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Daffodil flowers delay senescence in cut *Iris* flowers

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Abstract

Visible symptoms of tepal senescence in cut *Iris* × *hollandica* (cv. Blue Magic) flowers were delayed by placing one cut daffodil flower (*Narcissus pseudonarcissus*, cv. Carlton) in the same vase. Addition of mucilage, exuded by daffodil stems, to the vase water had the same effect as the flowering daffodil stem. The active compound in the mucilage was identified as narciclasine (using LC/MS, GC/MS, ^{1}H and ^{13}C-NMR, and comparison with an authentic sample of narciclasine). The delay of senescence, either by mucilage or purified narciclasine, was correlated with a delayed increase in protease activity, and with a considerable reduction of maximum protease activity. Narciclasine did not affect in vitro protease activity, but is known to inhibit protein synthesis at the ribosomal level. Its effects on senescence and protease activity were similar to those of cycloheximide (CHX), another inhibitor of protein synthesis, but the effective narciclasine concentration was about 100 times lower than that of CHX. It is concluded that the delay of *Iris* tepal senescence by daffodil stems is due to narciclasine in daffodil mucilage, which apparently inhibits the synthesis of proteins involved in senescence.

Keywords: *Iris* × *hollandica*; *Narcissus pseudonarcissus*; Alkaloids; Cut flowers; Mucilage; Narciclasine; Programmed cell death; Protease; Senescence

1. Introduction

Senescence in plant parts is accompanied by organised disassembly of polysaccharides, proteins, lipids, and nucleic acids (Winkenbach, 1970a,b; Baumgartner et al., 1975; Matile, 1997). The disassembly results in production of sugars and amides, which are transported to other plant parts (Bieleski and Reid, 1992; Buchanan-Wollaston and Morris, 2000; Wagstaff et al., 2002). It is still unknown how the onset of these senescence-associated degradation processes is regulated (Beers et al., 2000; Swidzinski et al., 2002).

Flowers of *Iris* × *hollandica* are usually harvested when the tepal tips just emerge above the green sheath leaves. When placed in water at 20 °C, the flowers usually open and show visible senescence symptoms within four to five days. The senescence symptoms include liquid logging of the apoplast, and turgor loss (van Doorn and Stead, 1994; Celikel and van Doorn, 1995). Preliminary experiments showed that flower senescence in *Iris* was delayed after placing a narcissus stem in the water. We isolated and identified the active compound, and investigated its effect on protease activity.

2. Results

2.1. Effect of daffodils and daffodil mucilage on opening and tepal senescence in Iris flowers

Iris flowers (cv. Blue Magic) were harvested when the stems were 45–55 cm long and when about 1 cm of the tepals was visible. The flower bud was then firmly enclosed by the uppermost two green leaves. When placed in water at 20 °C, flower opening mainly occurred during the first day and was complete after two days.

Flower opening in *Iris* requires adequate elongation of the pedicel, the stem segment between the uppermost node and the flower base. Flower opening is mainly due to lateral movement, at the base, of the outer whorl of tepals.[1] When the tepal tips were just visible, removal of

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[1] Flower such as *Iris* and tulips have no green sepals and all coloured floral leaves are called tepals (in contrast to the coloured leaves in flowers with green sepals, which are called petals).

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the green sheath leaves resulted in immediate flower opening, showing that these leaves impede lateral tepal movement. If the green leaves were not cut off, the flowers were only able to open when the flower base had moved upwards, to a point where the green leaves no longer impeded lateral tepal movement. When the uppermost leaves were present and a Carlton daffodil was placed in the same vase (results not shown) or its stem mucilage was included in the vase water, the flowers did not open, due to inhibition of pedicel (and ovary) elongation growth (Fig. 1). In such flowers the effect of narcissi or their mucilage on *Iris* tepal senescence could not be investigated, because the flowers remained closed and the senescence symptoms were not visible.

The effects of a daffodil flower and daffodil mucilage was therefore studied in stems that had been cut just above the uppermost node. The latter (short stems) lacked the two sheath leaves. Placement of a Carlton daffodil (results not shown) of its stem mucilage (Fig. 1) inhibited pedicel growth in such short-stem *Iris* flowers, similar to the inhibition in long *Iris* stems with the sheath leaves. The opening of such short stem *Iris* flowers was only slightly inhibited by these treatments (Fig. 1).

