Daffodil production for galanthamine

Report for Our Land and Water

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Summary

Galanthamine is a bioactive that has proven effect to reduce the effect of Alzheimer's. A source of natural galanthamine is from daffodils, but only certain varieties grown under specific conditions produce enough galanthamine to make it economically viable to extract for commercial purposes. Agroceutical Products, UK, have shown that this is possible in Wales. This project was to investigate the production of daffodil galanthamine grown on small areas on sheep farms in New Zealand.

Daffodils grown at sites in the South Island of New Zealand all produced significant levels of galanthamine. These levels exceeded small exploratory trials conducted in 2020 and 2021. The concentration per plant varied across the sites, and the size of the plants varied, but the relationship between plant size and Galanthamine concentration with altitude / soil characteristics still requires further elucidation. Extrapolating the results of the trials in terms of plant density, plant growth and Galanthamine concentration we estimate production levels would be in the range of 310gm to 400gm/ha.

Additionally, in the project micropropagation was investigated as a method to rapidly increase the number of bulbs available, as propagation could be a limitation on future commercialisation in New Zealand. A micro-propagation technique was investigated as a potential source of daffodil germplasm for future scale up. While successful, this technique will need further work to be commercially viable.

Based on this season's results further work should be undertaken to better understand how galanthamine levels vary by site and season and to commence developing business models and structures for a New Zealand industry.

Background

The production of galanthamine (so called when from natural sources, or galantamine when made synthetically) in daffodils for the pharmaceutical industry has been pioneered by Agroceutical Products Ltd UK, growing trial plots in Wales. Daffodils produce a range of bioactives, including galanthamine, which is an effective Alzheimer's drug. While production has been demonstrated in high country farming in Wales, there is a need for whole year production and New Zealand has been selected as the Southern Hemisphere counterpart to Welsh production. In 2020, Agroceutical, Ngāi Tahu and Lincoln University undertook a pilot project using small plots in the South Island high country to investigate the feasibility, with the pilot scale continued in 2021. The production levels in some sites reached the target concentration, confirming that daffodil farming in sheep country should be economically feasible. This project with Ngāi Tahu ceased in 2022 (partly due to staff changes and Covid impacts). The next step in developing daffodil farming in NZ as a sustainable (economically and environmentally) viable addition to pastoral farming is on-farm, in system evaluation from propagation and production through to harvesting and extraction of galantamine.

Agroceutical have identified cultivars of daffodil that produce higher levels of galanthamine in their conditions and these cultivars will form the basis of the work in this project. Although some plant material is available in NZ, the use of micropropagation or vegetative propagation will be required to produce the volume of bulbs required for future commercial scale plantings.

It is proposed to use the practices and machinery developed by Agroceutical Products in Wales with minor modifications for New Zealand farming systems. This approach means that the daffodils will be

sown into existing sheep pasture which will continue to be grazed. The daffodils biomass will be harvested once a year around early flowering and the crop will be harvested annually for a number of years, probably spelled for a year and then harvested for further years. The biomass will be fermented, the juice drained off and galanthamine extracted from the juice.

Goal

This nine month project aimed to:

1) confirm the compatibility with NZ farming systems;
2) provide an economic and environmental evaluation for production at scale;
3) identify any differences in galanthamine production by site and identify preferred sites;
4) identify any issues with the land use and compatibility with sheep farming;
5) adapt a micropropagation method for future commercial plants; and
6) provide information to move to establishment of a NZ company.

In alignment with the aims of OLW Rural Professionals Fund, this project will accelerate this innovative on-farm pharmaceutical production system, providing an alternate land use compatible with sheep farming, utilising poorer quality land, requiring no additional farm inputs, diversifying revenue streams and improving the resilience of farm businesses. Initial plot trial research underpinning this project was in cooperation with Ngāi Tahu farming and will now partner with Wakatū Incorporation, providing opportunity for uptake of the outcomes by Māori, as well as the wider farming community.

