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For accurate reporting, materials have generally been referred to by the name of the commercial product. No endorsement is intended of products mentioned, nor criticism of those not mentioned.

PRACTICAL SECTION

OBJECTIVES AND BACKGROUND

There is a demand for snowdrop bulbs that has, in the past, been met from bulbs collected from the wild, which is no longer acceptable. However, the crop is difficult to exploit commercially: there are difficulties in obtaining good stocks, in growing snowdrop bulbs satisfactorily in the field, and in storing bulbs. This project addresses these three aims:

- *Micropropagation* - to develop *in vitro* systems that sustain high vegetative propagation rates and yield superior quality, uniform bulb stock;
- *Agronomy* – to develop novel growing systems for effective commercial production of snowdrop bulbs;
- *Storage* – to investigate more suitable bulb storage regimes for snowdrops.

Success with these objectives should enable the industry to increase snowdrop bulb sales, both of dry bulbs sold in bulk, and of choice species sold in growth.

SUMMARY OF RESULTS

The main aim of the of the micropropagation work is:

The initial work on *micropropagation* has concentrated on finding appropriate methods for achieving sterile cultures of bulb explants, using *Galanthus nivalis*, *G. nivalis* Flore Pleno and *G. elwesii*. High concentrations of hypochlorite were necessary to achieve surface sterilisation of explants. Hot-water treatment, prior to surface sterilisation, increased (rather than decreased) subsequent contamination. Treatment with a commercial product, 'Plant Preservation Mixture' (PPM), after surface sterilisation with lower concentrations of hypochlorite, gave increased control of contamination, although high concentrations of PPM initially inhibited the growth of explants. Following successful surface sterilisation, there were additional benefits of incorporating fungicides in the culture medium: a mixture of carbendazim and imazilil completely eradicated residual infection. Steeping explants in imazilil before inoculation was highly effective in controlling infection, particularly when combined with incorporating imazilil in the culture medium. There were early indications that *in vitro* bulbil formation was occurring in most treatments where microbial infection had been controlled.

Agronomy experiments were set up with *G. nivalis* to study the effects of shading, shelter, irrigation and mulching on plant, seed and bulb production. As well as using artificial shading materials, narcissus and cereal intercrops and oversowing with rye-grass are being evaluated. The first results will be available later in 2001. Bulb *storage* experiments are due to begin in 2001.

ACTION POINTS FOR GROWERS

The project is at an early stage and there are no action points for growers at this point.

PRACTICAL AND FINANCIAL BENEFITS FROM STUDY

Again, an assessment of these benefits must await the completion of further work.

EXPERIMENTAL SECTION

INTRODUCTION

Drifts of delicate bulbous species such as snowdrops (*Galanthus* spp.) are a romantic evocation of woodland glades, and it is not surprising that snowdrops are popular garden plants, their attractive white flowers being among the earliest of the year in many British gardens. Snowdrops are hardy in all areas of the UK, and are in demand for naturalising in gardens and growing in containers outdoors and indoors. The delicate size, form and colouring of the flowers continues to match gardening fashions (Lear, 1988). In 1985/86, 48 million snowdrop bulbs were (re-)exported from the Netherlands, illustrating the extent of trading (Langeslag, 1989). In recent years in excess of 20 million snowdrop bulbs have been imported into the UK annually from The Netherlands (figures from *Produktschap voor Siergewassen*). Commercial bulb growers and traders have been aware for many years of the potential sales for snowdrop bulbs but, unlike larger spring-flowering bulbs such as narcissus and tulip, they do not grow satisfactorily under standard agricultural systems. As a result, the market for snowdrops was satisfied for many years by the collection of wild bulbs from Turkey (*G. elswesii* and some *G. ikariae*), resulting in the extensive loss of natural populations (Zimmer and Girman, 1987). *G. elswesii*, especially, has been collected in quantity at least from the 1970s, and, although this species is unlikely to be rendered extinct, its distribution is becoming more restricted (S. Oldfield, personal communication). Local pressure to collect bulbs resulted in collection earlier in the year, before the bulb had had time to bulk up and before seed had set, and led to tonnes of undersized bulbs being dumped. Losses through habitat destruction and over-collecting have also been reported from the Ukraine (Budnikov and Kricsfalusy, 1994), while in the UK there have been well publicised cases of the illegal digging of snowdrops (e.g., Tendler, 2000). In 1987, Dutch imports of Turkish snowdrops reached 62 million (Lear, 1988). In addition, snowdrops have been 'farmed' from 'sustainable' woodland sources in France (Loire Valley), with an annual production of about 10 million bulbs (Ekim, 1984), as well as being obtained from England and elsewhere. Even at this level of production, demand did not appear to have been satisfied. Sales could be increased further, should acceptable stocks become available in sufficient quantities.

All *Galanthus* species are now strictly protected by listing on Appendix I of CITES, the Convention on International Trade in Endangered Species of Wild Fauna and Flora. Controls on the trade in wild-collected bulbs have reduced Turkish exports (for all flower bulbs) from 70 million bulbs in 1989 to 23 million in 1996, while international sponsors have also encouraged Turkish villagers to cultivate bulbs in substantial quantities in compensation (Robbins, 1996). Over the period 1990-1995, Turkish exports of snowdrop bulbs (about 85% *G. elwesii* and 15% *G. ikariae*) fell from 30 million to 6 million bulbs (Ekim *et al*., 1997). There have been attempts to grow-on undersized wild-collected bulbs in Turkey (Altan, 1985; Gokceoglu and Sukatar, 1986), and the export of bulbs (both wild collected and 'artificially propagated') from Turkey has now been more closely regulated (Ekim *et al*., 1992, 1997), with the collection of these *Galanthus* species forbidden in 1995 and 1996. Some snowdrops are grown in the Netherlands, but production areas do not appear in the appropriate statistics. *Galanthus* is a uniform genus with about 15 species native to Europe and Asia Minor, but there are many hybrids, forms and selections. The main snowdrops in commerce are *G. nivalis* (single and double forms), with its larger scented hybrids such as 'S. Arnott', and *G. elwesii*. While typically associated with growing in shady places, some species are

natives of drier Mediterranean areas. Hence, there is scope for exploiting a larger variety of snowdrop types.

The UK bulbs industry has become scrupulous in ensuring that the bulbs it sells are obtained from commercially farmed stocks. However, it is considered likely that only a small proportion of snowdrop bulbs traded originates from stocks cultivated in the Netherlands, so there may still be residual doubts over the provenance of many bulbs. Aside from environmental issues, there are also questions of quality. Away from western Europe, expertise in bulb growing may be less than optimal. Warmer climates may encourage pests and disease, though some (e.g., *Sclerotium bataticola*) may enter on imported snowdrop bulbs but do not survive under western Europe conditions (Moore *et al*., 1979). Bulbs lifted from these sources do not store or transport well. At collection, other species may be mixed in, deliberately or accidentally. Snowdrop bulbs from different climatic origins are known to exhibit variations in their growth and development (Langeslag, 1989), a finding also seen in phenological studies (Thran, 1990; Gromisz, 1993). Even samples collected in the vicinity of a single Turkish village were found to show considerable variation in their morphological characteristics (Korkut *et al*., 1994). The problems associated with Turkish bulbs were discussed by, for example, Ekim (1984), Oldfield (1984), Lear (1988) and Read (1989). On the other hand, producing bulbs such as snowdrops in the UK should offer the advantages of suitable climate, exposure to careful monitoring and quality control, and closeness to bulb handling expertise and markets – as well as the cachet which could be attached to English- or UK-grown bulbs. Carefully controlled production would produce healthier, better bulbs, increasing the attraction to potential buyers and justifying sales promotion. Snowdrops are high-value bulbs. Recently, based on a spot-check of catalogue prices, wholesale prices for 'ordinary' snowdrops (*Galanthus nivalis*) have been around £50 to £60 per thousand, about twice this for other species and double varieties, and in excess of £400 per thousand for scarcer species and varieties. For retail sales, prices from about £200 (for *G. nivalis*) to £700 (for choice species and varieties) per thousand have been noted. These figures are for sales as 'dry bulbs', and there would be added-value for pot-grown plants 'in the green' for this market.

Monographs on snowdrops include Stern (1956) and Davis (1999). Horticulture textbooks that include details of snowdrop growing include Bergman *et al*. (1978), Moore *et al*. (1979), Lane (1984), MAFF (1984), Langeslag (1989) and IBC (undated). An HDC-funded review of the potential for speciality ('minor') bulb production in the UK, including snowdrops, was carried out in 1988 (Project BOF 13; Hanks, 1988). This review indicated that little R&D on snowdrops had been reported from either the UK or the Netherlands. A recent major textbook on flower bulbs (De Hertogh and Le Nard, 1993c) highlighted the lack of information available on snowdrop agronomy, physiology and related aspects. The reviewers concluded that more information was needed on commercial production, for which tissue culture was said possibly to be an excellent way to increase planting stocks (Le Nard and De Hertogh, 1993). In 1998, a search of the horticultural literature (CAB Abstracts covering 1986 to January 1998) found only 63 references to snowdrops. Of these references, 36 (57%) covered biotechnological papers (mostly on lectins); six covered aspects such as cytology, histology and cell physiology, six covered taxonomy, six covered ecology or ecological physiology, one covered plant chemistry and two were general reviews: only three papers dealt with propagation and another three with horticultural aspects of growing the bulbs. A recent update (covering the period 1996 to October 2000) found 68 references to snowdrops, and the breakdown to subjects was similar to the earlier analysis. Of these, 48 (71%) covered biotechnology, mostly lectins; five covered

anatomy, morphology, etc., four taxonomy, and seven ecology or ecological physiology; only two covered propagation and two snowdrop growing or horticulture.