Senescence symptoms in the flag tepals of control *Iris* flowers were slight discoloration and the beginning of inward rolling, both at the distal tepal edge (called stage 1 of senescence). This was followed by further inrolling until the flag part had curled up to the yellow patch in the middle of the flag (stage 4 of senescence). Subsequently the whole tepal lost its purple colour, became yellowish white (stage 5 of senescence) and then desiccated.

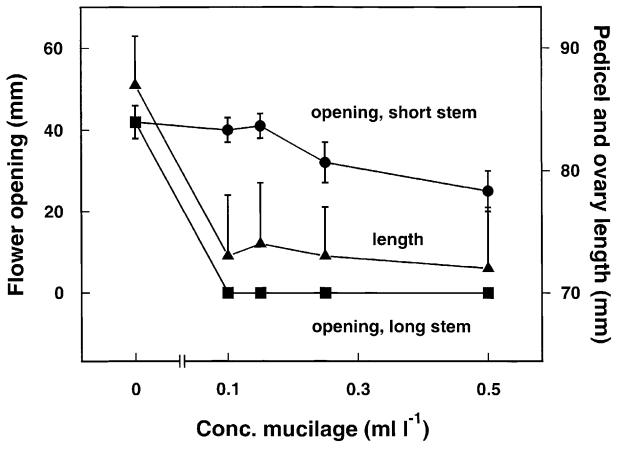

Fig. 1. Effect of various concentrations of mucilage from daffodil stems (*Narcissus pseudonarcissus*, cv. Carlton), placed in vase water, on the maxium of flower opening (● and ■, left axis) and elongation of the pedicel plus ovary (▲, right axis) in *Iris* × *hollandica*, cv. Blue Magic. Flowers were cut with a stem length of 45 cm (long stems) or at the pedicel base (short stems). The data on pedicel plus ovary length pertain to long stems. Data on floral opening in long stems of which the uppermost two green leaves were removed were similar to that of opening in short stems. Data are means ($n=10$) ± SD. When no SD bar is shown, the variation was smaller than the symbol.

Placing a cut Carlton daffodil flower in the vase water of an *Iris* flower (with a short stem) resulted in a remarkable delay of tepal senescence. It often had a small effect on the time to stage 1 of senescence (Table 1), although in some of the repeat experiments it did not affect this early stage. The daffodil flower considerably delayed or even prevented development of all subsequent senescence symptoms in *Iris* tepals. The treated *Iris* flowers eventually wilted and desiccated while retaining colour.

The same effects were produced by placing mucilage from the stems of Carlton daffodils in the water. At concentrations below 0.5 ml l^{-1}, mucilage considerably delayed tepal senescence, whereas toxicity symptoms (precocious desiccation) were observed at 1.0 ml l^{-1} or higher concentrations. A small delay in stage 1 of senescence was found in most experiments (Table 1). In other tests the mucilage only delayed senescence symptoms from stage 2. When the flowers were harvested earlier (when the bud was about to emerge) the mucilage always delayed the time to stage 1 of senescence. When included in the water from day 2 of placing the flowers in water, mucilage was no longer effective (results not shown).

Experiments with mucilage from Carlton daffodils were repeated with *Iris* × *hollandica* cvs. Professor Blaauw and Ideal. In these cultivars the mucilage produced a similar delay of the visible senescence symptoms as in cv. Blue Magic. Mucilage from the stems of *Narcissus pseudonarcissus* cvs. Golden Harvest, Ice Follies, and Moneymaker also delayed senescence in *Iris* × *hollandica* cv. Blue Magic, although the effects were observed at higher mucilage concentrations than in cv. Carlton. Mucilage of narcissi cv. Dick Wilde, Dutch Master, and Gigantic Star was ineffective in delaying *Iris* tepal senescence (results not shown).

2.2. Effect of enriched fractions and the active compound

An *n*-butanol extract was prepared from mucilage of Carlton narcissi, and flowers were placed in water containing both the *n*-butanol fraction and 0.25% (v/v) *n*-butanol for solubilization. This treatment usually delayed stage 1 of senescence by about 0.5 d (Table 1). All subsequent senescence stages were delayed by more than 0.5 day, in some experiments indefinitely as the petals did not inroll further and did not lose colour, but wilted and slowly desiccated instead. Control experiments with 0.25% *n*-butanol mostly showed little effect (Table 1), although in a few repeat experiments senescence was slightly delayed.

