**New gold in them thar hills: testing a novel supply route for plant-derived galanthamine**

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**Abstract**

**Introduction:** We tested the feasibility of an innovative dual-cropping approach to producing the plant alkaloid galanthamine, the main source of the Alzheimer’s disease treatment galanthamine

**Methods:** Experimental lines of daffodils were established under upland permanent pasture at four different sites. Triplicate lines of three different planting densities were prepared at each site. Above-ground biomass was harvested just before flowering.

**Results:** Over 80% of bulbs successfully established at each site. There was no effect of altitude or planting density on galanthamine concentrations in the green material, which were higher than anticipated.

**Discussion:**  The results confirm that planting daffodils under grass in upland areas could offer a novel and sustainable source of plant-derived galanthamine. Further research is now required to verify the commercial viability of this supply route.

*Keywords:* Galanthamine; *Narcissus;* alkaloid; less favoured areas

**1. Introduction**

Galanthamine is a long acting, selective and reversible acetylcholinesterase inhibitor that has been a licenced treatment for Alzheimer’s disease (AD) in the USA, across Europe and into Asia since 2000. The main source of galanthamine for the pharmaceutical industry has been the alkaloid galanthamine extracted from plants [[1](#_ENREF_1)]. Galanthamine occurs in several species of the Amaryllidaceae family, including *Galanthus nivalis*, *Leucojum* *aestivum*, *Lycoris radiate*, and *Narcissus* (daffodil) spp. However, with the exception of daffodils, the source plants are wild flowers not suitable for agricultural exploitation due to limitations in either resources or research, and consequently supplies have been limited. Opportunities for producing synthetic galanthamine have been explored [[2](#_ENREF_2), [3](#_ENREF_3)], but this has not proved to be a viable alternative.

Upland areas within the UK and northern Europe are characterised by poor growing conditions brought about by a combination of low temperatures, high rainfall, exposure to wind, thin soils, and a shortage of major nutrients. Consequently agricultural production in these areas is generally limited to grassland-based ruminant systems that are currently heavily reliant upon Government support payments to be economically viable [[4](#_ENREF_4)]. However, it has been reported study that daffodils grown at altitude may yield higher concentrations of galanthamine compared to bulbs grown under lowland conditions [[5](#_ENREF_5)]. Thus growing daffodils for galanthamine production in marginal areas could offer a novel solution to the issue of constrained galanthamine supplies, while simultaneously increasing the economic resilience and social sustainability of less favoured areas. Legistaltive constraints surounding ploughing of long-term grassland (the land cover accounting for by far the greatest proportion of upland farms [[6](#_ENREF_6)]) limit options for traditional cropping however. Furthermore, to date it has been the bulbs of *Narcissus* plants that have been used as material for extraction of galanthamine [[7](#_ENREF_7)], again requiring soil disturbance.

Our proof-of-principle study tested the feasibility of an innovative dual-cropping approach to producing daffodil-derived galanthamine based on integrating daffodil growing into existing marginal pasture and harvesting green above-ground growth rather than bulbs. Such an approach could offer a win-win-win scenario whereby i) AD patients have increased access to a proven treatment, ii) environmental impacts are minimised, and iii) traditional farming systems within marginal areas are maintained, with their economic viability increased. It has been theorised that the disparity in galanthamine concentrations reported within bulbs grown in lowland and upland conditions was a response to differing levels of environmental stress [[5](#_ENREF_5)], and abiotic factors are known to influence secondary metabolites in plants [[8](#_ENREF_8)]. The current experiment investigated such a premise further by quantifying galanthamine concentrations within green material grown under progressively more challenging field conditions, while at the same time exploring whether inter-specific competition for the limited supply of nutrients available within upland swards may also have a role to play in determining potential galanthamine yields.