Aims of the project

In the current project, cost-effective methods will be developed for the sustainable production of snowdrops in the UK. The project covers both the production of bulbs sold 'in the green' and as 'dry bulbs', because both systems have advantages and the availability of both would serve different sections of the market. The major problems of snowdrop production are (a) low multiplication rates, (b) they are difficult to grow commercially, and (c) the bulbs are difficult to store. The project therefore addresses the three objectives of micropropagation, agronomy and storage.

Micropropagation - to develop in vitro *systems that sustain high vegetative propagation rates and yield superior quality, uniform bulb stock.*
It is proposed to develop a micropropagation system for snowdrops, drawing on the QUB group's research experience with the related genus *Narcissus* (reviewed by Harvey *et al*., 1994, and Harvey and Selby, 1997) and with the initiation and growth of other plant storage organs *in vitro* (e.g., *Dierama* corms (Hughes, 1992) and potato microtubers (Harvey *et al*., 1991)). The main aims would be to explore ways of inducing and maintaining high shoot multiplication rates in 'shoot clump cultures' and promoting bulbil initiation and growth. Shoot multiplication rate is a key factor determining the speed with which novel crops can be brought to market and the volume of sales that can be expected. Bulbil initiation and growth is also an important aspect, since it will determine whether bulbs can achieve a size capable of flowering within one season. The post-micropropagation phase of acclimatisation also requires careful research, since this will determine whether bulbs are to be marketed as dry, dormant bulbs, or as actively growing plantlets. Acclimatisation of *in vitro* growing plantlets directly to the garden opens up a number of attractive possibilities for both the grower and the consumer. It would avoid the grower having to undertake the expensive acclimatisation of micropropagated plantlets in a conventional flat- or plug-based system. In addition, the consumer could plant out snowdrops 'in the green' over several months in late-spring or early-summer, with potentially a high degree of success compared with planting dry bulbs in the autumn.

Agronomy – to develop novel growing systems for effective commercial production of snowdrop bulbs.
From a study of snowdrop habitats in many countries, it was concluded that the main characteristics linking all situations were partial shade and a soil in which the roots always had some moisture, although less in summer than winter (Nutt, 1993). As snowdrops can grow well under trees, there is scope for farming them in a systematic manner in purpose-grown woodlands or orchards. While such a 'semi-natural' system might have appeal, from the point of view of its looks and environmentally aware approach, such a system could not be described as convenient or practical from a bulb-farming point of view, and other more novel approaches will be investigated. Since a woodland situation provides suitable conditions of shade, shelter and a relatively uniform environment, the cultivation of snowdrops on a field-scale, with suitable shading from inter-crops or artificial shade, is a possibility that will be explored. Initially, bulbs would be grown in the field in factorial experiments investigating the effects on production of different types and levels of shade. With some basic responses to shading established, artificial shading and inter-planted crops would be investigated to determine effective and practical growing systems. The preferred systems for

producing snowdrop stocks economically would then be evaluated using seed-, chip- and micropropagation-raised plants in addition to plants obtained conventionally as dry bulbs. To obtain additional information on the performance of snowdrops under different growing systems, plantings would be monitored in existing natural and 'semi-natural' sites such as woodlands, orchard and coppice.

Storage – to investigate more suitable bulb storage regimes for snowdrops.
For large-scale commercial, amenity or export sales, as opposed to retail sales to gardeners, the production of dry bulbs would offer advantages of convenience over transplanting 'in the green', provided improved bulb storage regimes were available. With our present state of knowledge, planting 'in the green' makes the conventional large-scale marketing of bulbs impossible, although it is useful for specialist mail-order sales. For large-scale sales, there would be advantages of investigating more effective storage regimes. In this project, the storage of snowdrop bulbs will be investigated in factorial experiments involving a range of bulb harvesting stages, storage temperature and humidity and storage conditions (such as the use of packing materials and bulb coatings).

Benefits of the project

Success in the project would result in many benefits for the industry as limitations that currently restrict the exploitation of snowdrops as a commercial crop are removed. The growers' knowledge and technology base would be greatly extended for a crop for which relatively little soundly based information is available at present, either for micropropagation or field-based production.

Dependence on wild bulbs would be eliminated through the development both of micropropagation and of practical farming systems, which in some cases might operate separately (depending on the required product) or, more likely, as the two stages in a new production system. This would give the industry a 'greener' image. Increased and sustainable production of snowdrops could substitute for imports, and, if successful, export markets could be created. Multiple retailers could be targeted with 'bulbs from environmentally sustainable UK stocks'. This could include uniform, weaned micropropagated plantlets sold as leafy rooted bulbs growing on a semi-solid culture medium in an aseptic plastic vessel. This would overcome the problem of the poor survival and flowering often found when dry, dormant bulbs are planted in autumn following lifting and storage. Such 'high-tech' marketing of this sort can appeal to gardeners, especially those who have struggled to establish snowdrops from conventionally marketed bulbs. Better guarantees of survival and disease-free status could also be given, and plants sold in this way could therefore command a premium price.

High vegetative multiplication rates would be achieved through micropropagation, compared with the currently available methods (seed, offsets, chipping and twin-scaling). Improved methods of field growing would enable low-input growing to take over effectively, where required, once initial stocks had been obtained *in vitro*. Growers could therefore commercialise choice or new cultivars rapidly, and then produce uniform stocks in bulk, replacing poorer types. This could also stimulate breeding snowdrops for improved cultivars. The development of micropropagation of snowdrops could therefore be viewed as extending an existing market, as well as for selling to fashion-conscious consumers requiring novel products. At present, sales of new bulb cultivars are often restricted to the high-value specialist market because of a shortage of

plant material. A better range of varieties could enhance garden centre and specialist sales. It would also enable the rapid bulking of stocks to provide material for high-volume sales, such as the amenity market.

Optimisation of *in vitro* bulb production conditions may allow large bulbs of flowering size to be formed in a few months. Using conventional bulb growing, snowdrop seedlings take four to five years to achieve flowering size (a circumference of 4-5 cm).

Through using micropropagation, production could be programmed to market the plants at the best time for sales or growing-on, probably in spring to mimic the practice of 'in-the-green' transfer of plants following flowering. With proper attention to detail and further research, it may be possible to produce plantlets with a relatively long shelf-life which could be successfully planted out by gardeners over several months. Using conventionally raised bulbs, improved bulb storage techniques would enable 'dry' bulbs to be marked over a longer period than at present, with improved results.

As is the case in most bulbs of the Amaryllidaceae family, snowdrops contain many alkaloids of potential medicinal interest. Snowdrops are also being used extensively in plant lectin studies and in other biotechnological research. This could represent another opportunity for bulb growers, given better production methods.

REVIEW OF *GALANTHUS* PROPAGATION AND AGRONOMY

Micropropagation

Very few studies of micropropagation of the genus *Galanthus* have been reported in the literature and none of these has been carried forward to a commercial stage. Moskov *et al.* (1980) briefly described work with the common snowdrop (*G. nivalis*) as a minor part of a larger study on other bulbous species. The only other studies on this genus were with *G. elwesii* (Girmen and Zimmer, 1988a, b, c) and *G. ikariae* (Tipirdamaz *et al*., 1999). The methodologies and main findings from these studies are reviewed briefly here.

Sterilisation techniques
Moskov *et al.* (1980) washed *G. nivalis* bulbs in soapy water before cutting them into bulb chip explants. These were surface-sterilised for 15min in a 7% calcium hypochlorite solution then rinsed in sterile distilled water. With *G. elwesii* (Girmen and Zimmer, 1988a) and *G. ikariae* (Tipirdamaz *et al.*, 1999), the bulbs were first prepared by removing the tunic and outer scales then washed thoroughly in water. Bulbs of *G. elwesii* were then surface-sterilised in 3 - 12% sodium hypochlorite for periods ranging between 5min to 20min. Surface-sterilisation of *G. ikariae* bulbs was achieved by first treating them with 96% ethanol for 3min then 40% sodium hypochlorite for 20min followed by rinsing in sterile distilled water. Bulb explants were then cut (see below) and inoculated onto culture media.

The effectiveness of sterilisation treatments was only reported for *G. elwesii*. Treatment with 12% sodium hypochlorite, for durations between 5min and 20min, was necessary to reduce microbial infection rates in bulb explants to 20%. Of other explant types tested, only seeds could be completely sterilised (Girmen and Zimmer, 1988a).

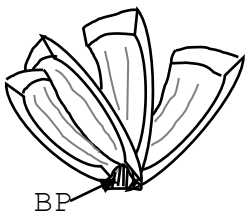
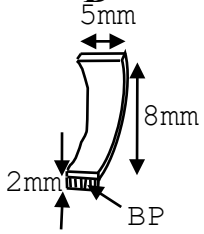
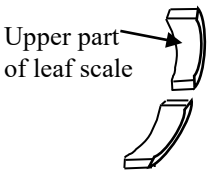
Choice of explant
In spite of the difficulties in their sterilisation, bulb section explants were the most commonly used due to their high regenerative ability. Normally, such explants were prepared by cutting surface-sterilised bulbs vertically into four to eight segments (*G. elwesii*) or four segments (*G. ikariae*) while ensuring that each segment contained a piece of basal plate tissue. After 16 weeks in culture 94% of bulb segment explants of *G. elwesii* formed new bulbils, compared with 40, 24 and 30% of leaf, peduncle and ovary explants, respectively (Girmen and Zimmer, 1988a). The numbers of new bulbils formed by the different explants showed a similar pattern. Regeneration from excised *G. elwesii* embryos was very slow.