Methods

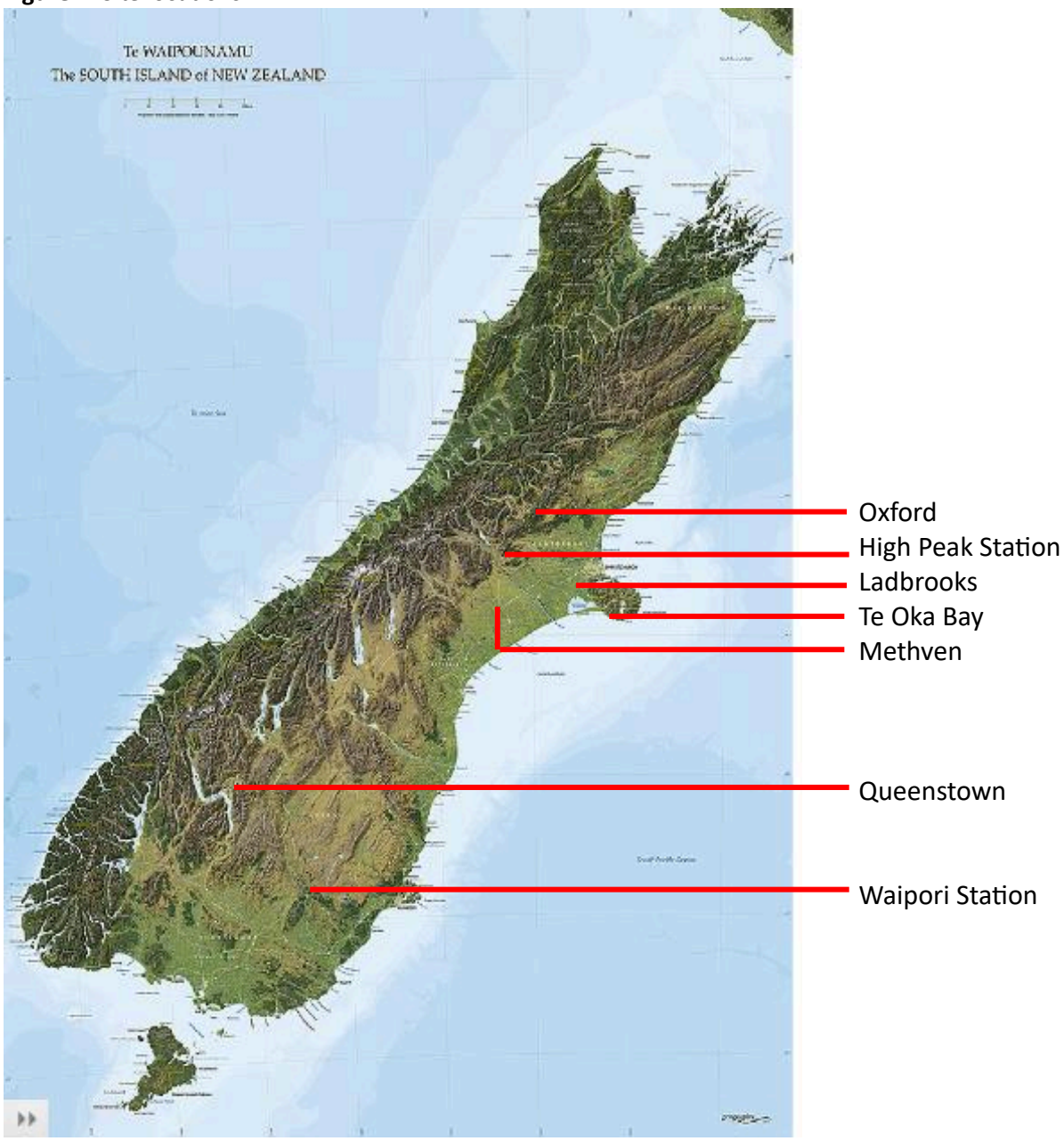
Bulbs

Three thousand daffodil bulbs were sourced from Bulbs Direct, a large bulb grower based between Auckland and Whangarei, at the time of purchase they were the only available source of a large quantity of the required variety of bulbs.

Sites

Seven sites across the South Island were selected for testing, with a range of altitudes, as high as 630 metres above sea level and including two sites close to sea level, one of which was a coastal site. The fertility of the sites also ranged from the high Olsen P of the cropping soils at Methven to the high country which typically has low fertiliser inputs.

Figure 1. *Site locations*



Planting

The technique for planting all sites except the very southern sites of Queenstown and Waipori Station was using a bulb auger run from a drill and generator. All sites had a minimum of 100 bulbs planted in a 1 m^2 configuration, the Methven site had a larger block of 10 m^2 planted. The High Peak Station and Oxford sites included rows of daffodils set at 70 cm apart which allowed yield to be assessed in a drill row scenario. The Queenstown and Waipori Station sites were planted by removing the layer of turf with a spade and planting 100 bulbs in the 1 m^2 area.

Table 1. *Summary of Site and Planting Details*

Site	Altitude	Fertility		Planting Details	
	m above sea level	pH	Olsen P	Planting Date	Planting Layout
Ladbrooks	8	6.5	33	18/04/23	100 bulbs planted in 1m^2
Te Oka Bay	6	6.2	9	2/05/23	100 bulbs planted in 1m^2
Methven	340	5.6	51	20/04/23	1000 bulbs planted in 10m^2
High Peak Station Site 1	450			28/04/23	100 bulbs planted in 1m^2
					3 x 50 rows of bulbs planted at 70 cm spacings
High Peak Station Site 2	490	5.5	15	28/04/23	100 bulbs planted in 1m^2
					3 x 50 rows of bulbs planted at 70 cm spacings
Oxford Site 1	345	5.7	8	21/04/23	100 bulbs planted in 1m^2
					3 x 50 rows of bulbs planted at 70 cm spacings
Oxford Site 1	395			21/04/23	100 bulbs planted in 1m^2
Queenstown	367	4.9	52	25/04/23	100 bulbs planted in 1m^2
Waipori Station 1	420	5.3	23	28/04/23	100 bulbs planted in 1m^2
Waipori Station 2	470	5.3	22	28/04/23	100 bulbs planted in 1m^2
Waipori Station 3	540	5.1	55	28/04/23	100 bulbs planted in 1m^2
Waipori Station 4	630	5.1	55	28/04/23	100 bulbs planted in 1m^2

Harvesting

The target harvest point was between 'Pencil Stage' and 'Goose-neck Stage'. Only a few plants were required for HPLC testing from the minimum of 100 planted at each site, making a reasonable window for sampling to achieve a sample of plants at this particular stage.