**2. Materials & method**

*2.1 Experimental design and plot preparation*

Lines of daffodils were sown into pasture at each of four different sites at the Pwllpeiran Upland Research Centre, Wales from 253 m a.s.l. to 430 m a.s.l. (Table 1). At each site bulbs of *N. pseudonarcissus* variety Carlton (size < 10; Grampian Growers, Montrose, UK) were planted at three different intervals: 5 cm, 10 cm and 15 cm apart. Triplicate lines of each treatment were organised into three separate blocks. Each line of daffodils was 8 m long, and lines were spaced 1 m apart. Planting lines were created using a single bolt-on tooth (15 cm × 10 cm wide) on the front bucket of a mini-digger (8026 CTS; JCB Ltd, Rocester, Staffordshire, UK). Bulbs were planted at the prescribed densities by hand, with the tops of the bulb the treatment distance apart.

*2.2 Measurements*

A 500 g soil sample was collected for each site by bulking 10 soil cores collected at random between daffodil lines. Sampling of the daffodil biomass was undertaken when the majority of flowers at a site reached the ‘gooseneck’ growth stage – i.e. were bent downwards to an angle of approximately 45⁰ but were unopened. The number of daffodil plants growing were counted along a 6 m length in the centre of each line. The corresponding growth was then harvested to a height of 3 cm using grass shears. The material cut from each line was weighed to determine fresh matter (FM) weight. Fifteen leaves and 15 flower stems were then selected from each bag at random for length measurements. To determine dry matter (DM) content a sub-sample was taken from each bag and oven dried to constant weight at 60 ⁰C. A separate sub-sample of approximately 100 g was taken for subsequent analysis to determine alkaloid concentrations.

*2.3 Alkaloid analysis*

Leaf sections of approximately 100 mg FM were homogenised in 500 µl of methanol adjusted to pH 8 with 25 % of ammonia added, and then a further 500 µl methanol added. The samples were left for at least 5 hr and then centrifuged at 13,000 r.p.m. for 1 min. An aliquot of 500 µl of the solution was removed and the solvent evaporated. The dry extract was dissolved in 500 µl mobile phase A (see below) prior to analysis by high-performance liquid chromatography. A Betasil C18 column (150 x 4.6 mm; particle size 5 μm) was used (Fisher Scientific UK Ltd, Loughborough, UK). The column was thermostatically maintained at 30 °C. Analyses were conducted with ultra-violet monitoring at 298 nm using a gradient method. The mobile phase consisting of 0.1% trifluoroacetic acid in pure water (mobile phase A) and acetonitrile (mobile phase B) was filtered through a membrane filter, degassed for 4 min before use and pumped to the column at the rate of 1 ml min-1. The data were collected and analysed using the Chrom Quest 5.0 HPLC database program (Thermo Fisher Scientific, Cramlington, UK).

*2.4 Data analysis*

Data were analysed using general analysis of variance with altitude and planting distance as treatment effects (Genstat (16th Edition); VSN International Ltd, Hemel Hempstead, UK). In this context ‘altitude’ was used as a collective term for the combination of factors relating to soil characteristics, climatic conditions and exposure which potentially influence the degree of environmental stress experienced.

**3.1 Results and discussion**

Soil nutrient status across the four sites was variable (Table 1). In terms of the key minerals, the concentrations recorded equate to moderate or high indices for K and Mg, but low or very low indices for P [[9](#_ENREF_9)]. The plant counts prior to harvest showed over 80% of the bulbs had successfully established (Table 2), demonstrating that planting under long-term pasture on comparatively poor soils is feasible. Planting distance inevitably had a significant effect on the biomass of herbage harvested (Table 2), but we found no effect of altitude on total DM yield or DM yield per bulb planted. Between-altitude differences in leaf and stem length followed a similar pattern to FM yield.