Tipirdamaz *et al.* (1999) tested four *G. ikariae* bulb explant types for their regenerative ability. These are described below and illustrated in Table 1:
A Bulbs divided vertically into four equal segments
B Individual 5mm wide bulb scales including a 2mm section of base plate
C Bulb twin scales
D Explants without base plate from the upper part of the scale leaves

Bulb segment explants (A) formed the most adventitious bulbils at both auxin levels tested (using the potassium salt of naphthalene acetic acid, KNA), but these were not significantly more productive than smaller explant types B and C. However, it would be possible to prepare many more B type explants than the four bulb segment explants from each mother bulb and thereby achieve a higher multiplication factor in the

initiation passage. In contrast to the findings of (Girmen and Zimmer, 1988a) with *G. elwesii*, leaf explants without base plate tissues (D) of *G. ikariae* did not regenerate new bulbils.

Table 1. Numbers of adventitious bulbils formed by different explant types of *G. ikariae* (drawings personal communication from R. Tipirdamaz, 2000) on media supplemented with different KNA concentrations (Tipirdamaz *et al.,* 1999). Explant types are described more fully in the text but types A and B were carefully prepared to leave base plate (BP) tissues attached.

Explant type	A	B	C	D
KNA concentration (mg/l)				
0.2	3.25[a]	2.98[a]	2.34[ab]	0.00
0.4	2.23[bc]	1.76[c]	1.87[c]	0.00

Culture media and physical conditions

Basal culture media used to grow *Galanthus* tissues are summarised in Table 2. All work reported so far used simple modifications of the revised MS medium (Murashige and Skoog, 1962).

Moskov *et al.* (1980) used combinations of 2,4-dichlorophenoxy acetic acid (1 or 2 mg/l) and kinetin (0.2 mg/l) to stimulate callus and bulbil production in *G. nivalis*. Work with *G. elwesii* and *G. ikariae* in the main used similar concentrations of KNA (0.1 mg/l for *G. elwesii* or 0.2 mg/l for *G. ikariae*) and benzylaminopurine (BA) (2 mg/l) to promote bulbil regeneration. In both these species KNA was reported to be more effective for bulbil induction than the free acid naphthalene acetic acid (NAA) (Girmen, 1986; Girmen and Zimmer, 1988a; Tipirdamaz *et al.,* 1999). With *G. ikariae* KNA also induced more rooting in explants than did NAA (Tipirdamaz *et al.,* 1999). No explanation for the greater effectiveness of the potassium salt is presented, although all the studies comparing these compounds calculated auxin concentrations on a weight rather than on a molar basis. Recalculation on a molar basis shows that 20% more auxin would be being supplied to the tissues with NAA compared with KNA and this difference could account for some of the reported changes in tissue response. Girmen and Zimmer (1988a) report that for bulbil formation with *G. elwesii*, indole acetic acid (IAA) is as effective as KNA and that BA increased the rate of bulbil formation compared with the alternative cytokinins kinetin and zeatin.

Carbohydrate source was extensively studied for both *G. elwesii* (Girmen and Zimmer, 1988b) and *G. ikariae* (Tipirdamaz *et al.,* 1999) at concentrations ranging between 10 and 60 g/l. With *G.elwesii* the highest rates of bulbil production was found with 20 – 30 g/l sucrose, maltose or fructose while glucose and lactose were less effective. No such clear relationship was found with *G. ikariae* using the same five carbohydrate sources, but 60 g/l sucrose gave the highest rate of bulbil production when combined with KNA.

Moskov *et al.* (1980) incubated *G. nivalis* cultures at 25°C in the dark in growth chambers maintaining the humidity at 50 – 60%. With *G. elwesii*, bulbil formation was possible at between 10°C and 25°C under both dark and light (16h photoperiod, 2,300 lux) conditions but bulbil production was reduced at 10°C and 15°C (Girmen and Zimmer, 1988b). Although bulbil production was highest at 20°C and 25°C, bulbil growth and development was significantly better at 20°C. At 25°C although large numbers of bulbils were formed these remained very small. Tipirdamaz *et al.* (1999) incubated *G. ikariae* cultures at 20°C with a 16h photoperiod and a light intensity of 2000 lux.

Table 2. Composition of media used to grow *Galanthus* species *in vitro*.

Species	Nutrients					pH (g/l)	Agar	Reference
	Macro	Micro	Vitamins (mg/l)		Carbohydrate (g/l)	Miscellaneous			
G. nivalis	MS	MS FeEDTA ($FeSO_4.7H_2O$ 27.85 mg/l & Na_2EDTA 37.25mg/l)	White (1963)		sucrose (30)	casein hydrolysate 0.5g/l	5.5-5.7	7	Moskov *et al.* (1980)
G. elwesii	MS	MS Fe chelate (Fetrilon)	myo-inositol glycine pyridoxine HCl thiamine HCl nicotinic acid	100 2.0 0.5 0.5 1.0	sucrose, glucose or fructose (20 - 30) best for bulbil formation, glucose & lactose less effective		4.5 - 7.0 tested. 5.0 - 5.5 optimal for bulbil production	?	Girmen and Zimmer (1988a, b)
G. ikariae	MS	MS	same as for *G. elwesii* above		wide range of sources tested, sucrose (60) best for bulbil production		5.5 used but 6.0 above optimal for bulbil production	6	Tipirdamaz *et al.* (1999)

Multiplication and genetic stability

Girmen and Zimmer (1988a) report an annual multiplication rate of 27,000 propagules arising from a single *G. elwesii* bulb. Their calculation assumes that two out of four bulb explants dissected from each mother bulb each produce 15 new bulbils in the 16 - 18 week initiation culture passage. Each of these bulbils was then divided into two new bulbil segments for the initiation of new bulbils in the second sub-culture, giving 60 segments per initial mother bulb. Further calculations are illustrated in Table 3. This method would therefore yield large numbers of very immature bulbils that are not ready for transfer to *in vivo* conditions at the end of one year.

Table 3. Annual multiplication schedule for *G. elwesii* according to Girmen and Zimmer (1988a). Tissues were transferred to fresh medium, with a growth regulator composition of 0.1 mg/l KNA and 2mg/l BA, every 4 weeks, but were only sub-divided at 16-18 week intervals. This assumes a multiplication of 15 between sub-divisions and new bulbils being split into two segments.

Time (weeks)	Total number of new bulbils	Number of bulbil segments for re-inoculation after splitting
16-18	30	60
32-34	900	1800
48-50	27,000	-

It is impractical to calculate potential multiplication rates for *G. nivalis* and *G. ikariae* since work on the former is mainly descriptive while studies on the latter only assessed growth and development up to the end of the initiation culture passage. At best, Tipirdamaz *et al.* (1999) report the formation of 7.1 adventitious bulbils from a single quarter bulb chip explant of *G. ikariae*, on a medium supplemented with 60 g/l sucrose, 0.2 mg/l KNA and 2.0 mg/l BA.

Nothing is reported for leaf or shoot proliferation *in vitro* since all three *Galanthus* species so far studied have only been found to regenerate non-sprouting bulbils directly either on explanted tissues, *in vitro* produced bulbils or from callus. This inability of *in vitro* grown tissues to produce leaves might be a reflection of the natural growth habit of *Galanthus* species that only produce two leaves per bulb each year. Photosynthate from these leaves is utilised in the growth of lateral bulb units, with the terminal flowering bulb unit setting seed before dying.

Both Moskov *et al.* (1980) and Girmen and Zimmer (1988c) reported callus formation associated with bulbil production, although in the case of *G. elwesii* this phase is very short. With this species irregular callus-like cell division takes place within storage parenchyma under the epidermis, producing large numbers of small cells that differentiate bulbil primordia.

Indirect formation of bulbils via callus tissues might be expected to carry a risk of genetic variation in the propagated plants (somaclonal variation). In general, callus or suspension

cultures lose regenerative ability with sub-culturing (Wang *et al*., 1999), and this is often attributed to genetic degeneration. However, this may not be the case with bulbous monocots. For instance, *Iris germanica* suspension cultures regenerated well after more than 3 years in culture and showed a high degree of genetic integrity (Wang *et al*., 1999). Likewise, *Lilium* x *formolongi* cultures regenerated plants after 4 years, a large random sample of which showed normal ploidy and phenotype (Godo *et al*., 1998). Therefore, indirect monocot regeneration system where the callus phase is short, such as with *Galanthus*, may yield mostly true-to-type plants.

