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CONTENTS Page no.

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.

GROWER SUMMARY

Headlines

- **Microprop set to boost quality and availability of snowdrops**
  Bulblet initiation has been successful in *Galanthus nivalis, G. elwesii, G. plicatus* and cultivars. Bulblet growth was greatly increased when activated charcoal was added to the medium. The bulblets were relatively easy to acclimatise to *in vivo* conditions.

- **Commercial field-scale production of snowdrops in the UK may be possible**
  Snowdrop bulbs have been considered difficult or impractical to grow in the field under commercial conditions. Field trials at HRI in Lincolnshire have shown that simple shading and mulching treatments can enhance snowdrop bulb yields.

Background and expected deliverables

There is a demand for snowdrop (*Galanthus*) bulbs that, 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 stocks;
- *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 meet and further stimulate the demand for snowdrops. This would apply to sales of (a) bulk sales of dry bulbs of commonplace material (*G. nivalis*), (b) choice material sold as pot-grown plants, and (c) unusual species and cultivars for the specialist market.

Summary of the project and main conclusions

<u>Micropropagation</u>

Bulblet initiation experiments were carried out using bulb chip explants from *G. nivalis, G. nivalis* cv Flore Pleno, *G. elwesii, G. plicatus* and *G. plicatus* cv Wendy's Gold. The main aims of these initiation experiments were to:

- Evaluate whether the methods developed previously in this project could be transferred successfully to *G. plicatus*.
- Control the physiological disorder 'hyperhydration' in *G. elwesii*.

G. plicatus responded to culture conditions in a similar way to *G. elwesii*, forming large bulblets (20 – 35 bulblets per chip explant over three culture passages) with a tendency to hyperhydrate. Cv Wendy's Gold tissues showed similar properties but were less prolific (11-13 bulblets per chip explant over three culture passages). Leaves of this cultivar were characteristically bright yellow in pigmentation.

Hyperhydration in *G. elwesii* was controlled by:

- Increasing the agar concentration from 7 g/l to 9 or 11 g/l which progressively decreased the levels of hyperhydration in the cultures.
- Changing the cytokinin in the medium from BA to kinetin which reduced hyperhydration at low agar concentrations.
- Changing basal medium from MS to G (a formulation devised earlier in this project) which gave some control of hyperhydration at low agar concentrations.

Factors affecting the bulblet growth to a size ready for transfer to *in vivo* conditions were explored further in several experiments. All experiments involved omission of plant growth regulator compounds from the culture medium and increasing either the carbohydrate (sucrose) supply and/or incorporating activated charcoal. Experiments were replicated with *G. nivalis* and *G. nivalis* cv Flore Pleno. The main findings were:

- Addition of activated charcoal greatly stimulated bulblet growth with *G. nivalis* and *G. nivalis* cv Flore Pleno.
- Increasing the sucrose concentration to 60 or 90g/l had little effect on bulblet growth even in the presence of activated charcoal.
- Cold treatment of cultures for 4 or 6 weeks at 5°C showed no improvements in bulblet growth.
- Dilution of the basal culture medium to half or quarter strength progressively reduced bulblet growth. G basal medium supported bulblet growth marginally better than MS medium.
- *G. nivalis* bulblets showed a consistent strong growth response to the addition of activated charcoal throughout a series of experiments whereas *G. nivalis* cv Flore Pleno bulblets showed a poorer growth response in later experiments. The decline in bulblet growth in cv Flore Pleno could only partially be explained by the medium dilutions used in later experiments. Greater production of competing organs (roots and bulblets) and (or) a decline in 'vigour' with repeated culture passaging by cv Flore Pleno might be responsible for declining bulblet growth rates.
- Longitudinal sections showed bulbs formed up to three normal looking scale leaves but formed no flowers.
- Activated charcoal stimulated initiation and elongation of roots.

Additional bulblet growth experiments were established to test the effects of:

- Alternative adsorption agents to activated charcoal.
- The plant growth regulator concentration in the bulblet multiplication phase.

Initial experiments with *G. nivalis* and *G. nivalis* cv Flore Pleno indicate that bulblets are acclimatise relatively easy to *in vivo* conditions.

Agronomy

Experiments were set up with *G. nivalis* to study the effects of shading, shelter and mulching on plant, seed and bulb production. Shading consisted of a single layer of Netlon Agroshade mesh stretched horizontally over the plots, 45cm above ground level. Windbreak consisted of a single layer of Netlon Tensar Windbreak 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 in November before emergence. Shading, windbreak and mulching extended beyond the appropriate plot, halfway into the adjacent guard areas.

The main findings were:

- Shading plots resulted in higher bulb yields after one, two and three years' growth.
- Bulb yields were also better where plots were mulched.
- Plots that had neither mulch, shading nor windbreak produced the poorest yields. In such plots, bulb yields declined from year one onwards.
- Using a windbreak protected new crops from leaf loss in adverse winter weather, but did not have a major effect on bulb yields.
- Plants in shaded plots produced more stems, seed pods and seeds.
- Both shading and mulching delayed the onset of leaf senescence.
- There were prospects for improving snowdrop growth by using simple shading materials, preferably in conjunction with a straw mulch.
- In the third year of the trial, plant growth declined, possibly due to the build-up of bulb disease (*Botrytis galanthina* is a widespread problem in snowdrops) or other limiting factors (inter-plant competition or nutritional factors).

In a second experiment, *G. nivalis* bulbs were grown in shaded or non-shaded plots, in plots inter-cropped with narcissus or cereal, or in plots over-sown with perennial rye-grass:

- In the first and second years, the highest numbers of shoots, stems, seed pods and seeds, and the highest bulb yields, were obtained in shaded plots. Yields were much poorer in inter-cropped plots and especially in plots sown with rye-grass.
- Inter-planted crops (cereal or narcissus) and, particularly, over-sown rye-grass, appeared too competitive to snowdrops to be used as alternatives to artificial shading.
- After three years of this experiment, the best yields were from plots grown under a medium-density shading.

In a fungicide experiment, bulbs of *G. nivalis* were either dipped in Benlate + Captan before planting, or were non-dipped as controls. After shoot emergence, the foliage was sprayed at 10 – 14 day intervals with Amistar, Benlate + Dithane 945, Folicur, Ronilan FL, Scala, Unix or Stroby WG fungicides, or remained non-sprayed as controls. Foliar senescence was delayed when Scala and Unix had been used, compared with unsprayed controls and plots sprayed with other fungicides. Bulb yields were significantly boosted where a pre-planting dip had been used but were not affected by spray programme. Mean bulb weight was significantly affected by both dips and sprays, compared with controls, with mean weight increased using a pre-planting dip, and when Unix, Stroby WG and, particularly, Scala, had been used. In contrast to the other fungicide sprays, using a Scala programme increased mean bulb weight irrespective of whether a pre-planting dip had been given or not.

Bulb storage

The effects of storage conditions on bulb quality were examined in an experiment with *G. nivalis*. Bulbs were lifted from the field in March (foliage still green), April (foliage beginning to senesce) and May (foliage fully died-down). Small batches of bulbs were stored

in controlled temperature stores running at 10, 13, 17 and 20°C and 65 – 75% relative humidity. Bulbs were held in polythene bags of silver sand, in open trays, in loosely closed polythene bags and in Perspex 'seed propagators', for 4, 8 or 12 weeks before being recovered and weighed and selecting healthy bulbs for growing-on.

- Storage in silver sand gave good protection from desiccation without resulting in mould growth, as did storage in loosely closed polythene bags.
- Storage at 13°C was satisfactory, irrespective of the dates bulbs were lifted from the field or the duration of storage.
- Storage in open trays resulted in desiccation, and storage in Perspex propagators resulted in excessive mould growth.
- When healthy bulbs were planted and grown-on, the best performance was from those stored in polythene bags (at 13 or 17°C) or in silver sand stored at 20°C.

Further storage experiments are under way. Investigating simple storage in a wide range of polythene bags of different gauges, bags of less than 150 g were considered to allow too much water loss from the bulbs. In another experiment, confectionery glazes are being tested as a way of reducing bulb desiccation. These bulbs are still being grown on, to assess the effects of storage conditions on plant vigour.

Financial benefits

An assessment of the benefits deriving from the project must await its completion. The success of micropropagation experiments, however, suggests there is every prospect of establishing good, sustainable bulb stocks that would stimulate demand, not only of the 'ordinary' *G. nivalis*, but also of *G. elwesii* and other species and cultivars. Additional benefits would accrue from better bulb husbandry and storage. Assuming an annual import of 20 million 'ordinary' single snowdrop bulbs, half to be sold retail and half to be sold wholesale, at current prices sales would be worth about £2 million annually, or considerably more for double-flowered or choice types.

Action points for growers

- The results from micropropagation experiments are encouraging, and reliable methodology is now available for the sterilisation, bulblet initiation, multiplication and growth phases of propagation. Thus growers interested in taking up snowdrop cultivation in due course might find it worthwhile to survey sources of good stocks or superior species or cultivars of snowdrops and how they would be marketed.
- The findings to date suggest that the erection of simple shading materials (eg Netlon Agroshade) over snowdrop crops would provide some protection and would delay leaf senescence, giving better bulb yields. Mulching with straw at a depth of 5cm also improves yields, and would also control weeds and conserve water; it is best to combine shading and mulching.
- A pre-planting fungicide bulb dip should be used, and a spray programme including Scala or Unix should be considered.

GENERAL INTRODUCTION

The background to this project was fully described in the first Annual Report. Briefly, there is in the UK a demand for snowdrop bulbs that was in the past met from bulbs collected from the wild, a practice no longer acceptable. The crop is difficult to exploit commercially because of 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.

The previous results have been described in detail in the previous three Annual Reports, and this report describes the additional results obtained up to the end of 2003.

MICROPROPAGATION

Materials and methods

Plant material

Bulbs for bulblet initiation experiments
Galanthus nivalis (5-6cm circumference), *G. nivalis* Flore Pleno (5+cm) and *G. elwesii* (7+cm) bulbs were supplied by Jacques Amand International in October 2001 and 2002, and were stored at room temperature. The same company supplied *G. elwesii* bulbs in October 2003. In autumn 2002 both John Shipton Bulbs (Carmarthenshire) and Monksilver Nursery (Cambridgeshire) supplied *G. plicatus* bulbs. The latter nursery also supplied *G. plicatus* cv Wendy's Gold, a variety that is in short supply and commands a premium price.

Bulblets for growth experiments
Bulblets multiplying *in vitro* were used to inoculate experiments aimed at finding the culture conditions needed to stimulate their growth to a size adequate for planting out *in vivo*. Cultures derived from bulbs purchased in 2000 and 2001 were used for *G. nivalis* and *G. elwesii*. However, with *G. nivalis* Flore Pleno only bulblets produced from bulbs purchased in 2001 were used, thus avoiding use of bulbs bought in 2000 that were found to be inauthentic Flore Pleno, with single rather than double flowers (see 2001 Report).

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. Apical bulb 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 given a preliminary surface sterilisation by shaking in 70% ethanol for 1min. Thereafter bulbs were surface sterilised singly in 100ml Erlenmeyer flasks (*G. nivalis*, *G. nivalis* Flore Pleno and *G. plicatus*) or in wide form 100ml beakers (*G. elwesii*) capped in aluminium foil and shaken at 130 r.p.m. on a reciprocating shaker. Whole bulbs were treated with 50% Domestos for 20min, rinsed five times in sterile de-ionised water, and trimmed top and bottom to remove tissues damaged by the sterilant. Trimmed bulbs were cut aseptically into chip explants and the chips treated with 4% PPM (in 50 mg/l $MgSO_4$) for 9h.

Bulb chips were approximately 8mm in height for *G. nivalis*, *G. nivalis* Flore Pleno and *G. plicatus* (all species yielding four chips per bulb) or 10mm in height for *G. elwesii* (yielding six chips per bulb). Four chips in 20ml of PPM was adequate to sterilise the smaller chips. Larger ones produced from *G. elwesii* bulbs required three chips in 30ml of PPM to improve surface sterilisation. 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

Galanthus medium (G) was described previously and prepared from Analar inorganic reagents and tissue culture tested organic compounds (Annual Report for 2002). Pre-prepared Murashige and Skoog (1962) basal macronutrients, micronutrients and vitamins (Sigma Aldrich Co. Ltd.) were used throughout, dissolved in water purified with an Elga Prima reverse osmosis apparatus (Elga Ltd). Unless otherwise stated G and MS media were supplemented with 30g/l sucrose, 1mg/l 6-benzyladenine (BA) and 0.1mg/l naththaleneacetic acid (NAA). 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 an air-conditioned growth room at a constant temperature of 18ºC. A photoperiod of 16h was provided by cool white fluorescent tubes giving a PAR of 100µ mol/m^2/s at bench height.

Bulblet initiation

Bulblet initiation in Galanthus plicatus

The ability of *G. plicatus* bulb chip explants to form adventitious bulblets *in vitro* was tested for the first time in an experiment inoculated on 5 December 2002. *G. plicatus* bulbs were obtained, freshly lifted, from two sources together with a well known cultivar of this species, Wendy's Gold. Tissues from *G. nivalis, G. nivalis* Flore Pleno and *G. elwesii* were also used for comparison and to establish fresh cultures of these species. Explants from each plant type were inoculated onto half strength MS and G media. The full experimental design was 2 basal media (0.5G + 0.5MS) x 6 snowdrop types (2 samples of *G. plicatus*, *G. plicatus* Wendy's Gold, *G. nivalis, G. nivalis* Flore Pleno and *G. elwesii*), 12 treatments in all. Each treatment was replicated with 10 bulbs of each snowdrop type, two chip explants from each bulb being inoculated onto each medium (240 cultures in total). For each explant type data from the two explants was meaned for each bulb before the data was subjected to ANOVA i.e. there were 10 independent replications.

Control of hyperhydration in G. elwesii

By the end of the second culture passage a physiological disorder known as hyperhydration (vitrification) developed in multiplying bulblet cultures in both 2000 and 2001. Hyperhydration is characterised by tissues becoming very swollen, translucent in appearance, deformed and brittle. This disorder is caused by an excessive uptake of water (Debergh *et al.,* 1992).

Although no systematic scoring of hyperhydration was attempted general observations were made. Occurrence of the disorder varied from explant to explant and even within different regions of the same explant. Whole scale leaf segments, present in the original bulb chip explant became very swollen and glassy in appearance. In the most extreme instances these

scales expanded to the extent were they pressed against both sides of the culture vessels (21mm i. d.). Tissues of newly initiated bulblets also became hyperhydrated forming swollen and distorted scales and leaves. Tissues of *G. elwesii* were much more prone to hyperhydration than *G. nivalis*. With this species hyperhydration occurred on all media tested. Hyperhydration is usually regarded as a problem because it is difficult to reverse and hyperhydrated plantlets are difficult to acclimatise to *in vivo* conditions. It was therefore important to develop methods to restrict hyperhydration particularly with *G. elwesii.*

A major factor controlling hyperhydration in plant tissues is the concentration and type of gelling agent used (Debergh, Harbaoui and Lemeur, 1981; Debergh, 1983). Choice of cytokinin can also influence hyperhydration with BA, the cytokinin used in the current study, being implicated as a cause of the disorder in some circumstances (Kataeva *et. al*., 1991; Debergh *et al.,* 1992). Experiments were therefore established to examine if manipulation of these factors could control hyperhydration in *G. elwesii*. Since it was also noted that hyperhydration in *G. elwesii* cultures was less frequent and severe on G medium compared to MS medium (Table 1.6 and Figure 1.2 in the 2002 Annual Report) effects of changing the basal medium were also tested.

