HDC Project BOF 48
Final Report (2005)

SNOWDROPS: DEVELOPING COST-EFFECTIVE PRODUCTION METHODS THROUGH STUDIES OF MICROPROPAGATION, AGRONOMY AND BULB STORAGE

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Project title: Snowdrops: Developing cost-effective production methods through studies of micropropagation, agronomy and bulb storage

HDC Project number: BOF 48

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Date commenced: April 2000

Date completion due: December 2004

Keywords: *Galanthus*, snowdrop, bulb, micropropagation, agronomy, storage

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Frontispiece. Four snowdrop types in the final stage of micropropagation photographed eight weeks after transfer into bulb growth-inducing conditions. Cultures had been introduced to culture three years previously and multiplied by regular subculture at 12-16 week intervals. Shown half life-size.

Galanthus nivalis

G. elwessii

G. plicatus

G. 'Wendy's Gold'

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GROWER SUMMARY

Headlines

- **Microprop set to boost quality and availability of snowdrops**

Successful microprop techniques have been developed for *Galanthus nivalis, G. elwesi, G. plicatus* and cultivars. Bulb initiation and bulb growth protocols are now robust enough to be exploited by the horticulture industry. The microprop plantlets can be successfully acclimatised to field conditions.

- **Commercial-scale production of snowdrops in the UK**

Field trials conducted at Warwick HRI, Kirton have shown that snowdrop growth under field conditions can be improved by using simple artificial shading, a straw mulch, and appropriate fungicide programmes.

Background and expected deliverables

There has long been a demand for snowdrop (*Galanthus*) bulbs that was met from bulbs collected from the wild, a practice no longer acceptable. The crop is however, 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 all three of these issues:

- *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 bulbs
- *Storage* – to find better bulb storage methods for snowdrop bulbs

Success with these objectives would enable the UK bulbs and ornamentals industry to meet and further stimulate the demand for snowdrops. This would give opportunities for increasing:

- Bulk sales of dry bulbs of commonplace material (*G. nivalis*), including for large-scale amenity planting
- Sales of a wider range of hardy species (e.g. *G. elwesii, G. plicatus*) for garden and other uses, including pot-plant sales
- The provision of choice and rare species and cultivars for the specialist market

Summary of the project and main conclusions

Micropropagation

The project included an intensive study developing techniques for the micropropagation of snowdrops, using *Galanthus nivalis, G. nivalis* cv 'Flore Pleno', *G. elwesii*, *G. plicatus* and *G. plicatus* cv 'Wendy's Gold'.

The initial work revealed the best ways to achieve sterile cultures of bulb explants of *G. nivalis*, *G. nivalis* 'Flore Pleno' and *G. elwesii*.

- High concentrations of hypochlorite were necessary for surface sterilisation of explants (tissue pieces removed from the bulb for culturing). However, hot-water treatment (HWT)

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of bulbs prior to surface sterilisation *increased* subsequent contamination, whereas HWT would be expected to reduce contamination.

- Treatment with a commercial product, 'Plant Preservation Mixture' (PPM) following surface sterilisation with lower concentrations of hypochlorite, increased the control of contamination, although high concentrations of PPM initially inhibited growth.
- Following successful surface sterilisation, there were additional benefits of incorporating fungicides into the culture medium, a mixture of carbendazim and imazilil completely eradicating residual infection. Also, steeping explants in imazilil before inoculation was highly effective in controlling infection, particularly when combined with incorporating imazilil in the culture medium. Fungicides, particularly imazalil, increased hyperhydration (see below). Steeping in a mixture of 20mg/l of both carbendazim and imazalil stimulated bulblet production, whereas use of either compound alone had no effect.

Explants of *G. nivalis* and *G. elwesii* initiated bulblets, but not shoots, after about 8 weeks in all culture conditions tested.

- Bulblets formed on the abaxial (outside) surface of scale leaves in both cases. In other ways, *G. nivalis* behaved differently to *G. elwesii*:
- Bulblets were formed basally in *G. nivalis* but randomly in *G. elwesii*;
- Fungicides in the culture medium reduced bulblet numbers with *G. elwesii* but not *G. nivalis*;
- *G. elwesii* tissues were more prone to hyperhydration (a physiological disorder also called vitrification) than those of *G. nivalis*. Fluridone (at 10µM) stimulated bulblet initiation; as fluridone is an inhibitor of abscisic acid (ABA), this indicates that ABA is not an important bulb initiation trigger in snowdrops.

The transfer to fresh culture medium of explants with bulblets initiated readily enabled multiplication, forming the basis for rapid propagation of selected bulbs.

- With *G. nivalis* and *G. elwesii*, bulblet numbers were increased by the division of bulb chip explants into three individual scale leaf explants. With these species scale leaf position within the bulb had little effect on their ability to regenerate bulblets. With *G. nivalis* 'Flore Pleno' there was no advantage in dividing chips into separate scales, owing to reduced bulblet production by the inner two scales compared with the outer scale.
- Plant growth regulators (PGRs) in the medium were not essential, and the use of the cytokinin BA alone inhibited bulblet production. However, the combination of BA plus the auxin NAA induced more bulblet production than when either was used alone.

A mineral analysis of snowdrop bulbs indicated that basal Murashige & Skoog (MS) medium, often used in the microprop of bulbous species, was probably not supplying minerals in the optimum ratios for snowdrops.

- MS medium was under-supplying phosphorus and copper and over-supplying potassium, zinc and manganese.
- This was rectified by using the mineral analysis to design a new *Galanthus* medium (G). A comparison of MS and G media at various dilutions showed that (a) greater numbers of bulblets were initiated throughout the dilution range on G medium with *G. nivalis* and *G. nivalis* Flore Pleno, whereas MS medium was superior with the full- and half-strength media with *G. elwesii*; (b) G and MS media supported similar bulblet multiplication rates throughout the dilution range, but dilution to one-eighth significantly reduced bulblet multiplication; (c) spontaneous root initiation was greater on G medium.

Hyperhydration in *G. elwesii* was controlled by:

- Increasing the agar concentration from 7 g/l to 9 or 11 g/l;
- Changing the cytokinin in the medium from BA to kinetin (at low agar concentrations);

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- Changing basal medium from MS to G (at low agar concentrations). Hyperhydration was not controlled by varying the iron or magnesium levels, as is the case in some species.

Attempts to initiate rapidly multiplying shoot cultures, rather than bulblets, were unsuccessful, bulblets continuing to be initiated regardless of the treatments imposed.
- Reducing the incubation temperature to 6ºC yielded no *de novo* bulblet or shoot initiation;
- Reducing the photoperiod from 16h to 8h marginally increased bulblet initiation rates.

Multiplication of bulblets was relatively slow but could be maintained even without removal or splitting of larger bulblets.
- New bulblets formed on scales of *in vitro*-formed bulblets and tissues derived from explant scales.
- Multiplication was increased by splitting larger bulblets of *G. elwesii*, but splitting was less effective with *G. nivalis*.

Removal of PGRs from the medium and the addition of activated charcoal stimulated bulblet growth and the initiation of roots. Improved growth of bulblets was achieved by increasing the sucrose supply in combination with addition of charcoal. Bulblets greater than 1cm in diameter, with up to three scale leaves but no flowers, could be produced by this method.

Several experiments explored ways to stimulate bulblet growth to a stage where bulbs could be successfully transferred to *in vivo* conditions.
- 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.
- All experiments involved the removal of PGR compounds from the medium and increasing either the carbohydrate supply and (or) incorporating activated charcoal in the medium.
- Addition of activated charcoal greatly stimulated bulblet growth with *G. nivalis, G. nivalis* 'Flore Pleno' and *G. elwesii* on a full-strength MS basal 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.
- Increasing the sucrose concentration to 60 or 90g/l had little effect on bulblet growth, even when used in combination with charcoal. Longitudinal sections showed that bulbs, stimulated to enlarge by the addition of extra sucrose and charcoal, formed up to three normal looking scale leaves but developed no flowers. Cold treatment of cultures for 4 or 6 weeks at 5°C showed no improvements in bulblet growth.

Additional bulblet growth experiments were established to test the effects of alternative adsorption agents to activated charcoal, including polyvinylpyrrolidone, Fuller's Earth, kaolin and a range of pectins. Unlike charcoal, none of these materials stimulated bulblet growth. The beneficial effect of charcoal appeared to work via shading, not absorption.

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 'Wendy's Gold'

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were characteristically bright yellow in pigmentation. *G. elwesii* and *G. plicatus* were much more prone to the physiological disorder hyperhydration than other snowdrop types tested. With *G. elwesii*, this could be corrected by increasing the agar strength to 11 g/l, by changing to G basal medium, by dilution of the MS or G media, or by using the alternative cytokinin, kinetin.

G. nivalis, *G. nivalis* cv 'Flore Pleno' and *G. elwesii* transferred readily to *in vivo* conditions in soil and did not display any dormancy problems.

A protocol for snowdrop micropropagation is given in Appendix A of the full report.

<u>Agronomy</u>

Field trials were conducted in which mulching, shading, shelter, intercropping and other techniques were applied to plots of *G. nivalis*, to simulate conditions that might be more appropriate to the species. Simple overhead shading with horticultural netting (50% shade) enhanced snowdrop growth, giving more vigorous growth with more stems, pods and seeds, delayed foliar senescence, and higher bulb yields. Using a low windbreak to provide shelter from the prevailing wind reduced leaf loss and increased growth, though to a lesser extent. A straw mulch (*ca*. 5cm deep) was effective in delaying senescence and enhancing growth and bulb yields, and was best used in combination with shading material. Hence, there appeared to be prospects for improving snowdrop growth by using simple shading materials and (or) 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, though in the second year bulb yields in the rye-grass plots had started to recover. Inter-cropping snowdrops with cereals or narcissus or over-sowing with perennial rye-grass were unsuitable as an alternative to using artificial shading materials, because these species were too competitive.

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 yield was 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 Unix, Stroby WG and, particularly, Scala, had been used. In contrast to the other fungicide sprays, using Scala programme increased mean bulb weight irrespective of whether a pre-planting dip had been given or not.

The acclimatization of micropropagated plantlets to field conditions was assessed by planting a number of clones of *G. nivalis*, *G. nivalis* 'Flore Pleno' and *G. elwesii* from culture vessels into soil in the field (into sheltered or non-sheltered plots) or in an unheated mesh tunnel. Growth of the different clones was very variable. In the field clonal vigour was not consistent between sheltered and non-sheltered plots: though several 'weak' clones grew better under shading, the reverse was true in other cases. Similarly, there was no reliable correspondence between vigour, whether a clone was planted in the field or in the

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mesh tunnel: several clones grew better in the tunnel, but other were more vigorous outside. In general, *G. nivalis* clones appeared more vigorous than the others and a wider range of clones performed better when grown under the mesh tunnel.

The effect of mycorrhizal fungi on micropropagated plantlets was investigated by growing 10 clones of *G. nivalis* in autoclaved or non-autoclaved growing medium amended with a proprietary mycorrhizal preparation, soil from established snowdrop sites, and soil from a non-snowdrop site. There was no evidence from the growth of the plants of benefits from mycorrhizal fungi.

<u>Bulb storage</u>

Bulb storage experiments were carried out in an attempt to find better ways of storing the small, vulnerable snowdrop bulbs.

- Storage of *G. nivalis* bulbs in silver sand at 13 or 17°C gave good protection from desiccation.
- Storage in loosely closed polythene bags at these temperatures was also successful in preventing desiccation and ensuring acceptable subsequent plant growth.
- The effects of modified atmosphere storage was tested by storing *G. nivalis* bulbs in perforated or non-perforated polythene bags of 90 to 700 gauge. 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 when using a perforated bag and as the polythene gauge decreased. Subsequent shoot production was similar in all treatments.
- Use of two confectionary glazes (Crystalac or Natureseal-PN) as pre-storage bulb dips was successful in reducing desiccation of the bulbs, but resulted in serious mould growth and in the bulbs clumping together encouraging damp and root and shoot growth.

Financial benefits

The results from the microprop experiments are encouraging, and reliable methodology is now available for the sterilisation, bulblet initiation, multiplication and growth phases of propagation. This suggests there is every prospect of establishing a programme producing high-quality, sustainable snowdrop stocks that would stimulate demand, not only of the 'ordinary' *G. nivalis*, but also of *G. elwesii* and other species and cultivars. The programme would be augmented by using the improved husbandry and bulb storage methods described. 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, and considerably more for double-flowered or choice types.

Action points for growers

- Businesses that are already growing snowdrops could utilise the information on growing the crop under 50% shade material, which will boost yields. A mulch could be used, with caution, but intercropping (or over-sowing with rye-grass) appeared to be unsuitable.
- Bulbs should be stored at 13-17ºC either in silver sand or in loosely closed polythene bags, as this was found superior to other conditions for reducing desiccation and other losses.
- Businesses interested in developing this crop should develop a business plan to exploit the findings in full, establishing superior micropropagated stocks as the starting point. The recommended protocol for snowdrop micropropagation is provided in this report.

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SCIENCE SECTION

INTRODUCTION

Drifts of delicate bulbous species such as snowdrops (*Galanthus* spp.) are a romantic evocation of woodland glades, and it is not surprising that snowdrops are popular garden plants, their attractive white flowers being among the earliest of the year in many British gardens. Snowdrops are hardy in all areas of the UK, and are in demand for naturalising in gardens and growing in containers outdoors and indoors. The delicate size, form and colouring of the flowers continue to match gardening fashions (Lear, 1988). In 1985-1986, 48 million snowdrop bulbs were exported (or re-exported) from the Netherlands, illustrating the extent of trading (Langeslag, 1989). In recent years in excess of 20 million snowdrop bulbs have been imported into the UK annually from The Netherlands (figures from *Produktschap voor Siergewassen*).

Commercial bulb growers and traders have been aware for many years of the potential sales for snowdrop bulbs but, unlike larger spring-flowering bulbs such as narcissus and tulip, they do not grow satisfactorily under standard agricultural systems. As a result, the market for snowdrops was satisfied for many years by the collection of wild bulbs from Turkey (*G. elswesii* and some *G. ikariae*), resulting in the extensive loss of natural populations (Zimmer and Girman, 1987). *G. elswesii*, especially, has been collected in quantity at least from the 1970s, and, although this species is unlikely to be rendered extinct, its distribution is becoming more restricted (S. Oldfield, personal communication). Local pressure to collect bulbs resulted in collection earlier in the year, before the bulb had had time to bulk up and before seed had set, and led to tonnes of undersized bulbs being dumped. Losses through habitat destruction and over-collecting have also been reported from the Ukraine (Budnikov and Kricsfalusy, 1994), while in the UK there have been well publicised cases of the illegal digging of snowdrops (e.g., Tendler, 2000). In 1987, Dutch imports of Turkish snowdrops reached 62 million (Lear, 1988). In addition, snowdrops have been ‘farmed’ from ‘sustainable’ woodland sources in France (Loire Valley), with an annual production of about 10 million bulbs (Ekim, 1984), as well as being obtained from England and elsewhere. Even at this level of production, demand did not appear to have been satisfied. Sales could be increased further, should acceptable stocks become available in sufficient quantities.

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

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in shady places, some species are natives of drier Mediterranean areas. Hence, there is scope for exploiting a larger variety of snowdrop types.

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

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

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Aims of the project

In the current project, cost-effective methods will be developed for the sustainable production of snowdrops in the UK. The project covers both the production of bulbs sold 'in the green' and as 'dry bulbs', because both systems have advantages and the availability of both would serve different sections of the market. The major problems of snowdrop production are:

- low multiplication rates,
- they are difficult to grow commercially,
- the bulbs are difficult to store.

The project therefore addresses the three objectives of micropropagation, agronomy and storage.

Micropropagation - to develop in vitro systems that sustain high vegetative propagation rates and yield superior quality, uniform bulb stock

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

Agronomy – to develop novel growing systems for effective commercial production of snowdrop bulbs

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

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different growing systems, plantings would be monitored in existing natural and 'semi-natural' sites such as woodlands, orchard and coppice.

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

Benefits of the project

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

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

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

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

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

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

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REVIEW OF *GALANTHUS* PROPAGATION, AGRONOMY AND STORAGE

Micropropagation

Introduction

Very few studies of micropropagation of the genus *Galanthus* have been reported in the literature and none of these has been carried forward to a commercial stage. Moskov *et al.* (1980) briefly described work with the common snowdrop (*G. nivalis*) as a minor part of a larger study on other bulbous species. More recently, Goddard (2000) also reported work with *G. nivalis*. The only other studies on this genus were with *G. elwesii* (Girmen and Zimmer, 1988a, b, c; Goddard, 2000) and *G. ikariae* (Tipirdamaz *et al.*, 1999; Tipirdamaz, 2003). The methodologies and main findings from these studies are reviewed briefly here.

Sterilization techniques

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

For *G. elwesii* we found that treatment with 12% sodium hypochlorite for durations between 5min and 20min was necessary to reduce microbial infection rates in bulb explants to 20% (ct. Girmen and Zimmer, 1988a). Of other explant types tested, only seeds could be completely sterilized. The largest part of Goddard's M.Sc. thesis (2000) focused on developing effective methods to sterilize bulb explants from both *G. nivalis* and *G. elwesii*, and the results of this study are summarised in Table 1.2. Hot-water treatment (HWT) followed by surface sterilization with sodium hypochlorite was effective only at a temperature of 54ºC, which severely damaged the bulb tissues. To control contamination, Goddard (2000) finally resorted to an initial bulb sterilization with sodium hypochlorite, followed by scale-leaf explant sterilization in an antibiotic solution and the use of fungicides in the culture medium. Even after this complex procedure, 11% of explants still became contaminated.

Choice of explant

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

Explants from leaves, flowers, floral scapes and bulb scales, prepared from actively growing plants grown under growth chamber conditions in vermiculite, rather than from resting bulbs, were tested for regeneration with *G. elwesii* and *G. nivalis* (Goddard, 2000).

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All types of explant from *G. elwesii* were capable of some combination of regeneration, ranging from callus and bulblets to somatic embryos. Of these, leaf explants were the most organogenic, and bulb scales the least. Parallel studies with *G. nivalis* were impossible to interpret, owing to high levels of microbial contamination. With this species, only leaf explants regenerated bulblets and somatic embryos.

Tipirdamaz *et al.* (1999) tested four *G. ikariae* bulb explant types for their regenerative ability. These are described below and illustrated in Table 1.1.

A Bulbs divided vertically into four equal segments
B Individual 5mm wide bulb scales including a 2mm section of base plate
C Bulb twin scales
D Explants without base plate from the upper part of the scale leaves

Bulb segment explants (A) formed the most adventitious bulblets at both auxin levels tested (using the potassium salt of naphthalene acetic acid, KNA), but these were not significantly more productive than smaller explant types B and C. However, it would be possible to prepare many more B-type explants than the four bulb segment explants from each mother bulb, and thereby achieve a higher multiplication factor in the initiation passage. In contrast to the findings of Girmen and Zimmer (1988a) with *G. elwesii*, leaf explants without base plate tissues (D) of *G. ikariae* did not regenerate new bulblets.

Table 1.1. Numbers of adventitious bulblets formed by different explant types of *G. ikariae* (drawings, personal communication, R. Tipirdamaz, 2000) on media supplemented with different KNA concentrations (Tipirdamaz *et al.,* 1999). Explant types are described more fully in the text but types A and B were carefully prepared to leave base plate (BP) tissues attached.

Explant type	**A**	**B**	**C**	**D**
KNA concentration (mg/l)	BP	5mm, 8mm, 2mm, BP		Upper part of leaf scale
0.2	3.25[a]	2.98[a]	2.34[ab]	0.00
0.4	2.23[bc]	1.76[c]	1.87[c]	0.00

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Table 1.2. Summary of surface sterilization experiments performed by Goddard (2000) on *Galanthus* bulbs*.

Sterilization treatment to:				*Medium*	*Contamination (%)*		*Remarks*
Bulb		*Scale leaf explant*			G. nivalis	G. elwesii	
Treatment	*Time (min)*	*Treatment*	*Time (h)*				
HWT 43.5°C then	60 – 120	-	-	BM	73 - 96	-	Bulbs treated either whole or
Domestos (10%)	10 – 20						halved in Domestos step
HWT (control RT)	60	-	-	BM	100	100	Each HWT was followed by
HWT (43.5°C)	60	-	-	BM	75	83	20min in 10% Domestos
HWT (49.0°C)	60	-	-	BM	5	39	54°C treatment damaged or killed
HWT (54.0°C)	60	-	-	BM	0	19	cells (soft & flaccid)
HWT (43.5°C)	60	-	-	BM	100	-	Each treatment was followed by
"	60	-	-	BM + B	35	-	20min in 10% Domestos
" + B	60	-	-	BM	40	-	
" + B	60	-	-	BM + B	5	-	Additional contamination
Contro1	?	-	-	BM	90	-	developed later indicating Benlate
HWT (control RT) + B	30	--		BM	40	-	was only fugistatic
Domestos (10%)	20	Control	24	BM	83		Explants were prepared from
"	"	N	24	BM+ B	100		bulbs that had been actively
"	"	N + R	24	BM	94		growing in pots for 2 months
"	"	N	24	BM+ SA	10		
"	"	N + R	24	BM+ SA	11		

*BM = basal medium, RT = room temperature, B = Benlate at 1g/l used as both a fungicide dip and in the medium, N = nystatin at 1g/l, R = rifampicin at 30μg/l, SA = Sportak Alpha at 1g/l.

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Culture media and physical conditions

Basal culture media used to grow *Galanthus* tissues are summarised in Table 1.3. All work reported so far used simple modifications of the revised Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). More recently, Goddard (2000) used the medium developed by Girmen and Zimmer (1988a, b; as outlined in Table 3.3) to culture both *G. nivalis* and *G. elwesii*, with some minor modifications to the vitamin composition.

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

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

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

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Table 1.3. Composition of media used to grow *Galanthus* species *in vitro*.