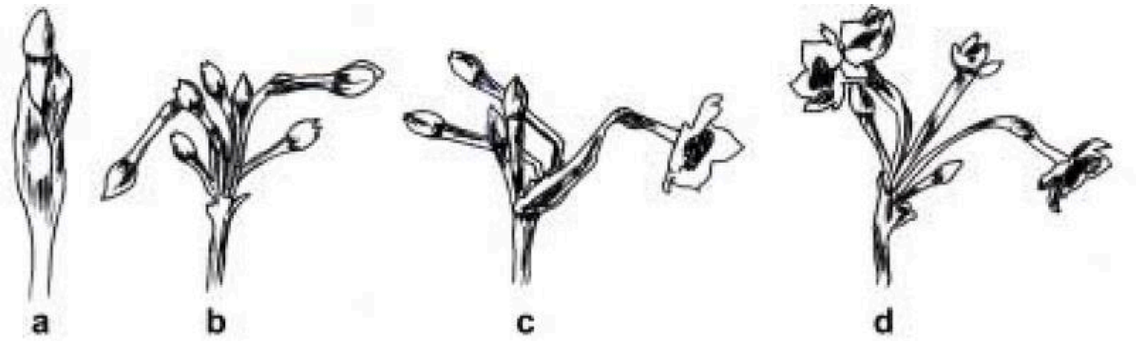


Figure 2. *Different harvesting stages, a) Pencil stage, b) Goose-neck stage, c) One floret open stage, d) Majority of florets open stage (from Jowkar and Kafi 2005).*

Table 2. *Summary of Harvesting Details*

Site	Harvesting Details	
	Harvesting Date	Plant Stage at Harvest
Ladbrooks	16/08/23	10 whole plants sampled between pencil and goose-neck stage
Te Oka Bay	15/08/23	10 whole plants sampled between pencil and goose-neck stage
Methven	4/09/23	10 whole plants sampled between pencil and goose-neck stage
	4/09/23	Bulk leaf sample taken to ground level (~200 grams)
High Peak Station Site 1	4/09/23	10 whole plants sampled between pencil and goose-neck stage
	4/09/23	Bulk leaf sample taken to ground level (~200 grams)
High Peak Station Site 2	4/09/23	10 whole plants sampled between pencil and goose-neck stage
	4/09/23	Bulk leaf sample taken to ground level (~200 grams)
Oxford Site 1	5/09/23	10 whole plants sampled between pencil and goose-neck stage
	5/09/23	Bulk leaf sample taken to ground level (~200 grams)
Oxford Site 1	5/09/23	10 whole plants sampled between pencil and goose-neck stage
Queenstown	11/09/23	10 whole plants sampled between pencil and goose-neck stage
Waipori Station 1		10 whole plants sampled between pencil and goose-neck stage
Waipori Station 2		10 whole plants sampled between pencil and goose-neck stage
Waipori Station 3		10 whole plants sampled between pencil and goose-neck stage
Waipori Station 4		10 whole plants sampled between pencil and goose-neck stage

Daffodil actives extraction for testing

Steps in extraction of bioactives from daffodils for testing for galanthamine levels are shown in Figure 3.

Figure 3. *Steps followed in the methanol extraction of galanthamine from daffodil leaves.*

Daffodils samples left for 15 min to reach room temperature (A), Cutting of bulbs from leaf (B) Weighing of the leaf part (C) Cutting of Leaf section (D) Weighing of leaf section (E) Crushing of leaf section with methanol using plastic pestle (F) Crushed leaf with methanol in sonicator (G) Methanol extracts after 5 hrs in sonicator (H) Methanol extracts after spinning ready for evaporation (I)

HPLC testing for galanthamine

For HPLC, 500 µL lHPLC mobile phase A (DI water + 0.2% TFA) was added into the extract HPLC vial, and the vial capped. The vials were vortexed and sonicated for 5 min, then vortexed again. For HPLC analysis, a 150X 4.6 mm, C18 column, 3µ ACE-111-1546, (Winlab, Scotland) was used and the column temperature was 40°C. The mobile phase was: A: 0.2% Trifluoroacetic Acid (TFA) in DI water; B: 100% Acetonitrile. A Flow rate of: 0.8 ml/min was used.