Agar concentration and basal medium
G. elwesii bulb chip explants were inoculated onto media solidified with either 7, 9 or 11 g/l Oxoid purified agar. Each agar concentration was repeated with both half strength G and MS media. In this experiment the general surface sterilisation treatment with PPM was modified to improve its efficacy. Explants were treated in groups of three in 30ml aliquots of 5% PPM. The full experimental design was 2 basal media (0.5G + 0.5MS) x 3 agar concentrations, 6 treatments in all. Each treatment was replicated with 26 bulbs, one chip explant from each bulb being inoculated onto each medium (156 cultures in total).

Agar concentration and cytokinin
G. elwesii bulb chip explants were inoculated onto media solidified with either 7, 9 or 11 g/l Oxoid purified agar. Each agar concentration was repeated with half strength MS media supplemented with either 1mg/l BA or an equimolar concentration of an alternative cytokinin kinetin (Kin). The full experimental design was 2 cytokinins (BA + Kin) x 3 agar concentrations, 6 treatments in all. Each treatment was replicated with 25 bulbs, one chip explant from each bulb being inoculated onto each medium (150 cultures in total).

Iron and magnesium concentration
Yadav, Gaur and Garg (2003) recently reported that increasing the concentrations of iron and/or magnesium effectively controlled hyperhydration and improved shoot multiplication in cultured plant tissues. The same media modifications were therefore tested for their ability to decrease hyperhydration in *G. elwesii*. Experiments employed full strength MS medium supplemented with 30g/l sucrose, 1 mg/l BA and 0.1 mg/l NAA. Normal control concentrations in the MS medium were 0.1mM for Fe and 1.5 mM for Mg. Two experiments were performed these being inoculated with:

1. *Bulb chip explants*
   The full experimental design was 2 Fe concentrations (0.1 and 0.15 mM) x 3

Mg concentrations (1.5, 2.25 and 3.0 mM) = 6 media treatments replicated 27 times (162 cultures in total). Replications comprised a single *G. elwesii* bulb divided into six chip explants.

2. *Hyperhydrated proliferating bulblet cultures*
The full experimental design was 3 Fe concentrations (0.1, 0.15 and 2.0 mM) x 3 Mg concentrations (1.5, 2.25 and 3.0 mM) = 9 media treatments replicated 25 times (225 cultures in total). Replications were inoculated with different clonal lines derived two years previously from *G. elwesii* bulbs.

Stimulation of bulblet growth

Sucrose and activated charcoal on half strength G basal medium
The effects of sucrose and activated charcoal were investigated further in two separate experiments, one with *G. nivalis* and the other with *G. nivalis* Flore Pleno. Both used half strength plant growth regulator free G medium. These experiments tested the effects of 30, 60 and 90 g/l sucrose with and without 5 g/l activated charcoal (6 media treatments in all). Twenty replicates were used for each snowdrop type (240 cultures in total).

Cold treatment
Since in nature pre-formed lateral bulb units are subjected to a winter cold treatment prior to them forming flowering, terminal bulbs in the spring the effect of cold treatment on subsequent bulblet growth was investigated. Bulblet clump cultures of *G. nivalis* and *G. nivalis* Flore Pleno were subjected to 0, 4, or 6 weeks cold treatments by incubating at 5ºC in a Sheerer CEL 512-37 growth chamber. Bulblet clumps from each treatment were inoculated onto half strength plant growth regulator free G medium containing 30 or 60 g/l sucrose plus or minus 5 g/l activated charcoal. The experiment was designed so that all cultures of each snowdrop type were inoculated at the same time with all three cold treatments. The full experimental design was 3 cold treatments x 2 sucrose x 2 charcoal concentrations x 2 snowdrop types, 24 treatments in all. Each treatment was replicated 15 times (360 cultures in total).

Medium strength
The effect of medium strength on bulblet growth was investigated to examine if this factor could explain differences in bulblet growth between different experiments where either full or half strength media had been used. The full experimental design was 2 basal media (MS and G) x 3 concentrations (full, half and quarter strength) x 2 snowdrop types (*G. nivalis* and *G. nivalis* Flore Pleno), 12 treatments in all. Each treatment was replicated 21 times (252 cultures in total).

Alternatives to activated charcoal (adsorption agents)
It was reported previously that the major factor influencing bulblet growth was the inclusion of activated charcoal in the culture medium (see Annual Report for 2002 and data presented in this Report). Several mechanisms have been proposed to explain benefits of activated charcoal (Pan and van Staden 1998). These include the adsorption of endogenous or synthetic plant growth regulator compounds from the medium. Adsorption of toxic metabolites,

released by plant tissues, might also be important. Activated charcoal could also function by darkening the culture medium and thereby inducing polarity in the cultured tissues. In addition, activated charcoal can alter the mineral composition of the medium by decreasing the supply of some minerals such as copper and zinc or increasing the supply others such as magnesium (Van Winkle, Johnson and Pullman 2003; Van Winkle and Pullman 2003).

A number of alternative adsorption agents were therefore tested to examine the mode of action of activated charcoal and to improve bulblet growth *in vitro*. Full strength G medium without plant growth regulators, supplemented with 60g/l sucrose was used throughout. Appropriate activated charcoal treatments and a control without adsorption agent were included for comparison.

The adsorption agents used were polyvinylpyrrolidone (PVP) Mol. wt. 10,000 (Sigma P-2307), Fuller's earth (FE) mesh size 30-60 (Sigma F-60), kaolin (KA)(Sigma K-1512) and Silicar CG-4, a silica based column chromatography medium (Mallinckrodt Ltd.). The particle size of the FE was reduced by grinding it in a pestle and mortar then passing it through a 300μm sieve. The full experimental design was 5 adsorption agents (PVP, FE, KA, SIL and AC) x 3 concentrations (0.2, 1.0 and 5.0 g/l) + control (no adsorption agent) x 2 snowdrop types (*G. nivalis* and *G. nivalis* Flore Pleno), 32 treatments in all. Each treatment was replicated with 20 bulblet clumps (640 cultures in total).

Alternatives to activated charcoal (pectins)

In second experiment a series of pectins were tested as alternatives to activated charcoal. One of the pharmacological function of dietary fibre, and pectins in particular, is to act as an intestinal sorbent (personal communication, Yuri Khotimchenko, Far East Branch of Russian Academy of Sciences, Vladivostok). This is reflected in a growing body of information on the pharmacological effects of pectin mediated by their adsorption of various elements and metabolites (Lewinska, Rosinski and Piatkiewicz 1994; Wu, *et. al.*, 2003; Umar *et. al.*, 2003).

Pectins used to stimulate bulblet growth were therefore chosen to give a range of adsorption characteristics. All were classed as low methyl pectins, having 50% or less esterification, after being chemically modified from citrus peel pectin that is naturally 74% methylated. Properties of these GENU® pectins are given in Table 1.1 and all were supplied by CP Kelco Ltd. Denmark. Full strength G medium without plant growth regulators and supplemented with 60g/l sucrose was used throughout. Since the pectins strongly modified the culture medium pH, and the medium further acidified after autoclaving, preliminary experiments were performed to find the initial pH values required to give a final media pHs from 5.1 to 5.6. All media were solidified with 7 g/l Oxoid purified agar and the effects of the pectin treatments on gel rigidity assessed using a penetrometer. Activated charcoal treatments and a control without adsorption agent were included for comparison. The full experimental design was 5 adsorption agents (the four pectins described in Table 1.1 plus AC) x 3 concentrations (0.2, 1.0 and 5.0 g/l) + control (no adsorption agent) x 2 snowdrop types (*G. nivalis* and *G. nivalis* Flore Pleno), 32 treatments in all. Each treatment was replicated with 15 bulblet clumps (480 cultures in total).

Table 1.1 Properties of GENU® pectins tested as alternative adsorption agents.

Chemical characteristic	Pectin type			
	LM-5 CS	LM-12 CG	LM-22 CG	LM-101 AS
Esterification (%)	5-10	35	50	35
Amidation (%)	0	0	0	15
pH of 1% solution	4.0-5.5	2.8-3.4	2.8-3.4	4.0-5.0

Plant growth regulator concentration in the multiplication phase
When bulblet clumps are transferred from bulblet multiplication conditions, where BA and NAA are supplied, to bulblet growth conditions, where no plant growth regulators are used, bulblet multiplication still takes place particularly with *G. nivalis* Flore Pleno (2002 Annual Report and data presented in this Annual Report). Continued production of new bulblets in the growth phase could be indicative in incomplete adsorption of growth regulators by the charcoal. New bulblets would be expected to compete with the transferred bulblets for nutrients and space and thereby reduce their growth. To investigate this possibility the effect of plant growth regulator concentrations in the multiplication phase on subsequent bulblet growth on charcoal media was assessed. The effects of plant growth regulator concentrations on bulblet multiplication were assessed in several multiplication passages as well as after transfer to bulb growth conditions.

Multiplying *G. nivalis* Flore Pleno cultures growing on half strength G medium were used as an inoculum. The full experimental design of the experiment was three plant growth regulator treatments [control (1 mg/l BA & 0.1 mg/l NAA), PGR/2 (0.5 mg/l BA & 0.05 mg/l NAA) and PGR/10 (0.1 mg/l BA & 0.01 mg/l NAA)], x 6 Flore Pleno genotypes (lines initiated from different bulbs) 18 treatments in all. In the first instance six replicates were used for each treatment but culture numbers were increased with passaging. At the end of the second culture passage on the three multiplication media bulblet clumps were transferred into both multiplication conditions (control, PGR/2 and PGR/10) for a third multiplication passage and into bulblet growth conditions (full strength G medium supplemented with 5g/l charcoal and 60 g/l sucrose).

Acclimatization of bulblets

On 12 September 2003 *G. nivalis* and *G. nivalis* Flore Pleno bulblets from the medium strength experiment described above were planted out into John Innes compost with drainage improved by the addition of either silver sand or perlite (2 : 1 compost : sand or compost : perlite). Larger single bulblets and clumps of bulblets were selected and soaked for 10 min in a fungicide solution (Derosal 0.4 g/l). Bulblets were planted to a depth of approximately three times their height. Smaller bulblets of each species, 2 mm or less in diameter, were also

fungicide treated then planted in a peat based compost with drainage improved with sharp sand (2 : 1). Larger bulbs were planted in tote boxes (36 x 51 cm) to give an adequate compost depth (c. 7cm) whilst the smaller bulbs were planted in conventional seed trays. All plants were grown in an open topped cold frame.

Bulblets were also grown on for larger scale field trials at Kirton. Half strength G medium was used supplemented with 60 g/l sucrose and 5 g/l activated charcoal. One pound glass honey jars filled with 90 ml of culture medium were used and each jar was inoculated with six bulblet clumps.

Results and Discussion

Initiation of bulblets

Bulblet initiation in Galanthus plicatus

Bulblet initiation was obtained from virtually all bulbs of *G. plicatus* in the trial and from all bulbs of the cultivar Wendy's Gold. Multiplication of bulblets in the first three culture passages was comparable to that of *G. elwesii* and *G. nivalis* but lower than that with *G. nivalis* Flore Pleno (Figure 1.1). The proliferating cultures of *G. plicatus* tended to look more like those of *G. elwesii* forming larger bulblets that had a greater tendency to become hyperhydrated (Figure 1.2). This is reflected in the numbers of hyperhydrated bulblets for this species shown in Figure 1.1. Thus, as with *G. elwesii,* efficient micropropagation of *G. plicatus* will also probably rely upon effective measures to control hyperhydration being developed.

Additionally, bulblets of Wendy's Gold were also very yellow in appearance (Figure 1.3). This yellowing was most apparent at the lower part of the expanding leaves. Yellow pigmentation is not unexpected considering the unusual, conspicuous yellow ovary colouration in this cultivar.

Figure 1.1 Bulblet production by various types of snowdrop over the first three culture passages. *G. plicatus* bulbs samples 1 & 2 were supplied by Monksilver Nursery, Cambridgeshire and John Shipton (bulbs) Carmarthenshire respectively. Bulb chip explants were grown on either full half strength MS (top Figure) or half strength G medium (bottom Figure). Shaded sections at the top of columns representing the third culture passage indicate the numbers of bulblets that were hyperhydrated.

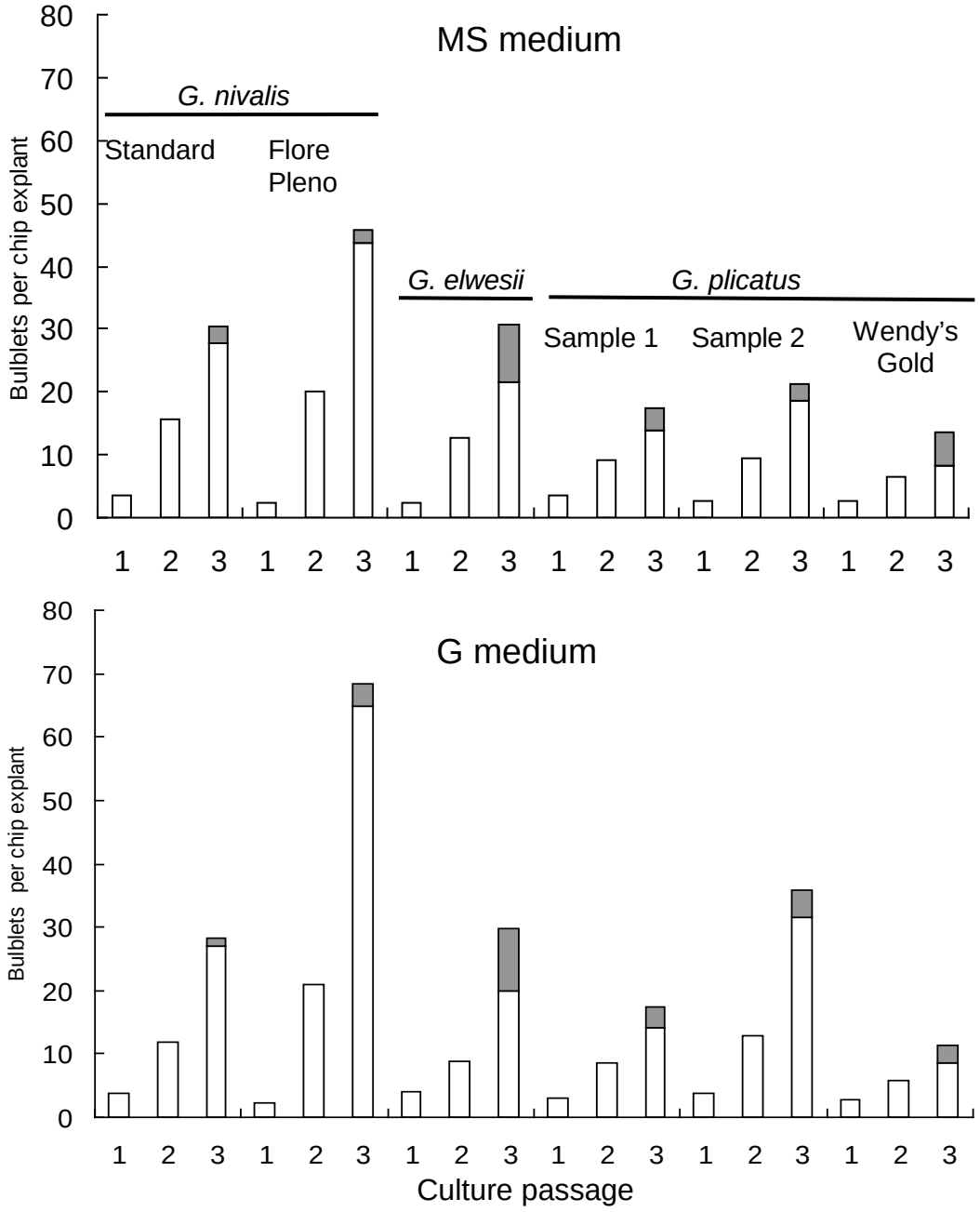


Figure 1.2. *G. plicatus* bulblets multiplying *in vitro*. Showing large *G. elwesii* like bulblets with some hyperhydrated tissues.

