Materials and Methods

Plant material

Bulbs of *Narcissus pseudonarcissus* cv. 'Carlton' were sourced from the Netherlands and supplied by Mark A. Vandervielt (New Generation Daffodils, Devon). Bulb stocks were retarded by warm storage throughout the year, as required, and vernalised in batches by dry cooling process of typically 10-16 weeks. Dry-cooling of bulbs was carried out at 9°C in a well ventilated cold temperature store, under darkness. Vernalised bulbs were held until required at temperatures of 2-5°C in a well ventilated cold room, under darkness. Prior to forcing, vernalised bulbs were rooted for 2-4 weeks in nutrient solution at 2-5°C, under dim lighting for 8hrs daily.

Trials were carried out throughout the year at Bangor University or at Henfeas Research Centre at Abergwyngregyn, Wales. Plants were grown in greenhouses under ambient lighting supplemented with 12 hr photoperiod provided by metal halide lamps, where necessary. Day-time temperatures were maintained at a minimum of 15°C. Shading and ventilation were adjusted throughout the year, as required. Alternatively plants were grown in controlled environment growth chamber under fluorescent lighting at 100μE m^{-2} s^{-1} with 12 hour photoperiod, day temperature of 17-20°C and night temperature of 14-16°C.

Hydroponic forcing

Bulbs were forced hydroponically, under greenhouse or growth room conditions, unless stated otherwise. Plants were grown in a custom-made hydroponic system. Hydroponic lids were constructed from sheets of polystyrene (5cm thickness, with 5cm diameter holes) and black plastic sheeting (3mm thickness, with 3cm diameter holes). Polypropylene plastic containers (15 or 30 L capacity) were used as hydroponic tanks. Bulbs were individually planted into holes in the hydroponic lids which remained suspended over the hydroponic tanks, which contained aerated nutrient solution. Aeration was provided by 40-120 L/min high-output air infusion pumps (Charles Austen Pumps Ltd, Byfleet UK) connected via a 12-way manifold to silicon tubing and ceramic airstones. Fresh water was pumped from cisterns into hydroponic tanks using a 120 L/min submersible water pump (Draper Tools Ltd, Chandler's Ford UK) and waste water was removed from tanks using 800W or 1200 W self-priming electric pumps (Clarke International, Epping UK).

The standard nutrient solution contained the following (in mg per litre): $Ca(NO_3)_2{\cdot}4H_2O$ (472.3), $NH_4H_2PO_4$ (115.0), KNO_3 (303.3), $MgSO_4{\cdot}7H_2O$ (123.2), NaFeEDTA (18.35), KCl (3.73), H_3BO_3 (1.55), $MnSO_4{\cdot}4H_2O$

(0.45), $ZnSO_4 \cdot 7H_2O$ (0.58), $CuSO_4 \cdot 5H_2O$ (0.12) and H_2MoO_4 (0.08). Elemental composition of the nutrient solution was as follows (in mg per litre or ppm) N (112), K (117), Ca (80), P (31), S (16), Mg (12) and Fe (2.80), Cl (1.77), Mn (0.11), B (0.27), Cu (0.03), Zn (0.13), Mo (0.05). Hydroponic solutions were made fresh from concentrated salt solutions diluted in tap water. Concentrated solutions were prepared as separate stock solutions, to avoid salt precipitation. The pH of the hydroponic solution was measured every 2-3 days using a portable pH probe and EC meter (Bluelab Corporation Ltd, Tauranga, New Zealand) and adjusted to pH 6.0-6.5 using solutions of concentrated KOH or HCl, unless stated otherwise. The solution was replenished weekly during the experimental growth period.

Sampling

Plants were weighed and measured upon sampling and transported in slotted polythene bags stored in large cardboard boxes, delivered to Agroceutical Products Ltd or stored in-house for analysis. Shoot, bulb and root fresh weight of plants was measured prior to tissue sampling. Two transverse sections of bulb tissue were taken using a cork borer (diameter 1cm). Bulb and leaf samples were stored at -20 °C until required. Bulb tissue was lyophilised in a freeze-drier and ground into a fine powder using a pestle and mortar. Shoot tissue was ground into powder under liquid nitrogen using a pestle and mortar and lyophilised in a freeze-drier. Ground and dried plant tissues was kept in sealed polythene bags and stored at -20 °C.

Experimental Trials

Anaerobic

Plants were grown hydroponically in non-aerated (anaerobic treatment) nutrient solution. Untreated controls were grown in vigorously aerated nutrient solution (oxygenated/aerobic control).

Chitosan

Plants were grown hydroponically and treated with foliar application of solutions containing chitosan. KaitoSol® (Agentra Ltd., Northampton UK) containing biologically active chitosan (12.5g/L) was diluted in water to final working concentrations containing 50 or 100 mg/L chitosan, and applied as foliar spray until run-off. Untreated controls were treated with water alone. Treatments were carried out weekly during the growth period.