The Solvent Gradient was:

Time (min)	Solvent A(%)	Solvent B(%)
0	90	10
14	60	40
15	90	10
20	90	10

1. Injector: 10.0 µl injection with needle wash at vial 91, thermo state kept at 4°C.
2. Detector: UV detector set at 298nm and 215nm, no reference
3. Scan store from 200nm to 400nm.
4. Limit of Quantification: 1.35mM at 215nm, 2.5mM at 298nm
5. Limit of Detection: 0.3mM at 215nm, 1.5mM at 298nm
6. Quantification used 215 nm.

To calculate the amount of galanthamine in each plant sample, the µM concentration of the 500 µl methanol sample analysed by HPLC was multiplied by the molecular weight of galanthamine (287.36) and volume of solvent (1 ml total), then adjusted for per L and 100 mg of starting material.

Statistical analysis

ANOVA was conducted on galanthamine (mg) per plant, comparing across the sites using Genstat 22nd edition.

Micropropagation of daffodils

Micropropagation of daffodils were done from April to October 2023 at Lincoln University. The aim of the study was to develop cost-effective methodology to vegetatively propagate daffodils. Dry bulbs were obtained from a commercial garden nursery. The variety of the daffodils was unknown.

The protocol by Ferdausi et al. (2020) was followed in the succeeding experiments. The experiments were initially done at the Bioprotection Research Center laboratory and later at the newly built Waimarie Research Center.

Results

Galanthamine concentrations

Daffodils grown across the South Island all had detectable levels of galanthamine in the subsamples (Figure 4). At most sites, the concentration in daffodil leaves exceeded the results from trials in 2021 and 2023. The exception was Leeston, which was not the variety planted at all other sites. The highest concentration was at High Peak at 490 m above sea level.

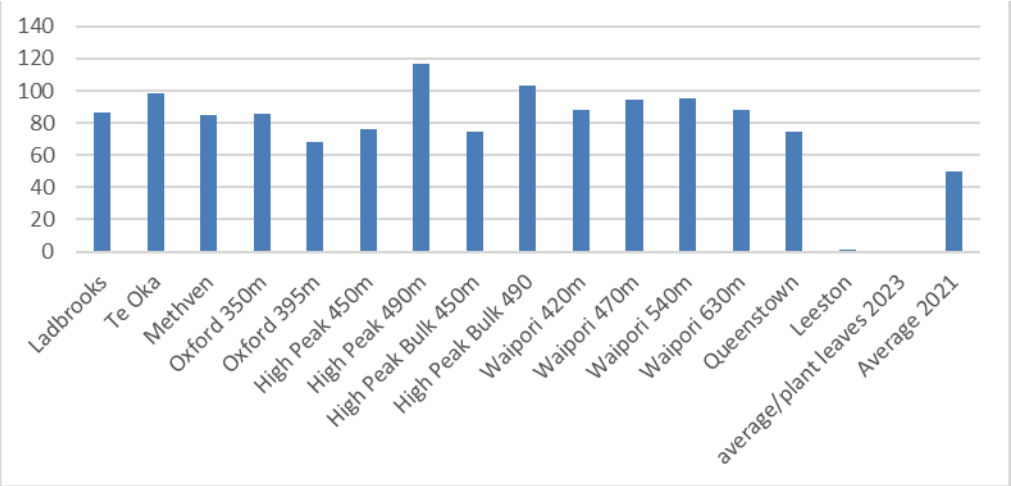


Figure 4. *Average galanthamine concentration (µM) in leaves of daffodils at each site. Average results from 2021 and 2023 are shown on the right for comparison.*

When adjusted for the weight of total above ground foliage, the Waipori (540 m asl) site had the highest total per plant (Figure 5). In general, the average per plant was higher in 2023 than in 2021 or 2020 (Figure 5).

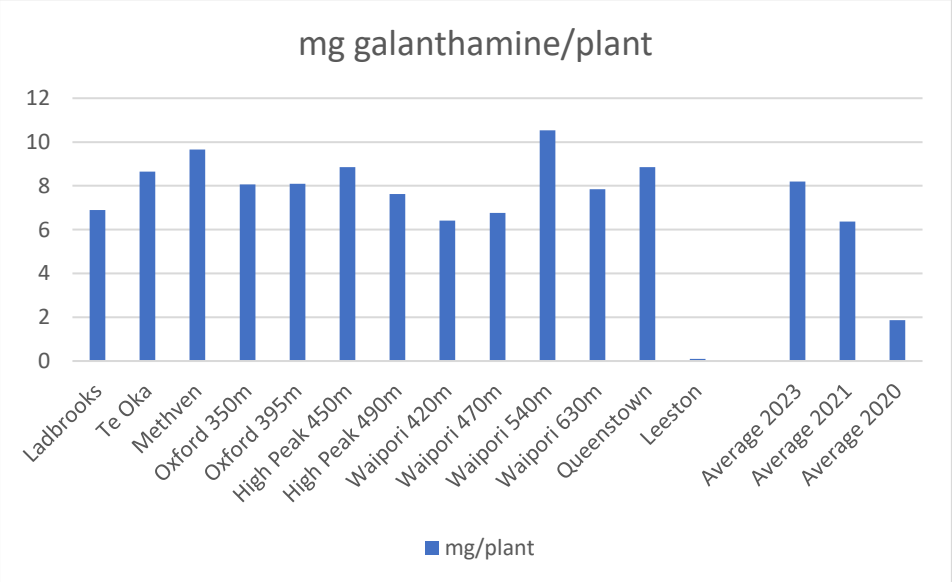


Figure 5. *Comparison of the average galanthamine content (mg) in total above ground foliage per plant at each site. Average across the sites is shown on the right, together with previous years samples.*