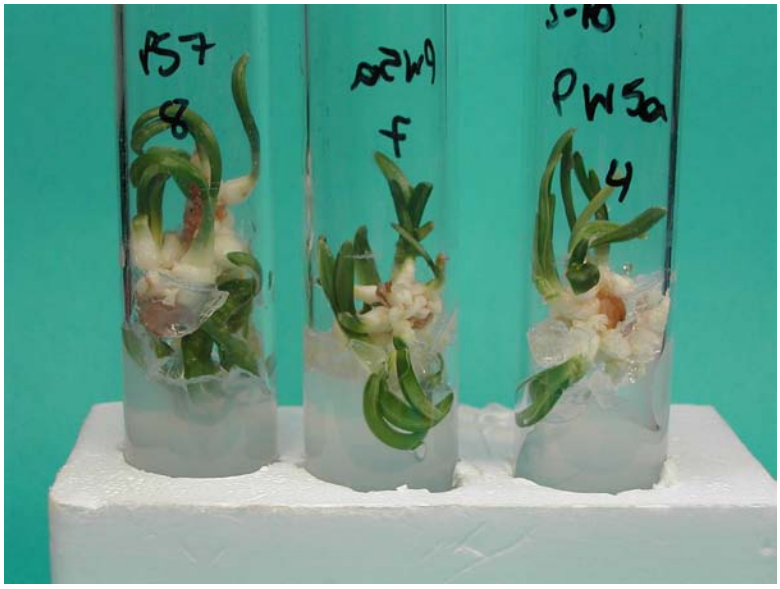
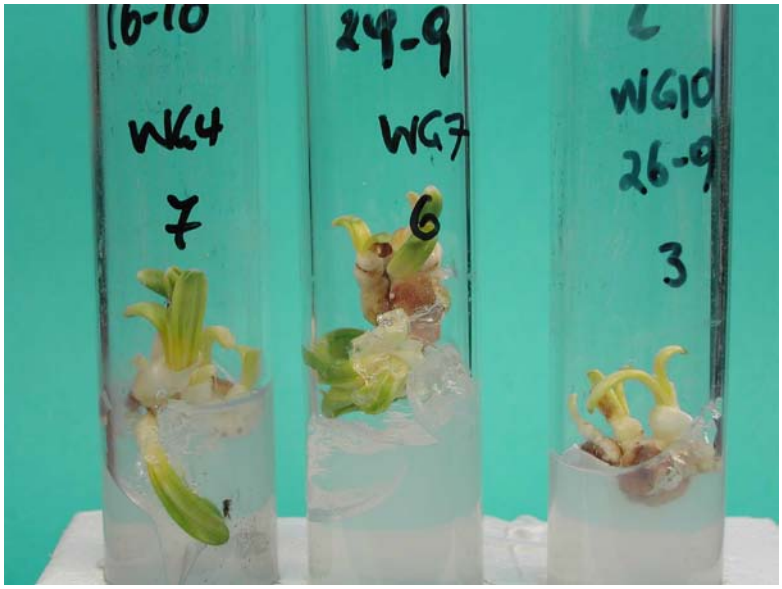


Figure 1.3. *G. plicatus* Wendy's Gold bulblets multiplying *in vitro*. Showing distinctive yellow pigmentation in the leaves.



Control of hyperhydration in *G. elwesii*

Agar concentration and basal medium

Composition of the basal culture medium did not effect the total numbers of bulblets formed by *G. elwesii* chip explants after three culture passages (Figure 1.4A). However, use of G medium gave significantly more normal (none hyperhydrated) bulblets than MS medium ($p < 0.05$). This is in broad agreement with lower levels of hyperhydration in *G. elwesii* bulblets grown on G media reported previously (2002 Annual Report). Similarly, tissues grown on G media were found to produce significantly fewer hyperhydrated bulblets than those on MS media if the data was normalised by square root transformation before the analysis of variance ($p = 0.016$). Thus a change from MS medium to G medium can give a limited control over bulblet hyperhydration.

Increasing the agar strength of the medium had more marked effects on the growth and development of the chip explants and also offered a measure of control over tissue hyperhydration. Total bulblet numbers significantly declined with increasing agar strength ($p < 0.05$) owing to a large and highly significant reduction in the numbers of hyperhydrated bulblets ($p < 0.001$) whilst the numbers of normal bulblets were unaffected (Figure 1.4A). Overall culture growth, as assessed by fresh weight (Figure 1.4B), was also markedly reduced by increasing the agar strength ($p < 0.001$). Increasing the agar strength also significantly reduced the total fresh weight of the smaller bulblets ($p < 0.001$) whilst the fresh weight of the largest bulblet was not significantly affected (Figure 1.4B).

Measurement of the water content of tissues, by a destructive harvest and drying down to constant weight at 70°C, was used in an effort to enumerate treatment effects on bulblet hyperhydration (Figure 1.4C). No significant differences were found in the water content of the largest bulblet, but increasing agar strength to 11 g/l was found to significantly reduce the water content of the other bulblets ($p < 0.01$). This is in agreement with the decreased numbers of hyperhydrated bulblets at high agar strengths and the observation that the largest bulblet in cultures was rarely hyperhydrated. Low hyperhydration in the largest bulblet is also reflected in the lower water content of this bulblet compared to the other bulblets in all treatments except the 11g/l agar treatment on G medium where bulblet hyperhydration was least.

No interactions between basal medium composition and agar concentration were found for any of the parameters assessed.

Agar concentration and cytokinin

Changing the cytokinin from BA to Kin significantly reduced the numbers of bulblets formed ($p < 0.05$) but also significantly reduced the numbers of hyperhydrated bulblets that were induced ($p < 0.05$) (Figure 1.5A). Control of hyperhydration with Kin was most pronounced at the lowest agar concentration (7 g/l) where use of BA gave very large numbers of hyperhydrated bulblets. This effect was statistically significant as indicated by the interaction between type of cytokinin and agar strength for the numbers of hyperhydrated bulbs per culture ($p < 0.05$).

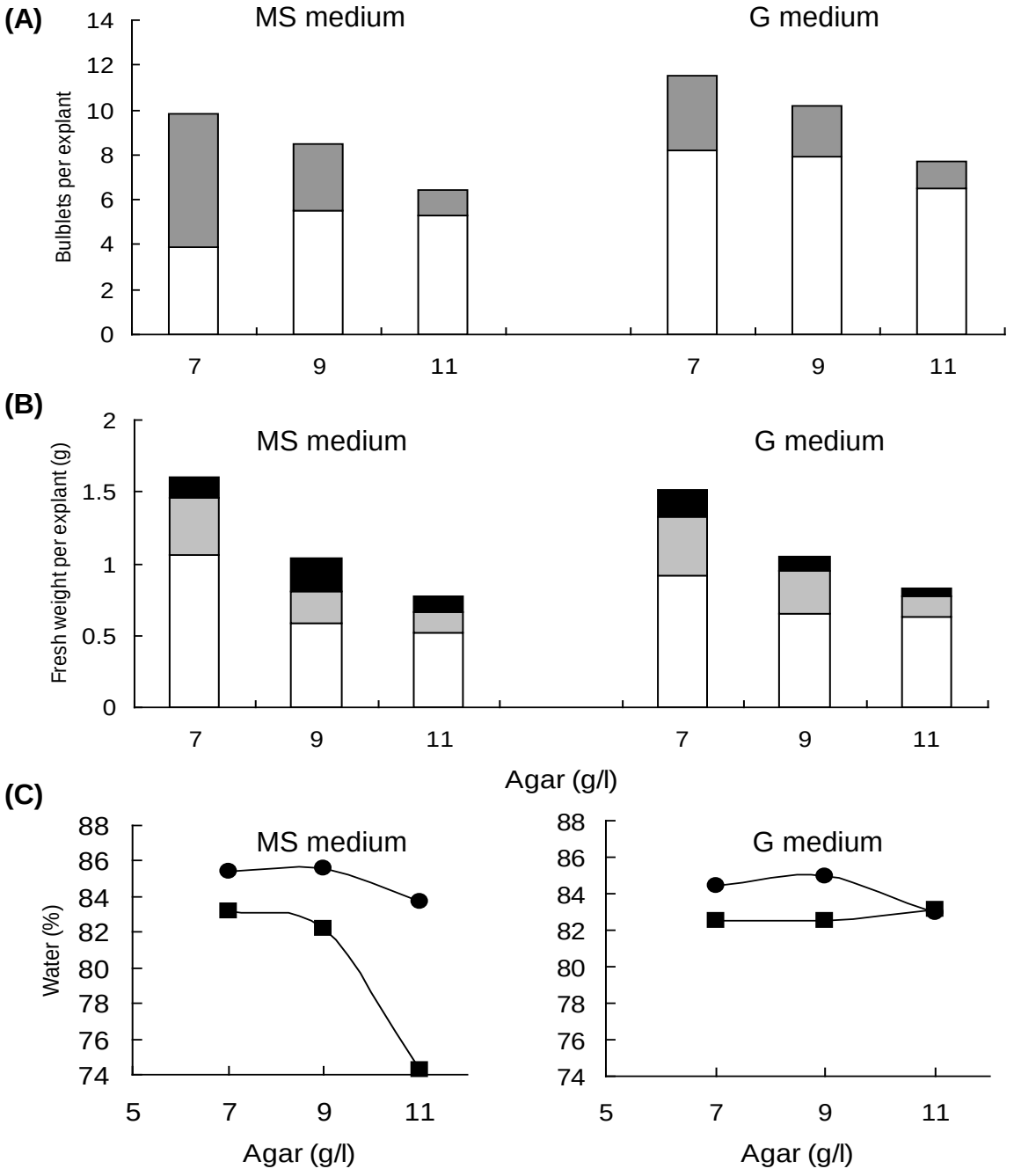
However, as in the previous experiment increasing the agar strength produced the most significant changes in chip explant response over the three culture passages before the experiment was harvested. Numbers of hyperhydrated bulbs were greatly reduced ($p < 0.001$) and normal bulblet numbers greatly increased ($p < 0.01$) when agar concentration was increased to either 9 or 11 g/l when BA was used as the cytokinin. These effects were much less pronounced when Kin was used as the cytokinin as indicated by the significant interactions between choice of cytokinin and agar strength. Increasing the agar strength also markedly reduced the overall growth of the cultures ($p < 0.001$) and the fresh weights of the bulblets excluding the largest bulblet ($p < 0.001$) (Figure 1.5B).

As in the previous experiment the water content of the largest bulblet was not significantly affected by increasing the agar concentration of the medium, reflecting the usual lack of hyperhydration in this bulblet (Figure 1.5C). As with the previous experiment increasing the agar strength significantly reduced water content of the smaller bulblets if BA was used as the cytokinin ($p < 0.05$). Water content was significantly lower in the smaller bulblets if Kin was used as a cytokinin even at the low agar strength ($p < 0.001$). With these smaller bulblets, taking into account data presented in Figures 1.4C and 1.5C, a water content above 82% would appear to be indicative of hyperhydration whilst water content below 82% would be expected for more normal bulblets. Separating hyperhydrated and normal bulblets and assessing the water content of each type could be used to confirm this.

Iron and magnesium concentration

Results to be presented in the final report.

Figure 1.4 Effects of agar concentration on the development of *G. elwesii* bulb chip explants on half strength MS or half strength G medium supplemented with BA after three culture passages. (A) Numbers of normal (open columns) and hyperhydrated bulblets (shaded columns). (B) Fresh weights of the largest bulblet (black), other bulblets (grey) and remaining tissues (open). The remaining tissues comprised expanded leaves, swollen scale and callus. (C) The water content of the largest bulblet (ν) and the remaining bulblets (λ).



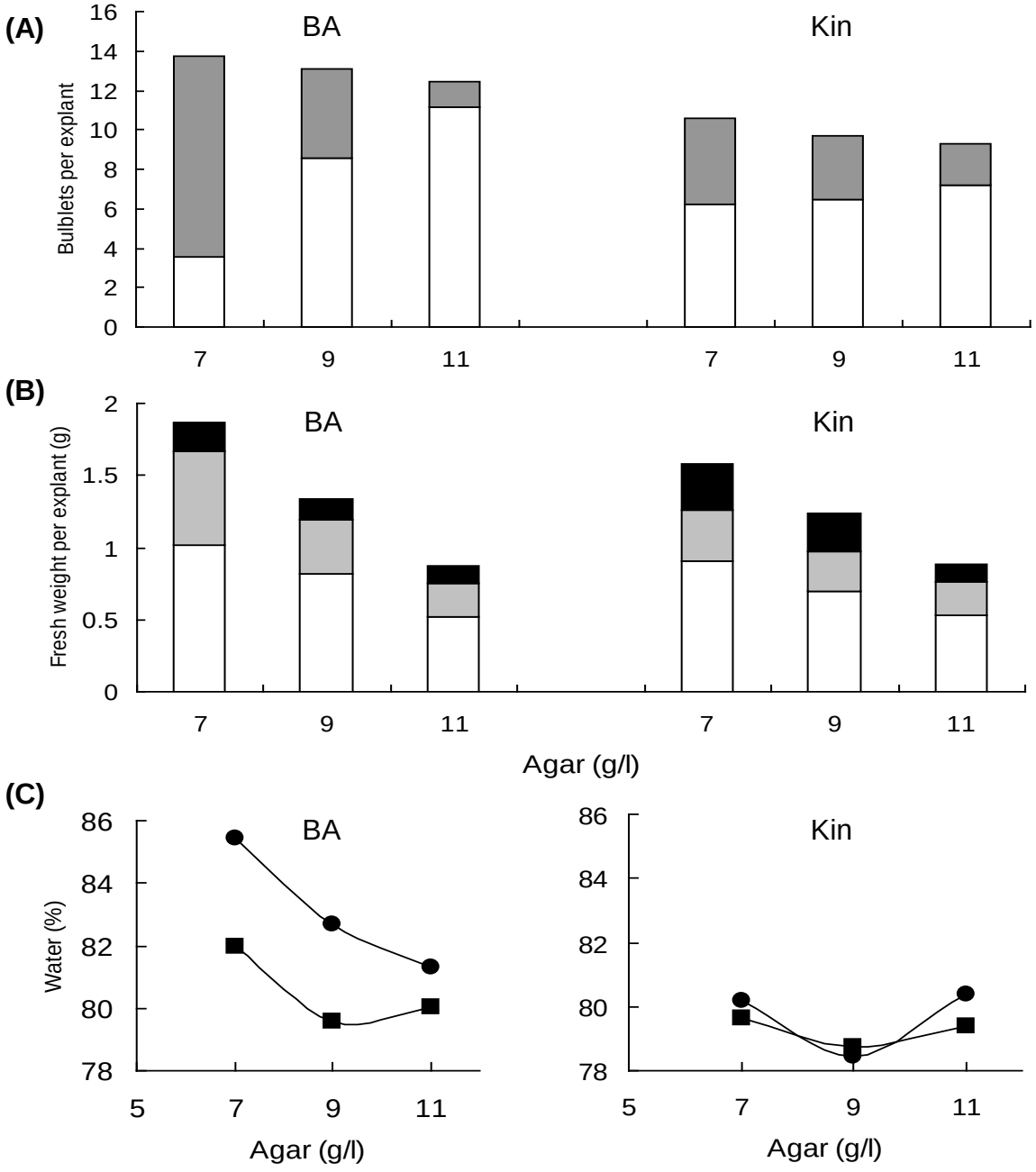
Analysis of variance summary for Figure 1.4

	d.f (m. v.)	Bulblet numbers					
		Total		Normal		Hyperhydrated	
		SED	sig	SED	sig	SED	sig
MEDIUM (M)	1	0.769	NS	0.706	*	0.494	NS
AGAR (A)	2	0.942	*	0.865	NS	0.605	***
M x A	2	1.332	NS	1.223	NS	0.856	NS
Residual	82 (8)						

	d.f (m. v.)	Fresh weight					
		Total		Largest bulblet		Other bulblets	
		SED	sig	SED	sig	SED	sig
MEDIUM (M)	1	0.091	NS	0.034	NS	0.033	NS
AGAR (A)	2	0.112	***	0.042	NS	0.040	***
M x A	2	0.158	NS	0.059	NS	0.057	NS
Residual	82 (8)						

	d.f (m. v.)	Water content (%)			
		Largest bulblet		Other bulblets	
		SED	sig	SED	sig
MEDIUM (M)	1	1.78	NS	0.347	NS
AGAR (A)	2	2.18	NS	0.425	**
M x A	2	3.08	NS	0.601	NS
Residual	82 (8)				

Figure 1. 5 Effects of agar concentration on the development of *G. elwesii* bulb chip explants on half strength MS medium supplemented with either BA or Kin after three culture passages. (A) Numbers of normal (open columns) and hyperhydrated bulblets (shaded columns). (B) Fresh weights of the largest bulblet (black), other bulblets (grey) and remaining tissues (open). The remaining tissues comprised expanded leaves, swollen scale and callus. (C) The water content of the largest bulblet (ν) and the remaining bulblets (λ).