TLC experiments showed 10 organic compounds in the *n*-butanol fraction. Reverse phase HPLC with UV detection at 230.5 nm yielded six peaks (results not shown). The *n*-butanol fraction was separated into the six corresponding fractions, of which only the second

Table 1
Effects of daffodil (*Narcissus pseudonarcissus*) flowers, mucilage from daffodil stems, narciclasine and cycloheximide on the time to tepal senescence in *Iris*×*hollandica*. At stage 1 tepals just begin to roll in, and at stage 4 the inrolling has advanced until the yellow part in the middle of the tepal, and discoloration has started. Treatments started on day 0 after harvest. Statistical differences ($P > 0.05$) per column are indicated by a different letter (a or b); $n = 10$

Treatment	Time to senescence symptoms (days from harvest)	
	Stage 1	Stage 4
Water control	4.0±0.2 a	5.3±0.3 b
n-Butanol control (0.25% v/v)	4.1±0.1 a	5.2±0.2 b
Placement of one daffodil cv. Carlton flower in vase water	4.4±0.2 a	6.8±0.3* a
Mucilage of daffodil cv. Carlton (0.3 ml l^{-1})	4.5±0.3 a	6.9±0.2* a
Active fraction from mucilage (equivalent to 0.6 μM narciclasine)	4.2±0.2 a	6.6±0.4* a
Active fraction from bulb extract (equivalent to 0.6 μM narciclasine)	4.3±0.3 a	6.9±0.3* a
Narciclasine (0.6 μM in 0.25% v/v n-butanol)	4.5±0.2 a	7.1±0.3* a
Cycloheximide (100 μM)	4.4±0.2 a	6.9±0.4* a

* In several repeat experiments stage 4 of senescence was not reached; development was halted at stage 2 or 3.

delayed senescence in *Iris* flowers (Table 1). The UV spectrum of this fraction exhibited absorption maxima at 203, 253 and 303 nm.

For comparison, the active compound was also isolated from bulbs of Carlton narcissi. Flower longevity experiments showed a similar effect of the fraction from stem mucilage and bulbs (Table 1). TLC showed identical R_f values for the active compound isolated from bulbs and stem mucilage. Both compounds were also compared using NMR, exhibiting identical spectra. ^{1}H-NMR spectra showed the presence of 13 protons, and ^{13}C-NMR spectra the presence of 14 C atoms. GC/EI-MS and LC/EI-MS analysis revealed the molecular ion at m/z 307, indicating the presence of an odd number of N-atoms in the molecule (results not shown). The data were identical to published spectral data of narciclasine (Evidente, 1991) and to data obtained from a native sample of narciclasine (results not shown).

Pure narciclasine, previously extracted from narcissus bulbs (Kreh, 1995), was included in the vase water of *Iris* cv. Blue Magic. It had the same effect on tepal senescence as daffodil mucilage (Table 1), and was effective at 0.10–0.20 mg l^{-1}. At higher concentrations, it produced the same toxicity symptoms as daffodil mucilage that had not been adequately diluted. When narciclasine was included in vase water on day 0 or day 1 after harvest it delayed senescence in the tepal edges, but when applied from day 2 onward it was without effect on the visible senescence symptoms.

2.3. Concentration of narciclasine in daffodil mucilage

For determination of the narciclasine concentration in Carlton daffodil mucilage, we used analytical HPLC with pure narciclasine as standard, and detection at 230.5 nm. The chromatograms of the mucilage samples from different sources were very similar. In four samples from different growers, we found 0.77–1.20 mg narciclasine per ml mucilage (intermediate values were 0.93 and 1.14 mg ml^{-1}).

2.4. Effects of mucilage and narciclasine on protease activity

As tepal senescence in *Iris* flowers started at the distal edges and slowly proceeded to the base, we only sampled tepal edges for determination of protease activity. The effect of narciclasine on in vitro protease activity was determined by assaying flowers that had been standing in water. The assay was conducted on day 4, when protease activity is high. Inclusion of narciclasine at 0.5 ml l^{-1} in the assay medium had no effect on total protease activity (results not shown).