Species	*Nutrients*						*pH*	*Agar* (g/l)	*Reference*
	Macro	*Micro*	*Vitamins* (mg/l)		*Carbohydrate* (g/l)	*Miscellaneous*			
G. nivalis	MS	MS FeEDTA ($FeSO_4.7H_2O$ 27.85 mg/l & Na_2EDTA 37.25mg/l)	White (1963)		sucrose (30)	casein hydrolysate 0.5g/l	5.5-5.7	7	Moskov *et al.* (1980)
G. elwesii	MS Fe chelate	MS glycine (Fetrilon)	myo-inositol 2.0 pyridoxine HCl thiamine HCl nicotinic acid	100 0.5 0.5 1.0	sucrose, glucose or fructose (20 - 30) best for bulblet formation, glucose & lactose less effective	tested.	4.5 - 7.0 5.0 - 5.5 optimal for bulblet production	?	Girmen and Zimmer (1988a, b)
G. ikariae	MS	MS *G. elwesii*	same as for above		wide range of sources tested, sucrose (60) best for bulblet production		5.5 used but 6.0 above optimal for bulblet production	6	Tipirdamaz *et al.* (1999) Tipirdamaz (2003)

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Multiplication and genetic stability

Girmen and Zimmer (1988a) report an annual multiplication rate of 27,000 propagules arising from a single *G. elwesii* bulb. Their calculation assumes that two out of four bulb explants dissected from each mother bulb each produce 15 new bulblets in the 16 - 18 week initiation culture passage. Each of these bulblets was then divided into two new bulblet segments for the initiation of new bulblets in the second sub-culture, giving 60 segments per initial mother bulb. Further calculations are illustrated in Table 1.4. This method would therefore yield large numbers of very immature bulblets that are not ready for transfer to *in vivo* conditions at the end of one year.

Table 1.4. Annual multiplication schedule for *G. elwesii* according to Girmen and Zimmer (1988a). Tissues were transferred to fresh medium, with a growth regulator composition of 0.1 mg/l KNA and 2mg/l BA, every 4 weeks, but were only sub-divided at 16-18 week intervals. This assumes a multiplication of 15 between sub-divisions and new bulblets being split into two segments.

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

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

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

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

Indirect formation of bulblets via callus tissues might be expected to carry a risk of genetic

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

Acclimatization

NAA has recently been shown to promote bulblet rooting in *G. ikariae* (Tipirdamaz, 2003). She found that rooting of bulblet cultures could be increased from 8.9% in the auxin-free control to 25.4% in cultures supplied with 0.5 mg/l NAA, when half-strength MS medium supplemented with 5 g/l activated charcoal and 30 g/l sucrose was used. Bulblets rooted in this way showed a 28% transplantation success when planted directly, without cold or gibberellic treatments, into a sand:soil:perlite (1:2:1) compost under growth chamber conditions. Other factors, such as medium strength, activated charcoal and sucrose concentration, did not significantly affect rooting of micropropagated bulblets of *G. ikariae*.

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

Other methods of propagation

Seed

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

Offsets, chipping and twin-scaling

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The use of offsets for multiplication is also slow, as they are produced only from large 'mother bulbs' (Le Nard and De Hertogh, 1993). The propagation rate can be increased to some extent by the use of bulb chipping or twin-scaling (Alkema and van Leeuwen, 1977a, b; Alkema, 1985a, b; Langeslag, 1989; Hanks, 1991; Aksu and Çelikel, 2003). Bulbs of *G. nivalis*, 4-5cm grade, were chipped successfully by cutting into eight segments (Hanks, 1987). However, the multiplication rate was relatively low for the labour input required, as the bulbs have few scales. It is reported that chipping has been used to propagate *G. elwesii* in the Netherlands, incubating the chips at 13-15°C (Langeslag, 1989). Van Leeuwen and van der Weijden (1997) reported on the chipping of *G. nivalis*, cutting bulbs into chips in the July to September period. The best yields were obtained by chipping as soon as possible after lifting, before mid-August, planting the chips immediately, and growing for two years.

Agronomy, bulb handling and bulb storage

'Commercial' production

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

Snowdrops from different sources may show different characteristics. Under Dutch conditions, the French type of *G. nivalis* produces smaller flowers and bulbs than the Dutch type (Langeslag, 1989). *G. elwesii* and Turkish *G. nivalis* do not grow well in the Netherlands (S. Oldfield, personal communication). Langeslag (1989) reported that bulbs collected from the wild are either lifted in late-March/early-April with the leaves still green, the bulbs being separated and re-planted immediately, or (more usually) they are harvested after the tunic (brown skin) has formed and re-planted in August/September. Commercial producers in the Netherlands have apparently used both systems, planting small bulbs (<4 cm grade) 5-10 cm deep and, as is usual in the Netherlands, mulching with straw (Langeslag, 1989). The bulbs are planted in shallow drills made up into beds. Snowdrops are said to prefer damp, heavy soil and to benefit from a generous autumn top-dressing. Sandy or loamy soils with a high organic matter content are used, high soil moisture is acceptable, and a pH about 7 ensures bulbs with good skins are produced. The foliage is susceptible to sun-scorch, so they are sometimes planted under trees or with some protection in the field in spring (Alkema, 1985a, b; Langeslag, 1989). *Galanthus* species from drier habitats, such as *G. graceus*, *G. corcyrensis* and *G. reginae-olgae*, would be expected to prefer different conditions.

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Two-year-down growing is recommended (Zandbergen, 1985). The bulbs may be lifted when the foliage has died down (late-May to early-June), using a riddle-type harvester with a fine screen, and collecting the bulbs into mesh-bottomed trays. The bulbs must be surface-dried to avoid rotting, but must not be allowed to dry out. Grading should be carried out immediately, and the small planting stock bulbs should be re-planted promptly.

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

Weed control

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

Cold tolerance

The freezing tolerance of snowdrops has been tested. Bulbs of *G. elwesii* with 4cm of leaf growth survived freezing to -7°C, but the flowers died when the temperature decreased below -5°C (Sakai and Yoshie, 1984). Bulbs of *G. nivalis*, acclimatised to 5°C, were severely injured by exposure to temperatures of -9°C, the roots and base plate being most sensitive to cold (Lundquist and Pellett, 1976).

Pests and diseases

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

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Physiology

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

Bulb storage

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

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

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MICROPROPAGATION

Materials and methods

Plant material

Bulbs for bulblet initiation experiments
G. 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 (*G. elwesii* only) in 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. All bulbs were stored at room temperature on receipt.

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 the bulbs bought in 2000 which were found to have only single 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. With a scalpel, basal bulb tissues were cut away 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 sterilization. Throughout bulbs were given a preliminary surface sterilization by shaking in 70% ethanol for 1min. Thereafter bulbs were surface sterilized 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 sterilize the smaller chips. Larger ones produced from *G. elwesii* bulbs required three chips in 30ml of PPM to improve surface sterilization. Following sterilization, explants were inoculated with their bases about 3mm into the agar-solidified culture medium. One explant was inoculated per culture vessel.

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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 100µmol/m^2/s at bench height.

Bulblet initiation and multiplication - Control of hyperhydration in *G. elwesii*

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 culture

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). Five replications were inoculated with each of five different clonal lines derived two years previously from *G. elwesii* bulbs.

Stimulation of bulblet growth

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 the benefits of activated charcoal (Pan and van Staden, 1998). These include the adsorption of endogenous or synthetic plant growth regulator compounds from the medium or of toxic metabolites, released by plant tissues. Activated charcoal could also function by darkening the culture medium and thereby inducing polarity in the cultured tissues. In addition,

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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 of others, such as magnesium (Van Winkle, Johnson and Pullman, 2003; Van Winkle and Pullman, 2003).

A number of alternative adsorption agents was therefore tested to examine the mode of action of activated charcoal in improving 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) MW10,000 (Sigma P-2307), Fuller's earth (FE) mesh size 30-60 (Sigma F-60), kaolin (KA)(Sigma K-1512) and Silicar CG-4 (SIL), 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 made up of five from each of four clones (640 cultures in total).

Alternatives to activated charcoal (pectins)
In a second experiment a series of pectins was 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 (Lewewinska, 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 2.1 and all were a gift from 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 pH values 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 2.1 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 made up of three from each of five clones (480 cultures in total).

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Table 2.1. Properties of GENU® pectins tested as alternative adsorption agents. Codes used to identify the pectins in Figures 2.4, 2.5 and 2.6 are shown in parenthesis.

Chemical characteristic	*Pectin type (CP Kelco code numbers)*			
	LM-5 CS (5)	*LM-12 CG (12)*	*LM-22 CG (22)*	*LM-101 AS (101)*
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

Effects of adsorption agents in light and dark

A further experiment was performed to examine if the main mode of action of activated charcoal was to darken the culture medium. This was achieved by comparing growth and development in both light and dark conditions. Complete darkness was imposed by individually wrapping half the cultures from each treatment in aluminium foil. Two concentrations of activated charcoal were used, 0.2 g/l that only partially shades the bases of cultured tissues and 1 g/l that excludes most light. These were compared with PVP treatments at the same rates that do not exclude light. Full strength G medium without plant growth regulators, supplemented with 60g/l sucrose, was used throughout.

The full experimental design was [2 adsorption agents (AC and PVP) x 2 concentrations (0.2, 1.0 g/l) + control (no adsorption agent)] x 2 light treatments (light and dark) x 2 snowdrop types (*G. nivalis* and *G. nivalis* 'Flore Pleno'), 20 treatments in all. Each treatment was replicated with 15 bulblet clumps made up of five from each of three clones (300 cultures in total).

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 and 2003 Annual Reports). 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 three 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 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

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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

Bulblets were 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. The results are described under the Agronomy section of the report.

Statistical Analysis

Data were subjected to analysis of variance using the software package 'Genstat V' release 3.1 (Lawes Agricultural Trust, 2002). Where appropriate, data were analysed with and without transformation. Non-transformed values are presented when diagnostic plots of residuals indicated that the assumptions necessary for analysis of variance were satisfied. Statistical significance in all the tables is presented as follows: NS = non significant, * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. In figures, since the analysis of variance requires the assumption of homogeneity of variance, a single error bar is shown for all treatments.

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Results and discussion

Control of hyperhydration in *G. elwesii*

Iron and magnesium concentration

1. Bulb chip explants.

The tissues in this experiment were cultured for one, three-month initiation passage followed by two, four-month multiplication passages. At the end of this period no obvious differences could be seen between the treatments in either bulblet production or the frequency or severity of hyperhydration in bulblets. Tissues from this experiment were discarded without further assessments being taken.

2 Hyperhydrated proliferating bulblet cultures

Increasing either Fe or Mg in the culture medium either alone or in combination had very little effect on either bulblet production or tissue hyperhydration (Table 2.2). Indeed doubling the Fe concentration normally used in MS medium to 0.2 mM significantly increased the percentage of bulblets scored as being hyperhydrated compared to the control. This increase was not reflected in greater amounts of water in the bulblet tissues in these treatments. Significant clonal differences were found in all parameters assessed (data not shown) but no significant interactions were found between any of the main factors. It is therefore unlikely that manipulation of Fe and Mg concentrations in the range suggested by Yadav *et al*. (2003) can be used to achieve control of hyperhydration in *G. elwesii* cultures in the same way as it can with carnation.

Stimulation of bulblet growth

Alternatives to activated charcoal (adsorption agents)

Incorporation of adsorption agents only had small effects on the formation of new bulblets under conditions favouring bulblet growth (Fig. 2.1). Of the alternatives tested only SIL inhibited bulblet production, perhaps by adsorbing chemical factor(s) important in bulblet differentiation. This slight inhibitory activity of SIL was not reflected in overall tissue growth (Fig. 2.1). All three concentrations of activated charcoal tested greatly promoted overall growth compared with the control, whilst none of the alternative adsorption agents differed significantly from the control. A similar pattern was seen in bulblet growth data in that none of the alternative adsorption agents stimulated bulblet growth (Fig. 2.2). Root production and growth were, as expected, greatly stimulated by activated charcoal, but not by any of the alternative adsorption agents (Fig. 2.3). These bulblet and rooting data strongly suggested that the mode of action of activated charcoal did not rely completely on its ability to adsorb compounds from the culture medium.

Alternatives to activated charcoal (pectins)

In a further experiment it was also shown that pectins could not substitute for activated charcoal to promote overall culture growth, bulblet or root development (Figs. 2.4 - 2.6). Indeed at the highest concentration all the pectins inhibited root growth (Fig. 2.6) whilst pectin 22 inhibited production of new bulblets (Fig. 2.4) compared with the control. Again this experiment suggested that the adsorptive properties of activated charcoal were not totally responsible for its ability to stimulate the growth of bulblets and roots.

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Effects of adsorption agents in light and dark

Growing the cultures in complete darkness had no significant effect on the production of new bulblets by either *G. nivalis* or *G. nivalis* 'Flore Pleno' (Fig. 2.7). Addition of PVP significantly ($p < 0.01$) reduced new bulblet production in the cultures to an average of 4.97 new bulblets from 6.17 and 7.18 in the control and activated charcoal treatments, respectively (LSD 5% = 1.284). In contrast, darkness ($p < 0.001$) and activated charcoal ($p < 0.001$) both significantly stimulated overall tissue growth (Fig. 2.7). Average tissue growth was 0.69 g FW (fresh weight) / culture in the light compared with 1.00 g FW/ culture in darkness (LSD 5% = 0.106), whilst the control, PVP treatments and activated charcoal treatments showed FW /culture of 0.76 g, 0.56 g and 1.17 g respectively (LSD 5% = 0.119). Assessment of overall culture fresh weight therefore indicated that the ability of activated charcoal to shade tissues from light might be important for its mode of action.

Assessments of bulblet growth were more difficult to interpret owing to the relatively poor response of the tissues to the addition of activated charcoal compared with other experiments (Fig. 2.8). Fresh weight of the largest bulblet failed to detect any significant effects of either adding adsorption agents or of growing the cultures in the dark when the two plant types were analysed together. When the plant types were analysed separately *G. nivalis* continued to show no significant main effects of either light or adsorption agents, whereas with *G. nivalis* 'Flore Pleno' fresh weight of the largest bulblet was significantly stimulated by both growing the cultures in darkness ($p < 0.01$; dark = 0.111 g, light = 0.079 g; LSD 5% = 0.0201 g) or by adding activated charcoal to the medium ($p < 0.001$; control = 0.073 g, PVP = 0.079 g and activated charcoal = 0.122 g; LSD 5% = 0.0275). Thus with *G. nivalis* 'Flore Pleno' activated charcoal and darkness again had similar effects. These effects were also reflected in the diameters of the largest and second largest bulblets of both plant types, where darkness and activated charcoal both marginally stimulated bulblet growth (Fig. 2.8). Interestingly, PVP stimulated neither overall tissue growth nor individual bulblet growth regardless of whether it was supplied in the light or dark. This suggested that the effects of activated charcoal cannot be reproduced by using an alternative adsorption agent that, like PVP, that does not shade, in the darkness. Nevertheless, this experiment would need to be repeated with plant tissues responding more typically to the inclusion of activated charcoal in the culture medium in terms of individual bulblet growth.

The strong rooting response to activated charcoal was much more typical of previous experiments (Fig. 2.9). This root promotion effect could be completely reproduced by growing the tissues in the dark in the absence of activated charcoal (Figs. 2.9 and 2.10).

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Table 2.2. The effects of Fe and Mg concentration on bulblet production and hyperhydration in proliferating bulblet cultures of *G. elwesii*. Bulblet numbers were subjected to square root transformation but untransformed data are also shown in parenthesis. All percentage data were subjected to angular transformation before analysis, and untransformed data are shown in parenthesis. For this and following Tables and Figures, summaries of the analyses of variance are given in Appendix B.

Parameter assessed	*Fe (mM)*			*Sig.*	*LSD (5%)*	*Mg (mM)*			*Sig.*	*LSD (5%)*
	0.10	*0.15*	*0.20*			*1.50*	*2.25*	*3.00*		
Total bulblets per culture	4.19 (18.47)	4.07 (17.41)	3.90 (16.07)	NS	0.258	4.06 (17.32)	4.17 (18.48)	3.93 (16.15)	NS	0.258
Hyperhydrated bulblets (%)	35.0 (37.5)	30.5 (31.7)	41.6 (46.1)	**	6.50	34.9 (37.5)	39.1 (44.0)	33.1 (33.6)	NS	6.50
Water in bulblets (%)	57.0 (70.0)	57.2 (70.4)	58.4 (72.1)	NS	1.83	57.6 (71.1)	58.3 (71.9)	56.7 (69.6)	NS	1.83
Water in other tissues (%)	60.9 (75.8)	59.2 (73.0)	61.5 (76.7)	NS	1.99	59.9 (74.1)	60.8 (75.5)	60.9 (75.9)	NS	1.99

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However, in darkness, root numbers and root growth could be further stimulated by the addition of activated charcoal, suggesting that activated charcoal had a complex mode of action over and above simply shading the tissues. An additional factor, not considered in these experiments, was that activated charcoal might function by improving the aeration of the culture medium. This is based on observations that tissues can develop better in liquid media, on inert supports such as glass wool, cotton or sand, than on an agar solidified medium (Yam *et al*., 1990; Bhattacharyya *et al*., 1994; Newell *et al*., 2003).

PVP again failed to substitute for activated charcoal in either light or dark conditions, indicating that adsorption of inhibitory compounds from the medium was not important in stimulating a rooting response.

Use of complete darkness was not an ideal way to reproduce the shading effect of adding activated charcoal to the medium. In complete darkness both the tissues above the medium as well as those below are screened from light. Thus complete darkness cannot induce polarity in plant tissues in the same way that basal darkening would. An improved experimental design would be to develop treatments that shade only tissues below the surface of the medium but do not have adsorptive properties, such as the use of graphite or aluminium foil (Yam *et al*., 1989; Sanchez *et al*., 1996 and references therein). As well as its polarising effect, shading might also protect photosensitive compounds, such as auxins and phenolic rooting co-factors, allowing their accumulation to physiologically active concentrations in basal tissues (Druart *et al*., 1982).

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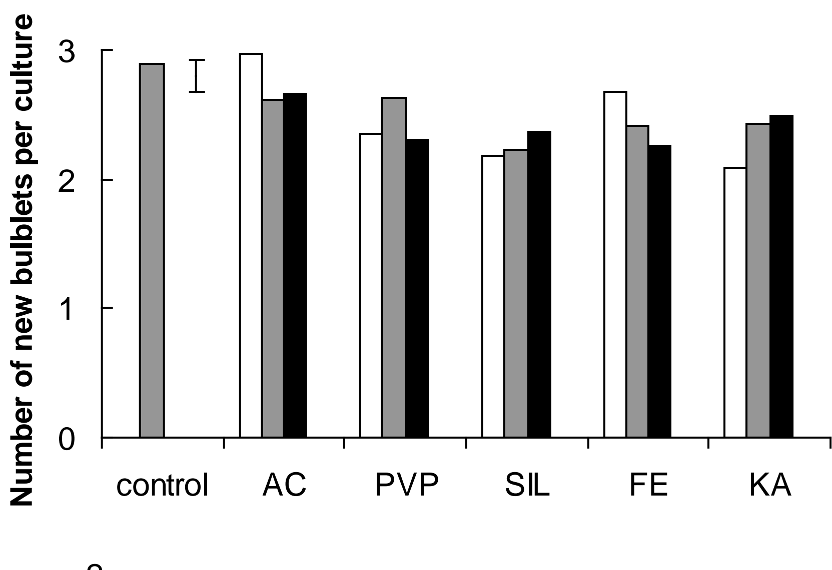
Figure 2.1. Effects of adsorption agents as alternatives to activated charcoal (AC) on the numbers of new bulblets formed and on the total culture fresh weight. The agents used were polyvinylpyrrolidone (PVP), Fuller's earth (FE), Silicar CG-4 (SIL) and kaolin (KA). Data are the mean of *G. nivalis* and *G. nivalis* 'Flore Pleno' and concentrations of adsorption agents were 0.2 g/l (open columns), 1.0 g/l (shaded columns) and 5.0 g/l (black columns). Bars show the SED.



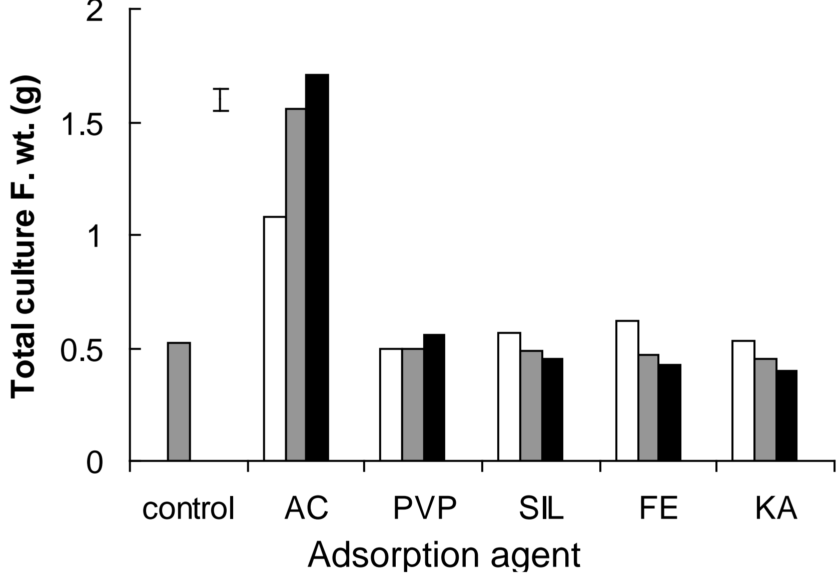


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Figure 2.2. Effects of adsorption agents as alternatives to activated charcoal (AC) on the growth of the largest and second largest bulblets. The agents used were polyvinylpyrrolidone (PVP), Fuller's earth (FE), Silicar CG-4 (SIL) and kaolin (KA). Data are the mean of *G. nivalis* and *G. nivalis* 'Flore Pleno' and concentrations of adsorption agents were 0.2 g/l (open columns), 1.0 g/l (shaded columns) and 5.0 g/l (black columns). Bars show the SED.