Analysis of variance summary for Figure 1. 5

	d.f (m. v.)	Bulblet numbers					
		Total		Normal		Hyperhydrated	
		SED	sig	SED	sig	SED	sig
CYTOKININ (C)	1	1.122	*	0.779	NS	0.714	*
AGAR (A)	2	1.374	NS	0.954	**	0.874	***
C x A	2	1.943	NS	1.350	*	1.236	*
Residual	82 (8)						

	d.f (m. v.)	Fresh weight					
		Total		Largest bulblet		Other bulblets	
		SED	sig	SED	sig	SED	sig
CYTOKININ (C)	1	0.098	NS	0.006	NS	0.041	*
AGAR (A)	2	0.120	***	0.007	NS	0.050	***
C x A	2	0.170	NS	0.010	NS	0.071	NS
Residual	82 (8)						

	d.f (m. v.)	Water content (%)			
		Largest bulblet		Other bulblets	
		SED	sig	SED	sig
CYTOKININ (C)	1	0.654	NS	0.557	***
AGAR (A)	2	0.801	NS	0.683	*
C x A	2	1.132	NS	0.966	NS
Residual	82 (8)				

Stimulation of bulblet growth

Sucrose and activated charcoal on half strength G basal medium

Data for *G. nivalis* and *G. nivalis* Flore Pleno were analysed separately because they represent separate experiments, employing the same media treatments, with the two species. The experiments were established about one week apart but results are presented side by side here for comparison (Figure 1.6). Results were broadly similar for both plant types. Increasing the sucrose concentration to as much as 90 g/l had minimal effects on bulblets growth as assessed by measuring bulblet diameters (Figures 1.6C and D) and bulblet fresh weights (Figures 1.6E and F). Bulblet multiplication was marginally but significantly stimulated ($p < 0.05$) by increasing the sucrose concentration with *G. nivalis* but not with *G. nivalis* Flore Pleno (Figures 1.6A and B).

In contrast, addition of activated charcoal stimulated bulblet multiplication (Figures 1.6A and B) and bulblet growth with both plant types over all sucrose concentrations (Figures 1.6C, D, E and F). These charcoal effects on bulblet growth were all very highly significant ($p < 0.001$). The magnitude of the bulblet growth response was very similar to that reported previously for *G. nivalis* but was much less pronounced for *G. nivalis* Flore Pleno (2002 Annual Report). Such disappointing growth by the Flore Pleno cultures in the current experiment may be due to the use of half strength G medium in place of the full strength MS medium used previously. Flore Pleno also differed from *G. nivalis* in that on the charcoal containing bulblet growth media it formed larger numbers of competing organs that could have reduced overall bulblet growth. This "over production" of organs by Flore Pleno can be seen for both bulblets (Figure 1.6B) and roots (Figure 1.6H). Increased organ production by Flore Pleno compared to *G. nivalis* was not however reflected in overall culture fresh weight where *G. nivalis* was found to grow notably better (Figures 1.6E and F). Thus although Flore Pleno produced greater numbers of organs these were very much smaller on average than their *G. nivalis* equivalents.

Increasing the sucrose concentration had no effect on root numbers but significantly decreased root length with both plant types ($p < 0.01$). Root length also showed significant interactions between charcoal and sucrose concentration for *G. nivalis* ($p < 0.05$) and *G. nivalis* Flore Pleno ($p < 0.01$) owing to the very low production and elongation of roots in the absence of charcoal.

Cold treatment

Although no statistical comparison could be made between the cold treatments, owing to their lack of replication in time, results were analysed separately and are presented side by side here for comparison. Cold treatment of the bulblets was found to have very little effect for bulblet growth parameters regardless of the culture media conditions used (Figure 1.7). As found in the previous experiment inclusion of charcoal in the medium had a much stronger effects on bulblet growth than increasing the sucrose concentration to 60 g/l. Similarly, bulblet growth was better with *G. nivalis* than *G. nivalis* Flore Pleno. This difference between plant types was particularly evident in the fresh weights of the largest bulblets (Figure 1.7 lowest row of figures). Again the relatively poor growth of the Flore Pleno bulblets might be explained by the use of half strength G medium.

Addition of charcoal to the bulb growth medium significantly stimulated the formation of bulblets and roots as well as overall culture growth and root elongation with both plant types regardless of the cold treatment applied or, in most instances, the sucrose treatment used (Figure 1.8). There were interactions between charcoal treatment and species, caused by *G. nivalis* Flore Pleno responding much more strongly to charcoal than *G. nivalis*. Thus like in the previous experiment Flore Pleno produced more competing organs, both bulblets and roots, that could have restricted the growth of the bulblets initially present in the inoculum. In contrast to the previous experiment the species varied very little in overall culture growth.

Medium strength

The effect of medium strength on bulblet growth was investigated in order to examine if this factor could explain differences between experiments where *G. nivalis* Flore Pleno bulblets had grown less well on half strength G medium (two experiments above) compared to on full strength MS medium (2002 Annual Report). Reducing the medium strength was found to significantly reduce the diameters of the largest and second largest bulblets as well as the fresh weight of the largest bulblet (all $p < 0.001$). These effects were found with both *G. nivalis* and *G. nivalis* Flore Pleno regardless of which basal culture medium was used (Figure 1.9). Thus diluting the basal media used for bulblet growth could explain some of the differences found between experiments. However, the magnitude of differences found between media strength treatments was relatively small compared to the differences found between experiments. For instance, the average fresh weight of the largest bulblets of Flore Pleno in 2002 was 0.42 g (on MS medium with 60 g/l sucrose and 5 g/l charcoal) compared to values between 0.08 and 0.16 g for bulblets growing on quarter and full strength media respectively in the current experiment (Figure 1.9C). Similar comparisons can be made for bulblet diameters. It is therefore unlikely that changes in medium strength alone can fully account for differences found between experiments.

In addition, with both plant types G medium supported bulblet growth marginally but significantly better than did MS medium (Figure 1.9). This was shown for diameters of the largest ($p < 0.05$) and second largest bulblets ($p < 0.001$) as well as the fresh weight of the largest bulblet ($p < 0.05$). Thus changing from MS to G medium could not explain poorer performance in bulblet growth between experiments.

If neither basal medium composition nor overall medium strength can explain fully the poorer growth of Flore Pleno bulblets in experiments reported here compared to in 2002 then another factor(s) must be responsible. One possibility is that the age of the cultures from bulblet initiation might be important. This factor would directly relate to the number of culture passages that the tissues had passed through before being used in bulblet growth experiments. Continuous micropropagation is well known to result in the miniaturisation of plantlets with many plant subjects as well as to an increase in multiplication rates and partial rejuvenation of tissues. Rejuvenation is often seen as an increased rooting ability of micropropagated shoots, of mainly woody plants, that have been repeatedly passaged *in vitro*. Similar progressive changes like rejuvenation or miniaturisation may be taking place in *Galanthus* cultures with repeat passaging and these could affect bulblet growth. In this context it is interesting to note that ageing phenomena are known to occur with bulbous species as evidenced by the reduced yield of some very old tulip cultivars that have been multiplied in the field over many years (de Klerk, personal communication). Whatever the explanation is for poorer bulblet growth in Flore Pleno it is clear that *G. nivalis* is not affected to the same extent since bulb growth in this species was consistent over several experiments from year to year. Changes with passaging that influence bulblet growth might be slower with this species.

A common method to combat tissue miniaturisation with repeated *in vitro* passaging is to reduce the plant growth regulator status of the medium at some stage after the induction passage. Reducing plant growth regulator concentrations can also be expected to reduce the

carry over of these compounds from the multiplication phase to the bulblet growth phase making the charcoal treatment more effective. This approach at maintaining good bulblet growth in Flore Pleno cultures is being investigated in an experiment described in the materials and methods section and will be reported on in the Final Report.

As with the previous experiments the *G. nivalis* cultures formed significantly fewer bulblets than the Flore Pleno cultures ($p < 0.001$) but produced greater overall tissue fresh weight ($p < 0.001$) Figures 1.10 A and B). There was little difference between plant types for root numbers or root growth although decreasing the medium strength was found to significantly reduce root numbers ($p < 0.01$) and the length of the longest root ($p < 0.01$) with both plant types and basal media (Figure 1.10 C and D). Root numbers were also significantly increased on the G medium compared with the MS medium ($p < 0.001$).

Improved root growth can be viewed in two ways. In a situation where nutrients are finite, as in *in vitro* culture, roots could be regarded as organs competing for resources with the growing bulblets. Alternatively, and probably more correctly, enhanced root production might benefit bulblet growth by scavenging particularly immobile nutrients such as Fe from the medium. On a charcoal supplemented medium root induction and growth is very prolific with roots reaching the base of the culture vessel within a few weeks. This would be expected to enhance uptake of nutrients from the culture medium and thereby boosting bulblet growth.

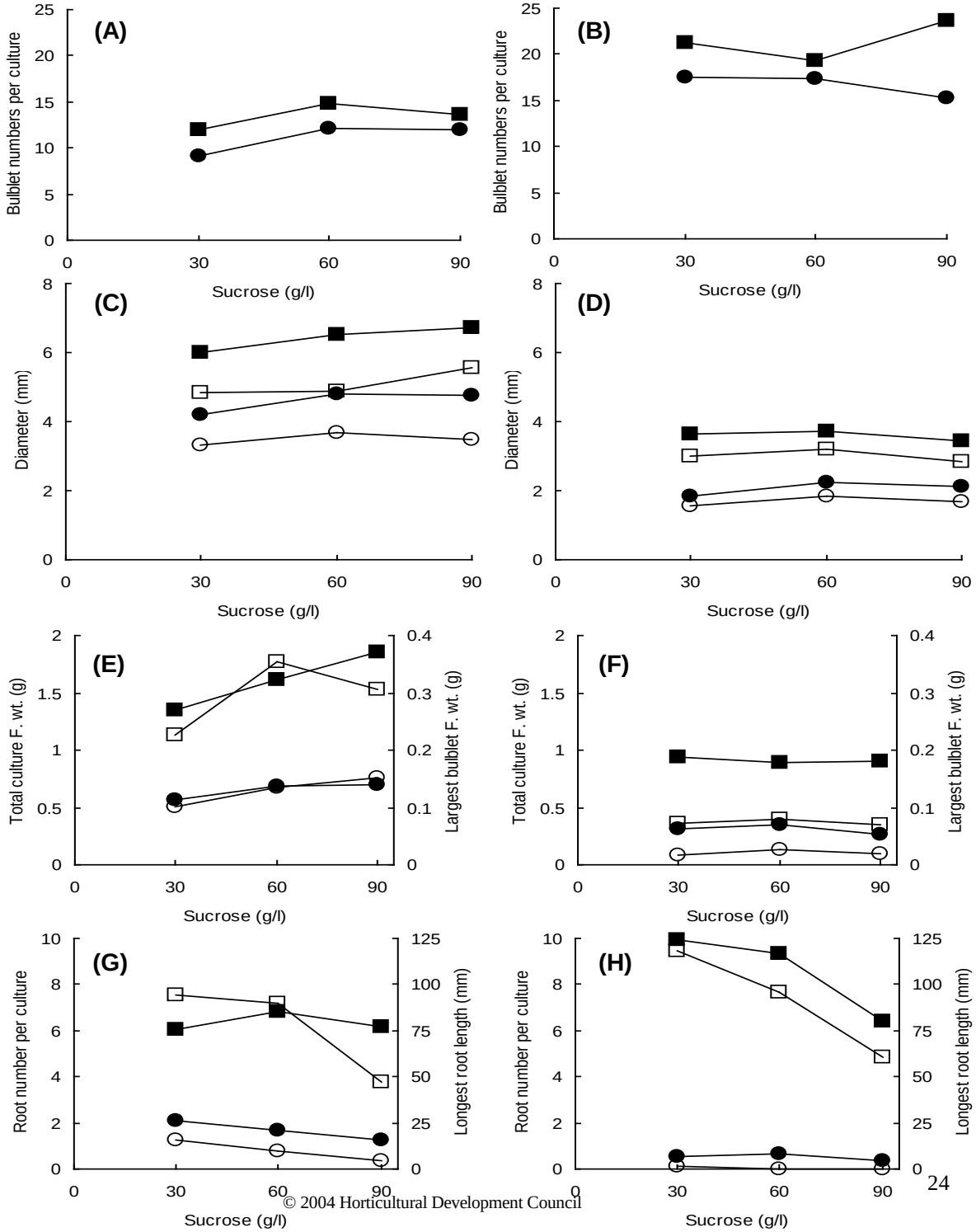
Alternatives to activated charcoal (adsorption agents)

Results to be presented in the final report.

Alternatives to activated charcoal (pectins)

Results to be presented in the final report.

Figure 1.6 The effect of sucrose concentration and charcoal [0 g/l charcoal (circles) & 5 g/l charcoal (squares)] on bulblet growth in half strength G medium for *G. nivalis* (left column of Figures) and *G. nivalis* Flore Pleno (right column of Figures). In **C** & **D** closed symbol = largest bulb diameter, open symbols = second largest bulb diameter. In **E** & **F** closed symbols = total culture F. wt., open symbols = largest bulblet F. wt. In **G** & **H** closed symbols = root numbers per culture, open symbols = longest root length.