The presence of narciclasine in the vase water of *Iris* flowers delayed and attenuated the increase in protease activity in the tepal edges (Fig. 2A). This effect was found if narciclasine was applied on day 0 or day 1 (NC 0 and NC 1 in Fig. 2A), but was absent when it was applied on day 2. The same effect of the day of application was found with inclusion of mucilage in the vase water (results not shown).

2.5. Comparison with effects of cycloheximide

Cycloheximide (CHX) in the vase solution also delayed the time to senescence, and produced effects similar to those of narciclasine. However, the effective CHX concentration was about 100 times higher than that of narciclasine (Table 1). CHX only delayed senescence if included in the vase water before day 2. CHX had a similar effect on protease activity as narciclasine, and this was also limited to application before day 2 (Fig. 2B).

3. Discussion

Cut daffodils, placed in water with other cut flowers, usually have a negative effect on flower quality. In cut tulips, for example, a daffodil stem (or daffodil mucilage) in the vase water greatly hastened the time to leaf yellowing (van Doorn, 1998). In contrast, in the present experiments with *Iris* flowers the presence of a daffodil (or its stem mucilage) delayed tepal senescence. It is not clear why the mucilage has such an opposite effect in various species. Although daffodil mucilage delayed Iris tepal senescence at 0.1–0.5 mg ml^{-1}, it was toxic at 1 mg ml^{-1}. Some tissues may be much more sensitive to mucilage toxicity than others.

The active compound in daffodil stem mucilage and daffodil bulbs was identified as narciclasine, an alkaloid previously isolated from daffodil bulbs (Ceriotti, 1967; Piozzi et al., 1968; Mondon and Krohn, 1975). Narciclasine has apparently not been described in the aboveground parts of any plant species. Pure narciclasine, isolated from daffodil bulbs, produced the same effects on tepal senescence and pedicel elongation growth as daffodil mucilage, confirming the action of the identified compound.

In cv. Carlton the narciclasine concentration in mucilage varied, in four random samples, by a factor of 1.5. Generally, more mucilage was required to produce an effect in *Iris* flowers than was expected on the basis of its narciclasine concentration. This may be due to a partial xylem occlusion by the mucilage. Inhibition of water uptake by mucilage of Carlton daffodils was found in cut rose flowers (van Doorn, 1998). As the presence of the mucilage of some daffodil cultivars other than cv. Carlton had little effect on *Iris* tepal senescence, the concentration of mucilage narciclasine may be cultivar-dependent.

Daffodil mucilage and narciclasine considerably delayed—and often halted—all senescence symptoms from stage 2 onward. The same was observed after treatment with CHX, but the effective concentration of CHX was about 100 times higher than that of narciclasine (Table 1). Although it may also have other effects, narciclasine is a rather specific inhibitor of protein synthesis. It interacts with the peptidyl transferase center of eukaryotic ribosomes, thereby preventing peptide bond formation (Jimenez et al., 1976; Vázquez, 1979). CHX also acts on protein synthesis but, in contrast, mainly inhibits protein synthesis at the initiation phase, although it also has an effect on chain elongation and termination (Vázquez, 1979). The mode of action of narciclasine in delaying senescence is not known but it may be hypothesised that it acts by inhibiting protein synthesis. Results from other experiments do not contradict the idea that narciclasine generally operates by reducing protein synthesis. For example, narciclasine has been reported to inhibit antimitotic and antiviral activity, and to prevent both gibberellin-induced amylase activity in seeds and cytokinin-induced expansion of cotyledons (Bi et al., 1998). It also inhibited the activity of key enzymes in both glyoxysomes and peroxisomes (Bi et al., 2003). These two organelles are involved in conversion of lipids to sugars in various organs, including senescing petal cells (Graham et al., 1994), Narciclasine, therefore, seems to have a very broad inhibitory effect.

We used protease activity as a measure of the degradation processes prior to visible senescence. Both narciclasine and CHX delayed and attenuated the increase in protease activity (Fig. 2). They similarly inhibited lipid breakdown (unpublished results).