Acclimatisation
Acclimatisation of *in vitro* grown plants to *in vivo* conditions, *in vitro* rooting, and the dormancy status of *in vitro* produced bulbs, have not been studied for *Galanthus.*

Other methods of propagation

Under natural conditions snowdrops are propagated mainly by seed, although a small amount of vegetative propagation takes place via offsets (Le Nard and De Hertogh, 1993). Commercially, most species and cultivars can be propagated from seed, except for double-flowered varieties which have to be propagated vegetatively, usually from offsets (ADAS, 1984). Seeds are sown in frames or beds in summer, immediately they are ripe, and should be kept moist and shaded; there are no problems with dormancy (ADAS, 1984; Bryan, 1989; Langeslag, 1989). However, little is know about the optimal conditions for germination: while seed of *G. nivalis* does not apparently need cold or light for germination, in trials with *G. elwesii* germination took place only following a 5-month light pre-treatment followed by transfer to 20°C in light, and then only slowly (Zimmer and Girman, 1987). Viviparous germination has been reported (Rees, 1992), and has been observed at Kirton. Bulbs produced from seed take four to five years to reach flowering size (about 4 cm circumference), and are therefore a relatively slow way of multiplying stocks. The figure of 1 to 2 years for the production of marketable bulbs of *G. elwesii* from seed, quoted by De Hertogh and Le Nard (1993a) and Le Nard and De Hertogh (1993), refers to the production of bulblets for planting stocks (3cm up), not for bulbs of flowering size. Information from an HDC-funded project on seed-raised *Narcissus* species (BOF 34; Hanks *et al*., 1998), dealing with growing in modules, etc., may be relevant to snowdrop production.

The use of offsets for multiplication is also slow, as they are produced only from large 'mother bulbs' (Le Nard and De Hertogh, 1993). The propagation rate can be increased to some extent by the use of bulb chipping or twin-scaling (Alkema and van Leeuwen, 1977a, b; Alkema, 1985a, b; Langeslag, 1989; Hanks, 1991). Bulbs of *G. nivalis*, 4-5cm grade, were chipped successfully by cutting into eight segments (Hanks, 1987). However, the multiplication rate is relatively low for the labour input required, as the bulbs have few scales. It is reported that chipping has been used to propagate *G. elwesii* in the Netherlands, incubating the chips at 13-15°C (Langeslag, 1989). Van Leeuwen and van der Weijden (1997) reported on the chipping of *G. nivalis*, cutting bulbs into chips in the July to September period. The best yields were obtained by chipping as soon as possible after lifting, before mid-August, planting the chips immediately, and growing for two years.

Agronomy and bulb handling

As might be expected for a small, delicate bulbous species, snowdrops grow very poorly when grown commercially on a field scale in the same way as more robust types of bulbs. In herbicide trials at Kirton, Lincolnshire, the average weight increase for *G. nivalis* was only 18% (Wallis, 1975), while *G. elwesii* grown in netting at Rosewarne, Cornwall, gave very little increase in weight (Tompsett, 1985). Attempts to grow snowdrops commercially in the Western Isles were disappointing (Anon., 1998). Trials in Turkey using *G. elwesii* bulbs (4-5cm grade) demonstrated that disturbance due to lifting a proportion of the bulbs reduced the number, but not the size, of the bulbs produced (Altan, 1985). When wild-collected but under-sized (3-4cm grade) bulbs were replanted (in irrigated or non-irrigated, manured plots), up to 75% of the bulbs reached an exportable size (up to 6-7cm) in one year (Gokceoglu and Sukatar, 1986). Further studies of commercial growing in Turkey (Arslan *et al*., 1997) showed the suitability of small family enterprises, but large-scale production led to problems due to the build-up of pests and diseases and the lack of suitable mechanisation.

Snowdrops from different sources may show different characteristics. Under Dutch conditions, the French type of *G. nivalis* produces smaller flowers and bulbs than the Dutch type (Langeslag, 1989). *G. elwesii* and Turkish *G. nivalis* do not grow well in the Netherlands (S. Oldfield, personal communication). Langeslag (1989) reported that bulbs collected from the wild are either lifted in late-March/early-April with the leaves still green, the bulbs being separated and re-planted immediately, or (more usually) they are harvested after the tunic (brown skin) has formed and re-planted in August/September. Commercial producers in the Netherlands have apparently used both systems, planting small bulbs (<4 cm grade) 5-10 cm deep and, as is usual in the Netherlands, mulching with straw (Langeslag, 1989). The bulbs are planted in shallow drills made up into beds. Snowdrops are said to prefer damp, heavy soil and to benefit from a generous autumn top-dressing. Sandy or loamy soils with a high organic matter content are used, high soil moisture is acceptable, and a pH about 7 ensures bulbs with good skins are produced. The foliage is susceptible to sun-scorch, so they are sometimes planted under trees or with some protection in the field in spring (Alkema, 1985a, b; Langeslag, 1989). Two-year-down growing is recommended (Zandbergen, 1985). The bulbs may be lifted when the foliage has died down (late-May to early-June), using a riddle-type harvester with a fine screen, and collecting the bulbs into mesh-bottomed trays. The bulbs must be surface-dried to avoid rotting, but must not be allowed to dry out. Grading should be carried out immediately, and the small planting stock bulbs should be re-planted promptly.

Weed control is by pre-crop-emergence application of paraquat, followed by chlorpropham in early winter. In UK literature, pre-planting incorporation of trifluralin and pre-crop-emergence chlorpropham + fenuron have had label approval in the past (ADAS, 1990). Herbicide trials with snowdrops were conducted on light silt and sandy loam soils in Lincolnshire (Wallis, 1975). Pre-emergence applications (in December) and post-emergence applications (early-February) of the following herbicides resulted in no visual damage or loss of yield compared with untreated controls: chlorpropham; chlorpropham + diuron; chlorpropham + linuron; pyrazone + chlorbufam; lenacil + linuron; and methazole.

The freezing tolerance of snowdrops has been tested. Bulbs of *G. elwesii* with 4cm of leaf growth survived freezing to -7°C, but the flowers died when the temperature went below -5°C

(Sakai and Yoshie, 1984). Bulbs of *G. nivalis*, acclimatised to 5°C, were severely injured by exposure to temperatures of -9°C, the roots and base plate being most sensitive to cold (Lundquist and Pellett, 1976).

Snowdrops suffer from fungal diseases (such as *Botrytis galanthina* and *Stagonospora curtisii*), nematodes (*Ditylenchus dipsaci* (both tulip and narcissus races) and *Pratylenchus penetrans*), aphids and large narcissus fly (Lane, 1984; Langeslag, 1989). Snowdrop bulbs can be treated with hot water (HWT) for 1 hour at 42°C, with added fungicide, to control grey mould (*B. galanthina*) (Moore *et al*., 1979). Fungicide dips may also be needed to control *S. curtisii*. *D. dipsaci* would be controlled by HWT, although there are no specific recommendations for snowdrops (Lane, 1984). Trials with *G. nivalis* (de Winter *et al*., 1986) showed no damage after HWT for 43.5°C for 4 hours, a previous storage at 25°C for 10 days giving a higher bulb yield.

Snowdrops have been grown in orchards in the Netherlands, and were formerly grown on the shady sides of hedges in Kennermerland (until the hedges were removed), and they can be shaded and sheltered by inter-planting with narcissus or by stretching nylon netting over the beds (Zandbergen, 1985). Although it is likely that expertise in snowdrop bulb production exists in private hands, this would be commercially sensitive, and little information therefore reaches the public domain. The shortage of supplies of snowdrops makes it likely that serious difficulties exist in developing practical farming systems for the bulbs.

Galanthus species from drier habitats, such as *G. graceus*, *G. corcyrensis* and *G. reginae-olgae*, would be expected to prefer different conditions.

G. nivalis begins to initiate flower buds in late-March soon after flowering, when soil temperatures are low (3-10°C), initiation is completed in late-May to June, and flowering taking place the next winter/spring (Luyten and van Waveren, 1952; Langeslag, 1989). Flowering date was strongly correlated with monthly mean near-surface air temperature in January to March (Maak and von Storch, 1997, 1998). In urban sites, warming due to natural climatic effects and man-made 'heat islands' led to earlier flowering, by 29 days over 40 years (Sachweh and Rotzer, 1997). Flowering date was found to be closely related to the duration of the frost-free period (Chen, 1997).

Bulb storage

Unlike many other bulbous ornamentals, it is well known that snowdrops do best if transplanted in full growth 'in the green' soon after flowering, much better than when the bulbs are stored dry and planted in the autumn (ADAS, 1984; Rees, 1989). The sale of potted plants in growth is very attractive, but there are also situations where the sale of dry bulbs would be more convenient. The current recommendations are to store snowdrop bulbs in peat or silver sand at 17°C, although this is only partly successful in preventing their drying out, and rapid re-planting is necessary (De Hertogh and Le Nard, 1993b; Langeslag, 1989). Rees (1989) pointed out that transplanting bulbs in the green before leaf senescence would reduce bulking up, which takes place at this time. He also considered that studies to improve dry storage would be worthwhile, since snowdrop bulbs dry out rapidly because of their large surface:volume ratio, few scales and poorly developed skin.

The best emergence was shown to follow storage at 13°C and in silver sand (de Winter, 1978), and trials showed that the success rate for flowering of conventionally stored snowdrop bulbs was low (de Winter, 1978; de Winter and van Leeuwen, 1985). The best flowering rate achieved was 50% when growing potted bulbs (5-6cm grade) in a heated glasshouse, although 95% had initiated flowers. Storage, especially at low temperatures (9°C), encourages shrivelling of the bud. Attempts have been made to retard snowdrops in a similar way to ice-tulips (Krinkels, 1987).