Analysis of variance summary for Figure 1.6

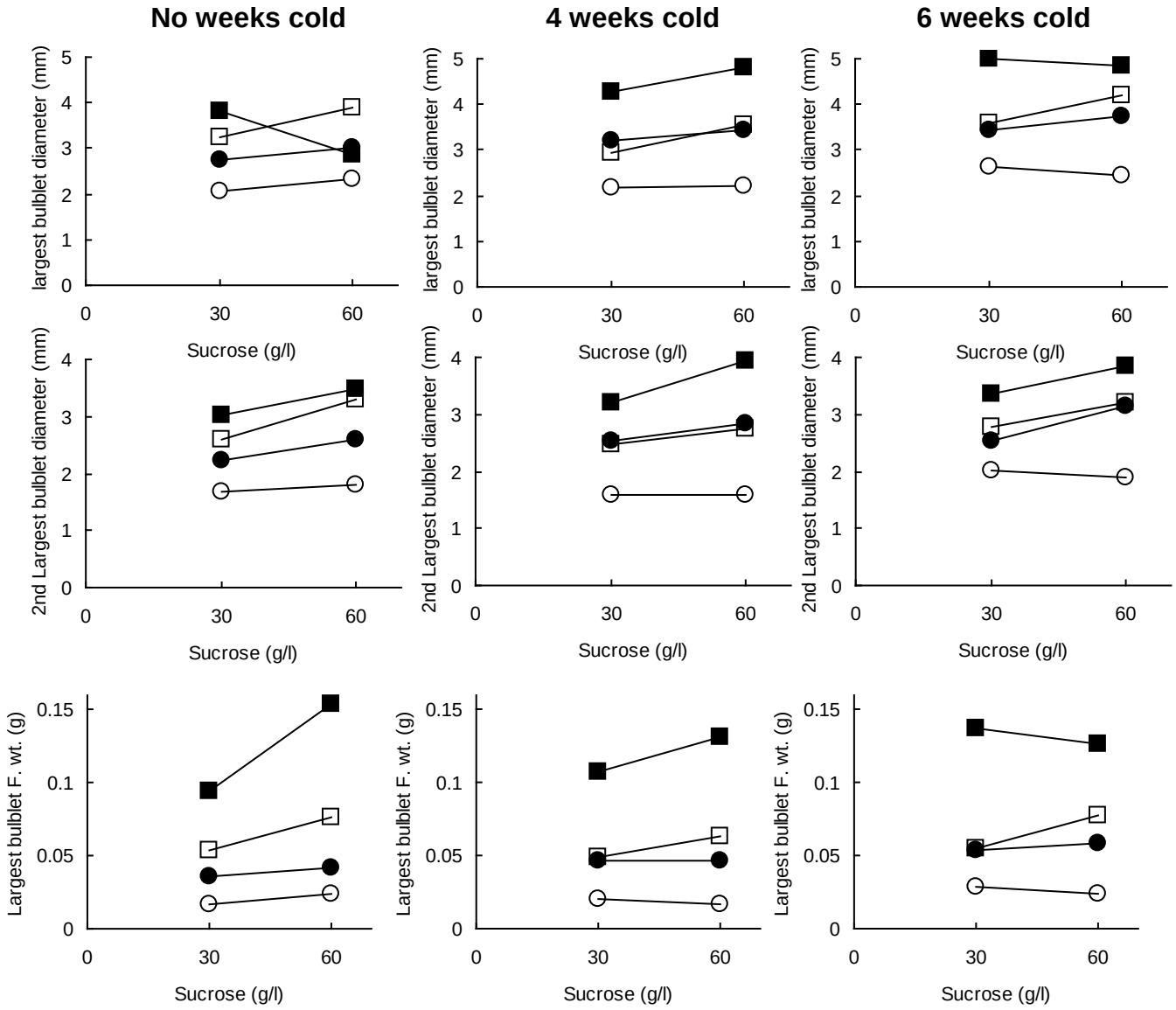
	d.f (m. v.)	***G. nivalis***		**Flore Pleno**	
		Bulblet numbers		Bulblet numbers	
		SED	sig	SED	sig
CHARCOAL (C)	1	0.586	**	1.046	***
SUCROSE (S)	2	0.718	*	1.281	NS
C x S	2	1.016	NS	1.811	NS
Residual	83 (2)				

		G. nivalis				**Flore Pleno**			
	d.f (m v)	Bulblet diameters				Bulblet diameters			
		Largest		2nd largest		Largest		2nd largest	
		SED	sig	SED	sig	SED	sig	SED	sig
CHARCOAL (C)	1	0.206	***	0.148	***	0.103	***	0.100	***
SUCROSE (S)	2	0.253	NS	0.181	NS	0.126	NS	0.121	NS
C x S	2	0.357	NS	0.256	NS	0.178	NS	0.171	NS
Residual	83 (2)								

	d.f (m v)	Fresh weight				Fresh weight			
		Total culture		Largest bulb		Total culture		Largest bulb	
		SED	sig	SED	sig	SED	sig	SED	sig
CHARCOAL (C)	1	0.075	***	0.030	***	0.051	***	0.005	***
SUCROSE (S)	2	0.092	NS	0.037	NS	0.062	NS	0.005	NS
C x S	2	0.130	NS	0.052	NS	0.088	NS	0.008	NS
Residual	83 (2)								

	d.f (m v)	Root				Root			
		Numbers		Length		Numbers		Length	
		SED	sig	SED	sig	SED	sig	SED	sig
CHARCOAL (C)	1	0.495	***	4.98	***	0.512	***	5.08	***
SUCROSE (S)	2	0.606	NS	6.10	**	0.627	NS	6.23	**
C x S	2	0.858	NS	8.63	*	0.887	NS	8.81	**
Residual	83 (2)								

Figure 1.7 The effect of sucrose concentration and charcoal [0 g/l charcoal (circles) & 5 g/l charcoal (squares)] on bulblet growth in half strength G medium for *G. nivalis* (solid symbols) and *G. nivalis* Flore Pleno (open symbols). After inoculation cultures were subjected to either no cold treatment (left column of figures), or to four weeks (centre column of figures) or 6 weeks cold treatment (right column of figures) at 5°C before being transferred to the normal growth room conditions.



Analysis of variance summary for Figure 1.7

Largest bulblet diameter

	d.f	**No Cold**		**Four weeks cold**		**Six weeks cold**	
		SED	sig	SED	sig	SED	sig
SPECIES (SP)	1	0.148	***	0.140	***	0.139	***
SUCROSE (S)	1	0.148	**	0.140	NS	0.139	NS
CHARCOAL (C)	1	0.148	***	0.140	***	0.139	***
SP x S	1	0.209	NS	0.198	NS	0.196	NS
SP x C	1	0.209	NS	0.198	NS	0.196	NS
S x C	1	0.209	NS	0.198	NS	0.196	NS
SP x S x C	1	0.295	NS	0.281	NS	0.278	NS
Residual	98						

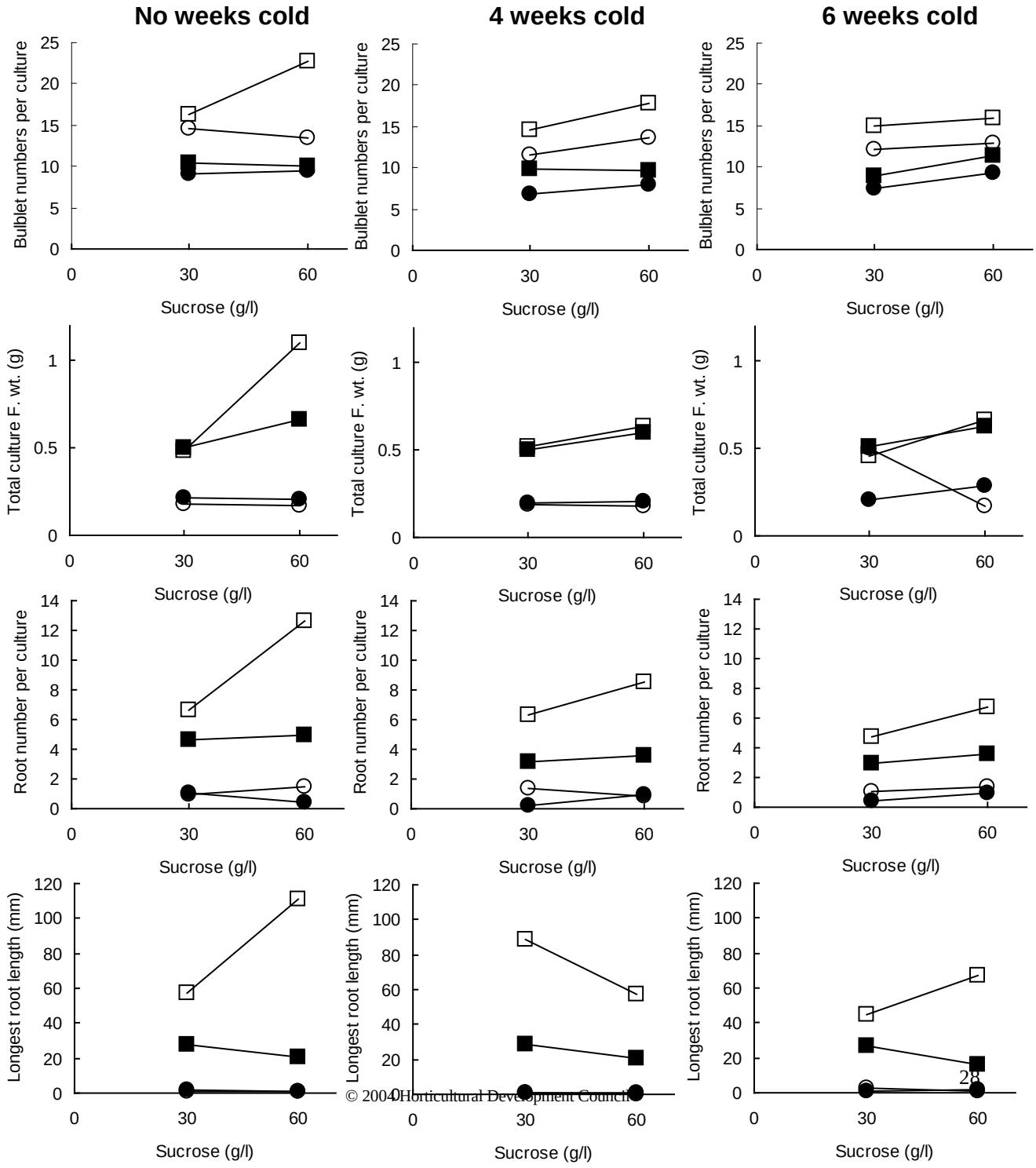
Second largest bulblet diameter

	d.f	**No Cold**		**Four weeks cold**		**Six weeks cold**	
		SED	sig	SED	sig	SED	sig
SPECIES (SP)	1	0.115	**	0.111	***	0.110	***
SUCROSE (S)	1	0.115	*	0.111	*	0.110	*
CHARCOAL (C)	1	0.115	***	0.111	***	0.110	**
SP x S	1	0.162	NS	0.157	NS	0.156	NS
SP x C	1	0.162	NS	0.157	NS	0.156	NS
S x C	1	0.162	NS	0.157	NS	0.156	NS
SP x S x C	1	0.229	NS	0.222	NS	0.221	NS
Residual	98						

Largest bulblet fresh weight

	d.f	**No Cold**		**Four weeks cold**		**Six weeks cold**	
		SED	sig	SED	sig	SED	sig
SPECIES (SP)	1	0.011	*	0.008	***	0.008	***
SUCROSE (S)	1	0.011	NS	0.008	NS	0.008	NS
CHARCOAL (C)	1	0.011	***	0.008	***	0.008	***
SP x S	1	0.015	NS	0.011	NS	0.011	NS
SP x C	1	0.015	NS	0.011	NS	0.011	NS
S x C	1	0.015	NS	0.011	NS	0.011	NS
SP x S x C	1	0.021	NS	0.015	NS	0.016	NS
Residual	98						

Figure 1.8 The effect of sucrose concentration and charcoal [0 g/l charcoal (circles) & 5 g/l charcoal (squares)] on bulblet numbers, culture growth and root development in half strength G medium for *G. nivalis* (close symbols) and *G. nivalis* Flore Pleno (open symbols). After inoculation cultures were subjected to either no cold treatment (left column of figures), or to four weeks (centre column of figures) or 6 weeks cold treatment (right column of figures) at 5°C before being transferred to the normal growth room conditions.



Analysis of variance summary for Figure 1.8

Bulblet numbers per culture

	d.f	No Cold		Four weeks cold		Six weeks cold	
		SED	sig	SED	sig	SED	sig
SPECIES (SP)	1	0.726	***	0.645	***	0.753	***
SUCROSE (S)	1	0.726	NS	0.645	NS	0.753	NS
CHARCOAL (C)	1	0.726	**	0.645	**	0.753	*
SP x S	1	1.027	NS	0.913	NS	1.065	NS
SP x C	1	1.027	*	0.913	NS	1.065	NS
S x C	1	1.027	NS	0.913	NS	1.065	NS
SP x S x C	1	1.453	*	1.291	NS	1.506	NS
Residual	98						

Total culture fresh weight

	d.f	No Cold		Four weeks cold		Six weeks cold	
		SED	sig	SED	sig	SED	sig
SPECIES (SP)	1	0.056	NS	0.039	NS	0.069	NS
SUCROSE (S)	1	0.056	*	0.039	NS	0.069	NS
CHARCOAL (C)	1	0.056	***	0.039	***	0.069	**
SP x S	1	0.079	NS	0.055	NS	0.098	NS
SP x C	1	0.079	NS	0.055	NS	0.098	NS
S x C	1	0.079	*	0.055	NS	0.098	NS
SP x S x C	1	0.112	NS	0.078	NS	0.139	NS
Residual	98						

Root number per culture

	d.f	No Cold		Four weeks cold		Six weeks cold	
		SED	sig	SED	sig	SED	sig
SPECIES (SP)	1	0.615	**	0.393	***	0.443	*
SUCROSE (S)	1	0.615	NS	0.393	NS	0.443	NS
CHARCOAL (C)	1	0.615	***	0.393	***	0.443	***
SP x S	1	0.869	NS	0.556	NS	0.627	NS
SP x C	1	0.869	*	0.556	**	0.627	NS
S x C	1	0.869	NS	0.556	NS	0.627	NS
SP x S x C	1	1.229	NS	0.786	NS	0.887	NS
Residual	98						

Longest root length

	d.f	No Cold		Four weeks cold		Six weeks cold	
		SED	sig	SED	sig	SED	sig
SPECIES (SP)	1	3.64	***	4.17	***	4.21	**
SUCROSE (S)	1	3.64	*	4.17	NS	4.21	NS
CHARCOAL (C)	1	3.64	***	4.17	***	4.21	***
SP x S	1	5.15	**	5.89	NS	5.96	NS
SP x C	1	5.15	***	5.89	***	5.96	**
S x C	1	5.15	*	5.89	NS	5.96	NS
SP x S x C	1	7.29	**	8.33	NS	8.42	NS
Residual	98						

Figure 1.9 *G. nivalis* (closed symbols) and *G. nivalis* Flore Pleno (open symbols) bulblet growth on dilutions of MS (circles) and G (squares) media (1 = full strength). A and B shows the diameters of the largest and second largest bulblet respectively and C shows the fresh weight of the largest bulblet with roots and leaves removed.