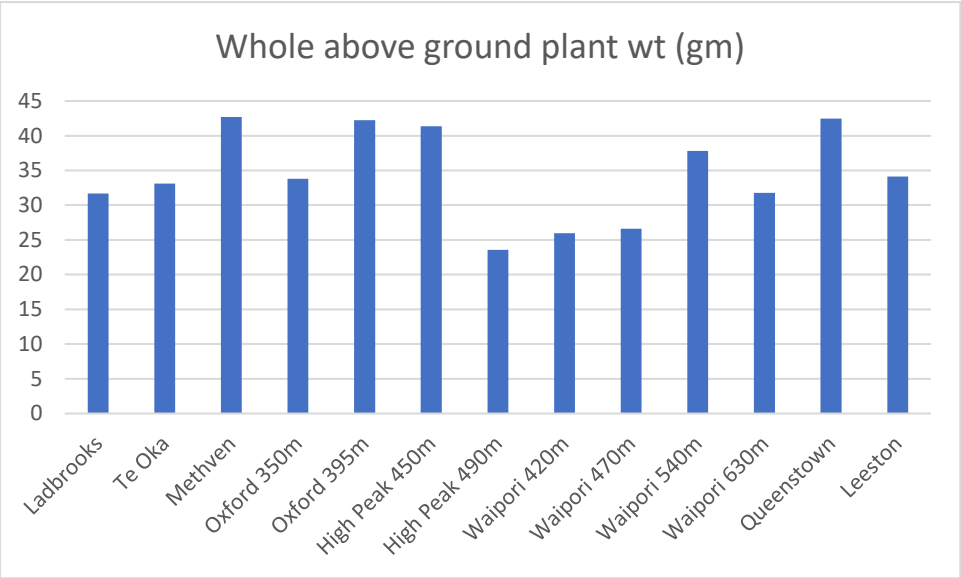


Figure 6. *Average weight of above ground foliage per pant at each site*

Daffodil yields

Yield assessments from spaced drill rows, to better simulate a paddock scale situation, yielded between 438 and 622 kg DM/ha from the High Peak and Oxford sites. A yield assessment was taken from the Methven site to show the yield potential from the higher sowing rate of 100 bulbs per m^2, as expected, the yield recorded was significantly higher from the higher sowing rate of bulbs.

Table 4. *Daffodil yields*

Site	Planting Layout	Dry Matter %	Yield extrapolated to kgDM/ha
Methven	1m2 harvested to ground level from 1000 bulbs planted in $10m^2$	10.8	2886
High Peak Station Site 1	70 cm x 3 drill rows harvested from 3 x 50 rows of bulbs planted at 70 cm spacings	12.0	438
High Peak Station Site 2	70 cm x 3 drill rows harvested from 3 x 50 rows of bulbs planted at 70 cm spacings	14.7	382
Oxford Site 1	70 cm x 3 drill rows harvested from 3 x 50 rows of bulbs planted at 70 cm spacings	11.6	622

Using the percentage of galanthamine in plants (Figure 7) and data in Table 4, yields could be expected to be in the range of 0.31 to 0.44 kg/ ha.

Correlation between weight of plant and galanthamine

Plants grew bigger at some sites (Figure 6), but there was little correlation between plant wet weight and concentration of galanthamine (Figure 7).