Cold

Plant were grown hydroponically in a controlled environment growth room and treated to repeated and intermittent exposure to cold stress. Cold stress was applied independently to shoots or roots, or in combination. For cold root treatments, the hydroponic nutrient solution was cooled to 1-3°C for 20 h daily using a refrigerated circulating water bath (Grant Instruments, Shepreth UK). For cold shoot treatments (0-1°C), ice was placed around growing shoots for 8 hours daily. For control treatments, root temperatures were maintained at 14-16°C and shoots at 17-20°C. Cold stress treatments were repeated twice weekly, on alternate days.

Drought

Plants were grown in 3 L pots in trays in a controlled environment growth room and gradually exposed to conditions of drought stress by withholding watering. Bulbs were planted in perlite/vermiculite media (1:1) which was saturated with nutrient solution (100% saturation point) or dried down to 50% saturation point. For drought regimes 1 and 2, the media was allowed to dry out naturally during the growth period from initial saturation of 100% and 50%, respectively. For untreated controls, trays were topped up regularly with water and the media remained saturated throughout the duration of the experiment.

Herbicides

Plants were grown hydroponically and treated with sub-lethal doses of glyphosate and rimsulfuron (Sigma-Aldrich). Prior to planting, bulb roots were imbibed for 48 hrs in nutrient solution containing glyphosate or rimsulfuron at concentrations of 2, 10, 50 µM. Plants were then forced hydroponically and treated weekly with foliar application of glyphosate and rimsulfuron at 2, 10, 50 µM in 0.1% Triton X-100, until run-off. Glyphosate (N-(phosphonomethyl)glycine) is broad-spectrum systemic weedkiller acting through inhibition of enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Rimsulfuron (N-[(4,6-dimethoxypyrimidin-2-yl)carbamoyl]-3-(ethanesulfonyl)pyridine-2-sulfonamide) is a systemic herbicide which acts by inhibition of acetolactate synthase (ALS).

Jasmonates

Concentrated solutions of methyl-jasmonate (MeJ), cis-jasmone (CisJ) and jasmonic acid (JA) (Sigma-Aldrich) were solubilised in EtOH and diluted in 0.1% Triton-X100 to final concentrations of 50µM, 500µM and 5mM. Solutions were applied as foliar sprays using a hand sprayer, until run-off. Untreated controls were treated with an equivalent volume of EtOH diluted in 0.1% Triton-X100. For hydroponic treatment, MeJ was solubilised in EtOH and added directly to the hydroponic solution at final concentrations of 25µM

or 250µM. Untreated plants and controls were treated with an equivalent volume of EtOH added to the hydroponic solution. Immediately after treatments, plants were wrapped in polythene bags for 24hrs, after which the bags were removed under well-ventilated conditions. Treatments were carried out once weekly during the forcing period.

Metals

Plants were grown hydroponically in nutrient solutions containing elevated concentrations of aluminium and manganese. For aluminium toxicity trials, plants were grown in nutrient solution supplemented with 500µM $CaCl_2$ and 50 µM $AlCl_3$, at pH 4.5. For untreated controls, $AlCl_3$ was omitted from the solution. For manganese toxicity trials, plants were grown in nutrient solution supplemented with 50µM $MnSO_4 \cdot 4H_2O$ and untreated controls were grown in standard nutrient solution, containing 2µM $MnSO_4 \cdot 4H_2O$.

Nutrients

Treatments were started mid-way through the vernalisation process and continued through to hydroponic forcing in the greenhouse. During the vernalisation component, bulbs were planted in cell trays and rooted in the various nutrient solutions at 9°C, under dim lighting for 8 hr daily. The standard (control) nutrient solution containing the following macronutrients (in mg per litre): $Ca(NO_3)_2 \cdot 4H_2O$ (590.4), $NH_4H_2PO_4$ (115.0), KNO_3 (202.2), $MgSO_4 \cdot 7H_2O$ (493.0), K_2SO_4 (174.3), with micronutrients as standard. The N, P and K content of the solution was modified according to experimental requirements, as detailed below. Sulphur (S) nutrient content was used to a degree of freedom. For nutrient deficient treatments, plants were grown in water alone. The double nutrient treatment contained twice the concentration of macro and micronutrients as the standard solution (control).