EXPERIMENTAL: MICROPROPAGATION

Materials and methods

Plant material
G. nivalis (5/6cm), *G. nivalis* Flore Pleno (5+cm) and *G. elwesii* (7+cm) bulbs were supplied by Jacques Amand International in October 2000 and stored at room temperature. Additional *G. nivalis* bulbs were sampled from the same batch as used in the Kirton field trials. These were used in later micropropagation experiments to provide planting stocks, of the same genetic origin as those used in the shading and bulb storage trials, for comparative field trialing at Kirton. Approximately 180 bulbs of each species were planted in 13cm (*G. nivalis*) or 17cm (*G. nivalis* Flore Pleno and *G. elwesii*) pots using a steam-sterilised loam of pH 5.8. The drainage in the compost for *G. elwesii* was improved by mixing the loam with coarse grit and sharp sand (3:1:1 loam:grit:sand). The bulbs were planted to a depth three times their height. For *G. nivalis* and smaller bulbs of *G. nivalis* Flore Pleno eight bulbs were planted per pot. Six and five bulbs per pot were planted for the larger *G. nivalis* Flore Pleno bulbs and *G. elwesii*, respectively. Initially these plants were grown in a well-ventilated unheated glasshouse, but were then transferred to a shaded cold frame and the pots embedded in sand to keep the root systems cool.

Bulb preparation for micropropagation
Healthy bulbs were selected and their tunics and any scale leaves showing discoloration or brown markings were removed by hand. Basal bulb tissues were cut away with a scalpel down to healthy white tissues, care being taken not to remove more base plate tissues than was necessary. For *G. nivalis* in all but experiment 1, trimming of basal tissues was restricted to the removal of roots and loose dead tissues, because the base plate tissues of these bulbs were too thin to allow trimming both before and after sterilisation. Apical bud tissues were also cut away 1mm below the region of scale leaf senescence so that only healthy white tissues remained. The bulbs were then ready for surface sterilisation. Throughout, bulbs were surface sterilised in pairs in 100ml Erlenmeyer flasks (*G. nivalis* and *G. nivalis* Flore Pleno) or singly in wide form 100ml beakers (*G. elwesii*). Sterilant was then added to completely cover the bulbs, the vessel capped in aluminium foil and shaken at 130 r.p.m. on a reciprocating shaker.

After sterilisation the bulbs were cut aseptically into explants. Bulbs were first trimmed top and bottom to remove tissues damaged by the sterilant. Basal trimming was such as to allow a minimum of 1mm of base plate to remain. Apical trimming then produced bulb sections approximately 8mm in height for *G. nivalis* and *G. nivalis* Flore Pleno or 10mm in height for *G. elwesii*. Longitudinal cuts were then made through the centre of the bulbs to produce four (*G. nivalis* and *G. nivalis* Flore Pleno) or six (*G. elwesii*) chip explants per bulb.

Following sterilisation, explants were inoculated with their bases about 3mm into the agar-solidified culture medium. One explant was inoculated per culture vessel.

Media preparation and culture conditions
Pre-prepared Murashige and Skoog (1962) basal macronutrients, micronutrients and vitamins (Sigma Aldrich Co. Ltd.) were used throughout dissolved in water purified with a Elga Prima reverse osmosis apparatus (Elga Ltd). This was supplemented with 30g/l sucrose and plant

growth regulators as described in individual experiments. BA, NAA and KNA were all cell culture quality tested (Sigma Aldrich Co. Ltd.). Media were adjusted to pH 5.6 with dilute KOH or HCl before adding 7g/l Oxoid purified agar. The agar was dissolved by heating, then 20ml aliquots of media were dispensed into boiling tubes. Tubes were enclosed with cotton wool bungs and autoclaved at 121ºC for 15 min.

Cultures were incubated in Fisons 600G3/THTL growth cabinets at a constant temperature of 18ºC. A photoperiod of 16h was provided by cool white fluorescent tubes supplemented at the red end of the spectrum with 40W incandescent bulbs giving a PAR of 100µmol/m^2/s at bench height.

Sterilisation methods

A series of four experiments was completed examining the effects of various surface sterilisation methods. Hypochlorite treatments were used either alone, or in combination with an initial hot-water treatment (HWT) of whole bulbs, or in combination with steeping treatments in Plant Preservative Mixture (PPM) or various fungicides applied to bulb chip explants. The effects of incorporating PPM or fungicides in the culture medium were also investigated. PPM, whose main active constituents are methylisothiazolinone and methylchloroisothiazolinone, is a specially formulated product used in plant tissue culture systems (Plant Cell Technology Inc.). PPM and the fungicides benomyl (Benlate 50% w/w a.i., Du Pont UK Ltd.), carbendazim (Derosal 80% w/w a.i., Hoechst UK Ltd.) and imazalil (100% imazalil sulphate, Janssen Pharmaceuticals Ltd.) were added to culture media prior to adjusting the media pH and autoclaving.

Experiment 1

In this experiment relatively mild hypochlorite sterilisation methods using dilutions of Domestos (Leaver Brothers Ltd.) were tested alone and combined either with HWT or PPM treatments. Domestos is manufactured to have a hypochorite concentration of 4.6%, but this can fall to 4.2% with time.

The six sterilisation treatments tested are described below:

1 Bulbs were shaken in 70% ethanol for 30sec then in 10% Domestos (0.42 - 0.46% hypochlorite) for 20min followed by six rinses in sterile distilled water.
2 The same as treatment 1 except the Domestos concentration was increased to 30% (1.26 - 1.38% hypochlorite).
3 Cleaned bulbs were HWT at 43ºC for 45min then sterilised as in treatment 1.
4 Cleaned bulbs were HWT at 43ºC for 45min then sterilised as in treatment 2.
5 Bulbs were sterilised as in treatment 1 and bulb chip explants prepared as described above. Explants were then steeped in 2% PPM for 9h. Chip explants were treated in groups of five, in 20ml aliquots of PPM solution prepared in 50mg/l $MgSO_4$, in 100ml Erlenmeyer flasks that were shaken at 100 r.p.m. Explants were inoculated directly to the culture media without rinsing.
6 The same as treatment 5 except the PPM concentration was increased to 4%.

Following sterilisation, explants were inoculated onto four culture media described below:

1 Basal medium lacking plant growth regulators (see media preparation above).
2 The same as medium 1 supplemented with 0.1mg/l NAA and 1mg/l BA.

3 The same as medium 2 supplemented with 20mg/l (a.i.) benomyl.
4 The same as medium 2 supplemented with 1ml/l PPM.

The full experimental design was 6 sterilisation treatments x 4 culture media x 3 snowdrop types, 72 treatments in total. Each treatment was replicated with six bulb chip explants (432 cultures in total).

Experiment 2
In this experiment surface sterilisation with higher hypochlorite and PPM concentrations was examined in combination with incorporating fungicides in the culture medium. The three sterilisation treatments used were:

1 Cleaned bulbs were shaken in 70% ethanol for 1min then in 50% Chloros (5% hypochlorite) for 20min followed by six rinses in sterile distilled water. Ten drops per litre of Tween 20 were added to the Chloros solution to act as a wetting agent.
2 The same as treatment 1 except the Chloros concentration was increased to 100% (10% hypochlorite).
3 Cleaned bulbs were shaken in 70% ethanol for 1min then in 50% Domestos (2.1 – 2.3% hypochlorite) for 20min followed by six rinses in sterile distilled water. Bulb chip explants were then prepared and steeped in 5% PPM for 9h as described previously (experiment 1 treatment 5).

Following sterilisation, explants were inoculated onto four culture media described below:

1 Same plant growth regulator supplemented medium number 2 as in experiment 1 (control).
2 The same as medium 1 supplemented with 20mg/l (a.i.) carbendazim.
3 The same as medium 1 supplemented with 20mg/l imazalil.
4 The same as medium 1 supplemented with 20mg/l (a.i.) carbendazim and 20mg/l imazalil.

The full experimental design was 3 sterilisation treatments x 4 culture media x 3 snowdrop types, 36 treatments in total. Each treatment was replicated with six bulb chip explants (216 cultures in total).

Experiment 3
Cleaned bulbs were shaken in 70% ethanol for 1min then in 50% Domestos (2.1 – 2.3% hypochlorite) for 20min followed by six rinses in sterile distilled water. Bulb chip explants were prepared and steeped in fungicide solutions for 1h (as described for PPM in treatment 5, experiment 1) then transferred without washing to the same four media used in experiment 2, as described below:

Steeping treatment	Media inoculated
1 Water control	All four media.
2 20mg/1 carbendazim	Control medium and 20mg/l carbendazim medium.
3 40mg/1 carbendazim	Control medium and 20mg/l carbendazim medium.
4 20mg/l imazalil	Control medium and 20mg/l imazalil medium.
5 40mg/l imazalil	Control medium and 20mg/l imazalil medium.

6	20mg/l carbendazim & 20mg/l imazalil	Inoculated onto control medium and the medium with a mixture of 20mg/l carbendazim & 20mg/l imazalil.
7	40mg/l carbendazim & 40mg/l imazalil	Inoculated onto control medium and the medium with a mixture of 20mg/l carbendazim & 20mg/l imazalil.

The full experimental design was 6 fungicide treatments x 2 media x 3 snowdrop types + water control (1treatment x 4 media x 3 snowdrop types), 48 treatments in all. Each treatment was replicated with six bulb chip explants (288 cultures in total).