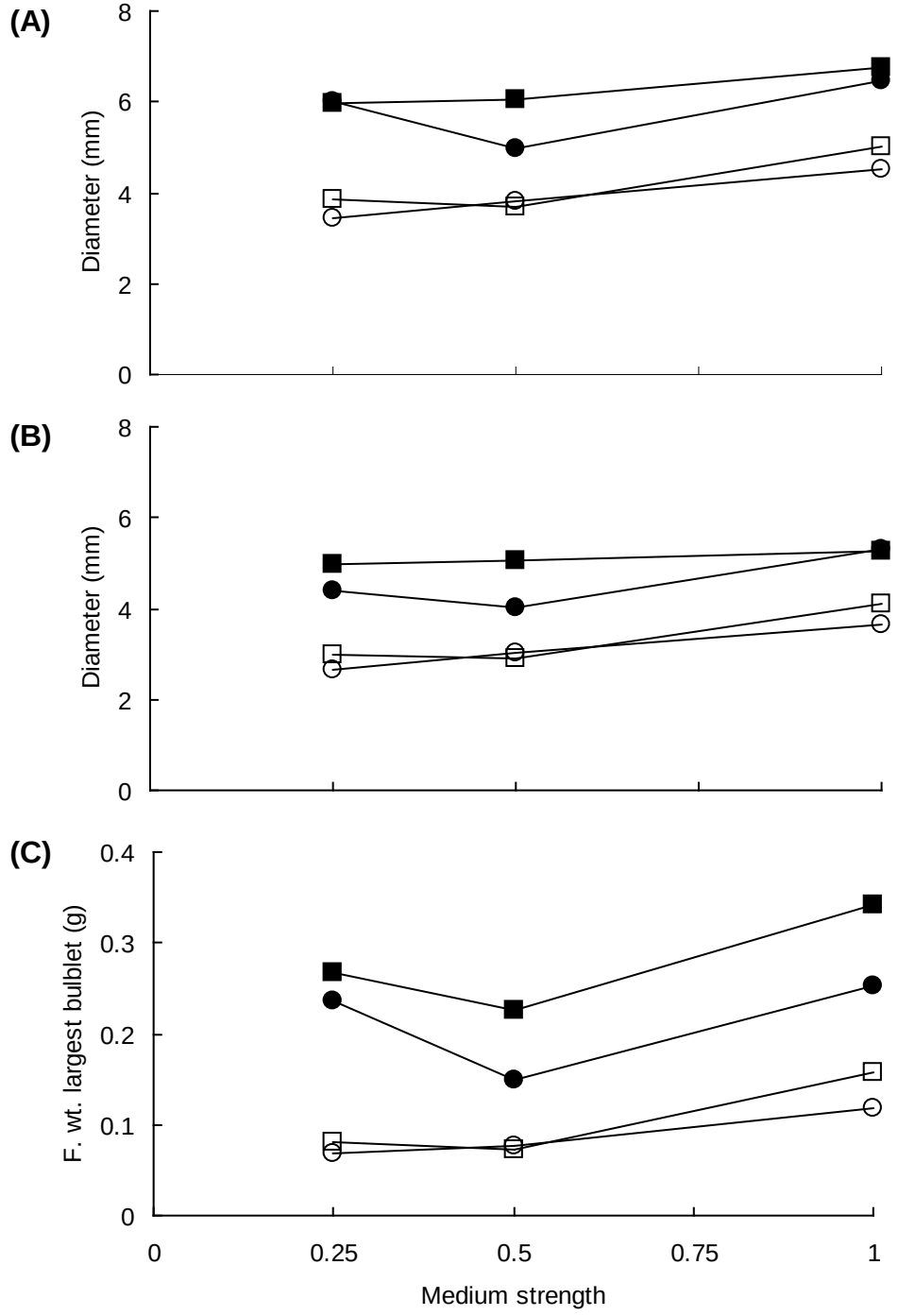
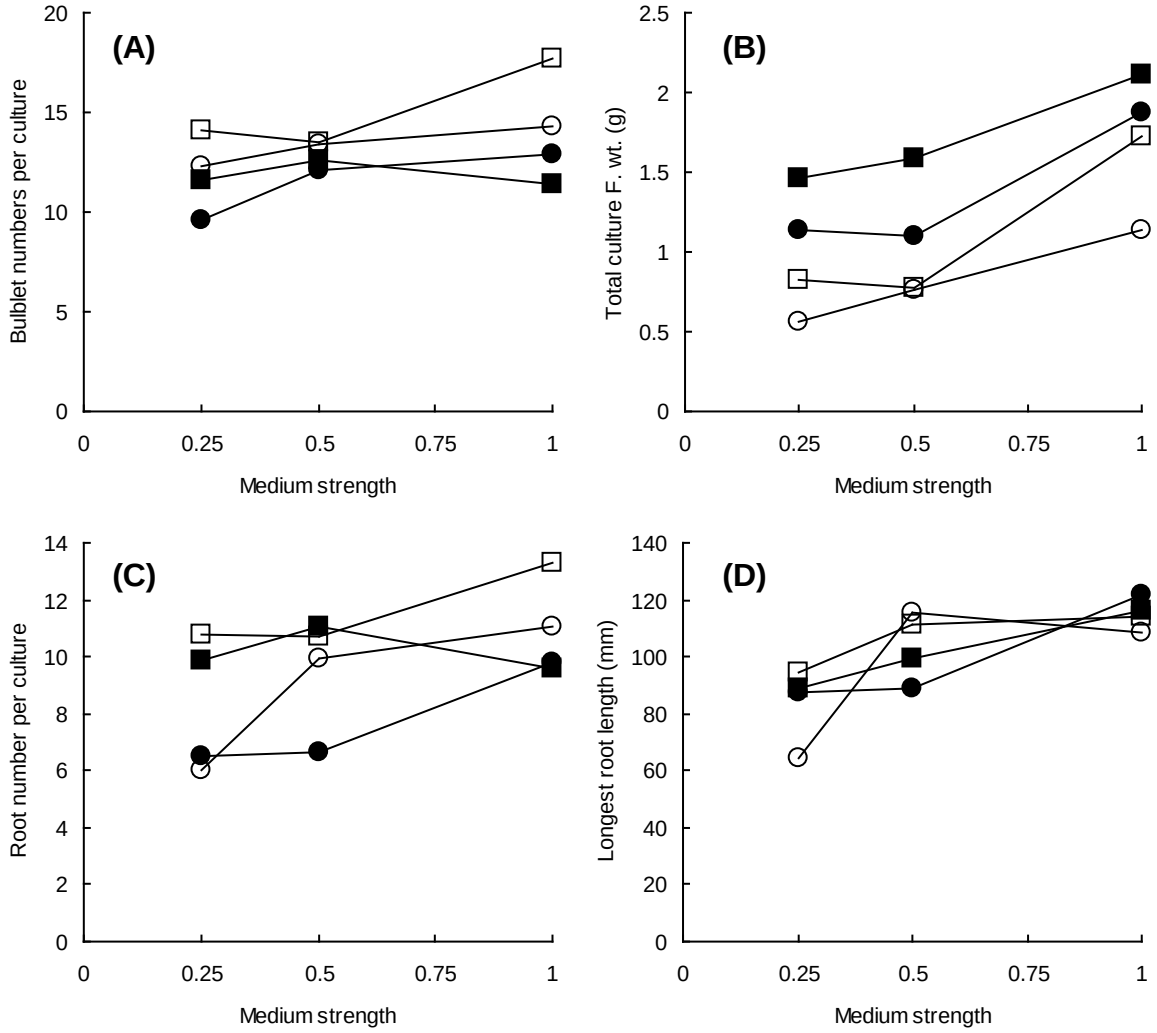


Figure 1.10 *G. nivalis* (closed symbols) and *G. nivalis* Flore Pleno (open symbols) bulblet clump and root growth on dilutions of MS (circles) and G (squares) media (1 = full strength). Cultures were inoculated with clumps of 3.99 and 4.05 bulblets for *G. nivalis* and *G. nivalis* Flore Pleno respectively and final bulblet numbers are shown in (A). Total culture fresh weight (B) and root numbers (C) and length of the longest root (D) are also shown.



Analysis of variance summary for Figure 1.9

	d.f (m. v.)	Bulblet diameter				Largest bulblet fresh weight	
		Bulblet 1		Bulblet 2			
		SED	sig	SED	sig	SED	sig
SPECIES (SP)	1	0.122	***	0.101	***	0.012	***
MEDIUM (M)	1	0.122	*	0.101	**	0.012	*
STRENGTH (S)	2	0.149	***	0.124	***	0.014	***
SP x M	1	0.172	NS	0.143	NS	0.016	NS
SP x S	2	0.211	NS	0.175	NS	0.020	NS
M x S	2	0.211	NS	0.175	NS	0.020	NS
SP x M x S	2	0.298	NS	0.248	NS	0.029	NS
Residual	218 (2)						

Analysis of variance summary for Figure 1.10

	d.f (m. v.)	Bulblet numbers		Culture fresh weight		Root			
						Numbers		Length	
		SED	sig	SED	sig	SED	sig	SED	sig
SPECIES (SP)	1	0.453	***	0.053	***	0.495	*	5.09	NS
MEDIUM (M)	1	0.453	NS	0.053	***	0.495	***	5.09	NS
STRENGTH (S)	2	0.554	*	0.065	***	0.607	**	6.23	**
SP x M	1	0.640	NS	0.075	NS	0.701	NS	7.19	NS
SP x S	2	0.784	NS	0.092	NS	0.858	NS	8.81	NS
M x S	2	0.784	NS	0.092	NS	0.858	NS	8.81	NS
SP x M x S	2	1.109	NS	0.130	NS	1.213	NS	12.46	NS
Residual	218 (2)								

Acclimatization of bulblets

Bulblets of both *G. nivalis* and *G. nivalis* Flore Pleno acclimatized relatively easily to the John Innes compost with drainage improved with ether peat or perlite (Figure 1.11 upper plate). Flore Pleno plantlets were similar to those shown in the figure except that the leaf growth was less extensive. Even small bulblets showed good growth when planted just below the surface of a peat based compost (Figure 1.11 lower plate). Bulblets developed *in vitro* do not appear to have any dormancy problems. This in accord with the high percentage of sprouting of even very small bulblets whilst still in either bulblet multiplication or bulblet growth phases of micropropagation.

Figure 1.11 Growth of *G. nivalis* bulblets, produced *in vitro*, in John Innes compost with the drainage improved with perlite (top plate). The bulblets were from the medium strength experiment and were planted on 9 September 2003 and photographed at the end of January 2004. The lower plate shows smaller bulblets less than 2mm in diameter planted in a peat based compost with the drainage improved with sand and planted at the same time.

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AGRONOMY

Materials and methods

Plant material

Forty 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. Since the bulbs supplied were somewhat variable, the largest and soundest were selected for experiment 1, the next best for experiment 2, and the remaining bulbs were planted as stock. At planting, bulb samples were taken and examined for the presence of *Botrytis galanthina* by Cheryl Brewster (then at 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*. Later, foliage samples examined by Dr Tim O'Neill (ADAS Arthur Rickwood) also failed to yield any fungal pathogens. Further bulbs (*G. nivalis* and *G. elwesii*) were bought from the same supplier in 2002 and 2003 and were stored until use in the same way as above.

General methods for field experiments

Field trials were set up in 2000, 2002 and 2003 at HRI Kirton, Lincolnshire, in an open field situation typical of the South Lincolnshire area. The soil was a coarse silty marine alluvium, and before planting the area used was fertilised as necessary, ploughed and cultivated following taking a soil sample for standard agricultural soil analysis. In 2000, the previous crop on the site used was barley, and in 2002 and 2003 the site used had been fallow, all therefore giving a MAFF N index of 0. In 2000 soil analysis revealed levels of pH 7.7, P index 4, K index 3 and Mg index 4, and, conforming to MAFF fertiliser recommendations for bulbs, no additional fertilisers were applied pre-planting, but N (70kg/ha) was applied by hand along the beds in winter (8 January 2001). In 2002 and 2003, similar nutrient levels and fertiliser rates were used.

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. Bulbs were planted, about 5cm deep, by hand using trowels. After planting, the area was raked over to tidy and consolidate the soil. When the experimental plots were planted, further bulbs were planted in rows in the same field to provide stocks for storage experiments; these received the same routine husbandry treatments as the experimental plots except that some areas were sprayed with herbicides to determine suitable materials.

Electric fencing and bird scarers were set up to deter predators. 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 in this report were applied at standard rates.

After the snowdrop foliage had died down in spring/summer 2001, the trials area was made tidy, shading were checked, etc. The following treatments were applied across the whole area: methiocarb (slug pellets, 5 November 2001), thiram (as a moss killer, 6 November 2001), and diquat + paraquat (13 November 2001). The pathways were treated with cyanazine herbicide on 19 November 2001, weed control in plots being by hand. Post-emergence herbicide and fungicide applications were applied as before. Similar procedures were used in 2002-2003.

Agronomy experiment 1: The effect of shade, shelter and soil moisture treatments

Along each bed, the plots were 2.475m-long and were separated by 0.825m-long unplanted 'guard' areas. On 25-27 September 2000, 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 the three years of the experiment. Since seed pods were collected and assessed each year from the appropriate sub-plots (see below), seed pods from other sub-plots were removed at the same time in order to avoid confusion about plant counts in subsequent years.

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) Windbreak: 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 windbreak levels x two 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 Netlon Agroshade mesh stretched horizontally over the plots, 45cm above ground level. Windbreak consisted of a single layer of Netlon Tensar Windbreak 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 in November before emergence. Shading, windbreak and mulching extended beyond the appropriate plot, halfway into the adjacent guard areas. In summer 2001, following complete die-down of the foliage, the remaining straw mulch was removed, being replaced in early-November. Similar procedures were followed in 2002-2003.

Crop production was assessed on one sub-plot (60 planted bulbs) of each plot annually for

three years starting in 2001. Seed pods were left *in situ* until ready for collection. The following records were made on each sub-plot:

- Number of shoots in January/February (recorded only from non-mulched plots in 2002 to avoid crop damage)
- Number of flower stems (February)
- Percentage of foliage die-back (24 May 2001)
- Number of seed pods and seeds (June)

Number and weight of bulbs <4 cm and ≥4 cm circumference (after lifting, cleaning and surface-drying bulbs in July 2001 and 2002)

Agronomy experiment 2: The effect of shading and inter-cropping

Plots were set up with six treatments:
(1) Control (no shading, no inter-cropping)
(2) Shading using 40% shade factor mesh, as above
(3) Shading using 70% shade factor mesh, as above
(4) 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
(5) One row of spring barley sown along each side of the planted area, 15cm from the edge of the planted area after planting bulbs
(6) Planting area over-sown with perennial rye-grass 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 inter-crop 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.

In October 2001 any remaining barley was removed and wheat was sown in its place. In November 2001 the rye-grass was cut close to ground level using a strimmer. Narcissus bulbs were left in place. Similar procedures were followed in 2002-2003.

The records taken were similar to those described for Experiment 1 (above).

Agronomy experiment 3: Growth of micropropagated plantlets

In autumn 2003, micropropagated plantlets of *G. nivalis*, *G. nivalis* cv Flore Pleno and *G. elwesii* were received from Dr Chris Selby. To compare the growth of micropropagated plantlets with that of commercially supplied bulbs, plots of both were planted in the field at Kirton (in shaded and sheltered plots and in unprotected plots) and in an unheated, mesh tunnel. Each plot was planted with six bulbs or six clusters of plantlets (i.e., the contents of one culture jar). Growth in these plots will be recorded in 2004.

Agronomy experiment 4: The effect of fungicide dips and sprays

Bulbs of *G. nivalis* (3-4 cm) were purchased in autumn 2002 and allocated to 54 lots of 100 bulbs each. Half of the bulb lots were dipped in Benlate (40 g per 10 litres) and Captan (100 g per 10 litres) for 15 minutes, and allowed to drain overnight at room temperature; the other half remained untreated at this stage. All bulbs were planted the next day, with 18 plots (nine dipped and nine non-dipped) in three randomised blocks. Each plot was 1m long and 0.15m wide. Otherwise, cultural methods were as described above. Ronilan FL (concentration as below) was applied to all plots except non-sprayed controls on 11 and 17 March 2003. Starting on 26 March 2003, fungicide spray programmes were applied, using the fungicides listed below. Eight sprays of these fungicides were applied in all, ending 27 May 2003. For the initial Ronilan FL sprays, a knapsack sprayer was used, thereafter an Oxford precision sprayer. A medium-quality spray nozzle, and a spray volume equivalent to 500 litres/ha, was used throughout:

- Untreated (control) (double replication)
- Amistar (25% a.i.), 1 ml/litre
- Benlate (50% a.i.) + Dithane 945 (80% a.i.), 0.5 + 1.5 g/litre
- Folicur (25% a.i.), 1 ml/litre
- Ronilan FL (50% a.i.), 1 ml/litre
- Scala (40% a.i.), 2 ml/litre
- Unix (75% a.i.), 0.67 g/litre
- Stroby WG (50% a.i.), 0.625 g/litre

After complete leaf senescence in all plots, the bulbs were recovered in June 2003, surface-dried, cleaned by hand and weighed and counted. The number and weight of bulbs from each plot were recorded after grading the bulbs to <4 cm and ≥4cm (circumference).