Nitrogen

The available nitrogen (N) content of the hydroponic nutrient solution was modified to contain 0, 8 or 16 mM total nitrogen. The standard nutrient solution contained 8mM total N. The nitrogen deficient solution (0mM N) was altered to contain (in mg per litre) $CaCl_2$ (277.5), K_2SO_4 (261.4) and KH_2PO_4 (136.1), whilst KNO_3, $NH_4H_2PO_4$ and $Ca(NO_3)_2 \cdot 4H_2O$ were omitted. For high nitrogen (16mM) treatment, the solution was altered to contain KNO_3 (404.4), $MgNO_3$ (512.8) and NH_4NO_3 (80.1), whilst $MgSO_4$ and K_2SO_4 were omitted. The supplied ammonium ($NH4^+$) to nitrate (NO_3^-) ratio was equivalent between treatments. A control treatment was carried out at 8mM N containing an equivalent Cl concentration as the 0mM treatment.

Phosphorus

The available phosphorus (P) content of the hydroponic nutrient solution was modified to contain 0, 1 or 3 mM total phosphate. The standard nutrient solution contained 1mM total P. The P deficient (0mM) solution was altered to contain KNO_3 (101.1), K_2SO_4 (261.4) and NH_4NO_4 (80.1), whilst $NH_4H_2PO_4$ was omitted. For high P treatment (3mM), the solution was altered to contain KH_2PO_4 (272.2) and K_2SO_4 was omitted.

Potassium

The available potassium (K) content of the hydroponic nutrient solution was modified to contain 0, 4 or 8 mM total K. The standard nutrient solution contained 4mM total K. For K deficient solution (0mM) the solution was altered to contain $MgSO_4 \cdot 7H_2O$ (246.5) and $MgNO_3$ (256.4), whilst KNO_3 and K_2SO_4 were omitted. For high K (8mM) treatment, the solution was altered to contain K_2SO_4 (522.8).

Ozone

Plants were grown in compost in 5L pots and exposed to elevated ozone in hemispherical solardomes (2m tall and 3m high) at the CEH Bangor Air Pollution Facility, North Wales, UK. Ozone supplied to the domes was produced from concentrated atmospheric oxygen (Workhorse 8, Dryden Aqua, UK) using a G11 ozone generator (Dryden Aqua) and supplied to each dome via polytetrafluoroethylene (PTEE) tubing. The solardomes were vented at a rate of two air changes per minute with charcoal filtered air injected with discrete levels of ozone according to the ozone profile (mean weekly target concentrations were 21, 28, 35, 42, 49, 56, 63 and 70 ppb). Computer software (LABVIEW version 7, Austin, TX, USA) determined ozone delivery to the solardomes via mass flow controllers (Celerion, Dublin, Ireland). Above canopy ozone concentrations were measured for 5 minutes in every 30 minutes in 7 of the solardomes using two ozone analysers (Model API 4ooA; Enviotech, St Albans, UK) of matched calibration. Ozone concentrations in one dome were continuously monitored using a dedicated ozone analyser (Thermoelectron, Model 49C, Reading, UK) and used for feedback control. If ozone concentrations exceeded target values by more than 20ppb, an automatic cut-off solenoid was triggered to preventing over-dosing. Ozone profiles used were based on current upland background concentrations of approximately 42ppb with incremental decreases and incremental increases superimposed on this background level.

pH

Plant were grown hydroponically and the pH of the nutrient solution was adjusted and maintained at pH 5, 6, 6.5, 7 or 8. Treatments were started mid-way through the vernalisation process and continued

through to hydroponic forcing in the growth room. During the vernalisation component, bulbs were planted in cell trays and rooted in the nutrient solutions at 9°C, under dim lighting for 8 hr daily.

Salicylates

Plant were grown hydroponically and treated with salicylic acid (SA) or methyl salicylate (meS). A concentrated solution of MeS (Sigma-Aldrich) was solubilised in EtOH and diluted in 0.1% Triton-X100 to a final concentration of 500µM or 5mM. SA and MeS solutions were applied as foliar sprays using a hand sprayer, until run-off. Untreated controls were treated with an equivalent volume of EtOH diluted in 0.1% Triton-X100. For hydroponic treatment, SA was added to the hydroponic solution at final concentrations of 100µM or 1mM. Immediately after treatment, plants were wrapped in polythene bags for 24hrs, after which the bags were removed under well-ventilated conditions. Treatments were carried out once weekly during the forcing period.

Salinity

Plants were grown hydroponically in nutrient solution supplemented with 50 mM or 100 mM NaCl to induce salinity stress. Salinity was increased daily in increments of 25 mM NaCl until the desired concentration was obtained.