It has been shown that the concentration of galanthamine in daffodils can vary between different varieties [[10](#_ENREF_10)]. The variety Carlton is considered to have potential as a commercial source of galanthamine due to relatively high concentrations of galanthamine in the bulbs, a large bulb size and good availability of large volumes of planting stock [[7](#_ENREF_7)]. Galanthamine concentrations in daffodil leaves have been found to be steady until flowering, before decreasing [[11](#_ENREF_11)]. Although higher concentrations of alkaloids could potentially be obtained from daffodil leaves at an earlier growth stage than the gooseneck stage, we judged that the total amount of biomass, and thus total yield of galanthamine, would not be so high. This is an aspect that may warrant further investigation. The galanthamine concentrations achieved during the current experiment were substantially higher than those recorded during the earlier study focussed on bulbs [[5](#_ENREF_5)], and higher than concentrations previously reported for above-ground daffodil biomass [[12](#_ENREF_12)]. Furthermore, by cutting green material there is potential for a single planting of bulbs to deliver harvests over multiple years.

There was no effect of planting distance on galanthamine concentrations. These results concur with those from an earlier study which found the concentration of galanthamine in other *Narcissus* cultivars to be unaffected by planting depth and density, bulb size or flower bud removal [[13](#_ENREF_13)]. Thus, overall, the results suggested that higher planting densities which would favour biomass yield would maximise galanthamine yield, although monitoring over multiple harvest years would be beneficial to determine whether further nutrient depletion from already poor quality soils becomes a factor over time.

**4. Summary and outlook**

This study has verified the feasibility of establishing daffodils under permanent pasture in upland areas as a means of producing plant-derived galanthamine. A number of different beneficiaries could potentially benefit from this novel production pathway. Further research is now required to verify the commercial viability of this supply route and develop management guidelines which maximise galanthamine yield.

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**References**

[1] Heinrich M, Teoh HL. Galanthamine from snowdrop - the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. J Ethnopharmacology. 2004;92:147-62.

[2] Trost BM, Toste FD. Enantioselective total synthesis of (-)-galanthamine. J Am Chem Soc. 2000;122:1262-3.

[3] Marco-Contelles J, Carreiras MD, Rodríguez C, Villarroya M, Garcia AG. Synthesis and pharmacology of galantamine. Chem Rev. 2006;106:116-33.

[4] Acs S, Hanley N, Dallimer M, Gaston KJ, Robertson P, Wilson P, et al. The effect of decoupling on marginal agricultural systems: Implications for farm incomes, land use and upland ecology. Land Use Policy. 2010;27:550-63.

[5] Morris P, Brookman JL, Theodorou MK. Sustainable production of the natural product galanthamine. Technical annex to DEFRA project NF0612 Final Report London: DEFRA; 2006.

[6] Fraser MD, Vale JE, Firbank LG. Effect on habitat diversity of organic conversion within the Less Favoured Areas of England and Wales. Agroecology & Sustain Food Syst. 2014;38:243-61.

[7] Berkov S, Georgieva L, Kondakova V, Atanassov A, Viladomat F, Bastida J, et al. Plant sources of galanthamine: phytochemical and biotechnological aspects. Biotechnol & Biotechnological Equip. 2009;23:1170-6.

[8] Ramakrishna A, Gokare AR. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signaling & Behav. 2011;6:1720-31.

[9] DEFRA. Fertiliser Manual, 8th Edition. TSO, Norwich; 2010.

[10] Torras-Claveria L, Berkov S, Codina C, Viladomat F, Bastida J. Daffodils as potential crops of galanthamine. Assessment of more than 100 ornamental varieties for their alkaloid content and acetylcholinesterase inhibitory activity. Ind Crop Prod. 2013;43:237-44.

[11] Lubbe A, Gude H, Verpoorte R, Choi YH. Seasonal accumulation of major alkaloids in organs of pharmaceutical crop Narcissus Carlton. Phytochemistry. 2013;88:43-53.

[12] Kreh M. Studies on galanthamine extraction from *Narcissus* and other Amaryllidaceae. In: Hanks GR, editor. Narcissus and Daffodil. London: Taylor & Francis; 2002. p. 256-72.