Environmental data

The following parameters were logged at 30-minute intervals during the growing seasons in representative plots:

- Soil temperature at bulb depth (Delta-T TM1 sensor)
- Air temperature at median leaf height (Delta-T TM1 sensor)
- Soil moisture at bulb depth (Delta-T M1M sensor ('Theta probes')
- Level of photosynthetically active radiation (PAR) at mid leaf height (Delta-T QS sensor)

Occasional obviously erroneous readings from the logger (due to equipment malfunctions) were deleted. Soil moisture at bulb depth was additionally recorded using Irrometer 'water sensors' checked at about weekly intervals. A selection of this environmental data was reported in the previous Annual Reports.

Results and Discussion

Main experiment

The results for the first two years of the experiment, 2000-2002, were presented in the previous Annual Reports. The third year's results are summarised in Tables 2.1 and 2.2.

In the first year, and despite initially uniform planting, fewer shoots emerged in the non-sheltered plots than in sheltered plots (suggesting that wind damage caused some loss of foliage), and fewer in mulched than in non-mulched plots (suggesting that the presence of the straw mulch impeded the emergence of weaker shoots). In the second year the main effect on shoot numbers was due to shading, with more shoots emerging in shaded plots than in non-shaded controls, presumably reflecting the overall better growth under shading seen in the previous growing season. The differential between control (non-shaded) and shaded plots increased in the third year, with shaded plots on average giving twice the number of shoots than control plots (Table 2.1). However, in all cases the number of shoots was substantially less than was found in the previous year, showing a gross loss of vigour in the third year.

In the first year there were no significant effects of treatments on the numbers of flower stems, which was as expected since flower initiation in snowdrops would have been determined in the previous summer. There were only minor significant effects of treatments in the second year. There was an effect of shading on the numbers of flower stems produced, with fewer stems in non-shaded than shaded plots. Minor significant interactions on stem and seed pod numbers were due to particularly weak growth in plots that had neither shade, shelter nor mulch. As in the case of shoot numbers, these results reflected the benefits to snowdrop growth of, particularly, shading in the first year. For year 3, as in the case of shoot numbers, the differential between numbers in control and shaded plots was increased, but the number of shoots and seed pods was less than that obtained in the second year. Foliage in shaded and mulched plots died-down slightly later than in non-protected plots.

In the first year, bulb yields (numbers and weights) were consistently higher in shaded than in non-shaded plots, although this effect did not always achieve statistical significance and there was, surprisingly, no clear 'dose-response' relationship between bulb yields and shading density. Results for the second year confirmed the beneficial effects of shading on bulb yield and, especially, on the mean weight of harvested bulbs. There were more significant benefits of mulching on bulb yield, mulching producing higher yields in the larger bulb grade and higher mean bulb weights. Statistically significant second-order interactions resulted from:

- Very poor yields in plots with neither shading, shelter nor mulch; and
- Very high yields in plots with mulch but neither shade nor shelter.

As observed for shoot and stem numbers, bulb yields decreased sharply in almost all cases in the third year, though the differential between treatments (better growth in shaded and mulched plots) was maintained (Table 2.2).

The beneficial effects of shading and mulching on snowdrop growth were substantial, but the addition of a side windbreak provided little extra benefit. As shading appeared to work by

prolonging the growing season (see first annual report and Table 2.1), this might also be achieved through fungicide spray programmes that delay foliar senescence (see below). However, there was little or no further growth in the third year of the experiment, indicating some severe restraint on growth when these bulbs were left down for a third year: whether this was due to over-crowding or other effects has to be determined. Figure 2.1. shows bulb yields after one to three years. The fact that yields had declined from year one to year two in the absence of mulch (except where dense shading was used), may explain why attempts at growing *G. nivalis* in a field situation may have failed. More significant to the project is that crop growth and bulb yields fell in the third year: this decrease was marked in the case of non-mulched plots, though in mulched plots bulb yields were at least only slightly reduced.

Table 2.1. Effects of shading, windbreak and mulch treatments on snowdrop growth in the third year (2003). Main effect means only presented.

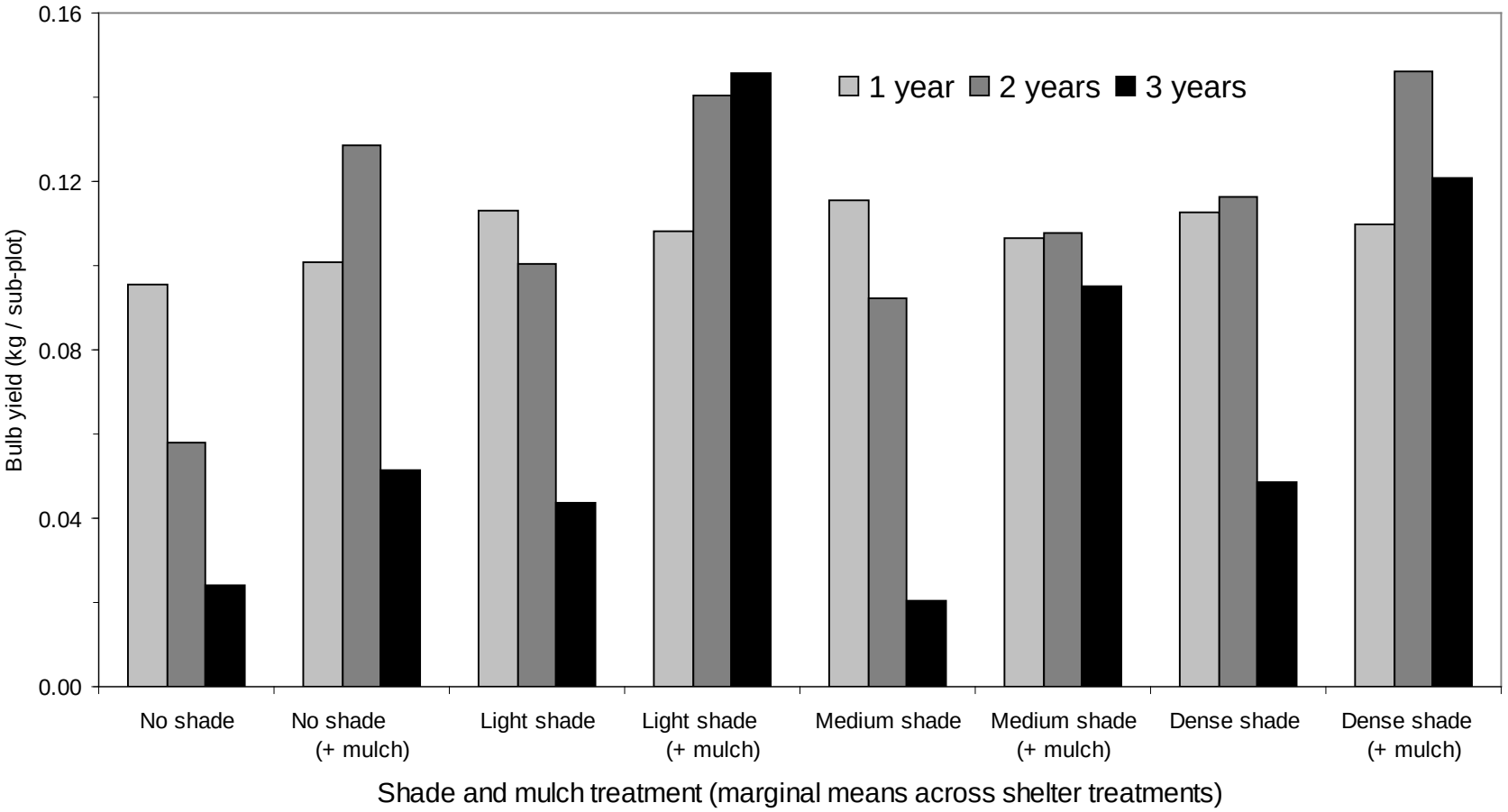
Factors and treatments	Numbers per sub-plot (60 bulbs planted)			% foliar die-back
	Shoots	Flower stems	Seed-pods	
Shading				
None	16.0	25.5	4.7	100
Light	33.4	42.2	11.6	96
Medium	26.2	36.5	6.8	99
Dense	39.9	45.5	11.9	96
SED (60 d.f)	4.43	5.33	2.96	1.9
Windbreak				
None	30.1	38.9	10.4	97
Yes	27.6	36.0	7.1	99
SED (60 d.f.)	3.13	3.77	2.09	1.4
Mulch				
None	29.9	34.8	9.4	99
Yes	27.8	40.1	8.1	97
SED (60 d.f.)	3.13	3.77	2.09	1.4
Analysis of variance summary [a]				
Shading (S)	***	**	*	ns
Windbreak (W)	ns	ns	ns	ns
Mulch (M)	ns	ns	ns	(*)
S x W	ns	ns	ns	ns
S x M	ns	ns	ns	ns
W x M	*	(*)	(*)	ns
S x W x M	ns	ns	ns	ns

[a] ns, not significant; (*), *, ** and ***, significant at the 10, 5, 1 and 0.1% levels of probability, respectively. These significance levels indicate that the observed differences between treatment means could have arisen by chance in 1 out of 10, 1 out of 20, 1 out of 100 and 1 out of 1000 occasions, respectively.

Table 2.2. Effects of shading, windbreak and mulch treatments on snowdrop bulb yield in the third year (2003). Main effect means only presented.

Factors and treatments	Bulb yields per sub-plot (60 bulbs planted)						Mean bulb weight (g)
	Numbers			Weight (g)			
	< 4cm	≥ 4cm	Total	< 4cm	≥ 4cm	Total	
Shading							
None	12.3	10.4	22.8	8.2	29.6	37.8	1.98
Light	18.9	25.4	44.3	14.6	80.0	94.6	1.77
Medium	15.9	16.1	32.0	10.6	47.3	57.9	1.59
Dense	20.3	25.4	45.8	12.5	72.3	84.8	1.71
SED (60 d.f)	3.36	4.88	7.55	2.75	17.37	19.36	0.463
Windbreak							
None	17.8	19.9	37.8	12.0	57.8	69.8	1.84
Yes	15.9	18.7	34.6	11.0	56.8	67.8	1.68
SED (60 d.f.)	2.37	3.45	5.34	1.95	12.28	13.69	0.327
Mulch							
None	12.6	10.6	23.2	7.7	26.5	34.2	1.62
Yes	21.2	28.0	49.2	15.3	88.1	103.4	1.90
SED (60 d.f.)	2.37	3.45	5.34	1.95	12.28	13.69	0.327
Analysis of variance summary							
Shading (S)	(*)	**	*	ns	*	*	**
Windbreak (W)	ns	ns	ns	ns	ns	ns	ns
Mulch (M)	***	***	***	***	***	***	***
S x W	ns	ns	ns	ns	ns	ns	ns
S x M	ns	ns	ns	ns	ns	ns	ns
W x M	ns	(*)	(*)	ns	(*)	*	(*)
S x W x M	ns	ns	ns	ns	ns	ns	ns

Figure 2.1. Snowdrop bulb yields after 1 - 3 years



Inter-crops experiment

The results for the first and second years of the experiment are detailed in the previous Annual Reports, and the third year's results are shown in Tables 2.3 and 2.4.

In the first year of the experiment there were trends but no statistically significant effects of treatment on the numbers of shoots, flower stems, seed pods or seeds obtained per sub-plot. In the second year the results showed that the adverse or beneficial effects of treatments on growth seen in the previous year were continuing to show clear effects on plant performance. There were significant effects of treatment, with best yields of stems and seeds under the medium-density shade, and much poorer yields where inter-crops, and especially over-sown rye-grass, had been used. In the third year, numbers of shoots and stems fell in all treatments, though much less so where a medium shading, cereal inter-crop or rye-grass had been used (Table 2.3). Foliar senescence was fastest in the control plots and most retarded in the plots with medium shading.

As in the first year, in the second there was a significant effect of treatment on total bulb yields and yield of the larger bulbs, but not on the yield of smaller bulbs. Corresponding with the effects on stem and seed production, the best yields were produced from plots under the higher density shade, with poorer yields from the inter-cropped plots. However, bulb yields (and the mean weight of harvested bulbs) were greater from the rye-grass plots than from the narcissus or cereal plots, indicating a marked recovery of the snowdrops over-sown with rye-grass, which were very poor in the first year. By the third year, bulb yields followed the trends of shoot and stem numbers: yields had fallen in the third year (except in the case of the cereal inter-crop, where yields had increased a little), but less so for the medium shade and rye-grass plots than for control of light shade plots.

The overall weaker growth of bulbs in this experiment, compared with experiment 1, was due to the better bulbs being used for the latter (see Materials and Methods).

In general these results confirm the beneficial effect of artificial shading on growth, seen in the first experiment. Inter-crops were too competitive for use with snowdrops.

Table 2.3. Effects of shading and inter-cropping on snowdrop growth in the third year, 2003.

Treatments	Numbers per sub-plot (60 bulbs planted)		% foliar die-back
	Flower stems	Seed pods	
None	24.4	5.2	100
Light shading	21.4	9.2	96
Medium shading	41.6	19.6	90
Narcissus intercrop	30.8	12.0	94
Cereal intercrop	31.8	8.6	95
Rye-grass oversown	20.0	5.6	94
SED (20 d.f.)	9.32	4.95	8.9
Significance	ns	(*)	ns

Table 2.4. Effects of shading and inter-cropping on snowdrop bulb yield in the third year, 2003.

Treatments	Bulb yields per sub-plot (60 bulbs planted)						Mean bulb weight (g)
	Numbers			Weight (g)			
	< 4cm	≥ 4cm	Total	< 4cm	≥ 4cm	Total	
None	3.8	7.8	11.6	2.4	20.0	22.4	2.0
Light shading	2.0	9.6	11.6	1.3	35.0	36.3	2.4
Medium shading	9.8	35.2	45.0	7.6	104.0	111.6	2.0
Narcissus inter-crop	5.2	14.6	19.8	3.6	43.0	46.6	1.6
Cereal inter-crop	5.0	21.2	26.2	3.4	54.0	57.4	2.2
Rye-grass over-sown	5.0	10.6	15.6	4.0	38.0	42.0	2.4
SED (20 d.f.)	2.21	15.16	16.21	1.86	50.30	51.1	0.66
Significance	*	ns	ns	(*)	ns	ns	ns

Fungicide trial

Table 2.5 gives the results of the fungicide trial. Foliar senescence was delayed when Scala and Unix had been used in the spray programme, compared with unsprayed controls and plots sprayed with other fungicides. At this stage, foliar senescence was not influenced by whether or not bulbs had received a pre-planting dip.

Bulb yield (both numbers and weights) was significantly affected by pre-planting dip but not by spray programme. Yields were significantly boosted where a pre-planting dip had been used. Mean bulb weight was significantly affected by both dips and sprays, compared with controls, with mean weight increased using a pre-planting dip, and when Scala sprays had been used (the effects of Unix and Stroby WG just failing to reach significance). In the case of mean bulb weight, there was a significant interaction between dip and spray treatments: a Scala spray programme increased mean bulb weight irrespective of whether a pre-planting dip had been given or not (Figure 2.2).

Botrytis galanthina is the main pathogen to attack snowdrops, and *Botrytis*-like sclerotia were found on many of the bulbs used in this project, though it was not possible to identify these to species level (see page 35). The Benlate + Captan pre-planting bulb dip appeared to have offered a useful measure of control of *Botrytis*. No plants with typical foliar symptoms of *B. galanthina* were seen in this experiment, so the fungicide sprays that improved bulb growth are presumed to have done so primarily as a result of delayed leaf senescence.