We made a cDNA microarray of *Iris* tepals harvested at several intervals from before opening to the first visible senescence symptoms. The expression of numerous genes was downregulated prior to full flower opening,

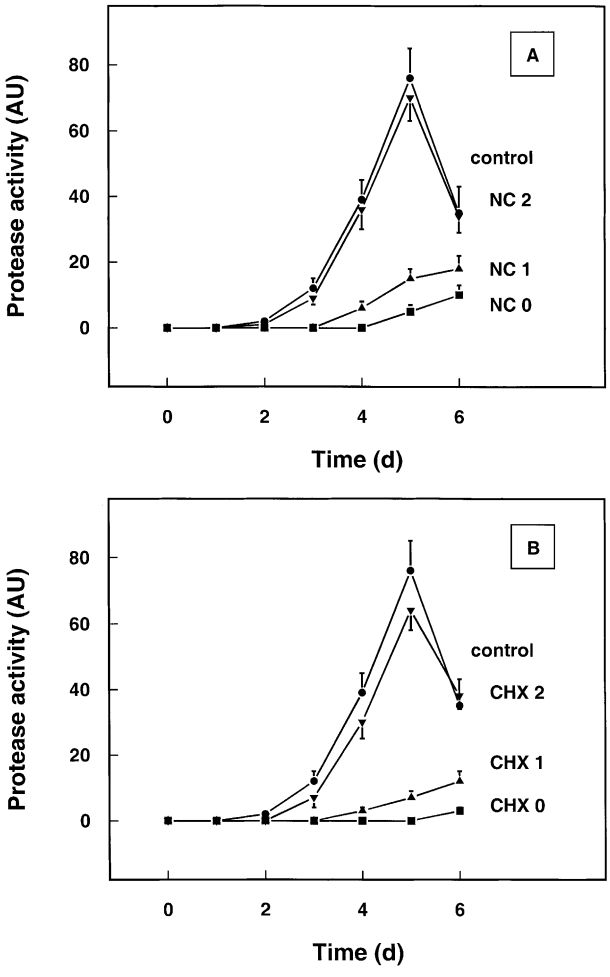

Fig. 2. Effect of inclusion of narciclasine (A) and cycloheximide (B) in the vase water on protease activity in tepal edges of cut *Iris×hollandica* flowers cv. Blue Magic, held at 20 °C. Day 0 refers to flowers in which the blue tepal tips were just visible. The compounds were added on day 0 (NC 0; CHX 0), day 1 (NC 1; CHX 1) or day 2 (NC 2; CHX 2). Data are means of three repeat experiments with different batches of flowers, and two powders per experiment, ±S.D.

whereas the expression of numerous other genes was upregulated prior to visible senescence (van Doorn et al., 2004). These results indicate that many new proteins were synthesized prior to visible senescence. The effect of CHX, a rather specific protein synthesis inhibitor, on the time to visible senescence indicates that it depends on de novo protein synthesis. CHX likely prevents translation of many genes whose expression is upregulated prior to visible senescence. Similarly, we hypothesize that narciclasine prevents synthesis of enzymes involved in senescence-associated degradation. CHX and narciclasine may also inhibit the synthesis of regulatory proteins, such as signal transduction and transcription factors. This may prevent the expression of numerous senescence-associated genes.

The effect of the CHX and narciclasine on stage 1 of senescence (the beginning of visible senescence at the distal tepal margins) was variable. In some experiments placement of a daffodil flower in the vase, or inclusion of daffodil mucilage, narciclasine, or cycloheximide in the vase water, had no effect on senescence stage 1. An effect of these treatments was always found when the flowers were cut at a slightly earlier stage of development. Apparently, proteins required for the development of senescence stage 1 had, depending on the experiment, already been synthesized or were about to be formed at the commercial harvest stage (flowers still closed; tepal tips just visible).

Narciclasine and CHX delayed senescence when given on day 0 and day 1, whereas they were no longer effective when applied on day 2. This indicates that the proteins required for the initiation of the senescence program (in the tepal edges) were synthesized before day 2. Narciclasine and CHX also no longer affected bulk protease activity when they were applied on day 2 or later. These results may be interpreted in at least two ways. The two compounds may inhibit the synthesis of signalling molecules that regulate the onset of the increase in bulk protease synthesis. Alternatively, they may inhibit the synthesis of protease precursors that are formed during day 0–2. Slow maturation of a cysteine protease involved in bulk protein degradation during senescence has been reported (Yamada et al., 2001). It is at this point not possible to distinguish between these two hypotheses. In vitro protease measurements showed no effect of CHX or narciclasine on protease activity, indicating that these compounds do not affect the protease proteins, after they have become active.