Experiment 4
Bulb chip explants were prepared from surface sterilised bulbs as described in experiment 3 then subjected to the treatments described below:
1 Inoculated directly to culture media.
2 Steeped in water for 1h.
3 Steeped in 40mg/l imazalil for 1h.

Explants were then inoculated onto a fungicide-free medium or a medium with 20mg/l imazalil. The full experimental design was 3 post-explant chipping treatments x 2 media x 2 snowdrop types (*G. nivalis* and *G. elwesii*), 12 treatments. Each treatment was replicated with 20 bulb chip explants with *G. nivalis* and 22 bulb chip explants with *G. elwesii* (252 cultures in total).

Results and discussion

Sterilisation methods
In experiment 1, mild hypochlorite treatments (0.44 - 1.32%) gave poor control of microbial infection in bulb chip explants of all three snowdrop types (Fig. 1). Higher hypochlorite concentrations (5 – 10%) used in experiment 2 improved control of contamination particularly if these were used in combination with fungicide in the culture medium (Fig. 2). This is in agreement with the findings of Girmen and Zimmer (1988a) using *G. elwesii* who reported that 12.5% hypochlorite was required to control contamination to levels of about 20% or less.

Preliminary HWT of bulbs at 43ºC for 45min before surface sterilisation increased the frequency of infection particularly with *G. nivalis* and *G. elwesii* (Fig. 1). A similar stimulation of microbial contamination by HWT, in bulb scale explants of *Lilium speciosum,* was explained in terms of high temperature activation of fungal spores or a more easy release of micro-organisms by plant tissues damaged by high temperature (Langens-Gerrits *et al.,* 1998). The HWT treatment time used here was inadequate to control infection but could potentially be increased. For instance, *Botrytis galanthina* was controlled in *Galanthus* species by a 1h HWT at 42ºC using a fungicide solution (de Winter *et al*., 1986) and HWT for as long as 4h at 43.5ºC caused no damage to *Galanthus* bulbs (de Winter *et al.,* 1986).

PPM treatment of chip explants following bulb surface sterilisation with a very low hypochlorite concentration (0.44%) gave a much-improved control of infection with all three snowdrop types in experiment 1 (Fig. 1). This encouraging result was improved further in Experiment 2 where concentrations of PPM and hypochlorite were increased (Fig. 2). The concentration 5% of PPM used in experiment 2 is the highest concentration recommended by

the manufacturers for use as a surface sterilant. Warnings are also given that PPM can cause an initial check in plant growth. PPM treated explants of *Galanthus* showed a marked inhibition of growth even after several weeks in culture and most tissues remained very white. Nevertheless, most PPM treated *Galanthus* explants eventually started to grow and regenerated new bulbils. This early adverse reaction to PPM strongly indicates that isothiazolones, as well as disrupting fungal and bacterial metabolism, also inhibit some higher plant biochemical processes. Smith *et al.* (1999) reported severe damage to bulb explants of *Hippeastrum* when PPM was used as a surface sterilant, to the point that all tissues were either killed or failed to grow. In contrast, using PPM to control infection by adding it to culture media, when much lower concentrations are needed (up to a maximum of 2ml/l), showed no phytotoxicity with various citrus tissue culture systems except for freshly isolated protoplasts (Niedz, 1998).

Inclusion of either benomyl or PPM in the culture medium showed little or no beneficial effects in experiment 1 (Fig. 1). Fungal contamination often originated high on the bulb chip explants, well away from the surface of the culture medium, and then spread down to infect the medium later. Thus fungal contaminants were probably not coming into contact with either PPM or benomyl until they were well advanced in their growth.

Although benomyl is regarded as being systemic it would not have moved into all parts of the bulb chip explants because there was no active transpiration stream. In the case of PPM the manufacturers recommend that plant tissues be completely immersed in the culture medium for the effective control of contamination. Such inoculation below the surface of the medium, with large explant tissues like bulb chips, might give rise to problems of anaerobiosis or tissue hyperhydration and was avoided.

In experiment 2, where more effective surface sterilisation methods were used, the benefits of incorporating fungicides in the culture medium were more clearly seen (Fig. 2). A mixture of carbendazim and imazalil completely eradicated any residual infection following both Chloros sterilisation treatments with all three snowdrop types. Likewise, use of a medium supplemented with imazalil alone was effective with all *G. nivalis* Flore Pleno and *G. elwesii* explants whilst residual infection in the *G. nivalis* cultures was unaffected by imazalil. Carbendazim alone was ineffective at controlling residual contamination. The differences between imazalil and carbendazim may be a reflection of the high water solubility of imazalil making it more mobile in plant tissues in the *in vitro* environment.

Results from experiments 1 and 2 indicated that in some circumstances there may be problems of antimicrobial agents not actually reaching sites of infection on explanted tissues if the agent is only incorporated in the culture medium. Whereas if the antimicrobial was very mobile, as in the case of imazalil in experiment 2, or explants are steeped in antimicrobials for a period before inoculation, as in the case of the PPM treatments in both experiments, then much improved infection control could be achieved.

To try and improve the access of fungicides to contaminant a further two experiments were therefore devised. These tested the efficacy of steeping explants in the fungicides carbendazim and imazalil before inoculation. These fungicides were chosen because they have dissimilar modes of action, were recommended for use in plant tissue culture by Shields *et al*. (1984), and are known to show beneficial hormone-like effects in plant culture systems

(Debergh *et al.,* 1993; Werbrouck and Debergh, 1996, and references therein). Thus, as well as controlling fungal infections, these fungicides may also improve some aspect of micropropagation in *Galanthus* by exerting plant growth regulator-like effects.

In experiment 3, with *G. nivalis* Flore Pleno and *G. elwesii*, both imazalil explant steeping and inclusion of imazalil in the culture medium was markedly more effective than using carbendazim (Fig. 3). Differences between these fungicides were less apparent with *G. nivalis*. Using a mixture of the two fungicides either as a steep or in the medium usually gave good control over infection. The only exception to this was with *G. nivalis* explants steeped in a 20mg/l fungicide mixture then inoculated onto a mixed fungicide supplemented medium. Here half the cultures became infected probably due to chance allocation of several badly contaminated bulbs to this treatment.

A combination of an imazalil steep and inclusion of imazalil in the culture medium gave effective infection control in both *G. nivalis* and *G. elwesii* in a repeat experiment (Fig. 4). Throughout experiments 3 and 4 uninfected explants remained very healthy in appearance regardless of either fungicide steeping or the presence of fungicides in the culture medium. Early indications are that *in vitro* bulbil formation was occurring in most treatments where microbial infection was controlled. This includes PPM and fungicide treated tissues, but an evaluation of bulbil induction rates will be reported on later. Similarly, other experiments examining media effects on bulbil initiation are also in progress.

Figure 1. The influence of various sterilisation methods on the percentage infection in bulb chip explants of snowdrops after 28d in culture (experiment 1). Following sterilisation, explants were inoculated onto a culture medium lacking plant growth regulators (open columns), a medium supplemented with 0.1mg/l NAA and 1mg/l BA (filled columns) or the same plant growth regulator supplemented media to which either 20mg/l a.i. benomyl (grey columns) or 1ml/l PPM (hatched columns) was added.