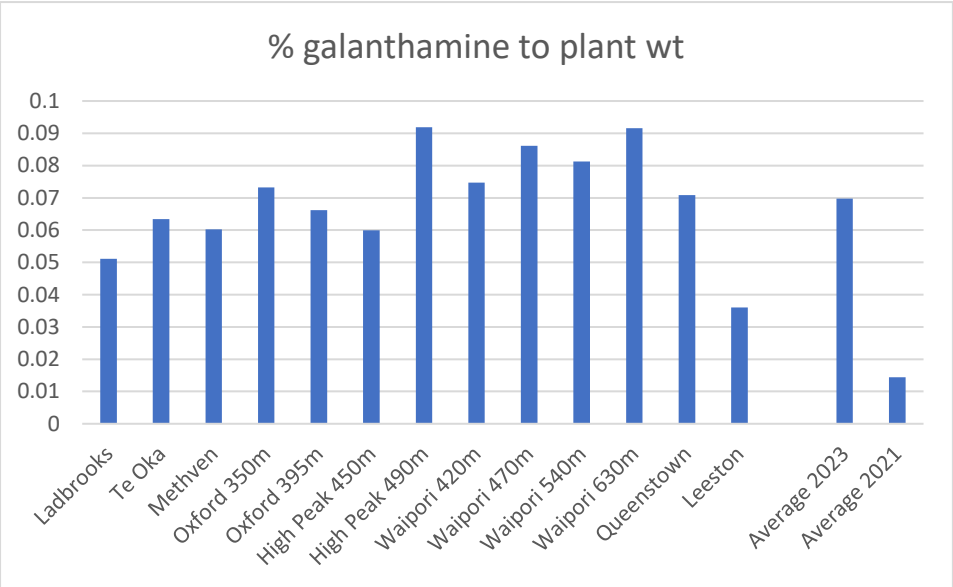


Figure 7. *Percentage of galanthamine per plant (wt:wt), average for each site*

The percentage of galanthamine per gm of plant foliage was higher in all samples than in 2021, averaging 0.07% compared to 0.0144 in 2021. (Figure 7).

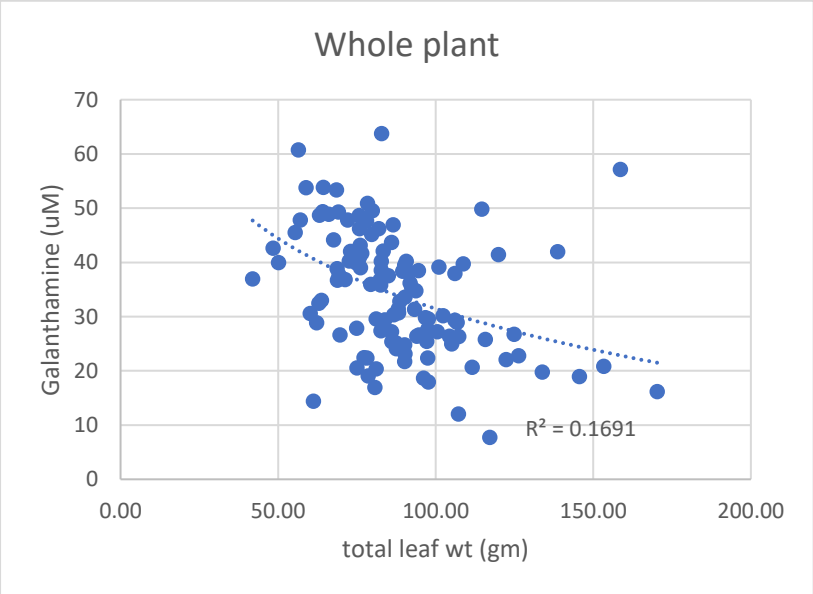


Figure 8. *Levels of galanthamine compared to total leaf weight for all plants*

Statistically the amount of galanthamine per plant differed significantly between sites (Table 3), although the Leeston site contributed strongly to the significance. When Leeston and 2 Waipori sites were removed (Waipori 540 m and 630 m had less samples than the others), the significance level was p=0.032 (Table 3), indicating some significant variation between the remaining sites.

Table 3. *ANOVA analyses*

ANOVA using all sites

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	462.06	12	38.504	5.1126	1.07E-06	1.8437
Within Groups	805.84	107	7.5312			
Total	1267.90	119				

ANOVA without 2 Waipori sites or Leeston

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	102.4969	9	11.38855	2.154478	0.032649	1.985595
Within Groups	475.739	90	5.285989			
Total	578.2359	99				

There was no relationship between altitude (Figure 9), pH or soil phosphate levels and galanthamine concentrations in the leaf or galanthamine production per plant. Within the samples from above 300 m there was a non- significant trend to an increase in galanthamine levels as altitude increased (r= 0.45).

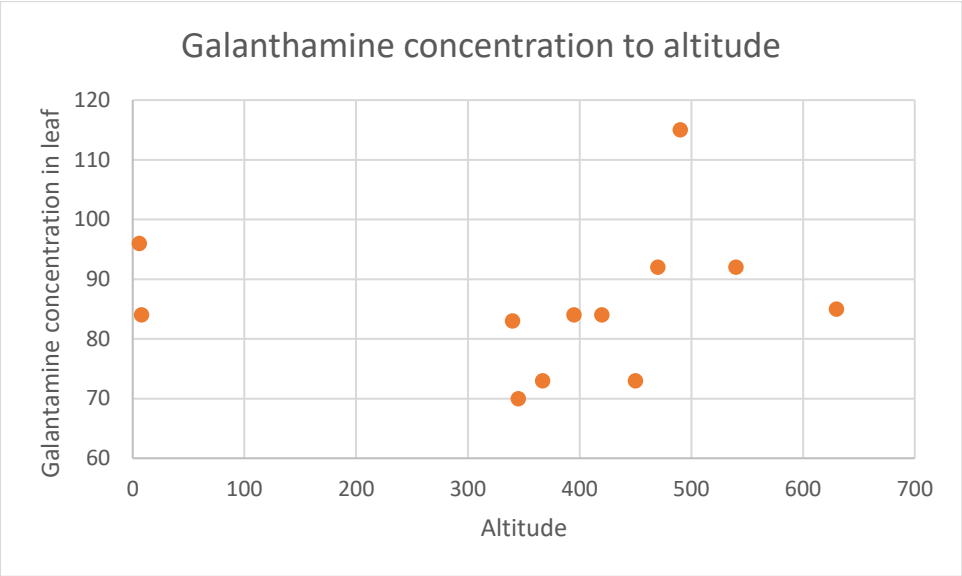


Figure 9: *Relationship between altitude and galanthamine concentrations in the leaf.*