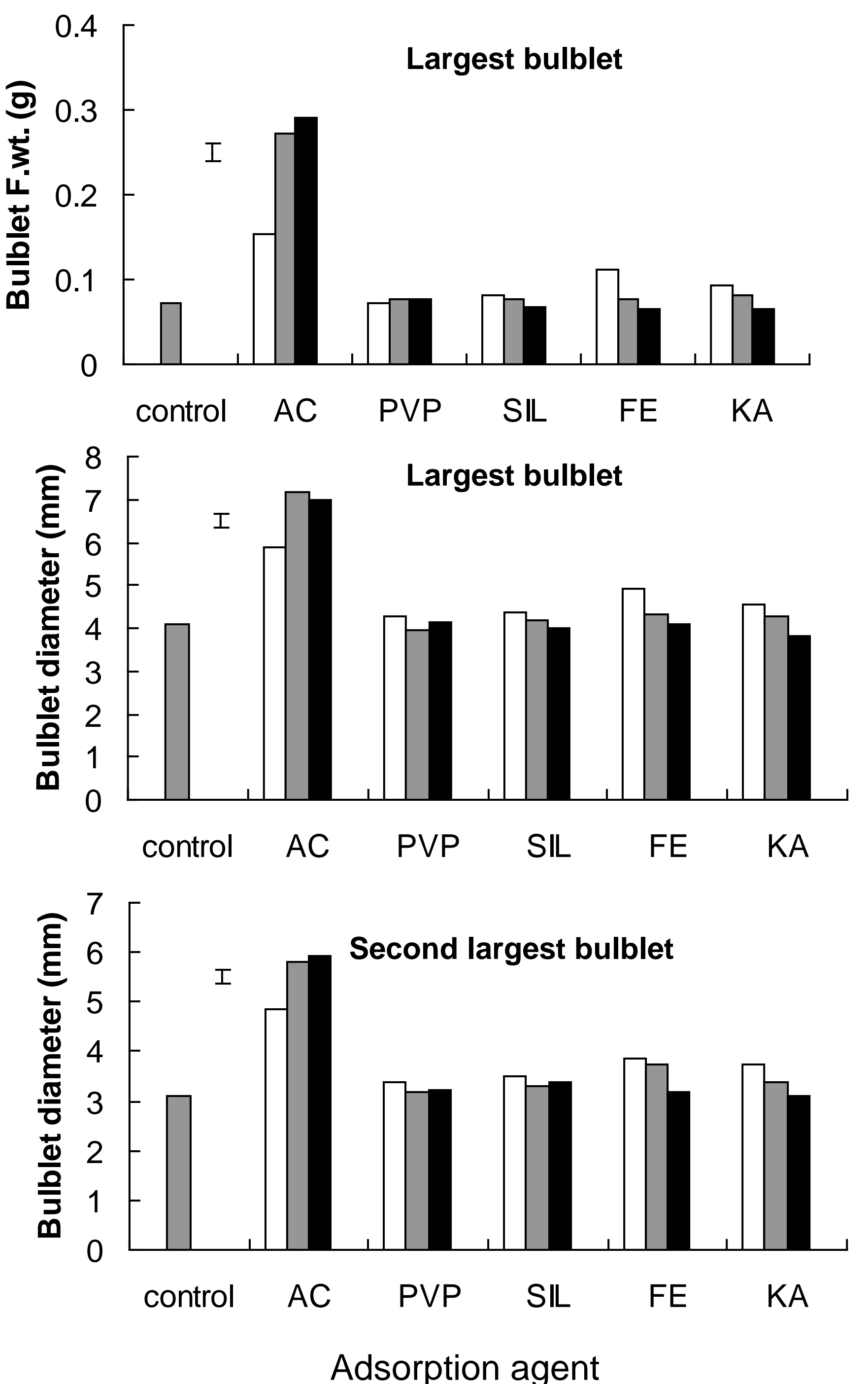

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Figure 2.3. Effects of adsorption agents as alternatives to activated charcoal (AC) root numbers and length of the longest root. The agents used were polyvinylpyrrolidone (PVP), Fuller's earth (FE), Silicar CG-4 (SIL) and kaolin (KA). Data are the mean of *G. nivalis* and *G. nivalis* 'Flore Pleno' and concentrations of adsorption agents were 0.2 g/l (open columns), 1.0 g/l (shaded columns) and 5.0 g/l (black columns). Bars show the SED.

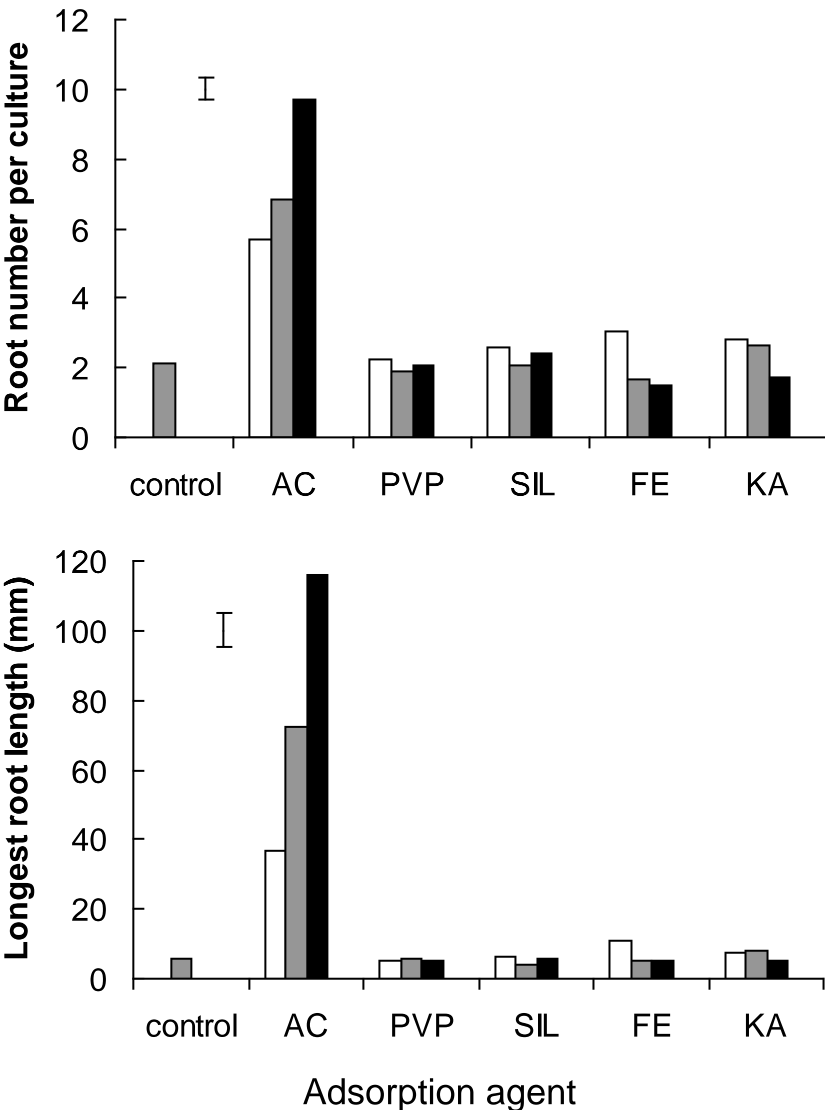

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Figure 2.4. Effects of pectins as alternative adsorption agents to activated charcoal (AC) on the numbers of new bulblets formed and on the total culture fresh weight. See Table 2.1 for chemical details of the pectins used. Data are the mean of *G. nivalis* and *G. nivalis* 'Flore Pleno' and concentrations of the pectins and AC controls were 0.2 g/l (open columns), 1.0 g/l (shaded columns) and 5.0 g/l (black columns). Bars show the SED.

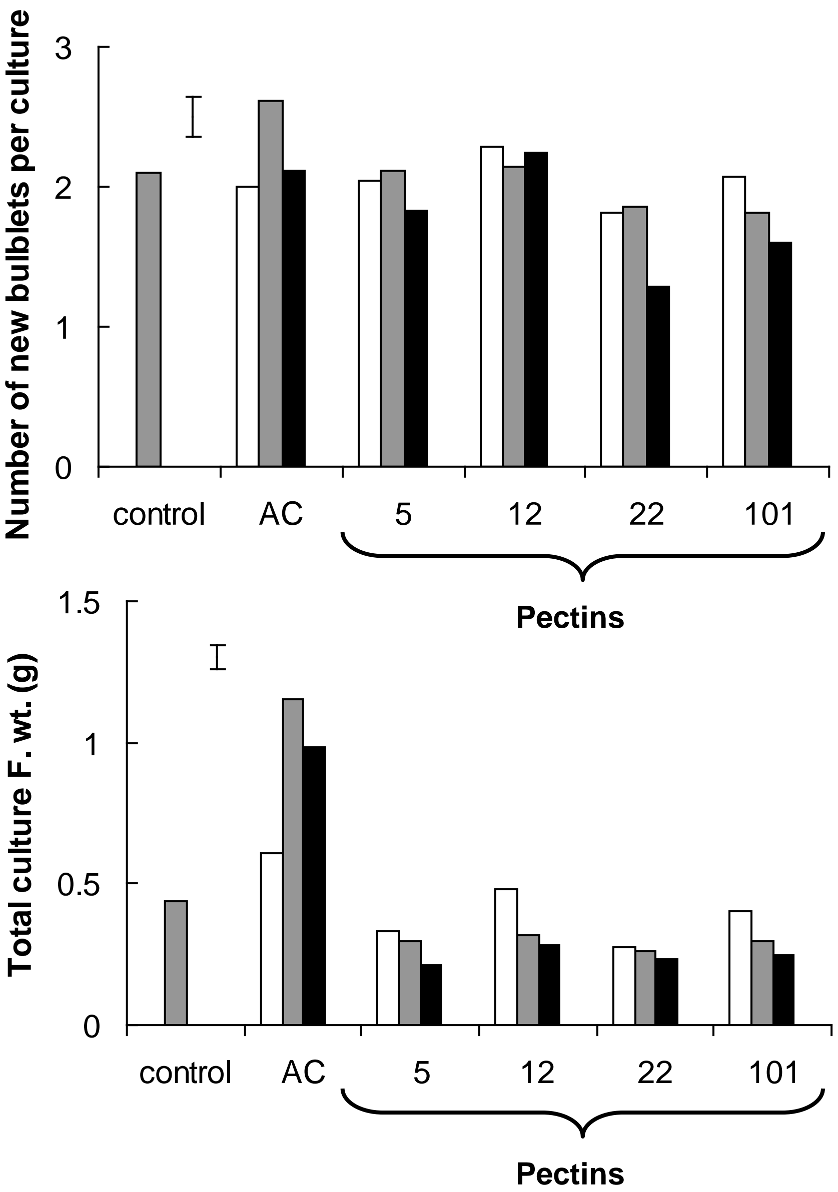

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Figure 2.5. Effects of pectins as alternative adsorption agents to activated charcoal (AC) on the on the growth of the largest and second largest bulblets. See Table 2.1 for chemical details of the pectins used. Data are the mean of *G. nivalis* and *G. nivalis* 'Flore Pleno' and concentrations of the pectins and AC controls were 0.2 g/l (open columns), 1.0 g/l (shaded columns) and 5.0 g/l (black columns). Bars show the SED.

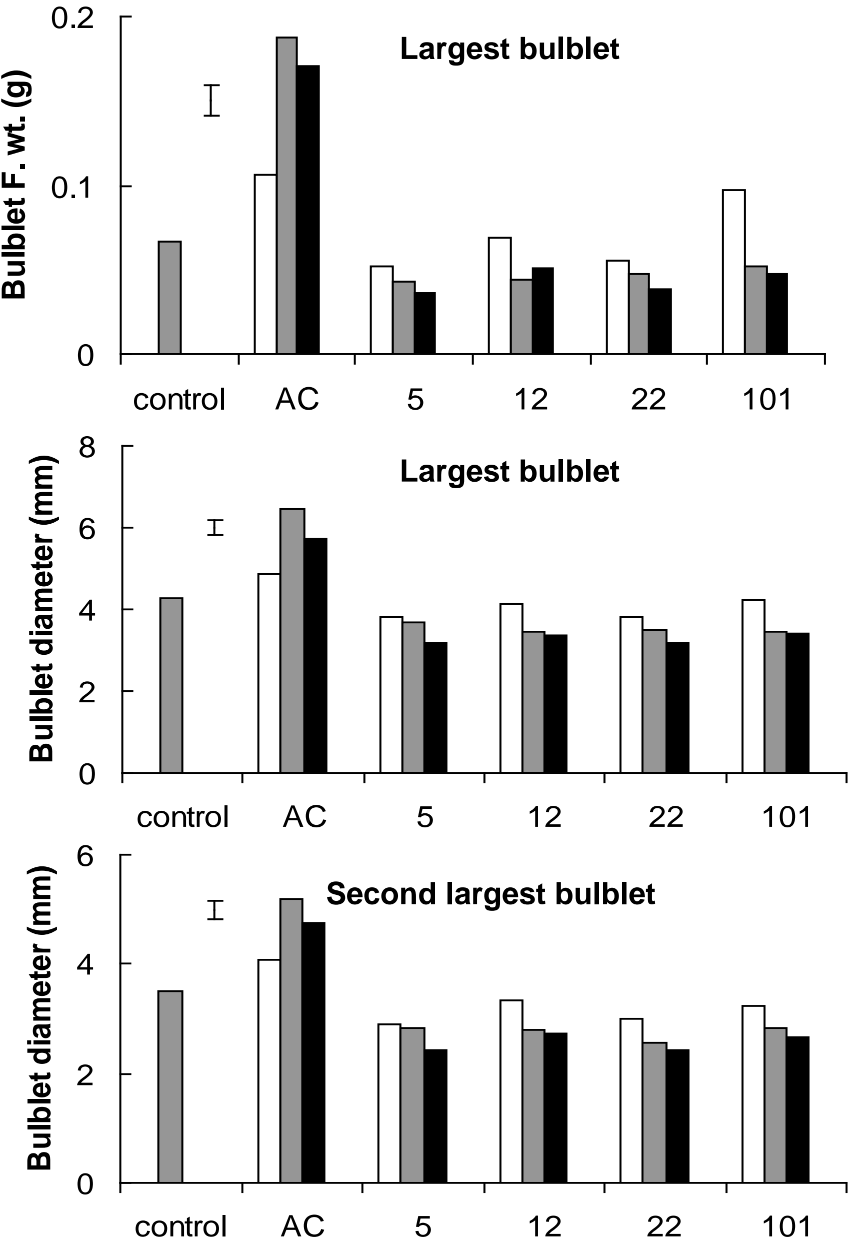

Figure 2.6. Effects of pectins as alternative adsorption agents to activated charcoal (AC) on the root numbers and length of the longest root See Table 2.1 for chemical details of the pectins used. Data are the mean of *G. nivalis* and *G. nivalis* 'Flore Pleno' and concentrations of the pectins and AC controls were 0.2 g/l (open columns), 1.0 g/l (shaded columns) and 5.0 g/l (black columns). Bars show the SED.

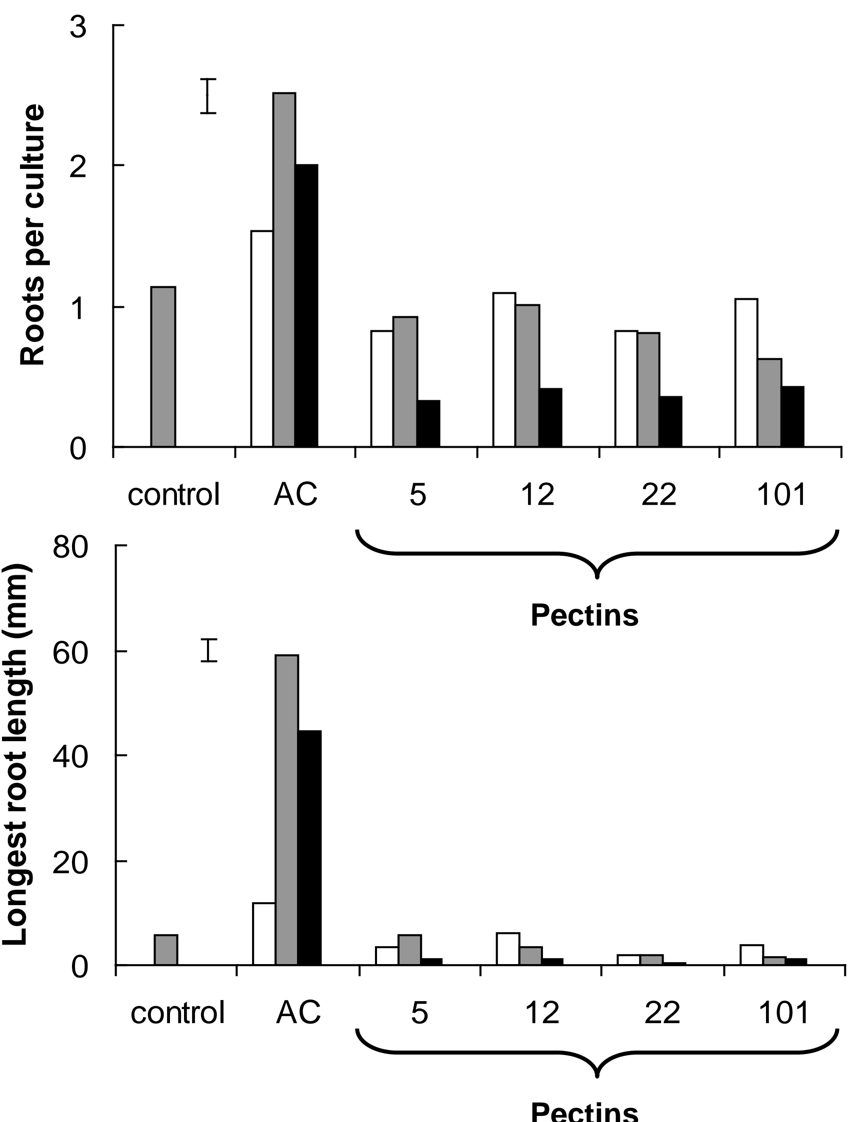

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Figure 2.7. Effects of the adsorption agents activated charcoal (AC) and polyvinylpoypyrrolidone (PVP) and culturing in light or darkness on the numbers of new bulblets formed and on the total culture fresh weight. The concentrations of the adsorption agents were 0.2 g/l (open columns) and 1.0 g/l (black columns). Bars show the SED.

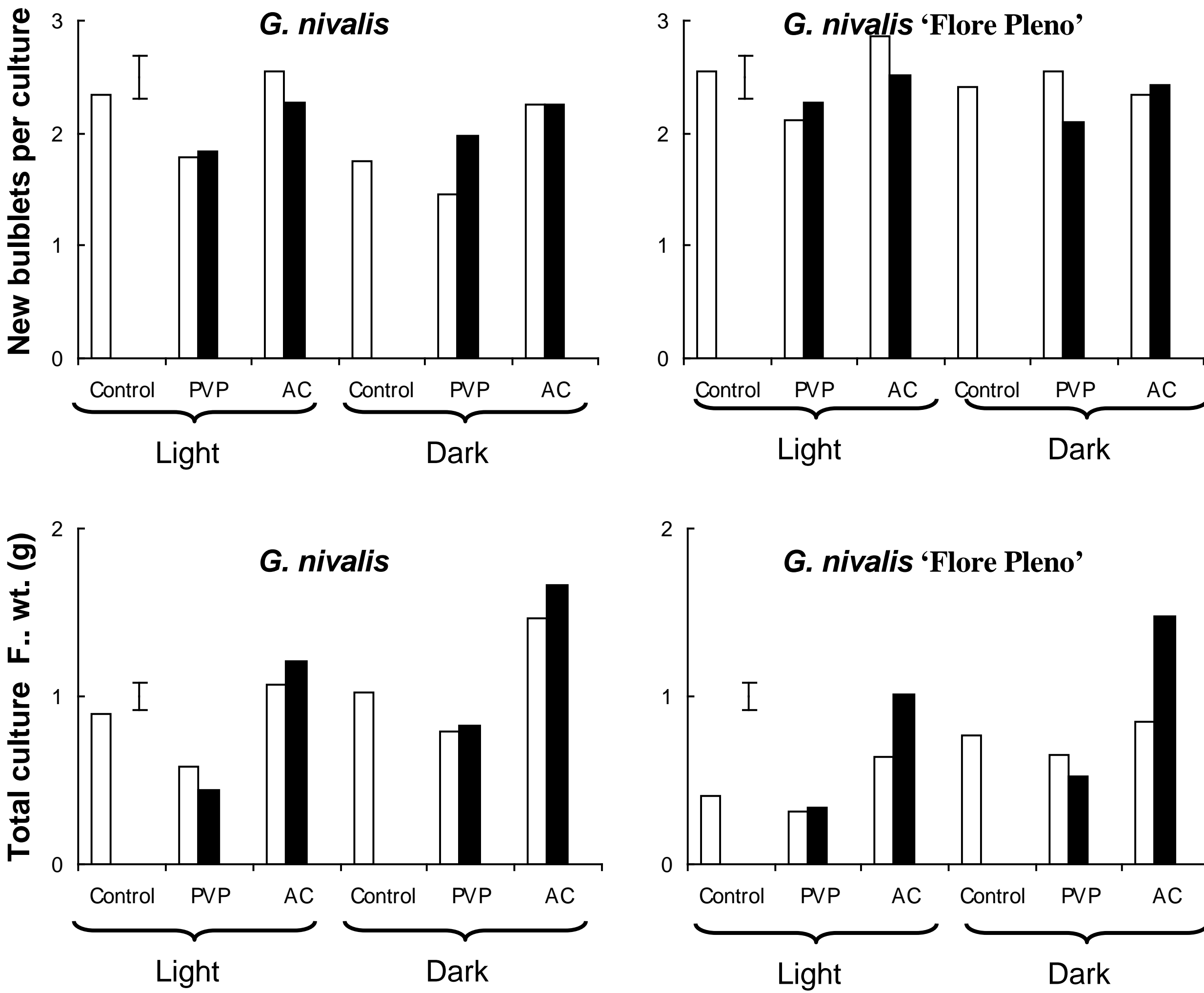


Figure 2.8. Effects of the adsorption agents activated charcoal (AC) and polyvinylpyrrolidone (PVP) and culturing in light or darkness on the growth of the largest and second largest bulblets. The concentrations of the adsorption agents were 0.2 g/l (open columns) and 1.0 g/l (black columns). Bars show the SED.