UV-B

Plants were grown hydroponically and exposed to varying levels of UV-B irradiation. UV light experiments were conducted in the greenhouse under conditions of ambient lighting supplemented with artificial UV light. Supplemental UV light was provided by TL20W/12 RS SLV UV-B broadband TL lamps (Philips Ltd, Guildford UK) powered by 240V 18-28W magnetic ballasts (MGC Lighting, Ipswich UK) suspended 65 cm above the plants. UV broadband lamps emitted light of UV-A (315-400nm), UV-B (280-315nm) and UV-C (100-280nm) wavelengths. The light source was filtered through layers (100-1000 µm total thickness) of cellulose acetate QW200 Ecomex® to attenuate UV-B irradiation levels, or through polyester PR172 Polymex® (100 µm thickness) to remove UV-B; transmission of UV-C light from the light source was prevented by both filter types (PSG Group Ltd, London UK). Supplemental UV light was provided by square-wave approach for 6 hours daily, centred on noon. Light filters were replaced between experimental trials and UV irradiance was routinely measured using a USB2000+UV-VIS-ES calibrated spectrophotometer (25µm slit, 1.5nm resolution) coupled with a 600µm UV-VIS fibre optic fitted with a CC-3-UV-S cosine corrected irradiance probe (Ocean Optics, Dunedin, Florida USA) and units given as Wm^{-2}. A low background level of UV-B radiation penetrated into the greenhouse and was typically in the range

of 0.05-0.1 Wm^{-2}. Both UV-A and UV-B light transmittance into the greenhouse varied depending on time of day and environmental conditions. UV-rated protective gloves, face-shield and overalls were worn at all times when working with the UV light source(s).

Wounding

Plants were grown hydroponically and treated repeatedly to wounding stress stimuli. For bulb wounding treatment, two transverse sections of bulb tissue were removed using a 5mm diameter stainless steel cork borer, avoiding the bulb central tissue containing the dormant flower. For leaf crushing treatment, leaves were crushed using a hemostat pressed perpendicular into the plane of the leaf, repeated along the length of each leaf. For leaf cropping treatment, leaf tissue was removed by hand, leaving 10cm of leaf length. For leaf wounding treatment, a stainless steel hole puncher was used to excise 3 mm diameter leaf discs at 5 cm intervals along the centre of each leaf. Immediately after all treatments, plants were wrapped in polythene bags for 24hrs, after which the bags were removed under well-ventilated conditions. All wounding treatments were carried out twice weekly throughout the forcing period, except bulb wounding which was carried out once at pre-planting.

Galanthamine extraction

Free alkaloids were extracted and quantified from dried samples by solvent extraction method. Briefly, 100mg dried sample was extracted in 2ml methanol basified with ammonia solution (pH8), for 2 hrs at room temperature with shaking. The sample was then centrifuged at 13,000 rpm for 10 mins. The supernatant was collected and centrifuged for a further 5 mins. The supernatant was collected and the internal standard, papaverine hydrochloride (Sigma-Aldrich), was added to the sample at a final concentration of 125 µg/ml and mixed thoroughly. Samples were stored in HPLC/GC vials (Chromasolv) until analysis by gas chromatography mass spectrometry (GC-MS) or flame-ionisation detector (GC-FID). GC-MS was run on a Clarus 680 gas chromatograph coupled with a Clarus 600C mass spectrometer. GC-FID was carried out with Autosystem XL gas chromatograph (Perkin Elmer, Waltham, Massachusetts USA). Samples were run on a VF-5MS 30 x 0.25 x 0.25 column (Agilent Technologies, Santa Clara, CA USA) with temperature gradient program (28min) as follows; 200 °C for 0 min, ramp 4.0°C per minute to 300 °C, held at 300° for 3 min, with injector and detector temperature of 260°C, injection volume of 1µl and a slit ratio of 50:1. Galanthamine concentration was quantified relative to the internal standard by relative quantification method and expressed as % content by weight per g of dried tissue. Galanthamine tissue yield was calculated as the product of tissue galanthamine content and tissue weight. Total galanthamine was calculated as the sum of bulb and shoot galanthamine yield. For GC-FID quantification, a linear

response of galanthamine was validated by standard curve method over a range of 0-500 µg/ml galanthamine standard. The relative response factor(s) for galanthamine and internal standard (RRF=1) were validated by calibration curve method across a concentration range representing observed galanthamine content of experimental samples.

$$Response\ Factor\ (RF) = \frac{Peak\ Area}{Concentration}$$

$$Relative\ Response\ Factor\ (RRF) = \frac{Response\ Factor\ Galanthamine}{Response\ Factor\ Internal\ Standard}$$

$$Concentration\ Galanthamine = \frac{Peak\ Area\ Galanthamine}{Peak\ Area\ Internal\ Standard} \times \frac{1}{RRF} \times Concentration\ Internal\ Standard$$

Statistics

Data was computed in MS Excel and statistical analyses were performed using SPSS software (IBM, Portsmouth UK) by analysis of variance (ANOVA) using Dunnett's 2 sided t-test. The Dunnett t test treats one group as a control and compares all other groups against it. Statistical tests in which the significance value was less than or equal to 0.05 ($p \leq 0.05$ or 5% probability level) were deemed significant.