Micropropagation of daffodils

The summary of results from three experiments is presented in Table 1. The number of fungal contaminations were reduced following the advice of Prof Xianmin Chang, Royal Agricultural University. Cross contamination between explants occurred because there were five explants cultured in a container. Experiments conducted from April to June had very high contamination and poor regeneration. Improvement in the regeneration and contamination levels was attained again following the advice and recommendation of Prof Xianmin Chang (personal communications July 19, 2023).

There were 18 total regenerants from the 3 experiments with shoots and shoots+ roots. Eleven (11) were sub cultured into fresh MS+low NAA (10 to 12 weeks after culture). A week after subculture, seven (15.5%) produced bulblets, 1 (2.2%) had shoot + roots and 3 (6.7%) with roots only.

Table 4. *Results of micropropagation of daffodils*

Culture media	Date cultured	Total number explants	Total number of contamination	Total number regenerants		
				# shoots	# Roots	# Shoots+ Roots
MS + high NAA*	8/08/2023	15	7 (47%)	3 (20%)	5 (33.33%)	
MS w/o sucrose	15/08/2023	15	10 (66%)	5 (33.33%)		
MS + low NAA*	23/08/2023	15	5 (33%)	6 (40%)		4 (26.67%)
Percent contamination was computed as # explants with contamination/total explants cultured x 100.						
Per cent regeneration = # of regenerants/total explants cultured x 100.						
Percent bulblets = # bulblets produced/total explants from 3 experiments x 100						
*Ferdausi et al 2020						

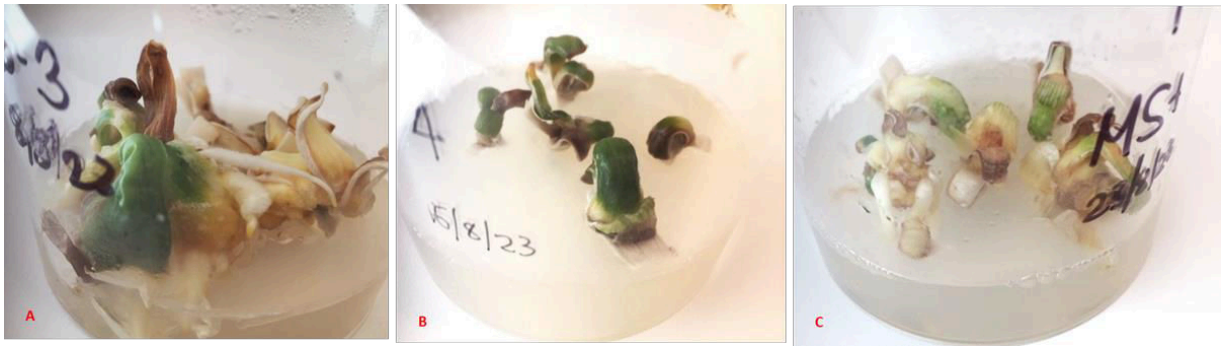


Figure 9. *Daffodil explants showing shoot and root (A and B) in MS+ low NAA and shoot regeneration (C) in MS without sucrose [8 weeks after culture (A), 7 weeks after culture (B) and 6 weeks after culture (C)]*