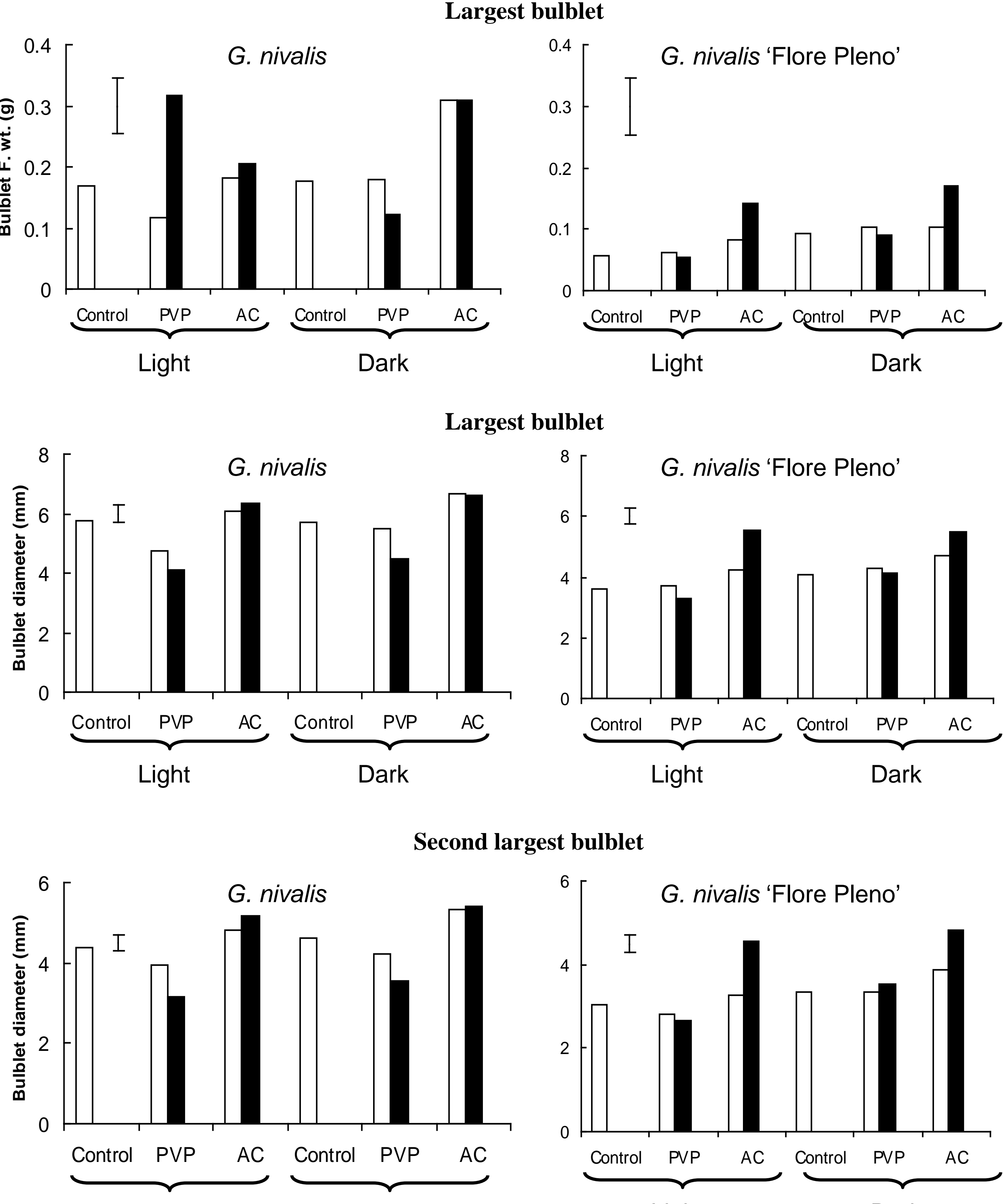


Figure 2.9. Effects of the adsorption agents activated charcoal (AC) and polyvinylpyrrolidone (PVP) and culturing in light or darkness on the growth of the largest and second largest bulblets. The concentrations of the adsorption agents were 0.2 g/l (open columns) and 1.0 g/l (black columns). Bars show the SED.

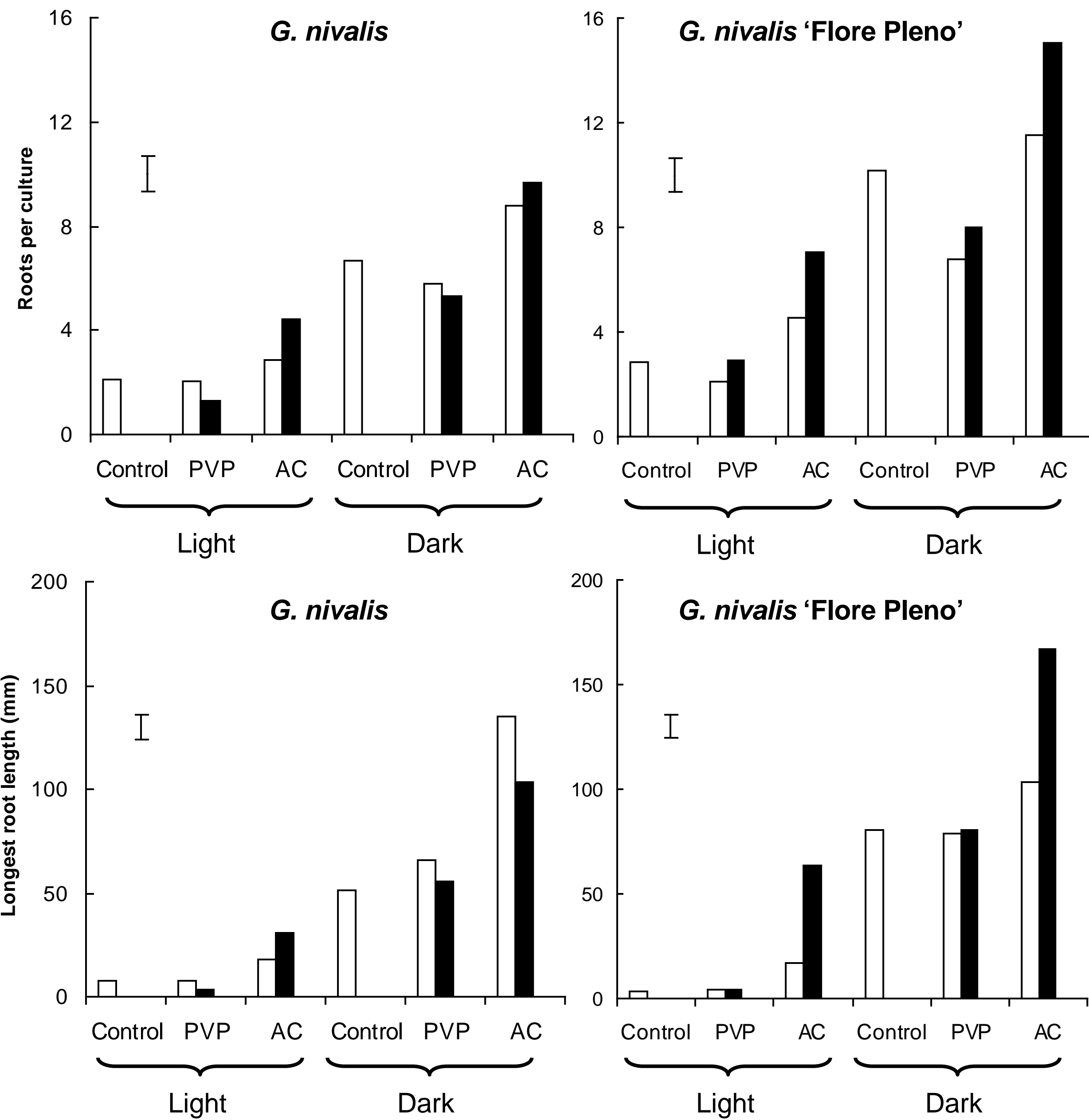
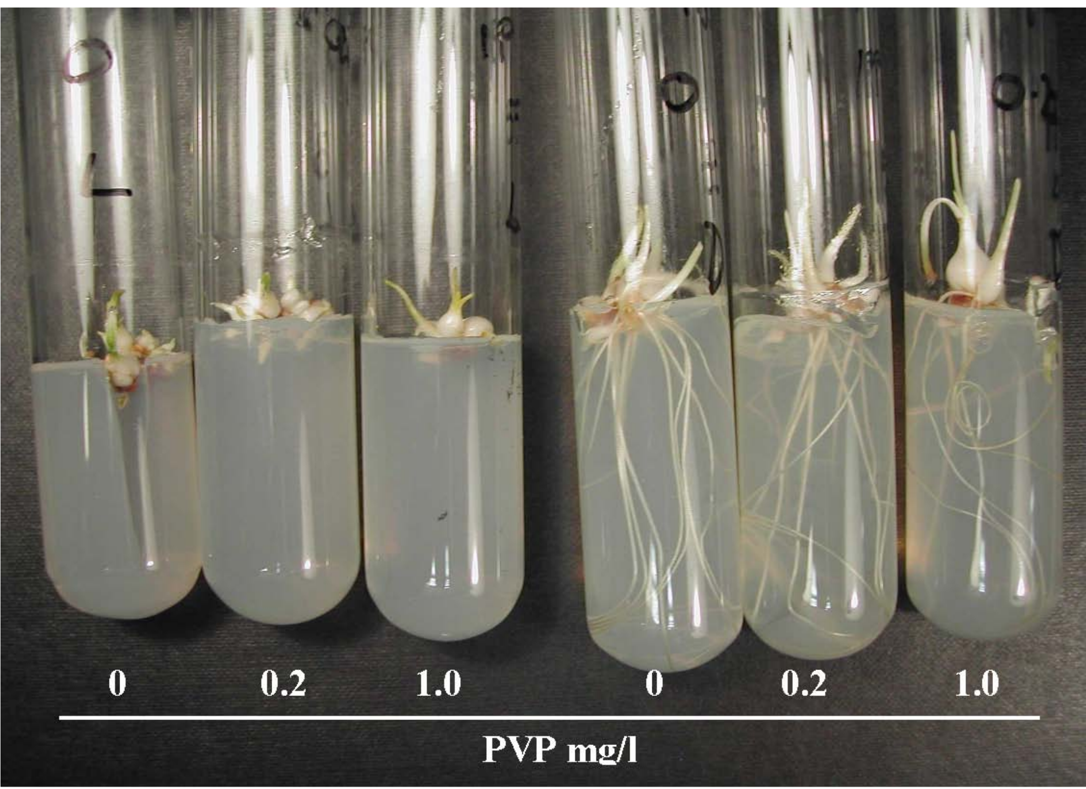


Figure 2.10. Stimulation of root production in bulblet clumps of *G. nivalis* 'Flore Pleno' clone 13 in constant darkness (right three tubes), compared with 16h photoperiods (left three tubes) on full strength G medium supplemented with 60 g/l sucrose.



Plant growth regulator concentration in the multiplication phase

Reducing the PGR concentration in the multiplication phase, particularly to a tenth (PGR/10) significantly reduced the multiplication of *G. nivalis* 'Flore Pleno' bulblets (Fig. 2.11). This was particularly evident in the third subculture passage of this experiment where bulblet multiplication in the control full-strength PGR conditions was in excess of x4 and more than double that found in the PGR/10 treatment. Although there were significant differences in multiplication rates between the six clones used, all clones reacted in the same way to reducing the PGR concentration as indicated by the lack of significant interactions between the clonal effect and the PGR concentration effect.

Just as in the multiplication phase, reducing the PGR concentration was also found to significantly reduce the subsequent growth of the tissues when transferred to bulblet growth conditions (Table 2.3). This was reflected in overall tissue fresh weight, multiplication of bulblets and the production of roots. The length of the longest root and the proportion of bulblets sprouting were unaffected by the PGR status in the multiplication phase. Likewise the growth of bulblets remained unaffected (Table 2.3 and Fig. 2.12). This indicates that carry over of PGRs in transferred tissues from the multiplication phase was important in determining the production of new organs but was not exerting a strong influence on the subsequent growth of the pre-formed or newly formed organs. It is therefore unlikely that the mode of action of activated charcoal, in stimulating the growth of bulblets, involves the adsorption of PGRs carried over from the previous passage. If adsorption of carry-over

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PGRs was important it would be expected that those tissues previously cultured with reduced PGRs, particularly the PGR/10 treatment, would have grown more quickly when transferred to the activated charcoal containing growth medium. Although not statistically significant, it can be seen that for bulblet diameters and root length the reverse might be true with the PGR/10 treatment producing least growth. This might be caused by higher carry-over of NAA in the control and PGR/2 treatments, stimulating bulblet growth and rooting. In support of this idea, NAA has recently been shown to promote bulblet rooting in *G. ikariae* (Tipirdamaz, 2003). She found that rooting of bulblet cultures could be increased from 8.9% in the auxin-free control to 25.4 % in cultures supplied with 0.5 mg/l NAA.

Figure 2.11. Effects of plant growth regulator concentration on bulblet multiplication (final bulblet numbers / inoculated bulblet numbers) in cultures of *G. nivalis* 'Flore Pleno' grown for three consecutive passages on half strength G medium. The three media used were a control, with the standard concentrations of BA and NAA (λ), PGR/2 (ν) and PGR/10 (σ). Data are the means of for six clones and bars show SED values calculated in separate ANOVAs for each culture passage.

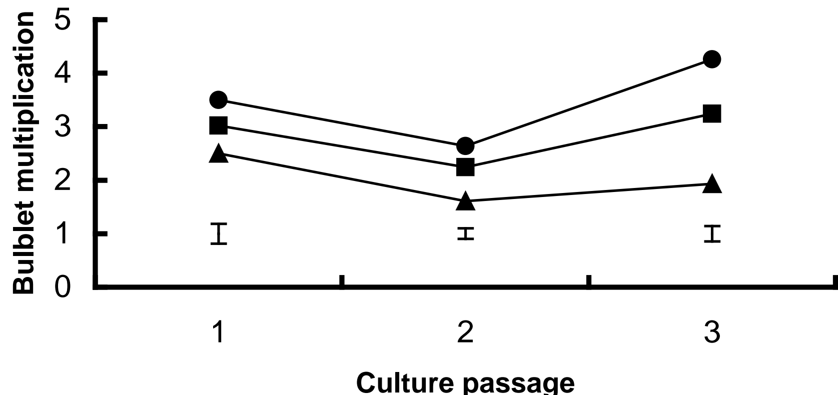

Table 2.3 Growth and development of *G. nivalis* 'Flore Pleno' bulblet cultures, grown initially on proliferation media supplemented with either the standard BA and NAA concentrations (control), PGR/2 or PGR/10 for two consecutive passages, before being transferred into bulb growth conditions (full strength G medium with 5 g/l activated charcoal and 60 g/l sucrose. Data are the means of for the six clones.

Parameter assessed	*Multiplication stage PGR strength*			*Sig.*	*LSD(5%)*
	Control	*PGR/2*	*PGR/10*		
Total culture FW (g)	1.77	1.46	0.97	***	0.254
Bulblet multiplication	2.73	2.13	1.96	**	0.446
Bulblets sprouting (%)	80.7	81.8	81.9	NS	5.80
Largest bulblet FW (g)	0.20	0.21	0.25	NS	0.050
Largest bulblet diameter (mm)	6.19	5.93	5.76	NS	0.594
2nd largest bulblet diameter (mm)	5.03	4.87	4.60	NS	0.495
Root numbers	15.76	15.06	9.20	***	2.375
Longest root length (mm)	115.3	130.3	100.1	NS	25.75

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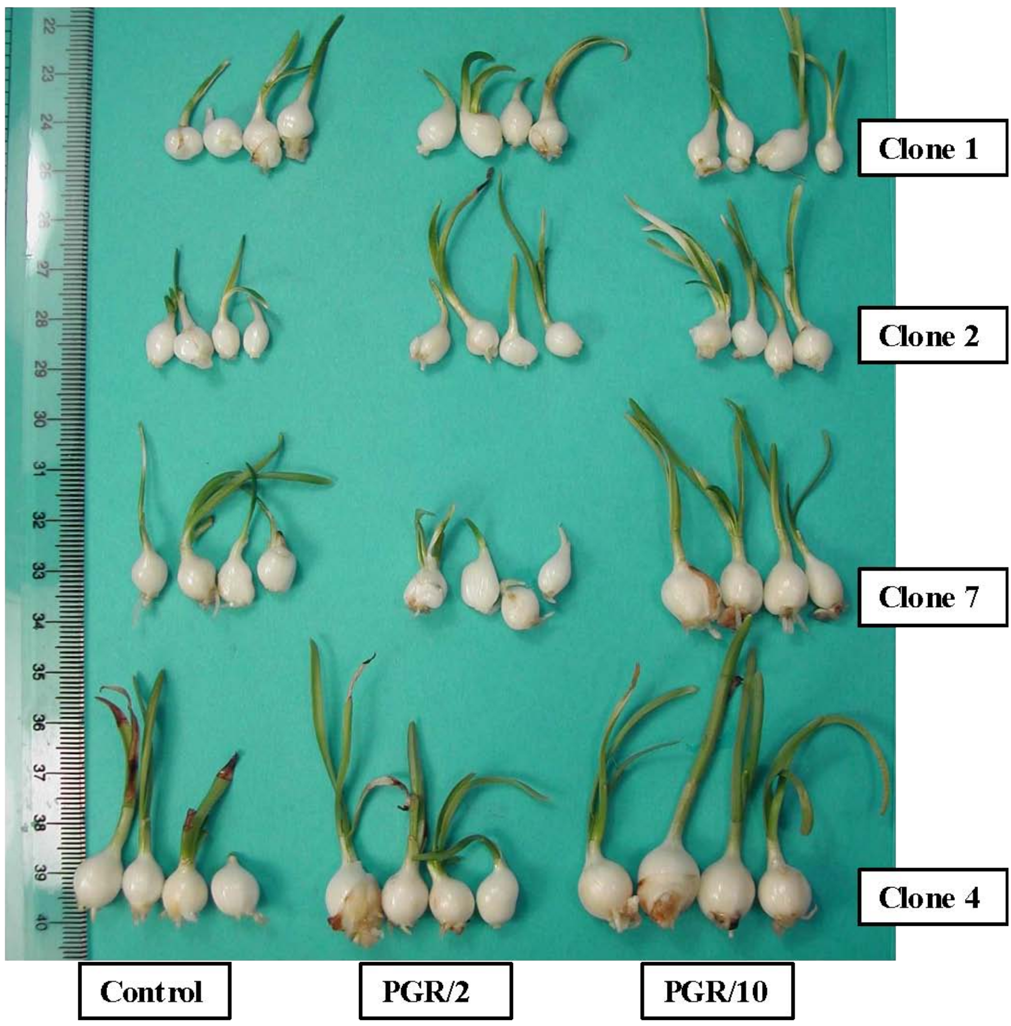
Figure 2.12. Examples of the largest bulblets from individual cultures of four *G. nivalis* ‘Flore Pleno’ clones. Bulblet clump cultures were first subjected to two passages on the standard proliferation medium (control), or the same media where the plant growth regulator concentrations were reduced to a half (PGR/2) or a tenth (PGR/10), before being transferred into bulblet growth conditions (G medium supplemented with 60 g/l sucrose and 5 g/l activated charcoal).



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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. 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.

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, and a much greater percentage had *Penicillium*-like mould. Difficulties were encountered in culturing *Botrytis* from the bulbs, because of this overwhelming presence of *Penicillium*, and it was not possible to confirm the presence of *B. galanthina*. Later, foliage samples with lesions were examined by Dr Tim O'Neill (ADAS Arthur Rickwood); while the cause was not identified, it was not typical of *B. galanthina.*

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 1.20m-wide beds separated by 0.60m-wide pathways, allowing for tractors on 1.83m '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-

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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.

When bulbs were due to be harvested, this was done manually because of the small size and complexity of the experimental layout, and to ensure a high recovery rate.

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 mesh stretched horizontally over the plots, 45cm above ground level. Windbreak consisted of a single layer of mesh (35cm high) held vertically on the windward (south-west) side of the plots 15cm from the edge of the planted area. Mulching consisted of a layer of straw about 5cm deep placed over the planted area 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.

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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: 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

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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).

Agronomy experiment 4: Acclimatization of micropropagated plantlets

As described in the Micropropagation section of the report, culture jars with clusters of snowdrop plantlets were supplied by Dr Chris Selby for field trials at Kirton in autumn 2003. Micropropagated plantlets were planted in the field at Kirton (both in sheltered plots and non-sheltered plots, protection being by both overhead shading and side windbreak shelter, similar to that used for agronomy experiment 1), and in an unheated, mesh tunnel.

Each plot was planted with six clusters of plantlets (i.e., the contents of one culture jar), using 23 clones of *G. nivalis*, five clones of *G. nivalis* 'Flore Pleno' and six clones of *G. elwesii* were used. Before planting, the shoot clusters were gently removed from the culture jars and the roots washed free of most agar medium using running cold tap-water. Shoot clusters were dipped in carbendazim fungicide for 15 minutes, and allowed to drain, before planting.

The soil at both field and tunnel sites was a fine-medium silty marine alluvium, ameliorated by the incorporation of peat and grit, fertilised by the addition of ammonium nitrate and potassium sulphate according to standard agricultural soil analysis and MAFF recommendations for bulb crops, and worked to a fine tilth. Shoot clusters were planted *ca.* 5cm deep, the sites were irrigated well, and the field plot area was covered using fleece for the first few weeks after planting. A design of three randomised blocks was used for each of sheltered field plots, non-sheltered field plots, and mesh tunnel plots.

