

Short Communication

New Gold in Them Thar Hills: Testing a Novel Supply Route for Plant-Derived Galanthamine

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Abstract. Many secondary plant compounds are synthesized in response to stressed growing conditions. We tested the feasibility of exploiting this feature in a novel strategy for the commercial production of the plant alkaloid galanthamine. Experimental lines of *Narcissus pseudonarcissus* were established under marginal upland permanent pasture at four different sites. Over 80% of bulbs successfully established at each site. There was no effect of altitude or planting density on galanthamine concentrations within vegetative tissues, which were higher than anticipated. The results confirm that planting *N. pseudonarcissus* under grass competition in upland areas could offer a novel and sustainable source of plant-derived galanthamine.

Keywords: Alkaloid, daffodil, galantamine, less favored areas, plant stress

INTRODUCTION

Galantamine is a long acting, selective and reversible acetylcholinesterase inhibitor that has been a licensed treatment for Alzheimer's disease (AD) in the USA, across Europe and into Asia since 2000. The main source of the pharmaceutical product galantamine (galan/t/amine) has been the alkaloid galanthamine (galan/th/amine) extracted from plants [1]. Galanthamine occurs in several species of the Amaryllidaceae family, including *Galanthus nivalis*, *Leucojum aestivum*, *Lycoris radiata*, and *Narcissus* (daffodil) spp. However, with the exception of *Nar-*

cissus spp, 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 galantamine have been explored [2, 3], but this has not proved to be a viable alternative.

There is a long-established relationship between the exposure of plants to stress and the production of a vast array of secondary compounds, a proportion of which have medical or other commercial values. In many instances, secondary metabolites are implicated in plant stress amelioration and so tend to increase during exposure to stresses [4–7]. Production of galanthamine has also been shown to increase in response to stress. For example, *Lycoris aurea* plants exposed to nitrogen stress (no added N) show markedly increased levels of the compound

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in the leaves [8]. To date, however, there has been no attempt to evoke similar stress-induced responses under commercial growing conditions.

Upland areas within the UK and northern Europe are characterized 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 [9]. However, it has been reported that *N. pseudonarcissus* grown at altitude may yield higher concentrations of galantamine compared to bulbs grown under lowland conditions [10]. Thus growing *Narcissus* spp. for galanthamine production in marginal areas could impose sufficient stress to increase galanthamine synthesis and so offer a novel solution to the issue of constrained galantamine supplies. This approach would simultaneously increase the economic resilience and social sustainability of less favored rural areas. Legislative constraints surrounding plowing of long-term grassland (the land cover accounting for by far the greatest proportion of upland farms [11]) 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 [12], again requiring soil disturbance.

Our proof-of-principle study tested the feasibility of an innovative dual-cropping approach to producing plant-derived galanthamine based on integrating *N. pseudonarcissus* growing into existing marginal pasture and harvesting green above-ground

vegetative plant materials rather than bulbs. By cultivating the crop in marginal growing conditions, we seek to exploit the previously observed increase in endogenous galanthamine biosynthesis of *N. pseudonarcissus* grown under such conditions [10] but also to enhance biosynthesis further through the imposition of interspecies competition with forage grassland plants. 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 minimized, and iii) traditional farming systems within marginal areas are maintained, with their economic viability increased.

MATERIALS AND METHODS

Experimental design and plot preparation

Lines of *N. pseudonarcissus* cv. Carlton (size < 10; Grampian Growers, Montrose, UK) 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, were planted at three different intervals: 5 cm, 10 cm, and 15 cm apart. Triplicate lines of each treatment were organized into three separate blocks. Each line of *N. pseudonarcissus* 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, Rotherham, Staffordshire, UK). Bulbs were planted at the prescribed densities by hand, with the tops of the bulb the treatment distance apart.

Table 1

Location and soil details for the four experimental sites where *N. pseudonarcissus* was established. Harvesting occurred when the majority of the plants at a site reached the gooseneck 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 3°48'13.82"W	52°21'12.58"N 3°47'48.16"W	52°21'33.80"N 3°48'37.53"W	52°21'43.42"N 3°49'11.70"W
Exposure	Comparatively sheltered	Comparatively sheltered	Poor protection from wind	Exposed hilltop
<i>Soil analysis results</i>				
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

Measurements

A 500 g soil sample was collected for each site by bulking 10 soil cores collected at random between the lines of *N. pseudonarcissus*. Sampling of the *N. pseudonarcissus* 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 45° but were unopened. The number of *N. pseudonarcissus* plants growing was counted along a 6 m length in the center 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°C. A separate sub-sample of approximately 100 g was taken for subsequent analysis to determine alkaloid concentrations.

Alkaloid analysis

Leaf sections of approximately 100 mg FM were homogenized 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 h 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 × 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 analyzed using the Chrom Quest 5.0 HPLC database program (Thermo Fisher Scientific, Cramlington, UK).

Data analysis

Data were analyzed 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.

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 potassium and magnesium, but low or very low indices for phosphorus [13]. The plant counts prior to harvest showed over 80% of the bulbs had successfully established (Table 2), demonstrating that

Table 2
Effect of altitude of planting site and planting distance on the establishment, yield, and galanthamine (GAL) concentration of biomass harvested from *N. pseudonarcissus* cv Carlton at the gooseneck 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	0.92 ^b	0.70 ^a	0.93 ^b	0.029	ns	<0.001
FM yield (g m ⁻¹)	95 ^a	126 ^b	100 ^a	96 ^a	6.4	177 ^c	82 ^b	54 ^a	5.6	<0.001	<0.001
DM content (g kg ⁻¹ FM)	148 ^b	119 ^a	135 ^b	139 ^b	7.3	133	131	142	6.4	<0.05	ns
FM yield per bulb (g bulb ⁻¹)	6.7 ^a	9.5 ^b	7.4 ^a	7.2 ^a	0.50	8.8 ^b	6.2 ^a	8.1 ^b	0.43	<0.001	<0.001
DM yield (g DM m ⁻¹)	13.2	15.0	13.8	13.3	0.96	23.2 ^c	10.7 ^b	7.6 ^a	0.83	ns	<0.001
Leaf length (mm)	149 ^a	182 ^c	170 ^b	153 ^a	3.7	166	163	161	3.2	<0.001	ns
Stem length (mm)	172 ^a	212 ^c	192 ^b	190 ^b	4.4	196 ^b	192 ^{ab}	187 ^a	3.8	<0.001	<0.05
Flower length (mm)	61 ^a	63 ^b	65 ^c	67 ^d	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 $p < 0.05$. FM, fresh matter; DM, dry matter. No statistically significant altitude effect × planting distance effect interactions were found.

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 *N. pseudonarcissus* can vary between different varieties [14]. 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 [12]. Galanthamine concentrations in *N. pseudonarcissus* leaves have been found to be steady until flowering, before decreasing [15]. Although higher concentrations of alkaloids could potentially be obtained from 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 focused on bulbs [10], and higher than concentrations previously reported for above-ground *N. pseudonarcissus* biomass [16]. These findings are in keeping with the imposition of greater plant-plant competition when growing in grassland eliciting a greater stress response, but further research is required to verify this relationship. 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 [17]. Thus, overall, the results suggest that higher planting densities which would favor biomass yield would maximize 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.

In summary, this study has verified the feasibility of establishing *N. pseudonarcissus* 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 that maximize galanthamine yield.

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