Considering the similarity of snowdrops and narcissus, it is perhaps surprising that these findings with snowdrops were different to results previously obtained in fungicide trials with narcissus in a recent HDC-funded project (BOF 41; O'Neill, Hanks & Wilson, in press). In snowdrops, this experiment showed a modest delay of leaf senescence and increase in bulb yield, and particularly so in response to the anilinopyrimidine fungicides Scala and Unix. Narcissus showed a considerable reduction in smoulder (*Botrytis narcissicola*) symptoms, delayed senescence and increased bulb yield in response to sprays of strobilurin fungicides (Stroby WG and Amistar). The strobilurin fungicide Stroby WG is known to have an effect on green leaf retention by delaying chlorophyll breakdown (Köhle *et al*., 1997), and a green leaf retention effect was observed with anilinopyrimidine fungicides on narcissus (BOF 41). In the present study, sprays of Ronilan consistently produced the poorest results in snowdrops, whereas in narcissus, Ronilan sprays were very effective in delaying senescence and increasing yield (BOF 41).

Table 2.5. Effects of pre-planting dip and fungicide spray programme on yield of *G. nivalis* over one year's growth. Main effect means only presented.

Factors and treatments	Bulb yields per plot				Mean bulb weight (g)	Foliage score[1]
	Numbers		Weight (g)			
	≥4cm	Total	≥4cm	Total		
Pre-planting dip						
Dipped	21.0	35.6	45.3	55.4	1.57	1.8
Not dipped	15.5	31.5	32.2	43.3	1.39	1.3
SED (36 d.f)	1.97	3.19	4.32	4.91	0.053	0.49
Spray programme						
None	18.3	36.0	37.8	50.3	1.41	1.6
Amistar	14.8	31.2	30.8	42.2	1.35	1.0
Benlate + Dithane	18.2	34.5	38.7	49.3	1.47	1.8
Folicur	19.5	35.3	41.2	52.2	1.48	0.5
Ronilan	13.0	28.0	25.5	36.3	1.28	0
Scala	21.8	35.0	51.5	60.3	1.77	3.8
Unix	18.3	32.3	40.0	50.2	1.56	2.5
Stroby WG	22.0	33.7	45.5	53.3	1.59	1.2
SED (36 d.f.)	3.63	5.85	7.93	9.03	0.097	0.91
Analysis of variance summary						
Pre-planting dip (D)	**	ns	**	*	**	ns
Spray programme (S)	ns	ns	ns	ns	**	*
D x S	ns	ns	ns	ns	(*)	ns

[1] Foliage scored from 5 (normal green foliage present) to 0 (no foliage remaining) on 13 June 2003

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Reference

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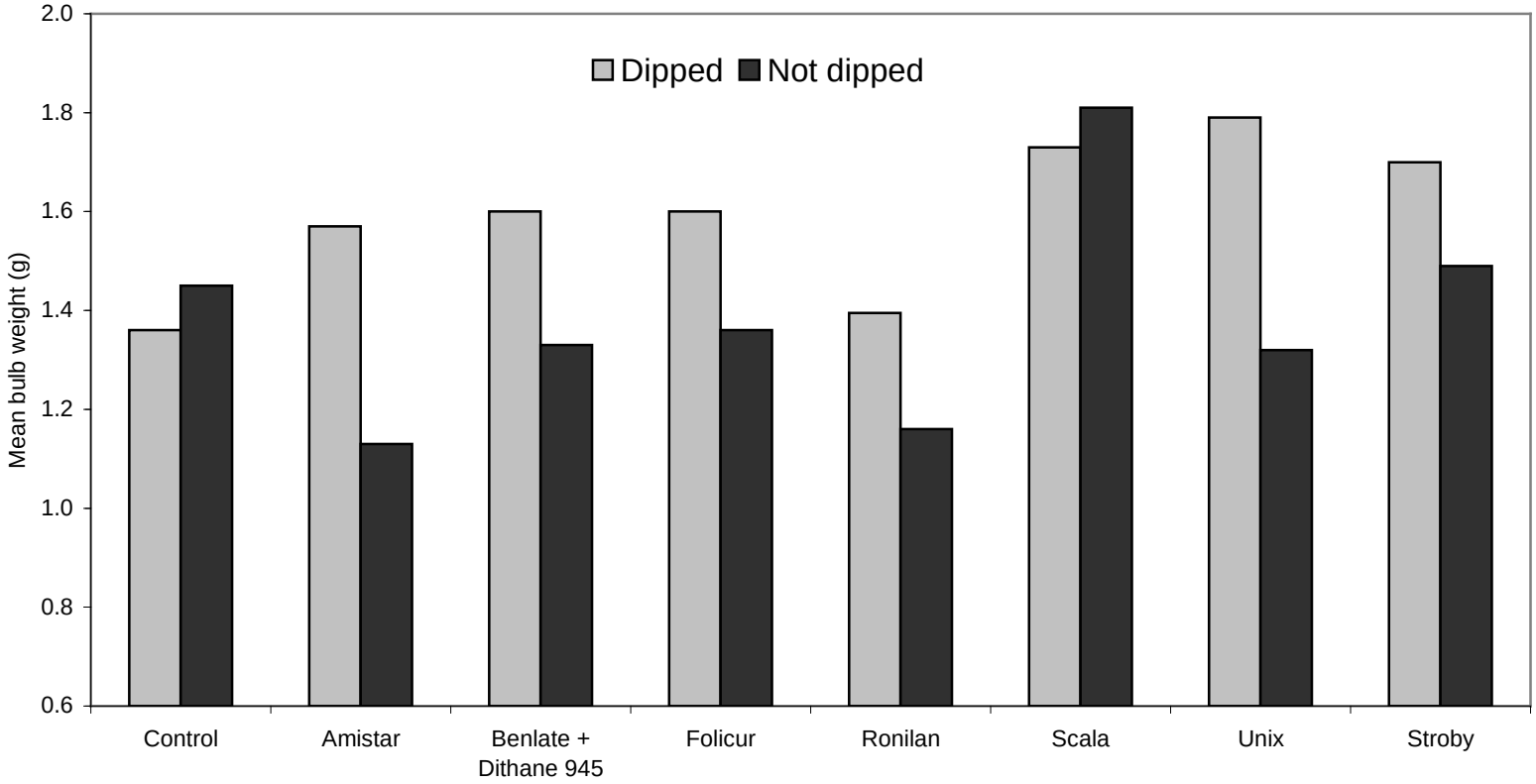
Figure 2.2. Mean bulb weight after fungicide bulb dip and spray treatments



BULB STORAGE

Materials and Methods

Experiment 1 – lifting date and storage temperature and medium

This experiment was reported in the previous Annual Report, and a brief summary is given below, under Results and Discussion.

Experiment 2 – modified atmosphere storage

Storage of produce in perforated or non-perforated polythene bags of different gauges (thickness) is a simple way of determining the effects of modified atmosphere (MA) storage. Bulbs of *G. elwesii* were purchased in autumn 2003, weighed in groups of five, and placed in 'polythene' (low-density polyethylene, LDPE) bags (90 – 700 gauge, 15 x 20 cm) which were then sealed. Half the bags of each gauge were perforated with a series of holes to facilitate some air exchange. The bags were stored at 13°C. The condition of the bulbs and their weights were assessed 4, 8 and 12 weeks after the start of storage, following which the bulbs were planted and grown on. Only the early results are available at this time, and these are discussed below.

Experiment 3 – use of confectionery glaze to reduce desiccation of bulbs

Bulbs of *G. nivalis*, purchased in autumn 2003, were allocated in groups of ten bulbs which were weighed. Groups of bulbs were either retained untreated as controls, or were immersed in the confectionery glazes Crystalac or Natureseal-PN (AgriCoat Industries Ltd) for 5 or 30 minutes before being allowed to drain free excess glaze. The bulbs were re-weighed and placed in polythene bags (250 gauge) that were loosely folded over and placed in a controlled temperature store at 13°C. The bulbs are being examined at 4 week intervals, and results will be reported later.

Results and Discussion

Experiment 1 – lifting date and storage temperature and medium

The effects of all three factors (harvest date, storage temperature and medium, and storage duration) were statistically significant. Of the three experimental factors, harvest date had the least impact on bulb weight: the overall means for the three dates were 6.9, 7.9 and 7.8g, respectively. Storage in 'damp' conditions was unacceptable due to fungal infection resulting in weight loss, and storage in open trays resulted in much desiccation. Standard storage in silver sand gave reasonable protection from desiccation, but storing bulbs in loosely closed polythene bags gave excellent weight retention, even over 12 weeks' storage. Within the temperature range studied, the lower the storage temperature the better the results. In silver sand and loosely closed polythene bags, storage at 13°C, the usual recommended temperature, was satisfactory.

A variable number of the stored bulbs were deemed suitable for potting and growing-on, any rotted or desiccated bulbs being discarded. For the means of the various treatment combinations, between 67 and 100% of the stored bulbs were deemed suitable for growing-on. Statistical analysis showed that storage temperature and medium, but not harvest date nor storage duration, significantly affected both the percentage of bulbs fit for re-planting and the percentage of these bulbs sprouting in the following spring. The highest percentage of sprouting bulbs was obtained following storage in polythene bags (at either temperature, 13 or 20°C) or in silver sand at 20°C.

Experiment 2 – modified atmosphere storage

The effects of polythene bag gauge, and whether the bag was perforated or not, on weight loss of snowdrop bulbs is shown in Table 3.1. All treatment factors significantly affected weight loss, though the duration of storage exerted the most significant effect, despite the wide range of polythene thickness included in the experiment. Overall, weight loss increased from 0.4 g following 4 weeks' storage to 1.6 g after 12 weeks' storage. Losses increased, overall, when using a perforated bag (1.0 g against 0.8 g for a sealed bag) and as the polythene gauge decreased (from 0.6 g for 700 gauge bags to 1.4 g for 90 gauge bags). At the end of 12 weeks' storage, most treatments had at least some bulbs with the start of shoot or root growth and with some mould growth. These bulbs are being grown on, and full results will be available after these have flowered and senesced in late spring 2004.

Experiment 3 – use of confectionery glaze to reduce desiccation of bulbs

This experiment is under way and will be reported later.

Table 3.1. Effect of polythene bag gauge and perforation on weight loss of snowdrop bulbs.

Gauge	Sealing	Weight loss (g/5 bulbs)		
		After 4 weeks	After 8 weeks	After 12 weeks
90	Sealed	0.56	1.12	2.48
	Perforated	0.60	1.15	2.68
120	Sealed	0.66	1.13	2.09
	Perforated	0.40	0.79	2.66
150	Sealed	0.98	0.86	1.74
	Perforated	0.42	1.00	1.60
200	Sealed	0.35	0.67	1.03
	Perforated	0.23	0.42	0.86
250	Sealed	0.24	0.69	0.96
	Perforated	0.27	0.60	1.41
500	Sealed	0.28	0.40	0.55
	Perforated	0.82	0.92	1.34
700	Sealed	0.10	0.25	0.58
	Perforated	0.14	0.76	1.91
SED (82 d.f.)			0.218	
Significance				
Gauge (G)			***	
Seal (S)			**	
Weeks of storage (W)			***	
G x S			***	
G x W			***	
S x W			***	
G x S x W			(*)	

GENERAL CONCLUSIONS

1. The continuing demand in the Bulb Trade for snowdrop (*Galanthus*) bulbs cannot be met from present sources. Furthermore, existing supplies are often of poor quality, with small, desiccated and diseased bulbs. Supplies in bulk are largely limited to 'ordinary' *G. nivalis*, with the choicer and more varied species and hybrids being highly sought after by galanthophiles but largely unappreciated by the wider gardening public.
2. In setting up the current project, three problems associated with the commercial exploitation of snowdrop bulbs were identified for study: a lack of good quality bulb stocks, the apparent unsuitability of snowdrop bulbs for normal commercial cultivation in the field, and the poor storage characteristics of the bulbs. These problems have been addressed through studies of micropropagation, agronomy and storage methods.
3. An intensive study of the micropropagation of snowdrops is being conducted, involving *G. nivalis*, *G. nivalis* cv Flore Pleno, *G. elwesii*, *G. plicatus* and *G. plicatus* cv Wendy's Gold. The main conclusions so far are:
   3.1. Tissues from all species and cultivars could be successfully sterilised by an ethanol rinse followed by treatments with hypochlorite and 'Plant Preservative Mixture'.
   3.2. Bulblets initiated on outer surfaces of bulb scales and division and transfer to fresh culture medium could readily multiply these, thus forming the basis for rapid propagation of selected bulbs. Bulblet numbers can be increased with *G. nivalis* and *G. elwesii* by individual culture of scale leaf explants as opposed to bulb chips.
   3.3. A mineral analysis of snowdrop bulbs indicated that basal MS medium, often used with bulbous species, was probably not supplying minerals in the optimum ratios for snowdrops. This could be rectified by use of a redesigned *Galanthus* medium (G).
   3.4. *G. elwesii* and *G. plicatus* were much more prone to the physiological disorder, hyperhydration, than other snowdrop types. With *G. elwesii* this could be corrected by increasing the agar strength to 11 g/l. To some extent by changing to the basal medium G or use of the alternative cytokinin, kinetin, reduced levels of hyperhydration.
   3.5. Removal of plant growth regulators from the medium and addition of activated charcoal greatly stimulated bulblet growth and the initiation of roots. Bulblets greater than 1cm in diameter could be produced by this method that had up to three scale leaves but no flowers. Giving the tissues cold treatments did not stimulate bulblet growth and dilution of the basal mineral medium reduced bulblet growth.
   3.6. Preliminary planting trials indicate the *G. nivalis* and *G. nivalis* cv Flore Pleno transfer readily to *in vivo* conditions and do not display dormancy problems.
4. In further micropropagation experiments, factors controlling hyperhydration in *G. elwesii*, bulblet growth and acclimatisation in a range of species, are being investigated.
5. Field trials have been conducted in which simple mulching, shading and shelter techniques have been applied to plots of *G. nivalis*, to simulate conditions that might be more appropriate to the genus. The main conclusions are:
   5.1. Simple shading, using horticultural netting, enhances snowdrop growth, giving higher bulb yields through more vigorous growth and delayed foliar senescence.
   5.2. A straw mulch was also effective in enhancing growth and bulb yields, and is best used in combination with shading material.

5.3. Inter-cropping snowdrops with cereals or narcissus bulbs, or over-sowing snowdrop plantings with perennial rye-grass, is unsuitable because these other species were too competitive.

5.4. In the above trials, snowdrop growth declined in the third year, suggesting that competitive, nutritional or disease factors may be operating.

6. A field trial on the effects of pre-planting fungicide dips and fungicide spray programmes showed that a dip (Benlate + Captan) was highly benefiicial, and that a spray programme using Scala or Unix improved bulb yield or size through delaying leaf senescence.
7. Field and tunnel trials are being conducted to compare the field growth of 'ordinary' bulbs and *ex vitro* plantlets.
8. Experiments have confirmed the value of storing *G. nivalis* bulbs in silver sand at 13 or 17°C. Storage in loosely closed polythene bags at these temperatures was also successful in preventing desiccation and ensuring acceptable subsequent plant growth. Polythene bags used for storage should be not less than 150 gauge.
9. The results obtained so far suggest that commercial snowdrop production could be enhanced through the availability of high quality nuclear planting stocks of a variety of species and cultivars (a) derived from micropropagation and (b) grown-on in the field under shading and (or) using a mulch.
10. While the machinery and handling aspects of snowdrop bulb growing is not a part of this project, this will need to be addressed.
11. In order to enhance the prospects for successful snowdrop bulb production and sales in the UK, an exploitation plan should be developed in 2004 involving researchers, micropropagation managers and specialist bulb growers.