After shoot emergence in spring 2004, shoot vigour was assessed on a score from 0, no shoots, through 1, few shoots, and 2, numerous shoots, to 3, many vigorous shoots. Once the foliage had senesced naturally in early summer 2004, bulbs from each plot were carefully recovered, cleaned and counted and weighed in two grades (bulbs under and over 1g in weight).

Agronomy experiment 5: Effect of mycorrhizae on micropropagated plantlets

Further snowdrop cultures jars supplied by Dr Chris Selby in autumn 2003 were used in an experiment to investigate the effects of mycorrhizae on snowdrop plantlets. Ten culture jars each of *G. nivalis* clones 3, 4, 11, 13, 15, 20, 22, 23, 25 and 26 were used. Before planting,

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the shoot clusters were washed free of agar medium, as in the preceding experiment, but in this case the shoot clusters were not treated in fungicide prior to planting.

There were 10 treatments:
1. Non-amended growing medium
2. Growing medium amended with soil from Walsingham Abbey grounds, Walsingham, Norfolk
3. Growing medium amended with soil from Anglesey Abbey grounds, Lode, Cambridgeshire
4. Growing medium amended with soil from Kirton
5. Growing medium amended with arbuscular mycorrhizal fungi (AMF)

6-10 As for treatments 1-6, but the growing medium and all amendments were autoclaved before use (30 minutes at 115°C).

Soil from Walsingham and Angelsey Abbeys was taken at bulb depth from areas on which large drifts of snowdrops had been growing for many years. Soil from Kirton was taken from an area not previously used to grow snowdrops. The AMF preparation (TerraVital Hort, PlantWorks Ltd) comprised a mixture of AMF found to be of benefit to a range of horticultural crops.

The base growing medium consisted of a mixture of medium sphagnum peat, proprietary John Innes No. 1 growing medium and horticultural sand, 3:2:1 v/v, amended with the following (amounts in kg/m^3): 0.3kg ammonium nitrate, 0.5kg potassium nitrate, 1.0kg single superphosphate, 1.5kg ground limestone, 1.5kg ground magnesian limestone and 0.3kg fritted trace elements (WM 255).

At planting, groups of six shoot clumps (the contents of one culture jar) were planted in washed 20-cm diameter (4 litre) plastic pots. Each pot was part-filled with 3 litres base growing medium (either autoclaved or not) and firmed, 50ml of appropriate soil or mycorrhizal preparation (either autoclaved or not) was sprinkled on, shoot clusters were placed evenly on the surface, and the pot topped-up with further base growing medium (either autoclaved or not) and firmed. All pots were placed on saucers in a glasshouse with a minimum maintained temperature of 3°C (frost-protection) and ventilated at 10ºC. All watering was via the saucers to avoid leaching of components.

After shoot emergence in spring 2004, shoot vigour was assessed on a score from 0, no shoots, through 1, few shoots, and 2, numerous shoots, to 3, many vigorous shoots. Once the foliage had senesced naturally in early summer 2004, bulbs from each plot were carefully recovered, cleaned and counted and weighed in two grades (bulbs under and over 1g in weight).

Statistical analysis

Data were subjected to analysis of variance. Statistical significance in all the tables is presented as follows: NS = non significant, (*) = p<0.10, * = p<0.05, ** = p<0.01 and *** = p<0.001. 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.

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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 mid leaf height (Delta-T TM1 sensor)
- Soil moisture at bulb depth (Delta-T M1M sensor ('Theta probes'). Soil moisture at bulb depth was additionally recorded using Irrometer 'water sensors' checked at about weekly intervals.
- Level of photosynthetically active radiation (PAR) at mid-leaf height (Delta-T QS sensor)

Results and discussion

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

In the first year of the trial[1], 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. Also, fewer shoots emerged in mulched than in non-mulched plots, suggesting that the presence of the straw mulch impeded the emergence of any 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 of control plots (Table 3.1). However, in the third year, in all cases, the number of shoots was substantially less than found in the previous year, showing a significant 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 at initiation 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 those 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. It was notable that the foliage in shaded and mulched plots died-down slightly later than in non-protected plots.

In the first year, bulb yields (measured as both numbers and weights of bulbs) 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:

[1] Detailed results from the first two years of the trial can be found in the previous Annual Reports.

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- 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 3.2).

The beneficial effects of shading and mulching on snowdrop growth were substantial, and the addition of a side windbreak provided extra benefit (Fig. 3.1). As shading appeared to work by prolonging the growing season (see first Annual Report and Table 3.1), this might also be achieved through fungicide spray programmes that delay foliar senescence (see below). Unexpectedly, there was little or no further yield increase 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 3.2 shows bulb yields over the three years of the trial. 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 might fail. 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 reduced only slightly.

Light, temperature and other environmental data logged in and around the plots of Experiments 1 and 2 were described in earlier Annual Reports. Typical findings are summarised here:

- Light – measured as photosynthetically active radiation (PAR) – was, on average reduced by 37, 38 and 57% by the light, medium and heavy shading, respectively. The similar PAR transmission of the light and medium materials explains the similarity in crop performance under these materials. For this purpose the percentage shading used may not be very critical, but overall a material with not less than 50% shading appears useful.
- Mean air temperature above the plots was raised by 1°C where shading and windbreak were used, compared with non-shaded plots, and minimum temperatures by about 2°C.
- Compared with control plots, mean soil temperature at bulb depth was decreased by about 2ºC where shading was used and more so with increasing density of shading. Mulching had a smaller effect in decreasing mean temperature. Heavy shading and, particularly, using rye-grass, greatly reduced the range of temperatures experienced at bulb depth.
- There was little effect of treatments on mean soil moisture levels, except for a reduction of 11% where rye-grass was used and an increase of 8% where narcissus were inter-planted, presumably reflecting water depletion by the rye-grass and a mulching effect along with relatively little water extraction by the narcissus. Mulching increased mean soil moisture by only 3%.

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

In the first year of the trial[2] 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 effects of treatments (either adverse or beneficial) on growth seen in the previous year were continuing to show clear effects on

[2] Detailed results from the first two years of the trial can be found in the previous Annual Reports.

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plant performance. There were significant effects of treatment, with best yields of stems and seeds under the artificial shading, 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 3.3). 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, however, these results confirm the beneficial effect of artificial shading on growth seen in the first experiment. It was clear that the inter-crops used were too competitive for use with snowdrops.

Fig. 3.1. Number of bulbs lifted per plot in 2003 in experiment 1, showing the additive effects of mulch, shade and shelter on yields.

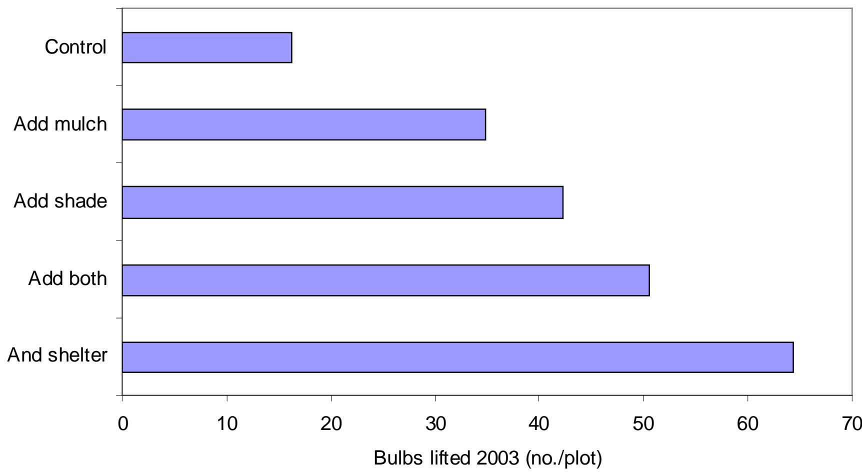

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Table 3.1. Effects of shading, windbreak and mulch treatments on snowdrop growth in the third year of the trial (2003). Main effect means only presented.

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				
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

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Table 3.2. Effects of shading, windbreak and mulch treatments on snowdrop bulb yield in the third year of the trial (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

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Figure 3.2. Snowdrop bulb yields after 1 - 3 years

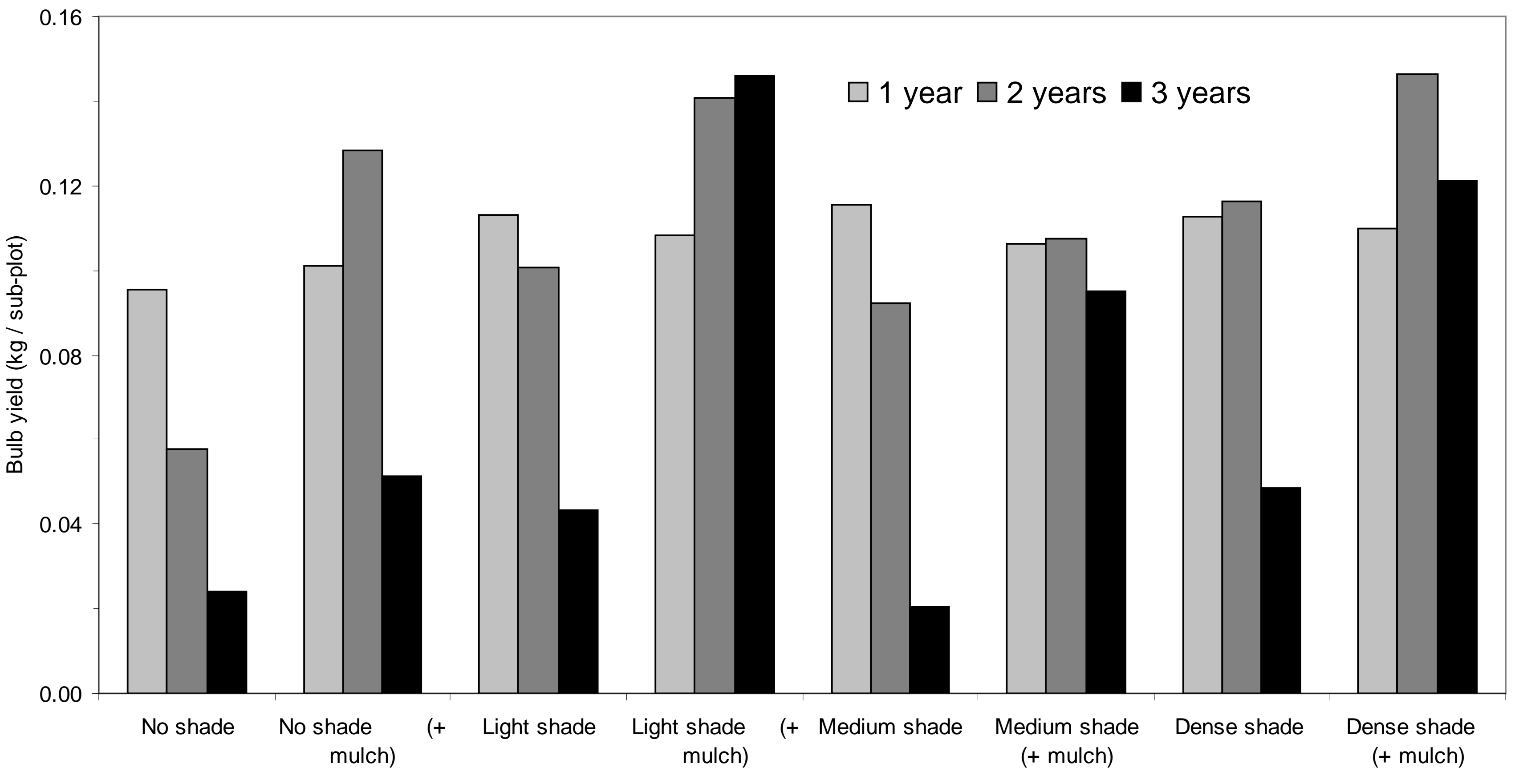

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Table 3.3. Effects of shading and inter-cropping on snowdrop growth in the third year, 2003.

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 3.4. 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

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Agronomy experiment 3: The effect of fungicide dips and sprays

Table 3.5 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 3.3).

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. 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, 2004). 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).

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Table 3.5. 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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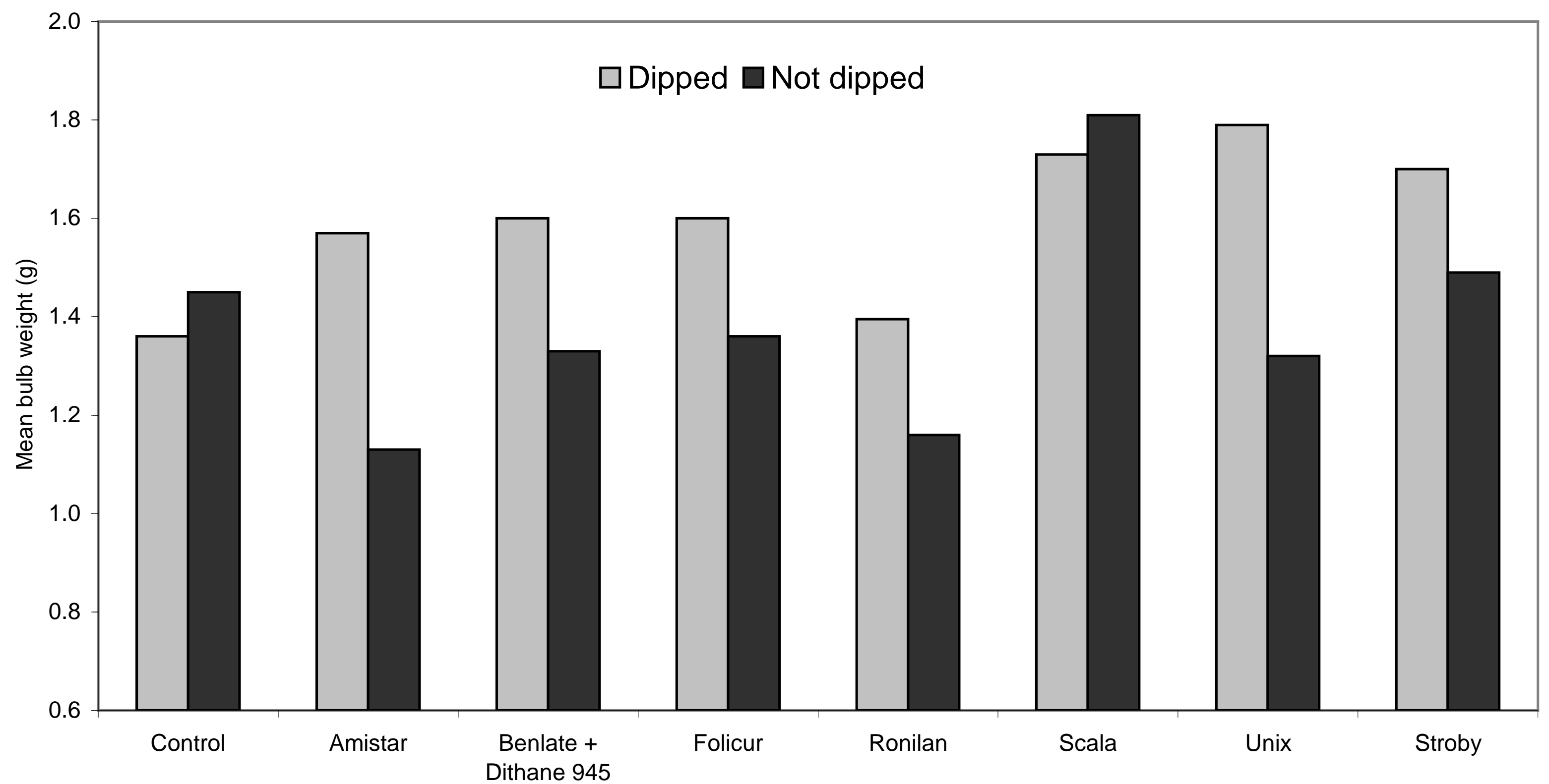
Figure 3.3. Mean bulb weight after fungicide bulb dip and spray treatments



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Agronomy experiment 4: Acclimatization of micropropagated plantlets

Growth of the various clones was very variable. Figures 3.4 and 3.5 show the shoot vigour scores, in the spring after planting, for the clones planted either in the field (with or without shading) and in an unheated mesh tunnel. In the field clonal vigour was not consistent between sheltered and non-sheltered plots: though several 'weak' clones grew better under shading, the reverse was true in other cases. Similarly, there was no reliable correspondence between vigour whether a clone was planted in the field or in the mesh tunnel: several clones grew better in the tunnel, but other were more vigorous outside. In general, *G. nivalis* clones appeared more vigorous than the others, and *G. nivalis* clones N10, N14, N16, N2 and N9 were consistently vigorous. These differences were also clear when the numbers and weights of bulbs recovered after one growing season were considered (Fig. 3.6 – 3.11). In general, a wider range of clones performed better when grown under the mesh tunnel.

Agronomy experiment 5: Effect of mycorrhizae on micropropagated plantlets

Many bulbous species are reported to be obligately mycorrhizal, and snowdrops might therefore benefit from a product containing a range of arbuscular mycorrhizal fungi (AMF) (Tim Dougall, PlantWorks Ltd, personnal communications). Indeed, vesicular-arbuscular mycorrhizae were reported in *G. nivalis*, although some time ago (von Kirchner *et al.*, 1908 and Peyronel, 1924, cited in Harley and Harley, 1987).

The average results for all clones for shoot vigour and the numbers and weights of bulbs recovered after one year are shown in Fig. 3.12 – 3.14. While the values for these three attributes correspond closely, the best growth (though not by far) was seen in treatment 1, the control, and overall there was poor growth in treatments 2 (snowdrop soil from Walsingham Abbey) and 4 (non-snowdrop soil from Kirton). On average, there was little difference in vigour between the autoclaved (treatments 6-10) and non-autoclaved (treatments 1-5) groups of treatments.

As expected from the previous results, inter-clonal differences in growth were significant, and the numbers of bulbs recovered after one year are illustrated for three example clones (11, 13 and 15) in Fig. 3.15 (the numbers and weights of bulbs recovered corresponded closely, and the results for weights are not presented). Plantlets in treatment 1 grew well in all three cases, but the apparently random relative growth across the ten treatments suggests that no clear mycorrhizal effects could have been operating; and in no case was any treatment substantially better than the control. Some interesting results did emerge for some clones: for example, in treatments 6 and 7 for clone 13, many small bulbs survived.

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Fig. 3.4. Shoot vigour score for micropropagated plantlets grown in the field with or without shading. Clones are described by species (E, *G. elwesii*; FP, *G. nivalis* Flore Pleno; N, *G. nivalis*) and number.

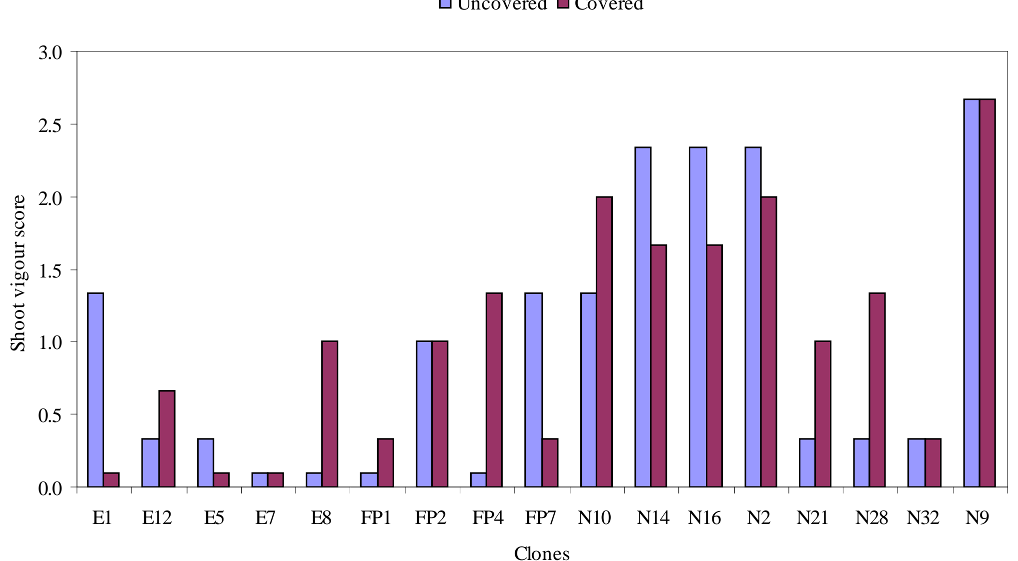

Fig. 3.5. Shoot vigour score for micropropagated plantlets grown in an unheated mesh tunnel.

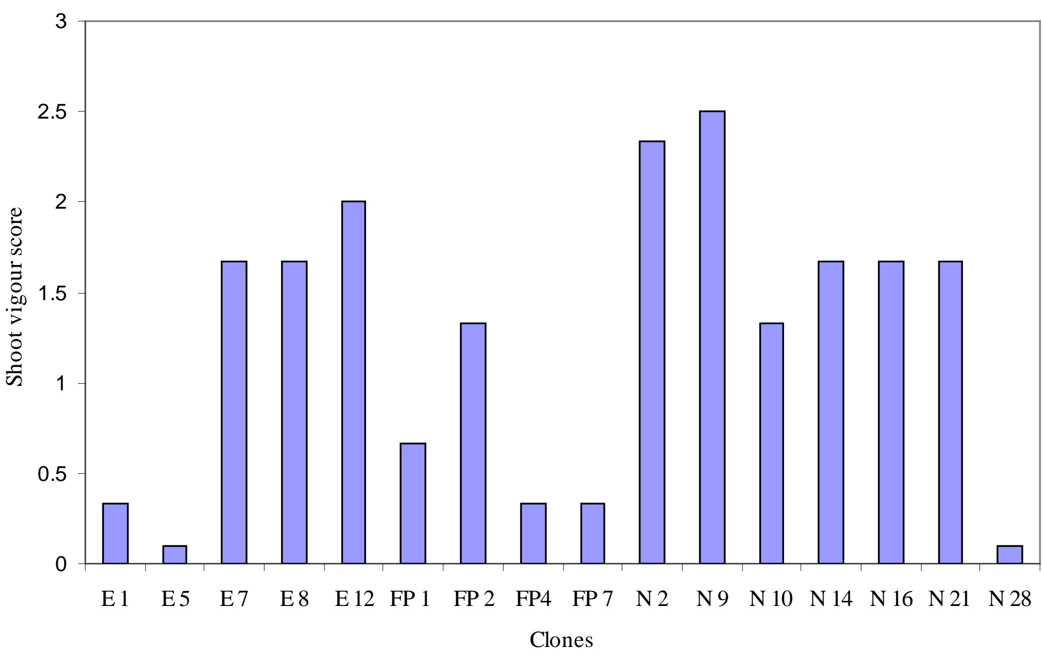

Fig. 3.6. Number of smaller and larger bulblets recovered for different clones grown in non-covered plots in the field.