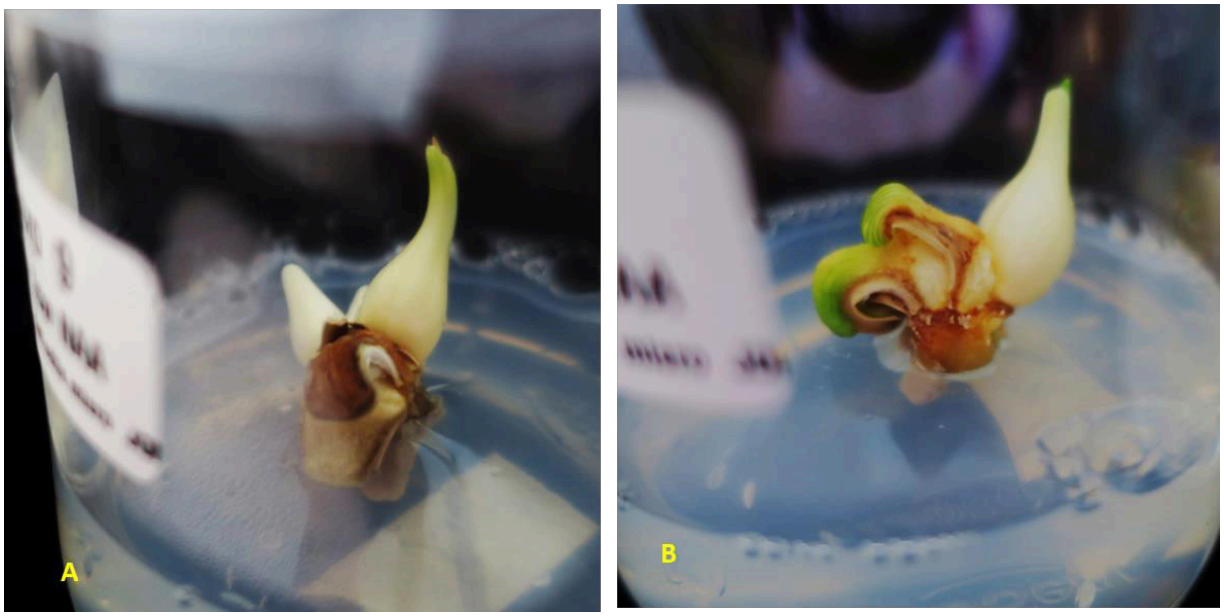


Figure 10. *Double (A) and single bulblet(s) 1 week after subculture (11 to 13 weeks after culture) in MS+ low NAA medium.*

Conclusions

The project aimed to 1) confirm the compatibility with NZ farming systems; 2) provide an economic and environmental evaluation for production at scale; 3) identify any differences in galanthamine production by site and identify preferred sites; 4) identify any issues with the land use and compatibility with sheep farming; 5) adapt a micropropagation method for future commercial plants; and 6) provide information to move to establishment of a NZ company.

1. The work has shown that daffodil production is compatible with NZ farming systems. In this work bulbs were planted in working high country sheep and beef farms, a foothills farm and a mixed cropping farm. At all sites the bulbs were compatible with the farm operation, however if cattle were being grazed they would need to be excluded from daffodil planting throughout the spring and any times were trampling could damage bulbs. Daffodil production may also be compatible with other farm systems such as grapes.
2. The levels of galanthamine produced were higher than in the previous years preliminary investigation. The results indicate levels in the range of 0.31 to 0.44 kg/ ha. The retail value of this level of galanthamine indicate that the returns to farmers should be high enough for daffodil growing to be integrated within pasture used for sheep grazing in harsh environments or on poor soils. Thus, any revenue from daffodils is expected to be primarily additional to the main land use. The environmental impacts of daffodil production are expected to be minimal as the bulbs can be planted with minimal soil disturbance and no other inputs are required while crops are expected to survive for a number of years.
3. The galanthamine levels were high at all sites. The higher levels appeared to be associated with higher stress sites which were colder or exposed if near sea level but there was no clear relationship with altitude, soil pH or phosphate levels. Further work is required to better determine any relationships between site and galanthamine levels.
4. This work did not identify any issues of compatibility between sheep farming and galanthamine production. If the daffodils are not harvested every year the unharvested areas in exposed paddocks may provide some protection particularly for ewes and lambs during lambing.
5. Micropropagation of daffodils was successful. However, further refinement of methods is required if large numbers of bulbs are needed to scale up galanthamine production.
6. The results from the last season indicate there is potential for galanthamine production in New Zealand. It is proposed to form an entity to further develop a business plan and the next steps for R&D in New Zealand. The entity will be designed with a business model that delivers the value as directly back to the farmers as possible. Examples of business models that are doing this, such as Lumina Lamb, are being reviewed to define their applicability for the new entity.

Next Steps

The results indicate that further work is required to understand the relationship between Genetics x Environment and Galanthamine levels. As daffodils store significant quantities of metabolites in the bulbs, then it is possible that environmental effects (e.g. micro-nutrient availability) are mitigated by the plant's stores laid down in previous growing conditions. To this end it would be both instructive and important from a commercialisation perspective to understand this relationship better by examining the alkaloid levels of the daffodils at these trial plots over multiple growing seasons.

The Micro-propagation system for multiplying germplasm for scale up will require further development work to provide an underpinning resource for the development of the commercial daffodil alkaloid industry in New Zealand.

In addition, the information secured from this season's trials should be further analysed and used as the basis for a commercialisation model for establishing daffodil alkaloid production as a new industry for New Zealand Agriculture.

References

Ferdausi, A., Chang, X., Hall, A. and Jones, M. (2020) Galanthamine production in tissue culture and metabolomic study on Amaryllidaceae alkaloids in *Narcissus pseudonarcissus* cv. Carlton. Industrial Crops and Products, 144, 112058. https://doi.org/10.1016/j.indcrop.2019.112058

Jowkar, M.M and Kafi, M. (2005) Effects of harvesting stages, 8-hydroxyquinoline citrate, silver thiosulphate, silver nitrate on the postharvest life of cut *Narcissus tazetta*. Acta Horticulturae 669 (669): 405-409 DOI: 10.17660/ActaHortic.2005.669.53