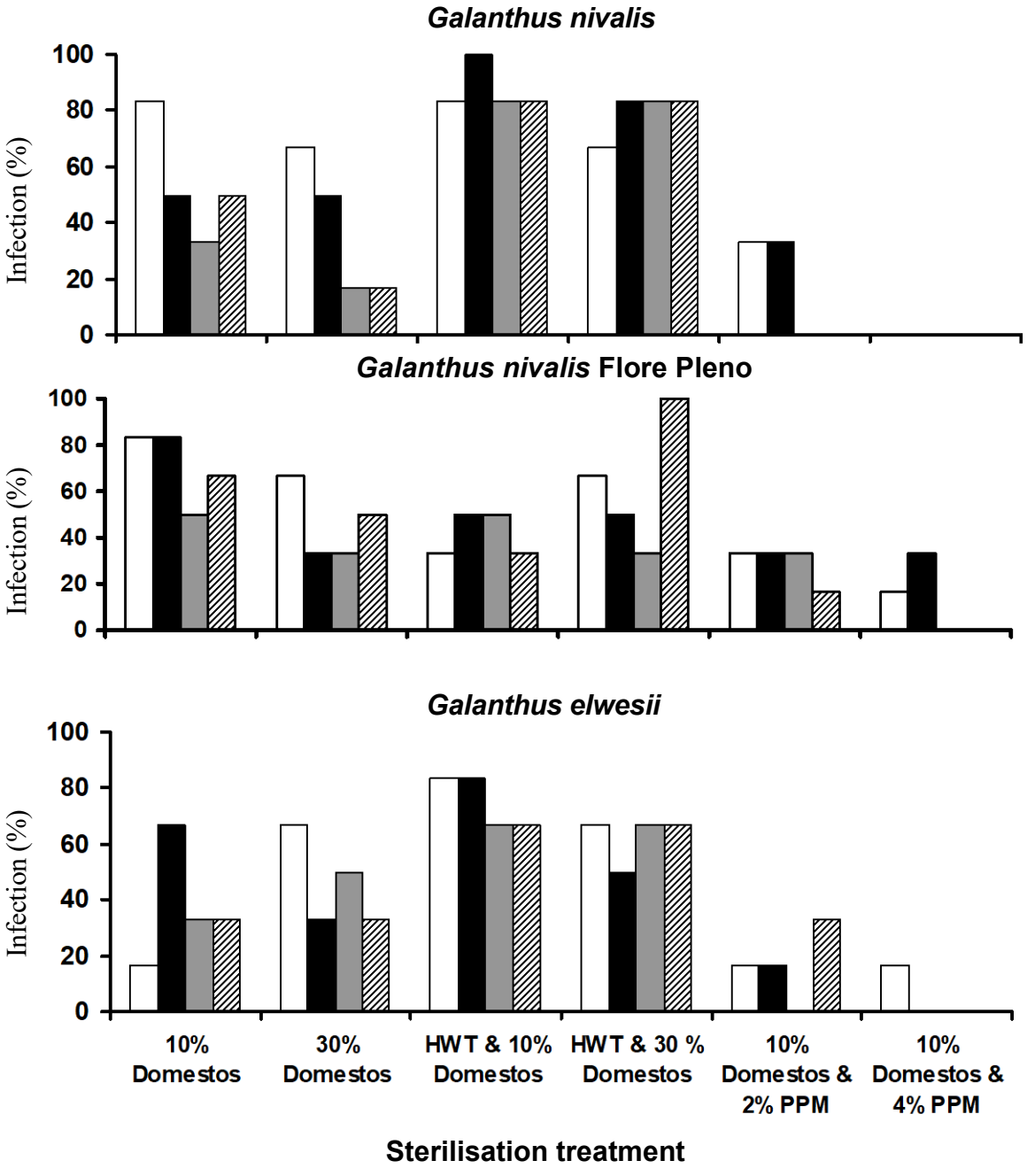


Figure 2. The influence of various sterilisation methods on the percentage infection in bulb chip explants of snowdrops after 28d in culture (experiment 2). Following sterilisation, explants were inoculated onto a culture medium supplemented with 0.1mg/l NAA and 1mg/l BA (open columns) or the same plant growth regulator supplemented media to which either 20mg/l carbendazim (filled columns), 20mg/l imazalil (grey columns) or a mixture of 20mg/l of both carbendazim and imazalil (hatched columns) was added.

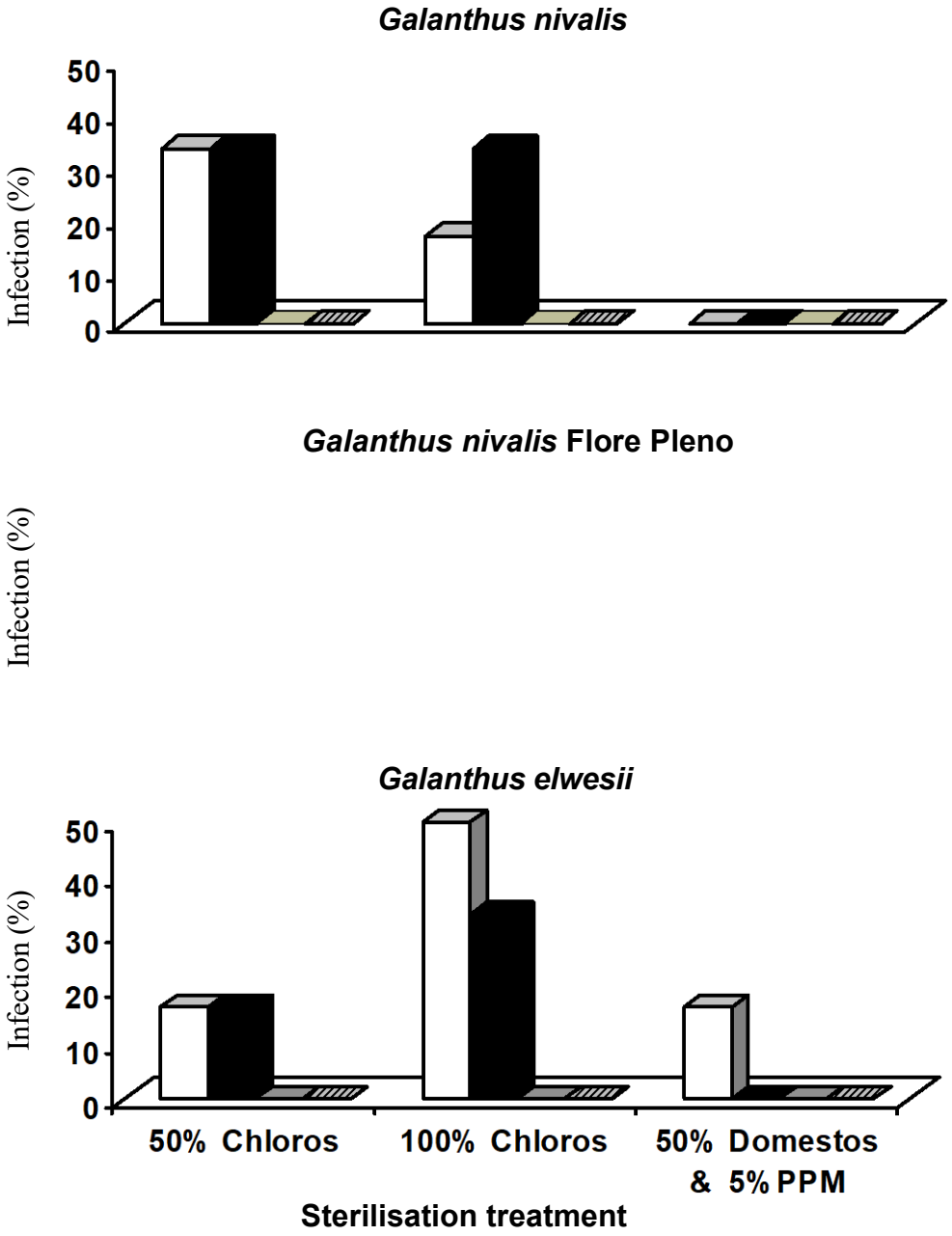


Figure 3. The influence of steeping snowdrop bulb chip explants in various fungicide solutions on microbial infection after 28d in culture (experiment 3). The fungicides carbendazim (C) and imazalil (I) were used alone and combined at both 20 and 40 mg/l. All bulbs were first surface sterilised by dipping in 70% ethanol for 1min then 50% Domestos for 20min followed by 6 rinses in sterile water before being cut into chip explants. After 1h steeping fungicide treated explants were transferred, without washing, to a culture medium supplemented with 0.1mg/l NAA and 1mg/l BA (open columns) or the same plant growth regulator supplemented media to which either 20mg/l carbendazim (filled columns), 20mg/l imazalil (grey columns) or a mixture of 20mg/l of both carbendazim and imazalil (hatched columns) was added.