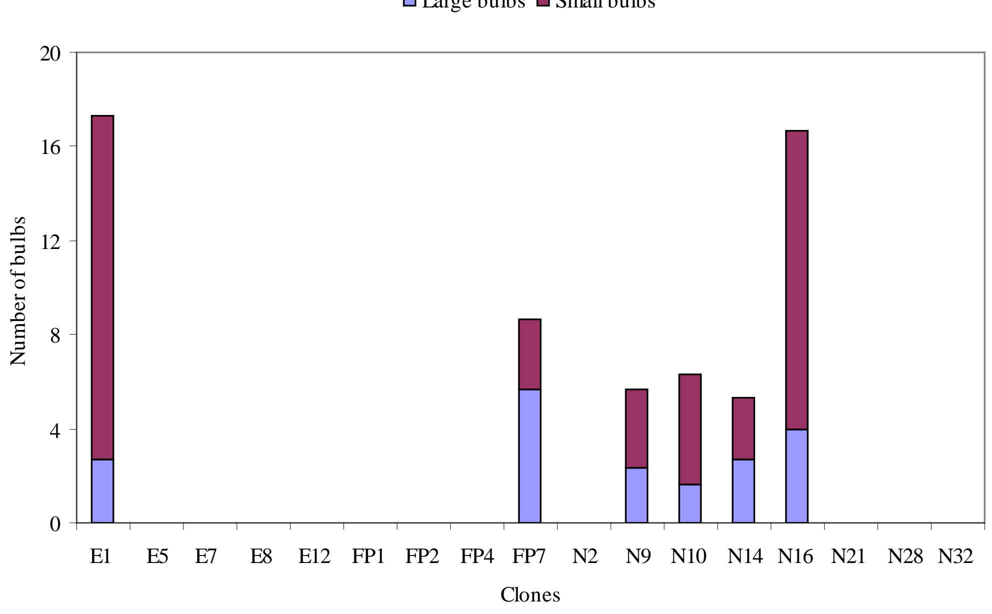

Fig. 3.7. Weight of smaller and larger bulblets recovered for different clones grown in non-covered plots in the field.

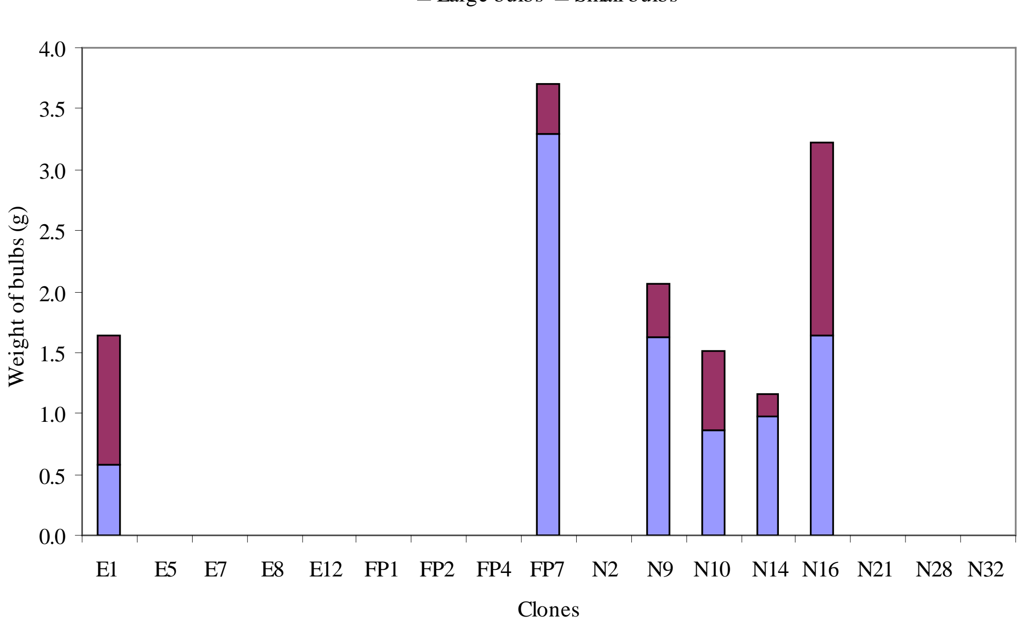

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Fig. 3.8. Number of smaller and larger bulblets recovered for different clones grown in covered plots in the field.

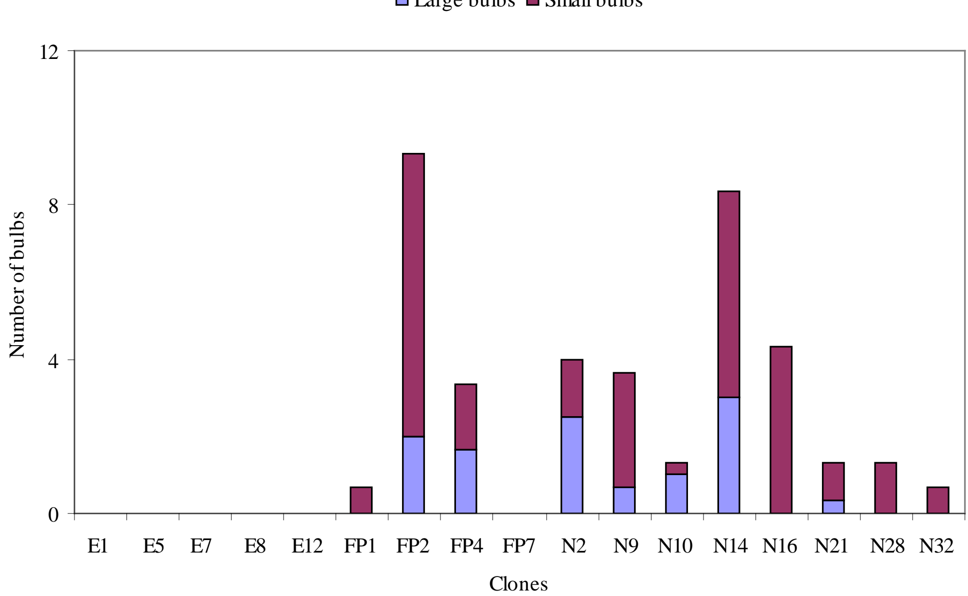

Fig. 3.9. Weight of smaller and larger bulblets recovered for different clones grown in covered plots in the field.

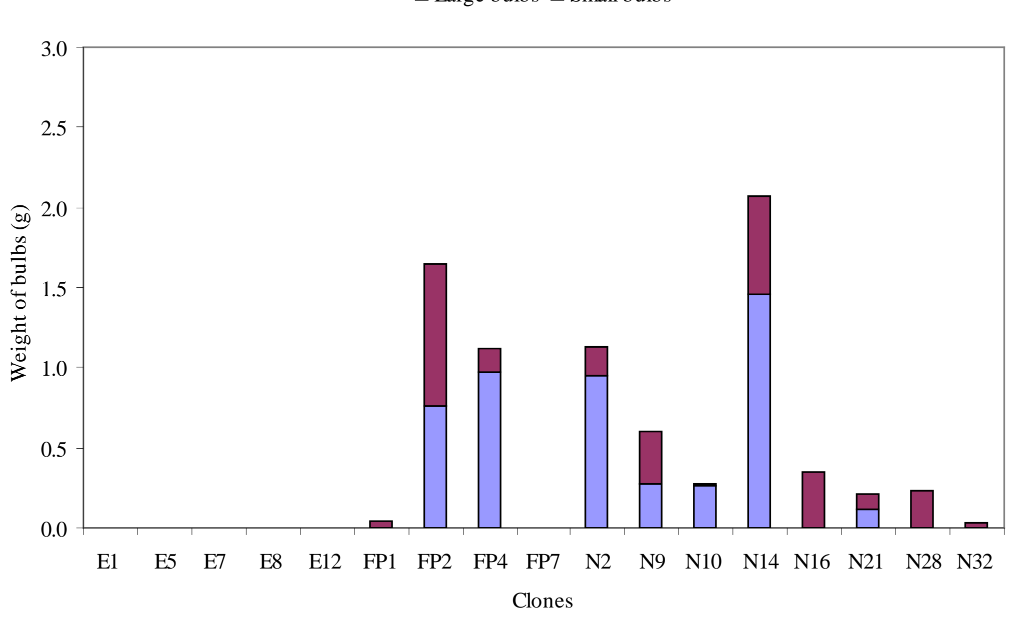

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Fig. 3.10. Number of smaller and larger bulblets recovered for different clones grown in a mesh tunnel.

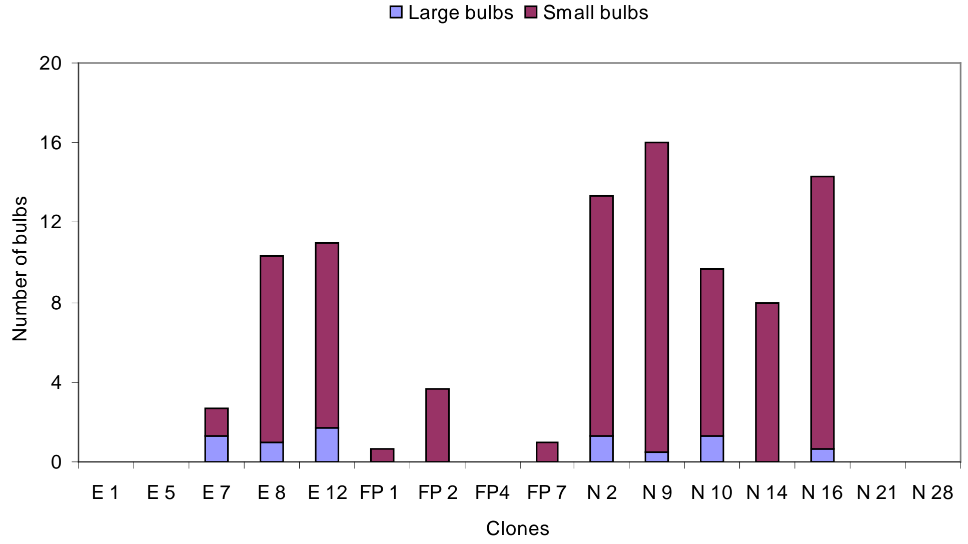

Fig. 3.11. Weight of smaller and larger bulblets recovered for different clones grown in a mesh tunnel.

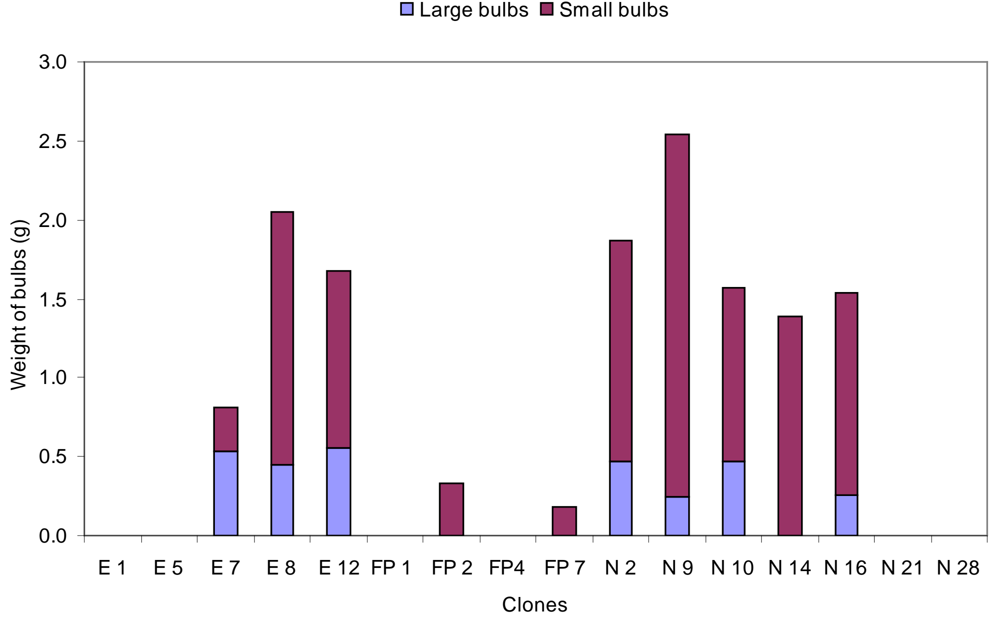

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Fig. 3.12. Shoot vigour score for micropropagated *G. nivalis* plantlets in mycorrhiza experiment. Data are averages for 10 clones.

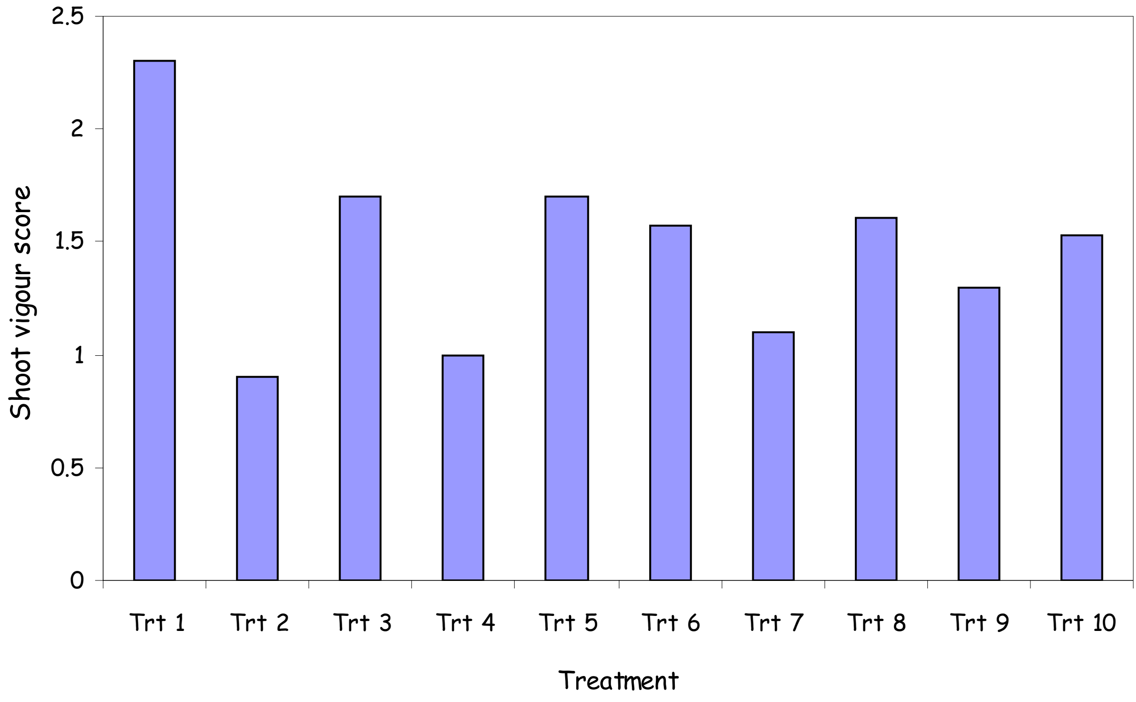

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Fig. 3.13. Numbers of bulbs recovered after one growing season for micropropagated *G. nivalis* plantlets in mycorrhiza experiment. Data are averages for 10 clones.

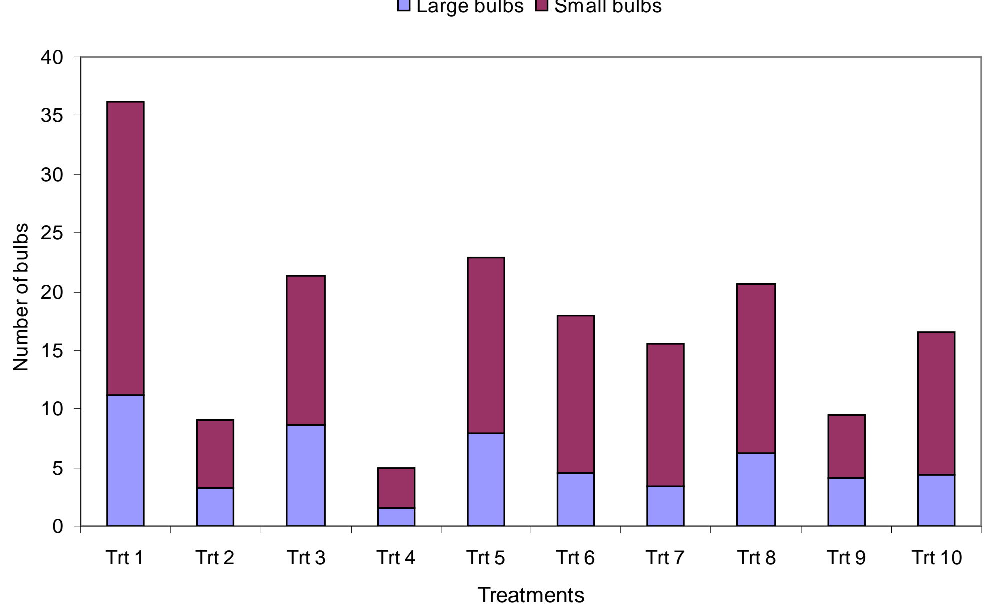

Fig. 3.14. Weights of bulbs recovered after one growing season for micropropagated *G. nivalis* plantlets in mycorrhiza experiment. Data are averages for 10 clones.

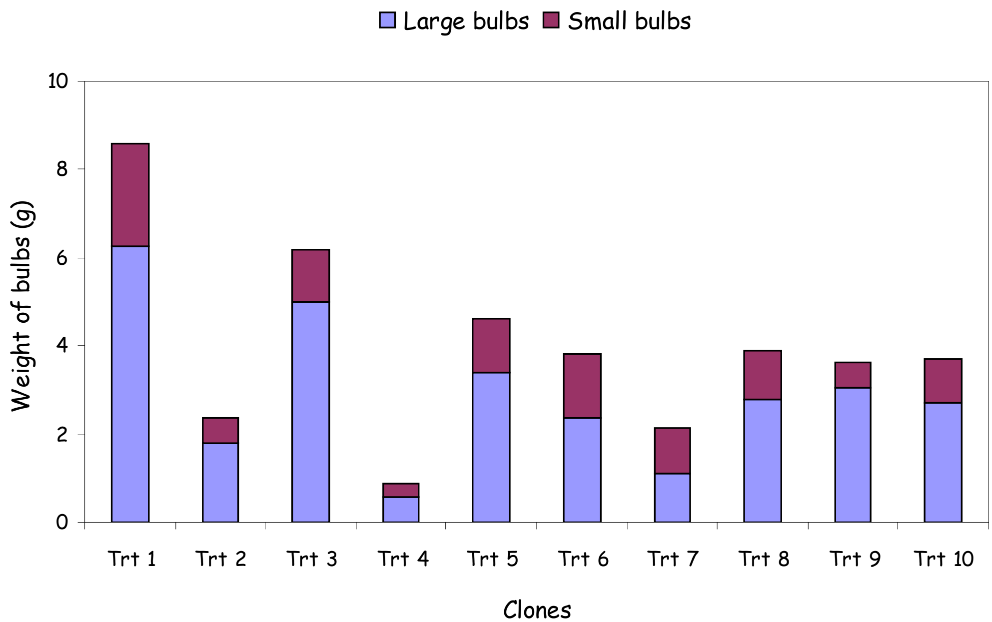

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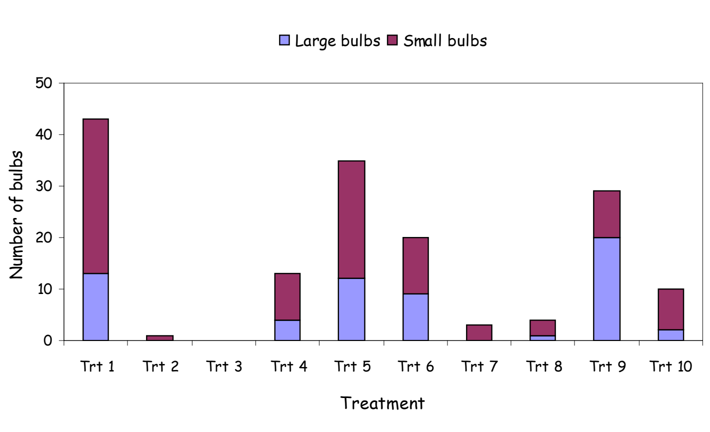
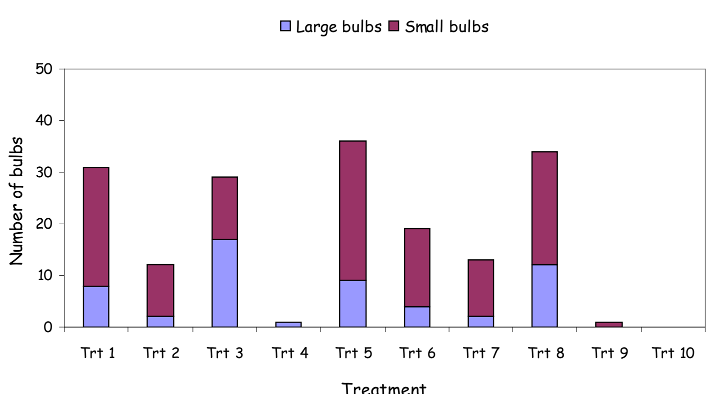
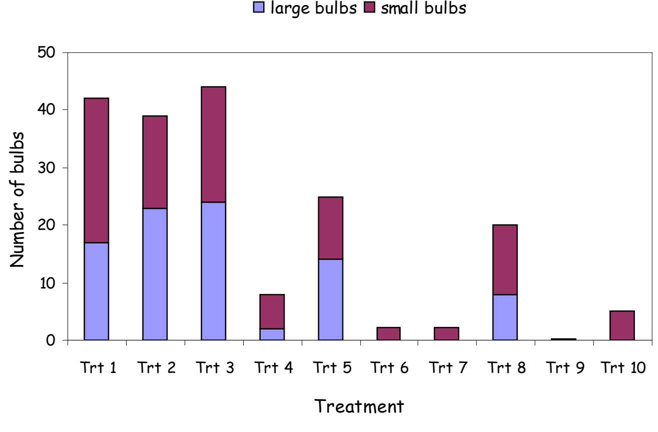
Fig. 3.15. Numbers of bulbs recovered after one growing season for micropropagated *G. nivalis* plantlets in mycorrhiza experiment. Clone 11 (top), 13 (middle) and 15 (bottom).