[13] Moraes Cerdeira RM, Burandt CL, Bastos JK, Nanayakkara NPD, Mikell J, Thurn J, et al. Evaluation of four Narcissus cultivars as potential sources for galanthamine production. Planta Medica. 1997;63:472-4.

**Table 1:** Location and soil details for the four experimental sites where daffodils were established. Harvesting occurred when the majority of the plants at a site reached the goose-neck growth stage.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Site** | **1** | **2** | **3** | **4** |
| Altitude (m a.s.l.) | 253 m | 284 m | 398 m | 430 m |
|  |  |  |  |  |
| Co-ordinates | 52°21'10.18"N | 52°21'12.58"N | 52°21'33.80"N | 52°21'43.42"N |
|  | 3°48'13.82"W 3°48'13.82"W 3°48'13.82"W | 3°47'48.16"W 3°47'48.16"W | 3°48'37.53"W | 3°49'11.70"W |
|  |  |  |  |  |
| Exposure | Comparatively sheltered | Comparatively sheltered | Poor protection from wind | Exposed hilltop |
|  |  |  |  |  |
| *Soil analysis results* |  |  |  |  |
| Phosphate-P (ppm) | 9.37 | 4.91 | 3.74 | 11.00 |
| K (meq %) | 0.42 | 1.67 | 0.34 | 0.63 |
| Mg (meq %) | 0.80 | 1.58 | 0.88 | 3.44 |
| Na (meq %) | 0.08 | 1.37 | 0.15 | 0.18 |
| Ca (meq %) | 1.70 | 6.24 | 3.81 | 13.30 |
| pH | 5.25 | 5.49 | 5.55 | 5.94 |
|  |  |  |  |  |
| Date harvested | 01-04-15 | 03-04-15 | 07-04-15 | 10-04-15 |

**Table 2:** Effect of altitude of planting site and planting distance on the establishment, yield and galanthamine (GAL) concentration of daffodil biomass harvested at the goose-neck growth stage.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Altitude** | | | | |  | **Planting distance** | | | |  | ***F* probability** | |
|  | **253 m** | **284 m** | **398 m** | **430 m** | **s.e.d** |  | **5 cm** | **10 cm** | **15 cm** | **s.e.d.** |  | **Site** | **Distance** |
| No plants/bulbs planted | 0.86 | 0.83 | 0.87 | 0.83 | 0.033 |  | 0.92b | 0.70a | 0.93b | 0.029 |  | ns | <0.001 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| FM yield (g m-1) | 95a | 126b | 100 a | 96a | 6.4 |  | 177c | 82b | 54a | 5.6 |  | <0.001 | <0.001 |
| DM content (g kg-1 FM) | 148b | 119a | 135b | 139b | 7.3 |  | 133 | 131 | 142 | 6.4 |  | <0.05 | ns |
| FM yield per bulb (g bulb-1) | 6.7a | 9.5b | 7.4a | 7.2a | 0.50 |  | 8.8b | 6.2a | 8.1b | 0.43 |  | <0.001 | <0.001 |
| DM yield (g DM m-1) | 13.2 | 15.0 | 13.8 | 13.3 | 0.96 |  | 23.2c | 10.7b | 7.6a | 0.83 |  | ns | <0.001 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Leaf length (mm) | 149a | 182c | 170b | 153a | 3.7 |  | 166 | 163 | 161 | 3.2 |  | <0.001 | ns |
| Stem length (mm) | 172a | 212c | 192b | 190b | 4.4 |  | 196b | 192ab | 187a | 3.8 |  | <0.001 | <0.05 |
| Flower length (mm) | 61a | 63b | 65c | 67d | 0.6 |  | 64 | 63 | 64 | 0.5 |  | <0.001 | ns |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| GAL concentration (% FM) | 0.045 | 0.036 | 0.049 | 0.043 | 0.0050 |  | 0.045 | 0.039 | 0.046 | 0.0043 |  | ns | ns |

Means within rows of treatments with different superscripts are significantly different at *P* < 0·05.

No statistically significant altitude effect × planting distance effect interactions were found.