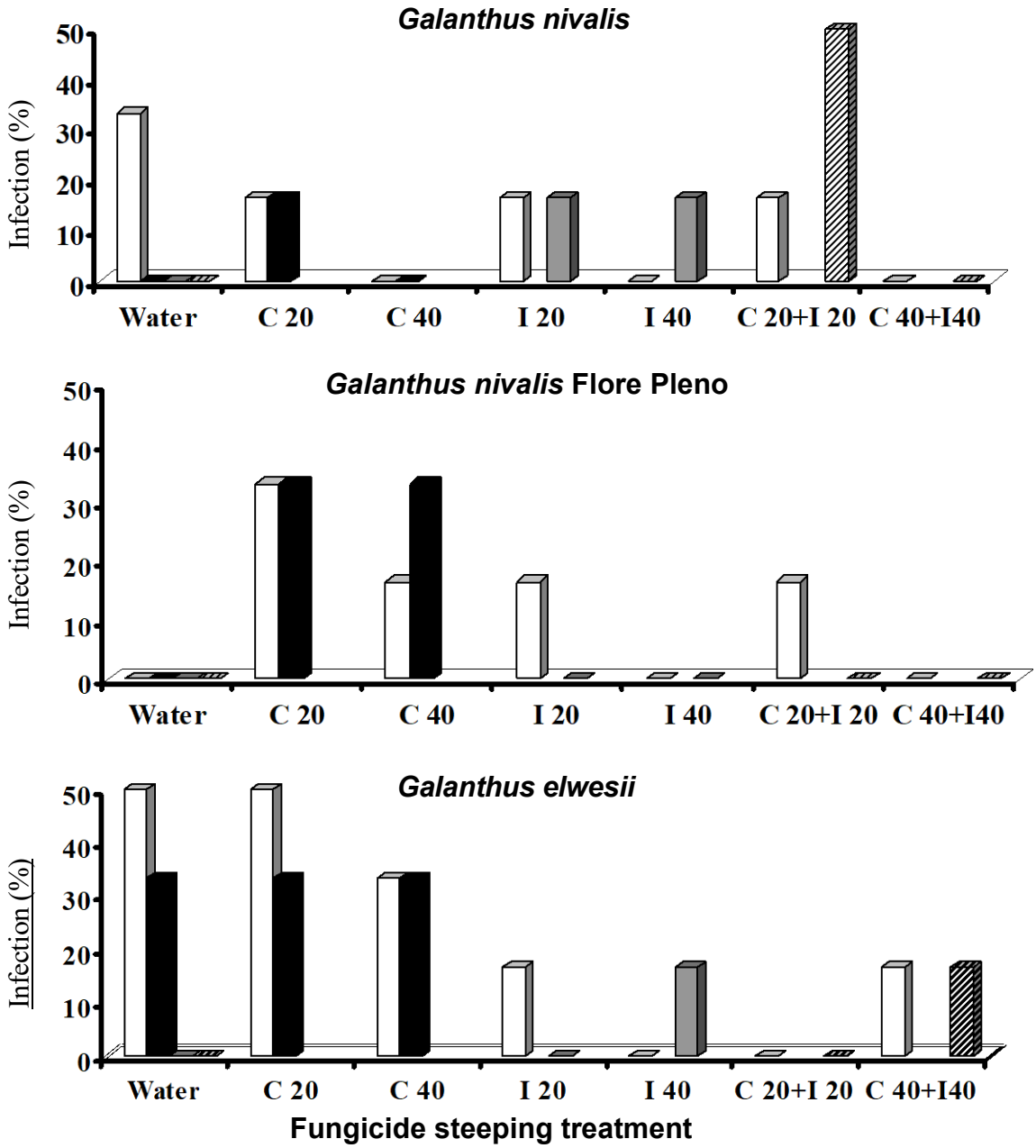
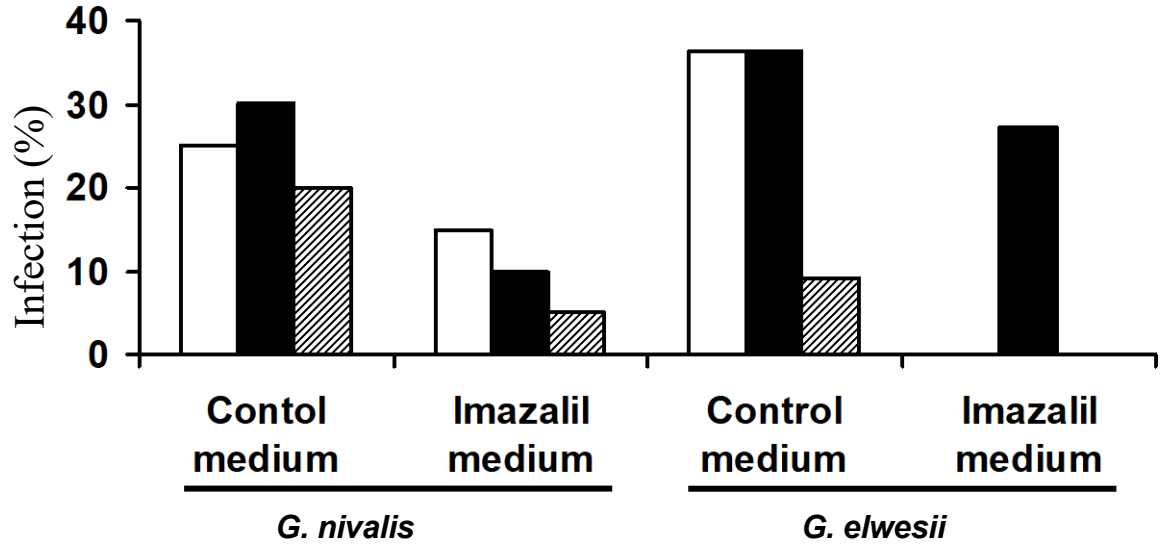


Figure 4. The influence of steeping snowdrop bulb chip explants in an imazalil solution on microbial infection after 21d in culture. All bulbs were surface sterilised by dipping in 70% ethanol for 1min then 50% Domestos for 20min followed by 6 rinses in sterile water. Bulb chips were then prepared and either inoculated directly on to the culture media (open columns), or steeped in sterile water (filled columns) or 40mg/l imazalil (hatched columns) for 1h before inoculating, without washing, on to the culture media. Two explants from each steeping flask were inoculated on to both a medium supplemented with 20mg/l imazalil and a medium without fungicide (control).



EXPERIMENTAL: AGRONOMY AND STORAGE

Materials and methods

Plant material
40 thousand bulbs of 'field-grown' *Galanthus nivalis*, grade 4–5 cm circumference, were purchased in August 2000 from a UK supplier. They were stored in net bags in a non-lit store at 15°C and about 70% relative humidity until planted.

At planting, bulb samples were taken and examined for the presence of *Botrytis galanthina* by Cheryl Brewster (HRI, Stockbridge House). About 10% of the bulbs had *Botrytis* sclerotia. Difficulties were encountered in culturing *Botrytis* from the bulbs, because of the overwhelming presence of *Penicillium*, and it was not practical to confirm the presence of *B. galanthina*.

General methods for field experiments
Field trials were conducted at HRI Kirton, Lincolnshire, in an open field situation typical of the South Lincolnshire area. The soil was a coarse silty marine alluvium. The previous crop was barley (giving a MAFF N index of 0). Before use the field was ploughed and cultivated. Standard agricultural soil analysis revealed the following levels: pH 7.7, P index 4, K index 3 and Mg index 4. Conforming to MAFF fertiliser recommendations for bulbs, no additional fertilisers were applied pre-planting, but N (70kg/ha) was applied in winter. Because of the precocity of the crop, the nitrogen application was made carefully by hand along the beds on 8 January 2001.

The layout of trials was based on 1.200m-wide beds separated by 0.600m-wide pathways, allowing for tractors on 1.830m 'wheelings' working over the beds. The beds were aligned approximately north-west to south-east, at right angles to the prevailing south-westerly winds. The beds were marked in and cultivated, and the position of plots was marked with canes and labels. Along each bed, the plots were 2.475m-long and were separated by 0.825m-long unplanted 'guard' areas. 204 bulbs were planted in each plot, 5-10cm deep and in six rows along the beds, with between-bulb spacings of 7.5cm between and within the rows (making a planted area 37.5cm wide across the bed). The bulbs in each plot comprised three, 60-bulb sub-plots, each sub-plot being separated by a row of six 'guard' bulbs. The sub-plots allowed for sampling in each of three years of the experiment. Bulbs were planted by hand using trowels on 25-27 September 2000. After planting, the area was raked over to tidy and consolidate the soil.

After planting, herbicide (diquat + paraquat) was applied across the whole area. After crop emergence, herbicide was applied carefully to pathways only using a knapsack sprayer, applying cyanazine 'pre-emergence' in early-December 2000, and chlorpropham + linuron 'post-emergence' in early-January 2001. The planted areas were maintained weed-free by hand weeding as required. A regular fungicide spray programme, consisting of alternating vinclozolin, iprodione and dichlofluanid, was applied. All pesticides listed were applied at standard rates.

When the experimental plots were planted, further bulbs were planted in rows in the same field to provide stocks for storage experiments in 2001; these received the same routine

husbandry treatments as the experimental plots.

Agronomy experiment 1: The effect of shade, shelter and soil moisture treatments
Plots were set up with three treatment factors:
(a) Shading: either no shading (control) or shaded with green polyethylene mesh (Netlon Agroshade), either 40, 50 or 70% shade factor
(b) Shelter: either no shelter, or plot sheltered on one side by black polyethylene mesh (Netlon Tensar Windbreak, 55% protection factor)
(c) Plots either (1) irrigated and mulched or (2) neither irrigated nor mulched

There were thus 16 treatment combinations (four shading levels x two shelter levels x two irrigation/mulching levels). The layout was a lattice square design for the 16 treatments in five squares, each of four rows and four columns; the one-, two- and three-year sub-plots were allocated randomly within plots. Shading consisted of a single layer of mesh stretched horizontally over the plots, 45cm above ground level. Shelter consisted of a single layer of mesh (35cm high) held vertically on the windward (south-west) side of the plots 15cm from the edge of the planted area. Mulching consisted of a layer of straw about 5cm deep placed over the planted area before emergence in December. Irrigation was provided from one line of drip tape (T-Tape, T-Systems International Inc, specification TSX 510-15-1000) placed centrally along the planted area (at the time of reporting, it has not yet been appropriate to apply irrigation). Runs of drip tape were connected across non-irrigated plots by plain pipe. Shading, shelter, mulching and irrigation extended beyond the appropriate plot, half-way into the adjacent guard areas.

Agronomy experiment 2: The effect of shading and intercropping
Plots were set up with six treatments:
(a) Control (no shading, no intercropping)
(b) Shading using 40% shade factor mesh, as above
(c) Shading using 70% shade factor mesh, as above
(d) One row of narcissus bulbs (cv. Carlton, 12-14cm grade) planted 15cm deep along each side of the planted area, 15cm from the edge of the planted area and using 26 bulbs per m, after planting snowdrop bulbs
(e) One row of spring barley sown along each side of the planted area, 15cm from the edge of the planted area after planting bulbs
(f) Planting area over-sown with perennial ryegrass after planting bulbs, extending 15cm beyond the edge of the planted area

The layout was a balanced row and column design with five replicates; the one-, two- and three-year sub-plots were allocated randomly within the plots. As in Experiment 1, shading and intercrop plantings extended beyond the appropriate plot, halfway into the adjacent guard areas. The initial barley sowing was lost due to predation, and so replaced by a further sowing and also by transplanting of module-raised seedlings. The intercrops will be removed and replanted annually as appropriate.

Agronomy experiments - crop records
Shoot, flower, seed and bulb production will be assessed on one sub-plot of each plot annually for three years starting in 2001.

Storage experiments
Storage experiments will be initiated in 2001, utilising the bulb stocks planted alongside the agronomic experiments.

Results and discussion

The first results from the agronomy experiments will be available later in 2001, and will be reported in subsequent annual reports.

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