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BULB STORAGE

Materials and methods

Storage experiment 1 – lifting date and storage temperature and medium

Stock bulbs of *G. nivalis*, planted in 2000, were lifted for use in a storage experiment on 27 March 2002 (foliage still fully green), 23 April 2002 (foliage in early stages of senescence) and 15 May 2002 (foliage completely died down). About 500 bulbs were lifted on each date.

On the day of lifting, bulbs were dipped in aqueous benomyl + captan (as 40g Benlate Fungicide + 100g Captan per 10 litres) for 15 minutes at ambient temperatures, and allowed to drain overnight at ambient temperatures under gentle air movement from a fan to produce surface-dry bulbs by the next morning. The remaining foliage was excised at the top of the bulb, and any damaged bulbs or bulbs falling outside the range 3-5 cm in circumference were discarded. Ninety lots of ten bulbs each were allocated at random and weighed. Nine lots were allocated to, and set up in, each of ten treatments:

Treatment no.	*Storage temperature (°C)*	*Storage medium*	*Typical humidity (% r.h.)*
1	10	Silver sand in polythene bag	-
2	13	Silver sand in polythene bag	-
3	17	Silver sand in polythene bag	-
4	20	Silver sand in polythene bag	-
5	13	Open seed tray	70
6	17	Open seed tray	70
7	13	Polythene bag (loosely folded)	90
8	17	Polythene bag (loosely folded)	90
9	13	Perspex propagator with damp paper	>90
10	17	Perspex propagator with damp paper	>90

The bags, trays and propagators were placed in controlled temperature rooms at the required temperatures and 65-75 % r.h. Three replicate lots of each treatment were removed from the stores after 4, 8 and 12 weeks, and the bulbs weighed. After weighing, the bulbs lifted after 4 or 8 weeks were placed in polythene bags of silver sand in the 13°C store, and storage was continued to a total period of 12 weeks.

After the 12-week period any rotted or desiccated bulbs were removed, and the remaining bulbs were counted and planted in a proprietary potting compost (Levington M2) in 14 cm-diameter flower pots and grown in a cold glasshouse (with frost protection heating set at 3°C and automatic ventilation at 10°C). The numbers of bulb shoots and flower stems was recorded for each pot in spring 2003, and the weight of bulbs recovered from each pot was recorded after complete foliage senescence.

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Storage experiment 2 – modified atmosphere storage

Storage of produce in perforated or non-perforated polythene (low-density polyethylene, LDPE) bags of different gauges (thicknesses) is a simple way of determining the effects of modified atmosphere (MA) storage. Stock bulbs of *G. elwesii* were lifted from the field at Kirton and dipped the same day in aqueous benomyl + captan (as 40g Benlate Fungicide + 100g Captan per 10 litres) for 15 minutes at ambient temperatures, and allowed to drain overnight at ambient temperatures under gentle air movement from a fan to produce surface-dry bulbs by the next morning. Bulbs of 4-5cm grade were allocated and weighed in groups of five and placed (without a filler) in polythene bags (90 – 700 gauge, 15 x 20 cm) which were then sealed using sellotape. Half the bags of each gauge were perforated with a series of holes to facilitate air exchange.

The bags were stored at 13°C and 65-75% r.h. The condition of the bulbs and their weights were assessed 4, 8 and 12 weeks after the start of storage. After examination at 4 and 8 weeks of storage, bulbs were placed into new polythene bags, covered with silver sand and replaced under the same conditions as before. After a total of 12 weeks' storage, all bulbs were assessed again. After discarding any rotted or damaged bulbs, the bulbs were planted (in their groups of 5) in 14cm-diameter flower pots of a proprietary growing medium (Levington M2) and grown in a glasshouse heated to 3C and ventilated at 10C. The numbers of shoots and flower-stems per pot was recorded in spring 2004. After senescence of the foliage in early summer 2004, the bulbs were recovered, allowed to surface-dry gently at ambient temperatures, and the number and weight from each pot was recorded.

Storage 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 one of two confectionery glazes, Crystalac or Natureseal-PN (AgriCoat Industries Ltd), for 5 or 30 minutes before being allowed to drain free of excess glaze. Crystalac is a material based on shellac and Natureseal-PN is a cellulosic preparation. The bulbs were re-weighed and placed in polythene bags (250 gauge, 15 x 20 cm) that were loosely folded over and placed in a controlled temperature store at 13°C. The bulbs were examined at 4 week intervals for 12 weeks.

Statistical analysis

Data were subjected to the analysis of variance as appropriate. For Experiment 1 recovered bulb weights were adjusted by using the initial weight (i.e. weight after lifting from the field) as a co-variate.

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Results and discussion

<u>Storage experiment 1 – lifting date and storage temperature and medium</u>

Bulb weight at the end of 4-, 8- or 12-week storage in different temperatures and media, following lifting bulbs at three dates, are shown in Figure 4.1. The effects of all three experiment factors (harvest date, storage temperature and medium, and storage duration) were statistically significant (at the 0.1% level), and second-order interactions (harvest date x storage temperature and medium x storage duration) were also significant (5% level; for analysis of variance summary, see Table 4.1). Of the three factors, harvest date had the least impact on bulb weight, the overall means for the three dates being 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 either silver sand or 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, after any rotted or desiccated bulbs were discarded. For the means of the treatment combinations, between 67 and 100% of the stored bulbs were deemed suitable for growing-on. These data, along with the subsequent performance of the bulbs, are summarised in Figure 4.2 (for analysis of variance summary see Table 4.1). Statistical analysis showed that storage temperature and medium, but not harvest date nor storage duration, significantly affected both the percentage of bulbs re-planted (at the 0.5% level) and the percentage of these bulbs sprouting in the following spring (0.1% level). The highest percentage of sprouting bulbs was obtained following storage either in polythene bags (at either 13 or 17°C) or in silver sand at 20°C. Figures 4.2 and 4.3 also shows a score that combines the effects of treatments on the percentage of bulbs fit for re-planting and the percentage that subsequently sprouted: these results confirm that the same three treatments also gave the best results, and that the effects of lifting date (crop stage) were relatively small.

<u>Storage experiment 2 – modified atmosphere storage</u>

The effects of polythene bag gauge, and whether the bag was perforated or not, on weight loss of snowdrop bulbs is shown in Table 4.2. 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 0.4g following 4 weeks' storage to 1.6g after 12 weeks' storage. Losses increased, overall, when using a perforated bag (1.0g against 0.8g for a sealed bag) and as the polythene gauge decreased (from 0.6g for 700 gauge bags to 1.4g 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. Subsequent shoot production was similar in all treatments (Fig. 4.3).

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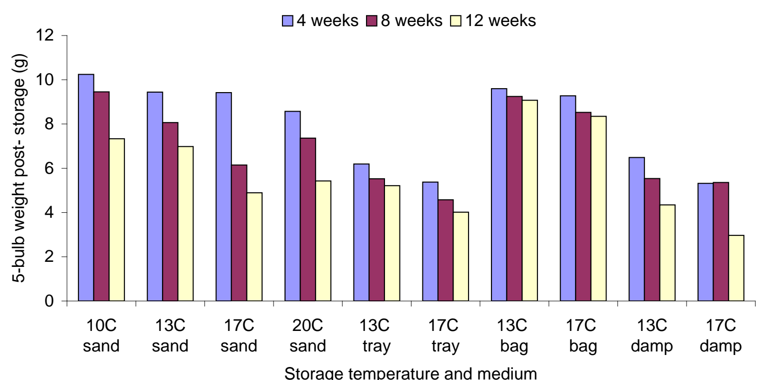
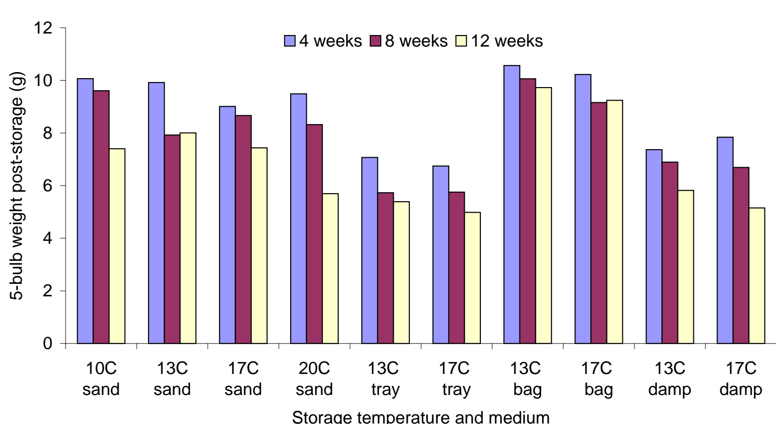
Fig. 4.1. Effect of storage conditions and duration on bulb weight.

(a) First bulb harvest date.



(b) Second bulb harvest date.



(c) Third bulb harvest date.

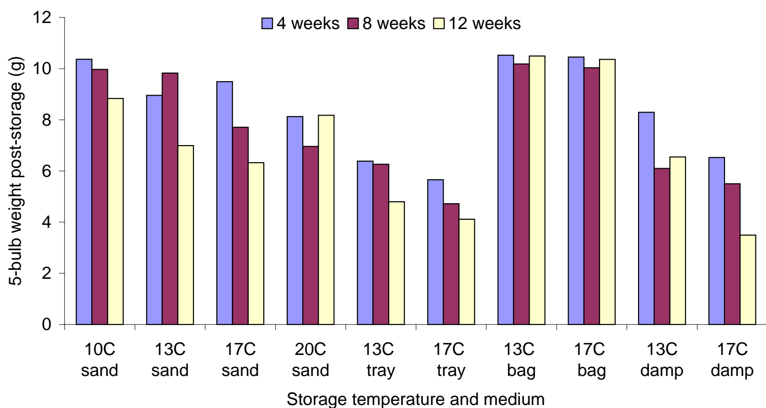


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Fig. 4.2. Effect of storage conditions on bulb performance*.

*Sprouting on 27 January 2003; score = (% re-planted x % sprouting)/100.

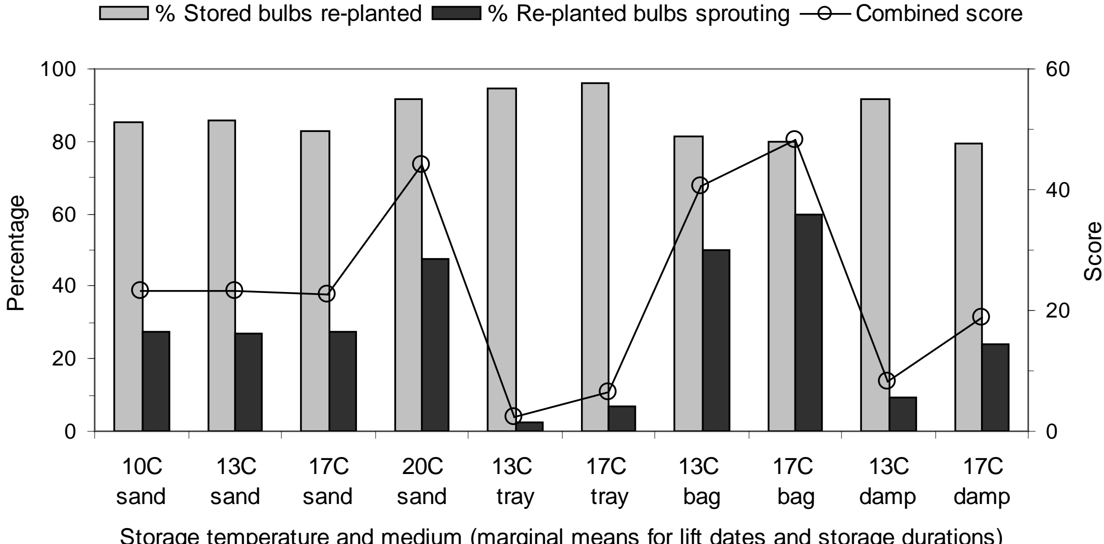

Fig. 4.3. Effect of storage conditions including storage duration on combined score ((% re-planted x % sprouting)/100).

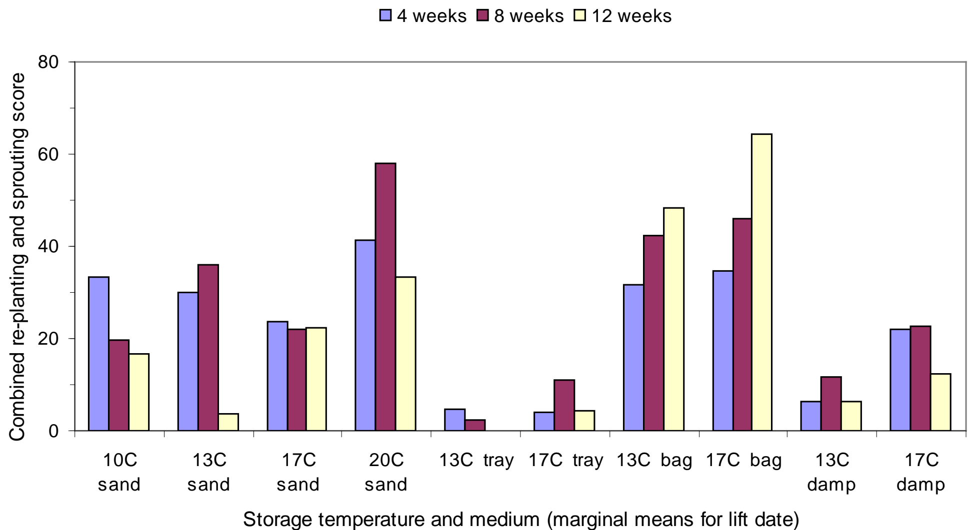

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Table 4.1. Analysis of variance table for storage experiment 1.

Factor or interaction	*d.f.*	*Probability*		
		Bulb weight after storage (g)	*Sound bulbs recovered after storage (%)*	*Bulbs sprouting (% of planted)*
Harvest date (HD)	2	<0.001***	0.581 ^{NS}	0.114 ^{NS}
Storage temp. and medium (STM)	9	<0.001***	0.006**	<0.001***
Storage duration (SD)	2	<0.001***	0.945 ^{NS}	0.052(*)
HD x STM	18	0.001**	0.208 ^{NS}	<0.001***
HD x SD	4	0.315 ^{NS}	0.189 ^{NS}	0.900 ^{NS}
STM x SD	18	<0.001***	0.285 ^{NS}	0.169 ^{NS}
HD x STM x SD	36	0.014*	0.902 ^{NS}	0.004**
Co-variate (initial bulb weight)	1	<0.001***	-	-

Table 4.2. 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			(*)	

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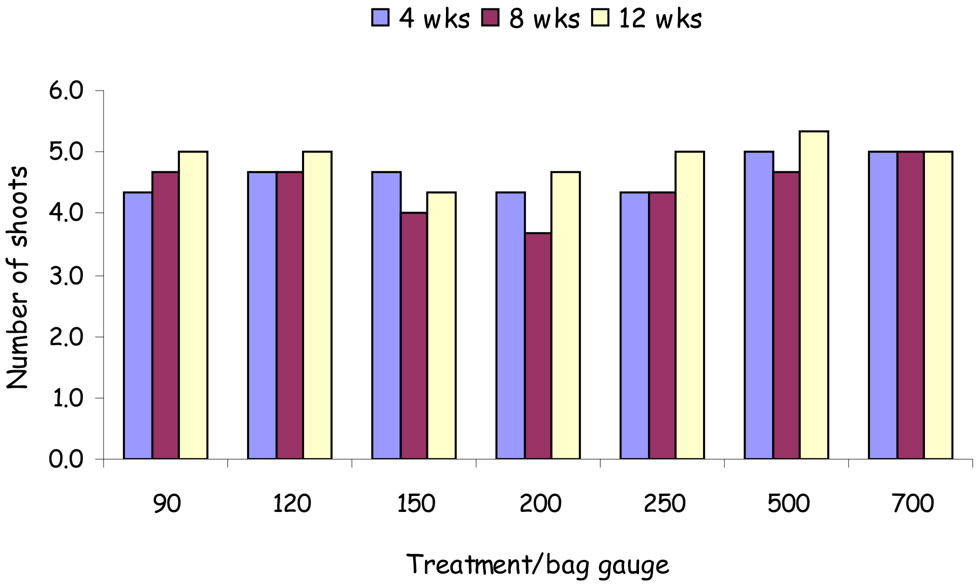
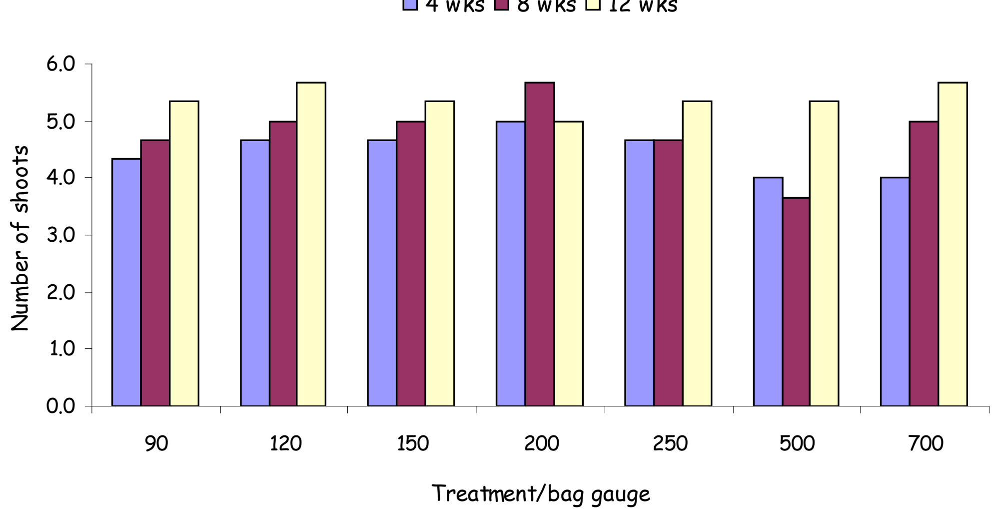
Fig. 4.3. Effect of polythene bag gauge on subsequent shoot production. Above, sealed bags; below: perforated bags.





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Storage experiment 3 – use of confectionery glaze to reduce desiccation of bulbs

Use of either of the two confectionary glazes was successful in reducing desiccation of the bulbs (Table 4.3), compared with non-treated bulbs. However, treatment with Crystalac (even for 5 minutes) resulted in serious mould growth, while treatment in Natureseal-PN resulted in the bulbs clumping together, encouraging damp and root and shoot growth. These bulbs were therefore not grown on further.

Table 4.3. Effect of confectionary glaze treatments on weight loss of snowdrop bulbs during storage.

Treatment	*% bulb weight loss after:*		
	4 weeks	*8 weeks*	*12 weeks*
Control	0.21	0.71	0.91
5 min Crystalac	0.51	0.97	1.02
5 min Natureseal-PN	0.49	0.85	1.03
30 min Crystalac	0.40	1.40	1.23
30 min Natureseal-PN	0.43	0.84	1.29

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DISCUSSION

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. In setting up this 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. Only very limited information for *Galanthus* spp. is available in the scientific and technical literature on these topics.

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 derived from micropropagation and grown-on in the field under shading and (or) using a mulch, or in an unheated mesh tunnel. The micropropagation study overcame initial problems of explant contamination, hyperhydration and slow bulb growth, and demonstrated successful propagation via shoot cultures for *G. nivalis* and other snowdrops. Shoot cultures from a number of *Galanthus* clones were successfully transferred to field plots and plots in a mesh tunnel, though marked inter-clonal variation in vigour was a prominent feature of the results. Unexpectedly, it was not possible to demonstrate a beneficial effect of introducing mycorrhizal fungi. The project has reached a point where protocols could be used to initiate a commercial snowdrop microprop programme. The agronomic studies showed the potential for using simple shelter and mulch to increase snowdrop bulb production under relatively 'normal' bulb growing practices. Similar beneficial effects of shading on bulb yield were claimed recently using a related species, *Leucojum aestivum* (summer snowflake) by Ayan *et al*. (2004). In this case, a 50% shade netting was stretched over the plots and vertically on the south and east side. However, this work was combined with gibberellin and auxin PGR treatments, and the shading effect seemed quite variable depending on the PGR treatment applied. Bulb storage studies confirmed known suitable conditions for small snowdrop bulbs for up to 12 weeks – in silver sand at 13-17°C – storage of small quantities in sealed polythene bags was also suitable. However, as it is likely that bulbs from microprop would be delivered as rooted shoot clumps, the storage of 'dry bulbs' would become less critical.

To exploit these findings, the following key elements are proposed:

1. A consortium should be established to decide how to manage the commercialisation of the project – bulb growers or nurserymen, microprop specialists and researchers. For example, should there be some setting-up of a co-operative venture, or should development funding be sought to finance the work?

