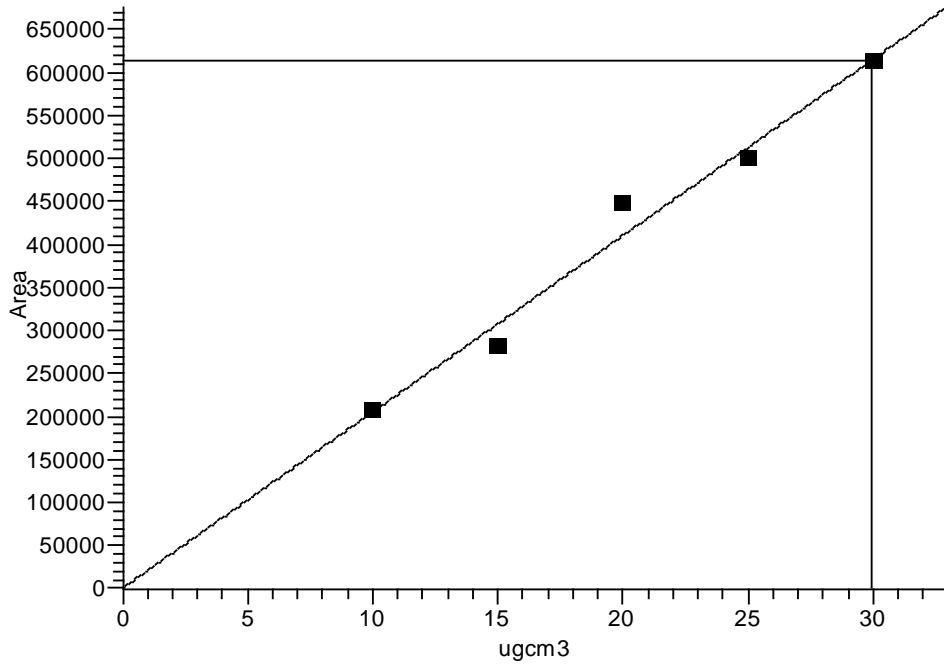
Rutin
Y = 9910.98*X   R^2 = 0.9939   W: Equal