2. The microprop protocols should be formalised and high-quality starting material of both commonly traded *Galanthus* (*G. nivalis, G. elwesii*), and of some other popular cultivars, should be sourced and established.

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3. For each species, a number of clones should be produced *in vitro*, and these should be screened for acclimatisation to soil and for vigour growing under 'normal' conditions. The best clones should be selected and multiplied further.
4. While initial stocks are being established, mechanisation aspects of bulb harvest should be investigated, perhaps using specialist Dutch small bulb or set harvesting machines or the technique of growing in nets.
5. Because of serious fungal disease problems in snowdrops, there is a need to improve diagnostics (there is confusion over *Botrytis* and *Stagonospora* symptoms) and develop improved pest and disease control protocols. Initially, a pre-planting fungicide bulb dip, and a spray programme including Scala or Unix, should be used.
6. These clones would be grown initially in an un-heated mesh tunnel, later transferring to the field where they would be grown under simple artificial shading.
7. For routine tunnel and field growing, husbandry protocols should be established using the information on shading, mulching, etc., presented here, augmented with further studies on herbicides or alternative methods of weed suppression. The aim would be to grow the bulbs two- or three-years-down, maximising leaf longevity in the initial years.

ACKNOWLEDGEMENTS

The authors would like to thank:

- Rod Asher (Warwick HRI) for his skilled help with the field trials
- Andrew Mead (Warwick HRI) for design and statistical advice on field trials
- Cheryl Brewster (formerly of HRI Stockbridge House) and Dr Tim O'Neill (ADAS) for examining bulbs for fungal diseases
- Dr Sally Watson (DARD) for statistical analysis of the micropropagation data
- Dr John Bailey and his staff (DARD) for the mineral analysis on bulb samples
- John Downing (Walsingham Estate Co.) and Richard Todd (Anglesey Abbey) and their staff for providing soil samples
- Tim Dougall (PlantWorks Ltd) for advice on mycorrhizal fungi
- Simon Matthews (AgriCoat Industries Ltd) for advice on confectionery glaze and for supplying samples of Crystalac and Natureseal-PN

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APPENDIX A

Protocol for the micropropagation of snowdrops

Bulb explant preparation and surface sterilization

Initial surface sterilization of whole bulbs
Bulbs should be in a non-dormant condition and just starting active growth (October-December). Healthy bulbs are selected and their tunics and any scale leaves showing discoloration or brown markings are removed by hand. Basal bulb tissues are cut away with a scalpel down to healthy white tissues, care being taken not to remove more base plate tissues than is necessary. With *G. nivalis* trimming of basal tissues needs extra care because the base plate tissues of these bulbs are very thin and it is difficult to trim both before and after sterilization and still leave base plate tissues on explants. Apical bulb tissues are also cut away 1mm below the region of scale leaf senescence so that only healthy white tissues remain. The bulbs are then ready for surface sterilization. Bulbs are surface sterilized in pairs in 100ml Erlenmeyer flasks (*G. nivalis* and *G. nivalis* 'Flore Pleno') or singly in wide-form 100ml beakers (*G. elwesii and G. plicatus*). Sterilant is added to completely cover the bulbs, the vessel capped in aluminium foil and shaken at 130 r.p.m. on a reciprocating shaker. Bulbs are first shaken for 1 minute in 70% ethanol, for 20 minutes in 50% Domestos and then rinsed five times in sterile de-ionised water.

Preparation of bulb chip explants
After an initial surface sterilization the bulbs are cut aseptically into explants. Bulbs are first trimmed top and bottom to remove previously cut tissues damaged by the sterilant. Basal trimming is such as to allow a minimum of 1mm of base plate to remain. Apical trimming produces bulb sections approximately 8-10 mm in height for *G. nivalis, G. nivalis* 'Flore Pleno', *G. plicatus* and *G. plicatus* 'Wendy's Gold' or 10-13mm in height for *G. elwesii*. Longitudinal cuts are then made through the centre of the bulbs to produce four (*G. nivalis, G. nivalis* 'Flore Pleno', *G. plicatus* and *G. plicatus* 'Wendy's Gold') or six (*G. elwesii*) chip explants per bulb.

Chip explants are shaken for 9h in 4% Plant Preservative Mixture (PPM) (Plant Cell Technology Incorporated, Washington, DC) prepared in a 50mg/l $MgSO_4$ solution. Explants are treated in groups of four in 20ml of PPM solution. With larger bulbed species like *G. elwesii* groups of three chip explants are treated in 30ml of PPM solution to compensate for their greater size and higher levels of infection. Explants are inoculated without rinsing with their bases about 3mm into the agar-solidified culture medium. One explant is inoculated per culture vessel.

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Culture conditions

Cultures are incubated at a constant temperature of 18ºC with 16h photoperiods provided by cool white fluorescent tubes giving a PAR of 100µmol/m^2/s at bench height.

Media composition and culture passages

Media used are described in Table A1. Micropropagation consists of three phases as defined below.

Bulblet induction
Chip explants are initially inoculated onto induction and proliferation medium (IM) and incubated for between 12 and 16 weeks. Bulblets are initiated on the outer surfaces of the scale leaf tissues.

Bulblet proliferation
Zones of healthy white bulblet scale tissues, preferably with newly initiated bulblets, are dissected from the chip explants and sub-cultured onto fresh IM. Sub-culture of browning tissues is avoided. Proliferating bulblet cultures are developed by routine sub-culture onto fresh IM every 12 to 16 weeks. After 3 or 4 passages rapidly proliferating bulblet cultures are stabilised and these can be sustained by sub-culturing for several years. The sub-culture technique involves cutting proliferating bulblet clumps into smaller groups consisting of about 2 to 6 bulblets and inoculating these basal ends down into fresh culture medium.

Bulblet growth
Proliferating bulblets need to be grown on to a larger size in preparation for their acclimatization back to *in vivo* conditions. This is achieved by transferring small bulblet clumps, prepared as described above, onto growth medium (GM) and incubating for 12 to 16 weeks.

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Table A1. Composition of *Galanthus* media devised from the mineral composition of bulbs.

Compound (mg/l) or pH	*Induction and proliferation medium**	*Growth medium**
KNO_3	430.5	861.1
NH_4NO_3	800.5	1600.9
KH_2PO_4	301.9	603.9
$MgSO_4.7H_2O$	242.5	485.1
$Ca(NO_3)_2.4H_2O$	677.1	1355.2
$FeSO_4.7H_2O$	27.8	27.8
Na_2EDTA	37.2	37.2
H_3BO_3	3.10	6.20
$ZnSO_4.7H_2O$	2.52	5.05
$MnSO_4.H_2O$	0.60	1.21
KI	0.42	0.83
$CuSO_4.5H_2O$	0.38	0.76
$Na_2MoO_4.2H_2O$	0.12	0.25
$CoCl_2.6H_2O$	0.012	0.025
Inositol	100	100
Thiamine HCl	0.1	0.1
Pyridoxine HCl	0.5	0.5
Nicotinic acid	0.5	0.5
Glycine	2.0	2.0
Sucrose	30000	60000
6-benzylaminopurine	1.0	-
Naphthalene acetic acid	0.1	-
Activated charcoal (Sigma C6289)	-	5000
pH	5.6	5.8
**Agar (Oxoid purified)	7000	7000

*Alternatively half strength Murashige and Skoog medium (MS) can be used for bulblet initiation and proliferation and full strength MS medium can be used for bulblet growth. Both these media need to be modified with PGRs and activated charcoal as with GM in this Table.
**Agar can be raised to 12,000 mg/l to help suppress hyperhydration in susceptible species like *G. elwesii.*

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APPENDIX B

Analysis of variance summaries for micropropagation results

Table 2.2.

	d.f.	*Bulblet numbers*	*Hyperhydrated bulblets (%)*	*Water in bulblets (%)*	*Water in other tissues (%)*
CLONE (C)	4	***	***	***	***
Fe (F)	2	NS	**	NS	NS
Mg (M)	2	NS	NS	NS	NS
C x F	8	NS	NS	NS	NS
C x M	8	NS	NS	NS	NS
F x M	4	NS	NS	NS	NS
C x F x M	16	NS	NS	NS	NS
Residual	176				

Table 2.3.

		Total culture FW		*Bulblet multiplication*		*Bulblets sprouting*		*Largest bulblet FW*	
	d.f. (m.v.)	*SED*	*Sig.*	*SED*	*Sig.*	*SED*	*Sig.*	*SED*	*Sig.*
PGR (P)	2	0.127	***	0.224	**	2.91	NS	0.025	NS
Clone (C)	5	0.180	**	0.317	NS	4.13	***	0.036	***
P x C	10	0.313	NS	0.549	NS	7.16	NS	0.062	NS
Residual	99 (3)								

		Largest bulblet diameter		*2nd largest bulblet diameter*		*Root numbers*		*Longest root length*	
	d.f. (m.v.)	*SED*	*Sig.*	*SED*	*Sig.*	*SED*	*Sig.*	*SED*	*Sig.*
PGR (P)	2	0.298	NS	0.249	NS	1.194	***	12.94	NS
Clone (C)	5	0.421	***	0.352	*	1.689	***	18.31	**
P x C	10	0.730	NS	0.161	NS	2.923	†*	31.71	NS
Residual	99 (3)								

†NS with square root transformed data

Figure 2.1.

	d.f. (MV)	*New bulblet numbers*		*Total culture FW*	
		SED	*sig.*	*SED*	*sig.*
SPECIES (SP)	1	0.0909	NS	0.0338	***
CONTROL (C)	1	0.1877	*	0.0698	*
SP / CLONE (CL)	6	0.1817	**	0.0676	NS
SP x C	1	0.2654	NS	0.0987	NS
AGENT (A)	4	0.1484	*	0.0552	***
RATE (R)	2	0.1149	NS	0.0427	NS
C x SP / CL	6	0.5309	NS	0.1974	NS
SP x A	4	0.2098	NS	0.0780	*
SP x R	2	0.1625	NS	0.0604	NS
A x R	8	0.2570	NS	0.0956	***
A x SP / CL	24	0.4197	NS	0.1560	NS
R x SP / CL	12	0.3251	NS	0.1209	NS
SP x A x R	8	0.3635	NS	0.1351	*
A x R x SP / CL	48	0.7269	NS	0.2703	NS
Residual	497 (11)				

Figure 2.2.

	d.f. (MV)	*Largest bulblet FW*		*Largest bulblet Diameter*		*2nd largest bulblet diameter*	
		SED	*sig.*	*SED*	*sig.*	*SED*	*sig.*
SPECIES (SP)	1	0.0068	***	0.116	***	0.099	***
CONTROL (C)	1	0.0140	**	0.239	*	0.205	***
SP / CLONE (CL)	6	0.0135	**	0.231	***	0.198	**
SP x C	1	0.0198	NS	0.337	NS	0.289	NS
AGENT (A)	4	0.0110	***	0.189	***	0.162	***
RATE (R)	2	0.0086	NS	0.146	NS	0.125	NS
C x SP / CL	6	0.0395	NS	0.675	NS	0.579	NS
SP x A	4	0.0156	*	0.267	NS	0.229	NS
SP x R	2	0.0121	NS	0.207	NS	0.177	NS
A x R	8	0.0191	***	0.327	***	0.280	***
A x SP / CL	24	0.0312	NS	0.533	NS	0.457	NS
R x SP / CL	12	0.0242	NS	0.413	NS	0.354	NS
SP x A x R	8	0.0270	NS	0.462	NS	0.396	NS
A x R x SP / CL	48	0.0541	NS	0.924	NS	0.792	NS
Residual	497 (11)						

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Figure 2.3.

	d.f. (MV)	*Root numbers*		*Longest root length*	
		SED	*sig.*	*SED*	*sig.*
SPECIES (SP)	1	0.073	***	3.42	*
CONTROL (C)	1	0.151	*	7.07	*
SP / CLONE (CL)	6	0.146	***	6.85	*
SP x C	1	0.213	NS	10.00	NS
AGENT (A)	4	0.119	***	5.59	***
RATE (R)	2	0.092	NS	4.33	**
C x SP / CL	6	0.426	NS	20.01	NS
SP x A	4	0.169	**	7.91	***
SP x R	2	0.131	NS	6.13	NS
A x R	8	0.206	***	9.68	***
A x SP / CL	24	0.337	NS	15.82	NS
R x SP / CL	12	0.261	NS	12.25	NS
SP x A x R	8	0.292	*	13.70	*
A x R x SP / CL	48	0.584	NS	27.39	NS
Residual	497 (11)				

Figure 2.4.

	d.f. (MV)	*New bulblet numbers*		*Total culture FW*	
		SED	*sig.*	*SED*	*sig.*
SPECIES (SP)	1	0.101	NS	0.0298	***
CONTROL (C)	1	0.208	NS	0.0615	NS
SP / CLONE (CL)	8	0.225	***	0.0666	NS
SP x C	1	0.294	**	0.0870	NS
AGENT (A)	4	0.164	***	0.0486	***
RATE (R)	2	0.127	*	0.0377	NS
C x SP / CL	8	0.656	NS	0.1946	NS
SP x A	4	0.232	NS	0.0688	*
SP x R	2	0.180	NS	0.0533	NS
A x R	8	0.284	NS	0.0843	***
A x SP / CL	32	0.519	NS	0.1538	NS
R x SP / CL	16	0.402	NS	0.1192	*
SP x A x R	8	0.402	NS	0.1192	NS
A x R x SP / CL	64	0.899	NS	0.2664	**
Residual		300 (18)		315 (3)	

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Figure 2.5.

	d.f. (MV)	*Largest bulblet* FW		*Largest bulblet* Diameter		*2nd largest bulblet diameter*	
		SED	*sig.*	*SED*	*sig.*	*SED*	*sig.*
SPECIES (SP)	1	0.0065	***	0.131	***	0.115	***
CONTROL (C)	1	0.0134	NS	0.271	NS	0.238	NS
SP / CLONE (CL)	8	0.0145	**	0.294	**	0.258	**
SP x C	1	0.0190	NS	0.383	NS	0.337	NS
AGENT (A)	4	0.0106	***	0.214	***	0.188	***
RATE (R)	2	0.0082	NS	0.166	*	0.146	NS
C x SP / CL	8	0.0424	NS	0.857	NS	0.753	NS
SP x A	4	0.0150	NS	0.303	NS	0.266	NS
SP x R	2	0.0116	NS	0.235	NS	0.206	NS
A x R	8	0.0184	***	0.371	***	0.326	*
A x SP / CL	32	0.0335	NS	0.678	NS	0.596	NS
R x SP / CL	16	0.0260	NS	0.525	NS	0.461	NS
SP x A x R	8	0.0260	NS	0.525	NS	0.461	NS
A x R x SP / CL	64	0.0581	**	1.174	NS	1.032	NS
Residual		314 (4)		315 (3)		314 (4)	

Figure 2.6.

	d.f. (MV)	*Root numbers*		*Longest root length*	
		SED	*sig..*	*SED*	*sig.*
SPECIES (SP)	1	0.086	*	1.47	NS
CONTROL (C)	1	0.178	NS	3.04	NS
SP / CLONE (CL)	8	0.193	***	3.29	NS
SP x C	1	0.252	NS	4.30	NS
AGENT (A)	4	0.141	***	2.41	***
RATE (R)	2	0.109	***	1.86	***
C x SP / CL	8	0.563	NS	9.62	NS
SP x A	4	0.199	NS	3.40	**
SP x R	2	0.154	NS	2.64	NS
A x R	8	0.244	**	4.17	***
A x SP / CL	32	0.445	NS	7.61	NS
R x SP / CL	16	0.345	NS	5.89	NS
SP x A x R	8	0.345	NS	5.89	NS
A x R x SP / CL	64	0.770	NS	13.18	NS
Residual	315 (3)				

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Figure 2.7.

	d.f. (MV)	New bulblet no.		Total culture FW	
		SED	*sig.*	*SED*	*sig.*
SPECIES (SP)	1	0.122	**	0.054	***
LIGHT (L)	1	0.122	NS	0.054	***
CONTROL (C)	1	0.153	NS	0.068	NS
SP / CLONE (CL)	4	0.212	***	0.094	**
SP x L	1	0.173	NS	0.077	NS
SP x C	1	0.216	NS	0.096	NS
L x C	1	0.216	NS	0.096	NS
AGENT (A)	1	0.137	**	0.061	***
RATE (R)	1	0.137	NS	0.061	*
L x SP / CL	4	0.299	*	0.133	**
C x SP / CL	4	0.374	NS	0.166	NS
SP x L	1	0.193	NS	0.086	NS
SP x A	1	0.193	NS	0.086	NS
L x A	1	0.193	NS	0.086	NS
SP x R	1	0.193	NS	0.086	NS
L x R	1	0.193	NS	0.086	NS
A x R	1	0.193	NS	0.086	**
L x C x SP / CL	4	0.529	NS	0.235	NS
A x C x SP / CL	4	0.410	NS	0.182	NS
SP x C x L x A	1	0.335	NS	0.148	NS
R x C x SP / CL	4	0.410	NS	0.182	NS
SP x C x L x R	1	0.335	NS	0.148	NS
SP x A x R	1	0.273	NS	0.121	NS
L x A x R	1	0.273	NS	0.121	NS
L x A x SP / CL	4	0.474	NS	0.210	NS
L x R x SP / CL	4	0.474	NS	0.210	NS
A x R x SP / CL	4	0.474	*	0.210	NS
SP x L x A x R	1	0.387	NS	0.171	NS
L x A x R x SP / CL	4	0.670	NS	0.297	NS
Residual		208 (28)		214 (22)	

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Figure 2.8.

	d.f. (MV)	*Largest bulblet* *FW*		*Diameter*		*2nd largest bulblet diameter*	
		SED	*sig.*	*SED*	*sig.*	*SED*	*sig.*
SPECIES (SP)	1	0.029	***	0.173	***	0.137	***
LIGHT (L)	1	0.029	NS	0.173	*	0.137	**
CONTROL (C)	1	0.036	NS	0.216	NS	0.171	NS
SP / CLONE (CL)	4	0.050	NS	0.299	***	0.238	NS
SP x L	1	0.041	NS	0.244	NS	0.194	NS
SP x C	1	0.051	NS	0.305	NS	0.242	NS
L x C	1	0.051	NS	0.305	NS	0.242	NS
AGENT (A)	1	0.033	NS	0.193	***	0.153	***
RATE (R)	1	0.033	NS	0.193	NS	0.153	NS
L x SP / CL	4	0.071	NS	0.423	NS	0.336	NS
C x SP / CL	4	0.089	NS	0.529	NS	0.420	NS
SP x L	1	0.046	NS	0.273	NS	0.217	NS
SP x A	1	0.046	NS	0.273	NS	0.217	NS
L x A	1	0.046	NS	0.273	NS	0.217	NS
SP x R	1	0.046	NS	0.273	*	0.217	**
L x R	1	0.046	NS	0.273	NS	0.217	NS
A x R	1	0.046	NS	0.273	**	0.217	**
L x C x SP / CL	4	0.126	NS	0.747	NS	0.594	NS
A x C x SP / CL	4	0.097	NS	0.579	NS	0.460	NS
SP x L x A	1	0.080	NS	0.473	NS	0.376	NS
R x SP / CL	4	0.097	NS	0.579	NS	0.460	NS
SP x L x R	1	0.080	NS	0.473	NS	0.376	NS
SP x A x R	1	0.065	NS	0.386	NS	0.307	NS
L x A x R	1	0.065	NS	0.386	NS	0.307	NS
L x A x SP / CL	4	0.112	NS	0.669	NS	0.531	NS
L x R x SP / CL	4	0.112	NS	0.669	NS	0.531	NS
A x R x SP / CL	4	0.112	NS	0.669	NS	0.531	NS
SP x L x A x R	1	0.092	NS	0.546	NS	0.434	NS
L x A x R x SP / CL	4	0.159	NS	0.945	NS	0.751	NS
Residual	214 (22)						

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Figure 2.9.

	d.f. (MV)	Root numbers		Longest root length	
		SED	*sig.*	*SED*	*si.g*
SPECIES (SP)	1	0.418	***	3.65	***
LIGHT (L)	1	0.418	***	3.65	***
CONTROL (C)	1	0.523	NS	4.56	***
SP / CLONE (CL)	4	0.725	***	6.32	***
SP x L	1	0.592	NS	5.16	*
SP x C	1	0.740	NS	6.45	NS
L x C	1	0.740	NS	6.45	*
AGENT (A)	1	0.468	***	4.08	***
RATE (R)	1	0.468	NS	4.08	*
L x SP / CL	4	1.025	***	8.94	***
C x SP / CL	4	1.281	NS	11.18	NS
SP x L	1	0.662	NS	5.77	NS
SP x A	1	0.662	NS	5.77	NS
L x A	1	0.662	NS	5.77	***
SP x R	1	0.662	NS	5.77	***
L x R	1	0.662	NS	5.77	NS
A x R	1	0.662	NS	5.77	**
L x C x SP / CL	4	1.812	NS	15.81	NS
A x C x SP / CL	4	1.403	NS	12.25	NS
SP x L x A	1	1.146	NS	10.00	NS
R x SP / CL	4	1.403	NS	12.25	NS
SP x L x R	1	1.146	NS	10.00	*
SP x A x R	1	0.936	NS	8.16	***
L x A x R	1	0.936	NS	8.16	NS
L x A x SP / CL	4	1.621	NS	14.14	NS
L x R x SP / CL	4	1.621	NS	14.14	NS
A x R x SP / CL	4	1.621	NS	14.14	NS
SP x L x A x R	1	1.323	NS	11.55	NS
L x A x R x SP / CL	4	2.292	NS	20.00	NS
Residual	214 (22)				

Figure 2.11.

	d.f. (m.v.)	Passage 1		Passage 2		Passage 3	
		SED	*Sig.*	*SED*	*Sig.*	*SED*	*Sig.*
PGR (P)	2	0.372	*	0.198	***	0.286	***
Clone (C)	5	0.528	*	0.279	**	0.404	**
P x C	10	0.925	NS	0.484	NS	0.701	NS
Residual	99 (3)						

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