It is concluded that cut daffodil flowers, if placed in water in the same vase, can delay tepal senescence in cut *Iris* flowers. The effect is due to narciclasine, an alkaloid known to inhibit protein synthesis, in daffodil mucilage. Delayed senescence was accompanied by a delay and attenuation of protease activity. The results indicate that the increase in protease activity prior to visible senescence depends on de novo protein synthesis.

4. Experimental

4.1. Plant material

Iris flowers (*Iris* × *hollandica* Tub., cv. Blue Magic) were cut in greenhouses of commercial growers, usually when their tepals had just emerged. In a few experiments flowers were cut when the tepals were about to emerge. Immediately after harvest the flowers were placed in water and stored at 4 °C before being transported to the laboratory, which occurred the same morning. Transport occurred with the stem ends in water. In the laboratory the stems were recut in air to a length of 50 cm, or were cut above the uppermost node (short stems). Unless otherwise indicated, the experiments were carried out with short stems. These stems have no green leaves. Flowers were placed individually in vials in a climate-controlled room at 20 °C, 60% RH, and a photosynthetically active photon flux of 15 μmol m^{-2} s^{-1} (Philips TDL 36W/84 cool white fluorescent tubes) from 7 a.m. to 7 p.m. The short stems were placed in vials that contained 15 ml deionised water. Senescence symptoms in the flag tepals were observed two times per day, and were expressed in days after harvest.

Daffodil flowers (*Narcissus pseudonarcissus*) were also obtained from commercial growers. Flowers were harvested either with their bulbs or without bulbs and brought dry to the laboratory. The bulbs were cut off or the stems (without bulbs) were recut in air. The flowers were then placed in water together with *Iris* flowers. Other narcissi were used for extraction of mucilage, which was either allowed to drip from stems that had been re-cut, or which was squeezed out of the stems.

4.2. Flower opening

The degree of opening in *Iris* flowers was determined by measuring the distance between the tips of the flag tepals, using a ruler. Two measurements were made on each flower, and 10 flowers were used per treatment. Tests were made both with long (45 cm) and short stems.

4.3. Protease activity

One gram of freshly cut material from the distal tepal edges was ground to a fine powder in liquid nitrogen, and placed in 5 ml extraction medium (25 mM Hepes at pH 8.2, 3.5% NaCl and 2 mM DTT). The solution was desalted over a PD-10 column (Pharmacia, Uppsala, Sweden). After desalting, 20 mM $CaCl_2$ was added to stabilise the proteins. Resorufin-labelled casein was used as a substrate (Fernandez et al., 1999). The incubation medium consisted of 70 μl extract, 35 μl incubation buffer (400 mM citrate/phosphate buffer at pH 5.2) and

35 μl of substrate (resorufin-labeled casein, final concentration 0.1%). The reaction was performed at 37 °C for 30 min, and was halted by adding 336 μl trichloroacetic acid (5%). After centrifugation, 400 μl of the supernatant were mixed with 600 μl of 0.5 M Tris–HCl at pH 8.8. Absorption was measured at 574 nm. The data were corrected for absorption in controls without substrate. Protein levels were determined as described previously (Celikel and van Doorn, 1995).

4.4. Chemicals

The flowers were treated with chemicals directly after harvest. Ten replicate short-stem flowers (stem length about 15 cm) individually placed in vials, were used for each concentration tested. Cycloheximide (CHX) was obtained from Merck (Darmstadt, Germany) and was >97% pure. The treatments were given continuously, unless specified otherwise. Pure narciclasine was isolated from *Narcissus pseudonarcissus* cv. Carlton as described previously (Kreh, 1995), and was a gift from Prof. Matusch (Department of Pharmaceutical Chemistry, University of Marburg, Germany). Treatments started at the onset of placing the flowers in water and were given continuously, unless specified otherwise.

4.5. Extraction and isolation

5 ml of mucilage from Carlton daffodil stems were washed 3 times with 20 ml water and extracted with 20 ml *n*-butanol. A brown droplet of syrup, containing the active compound, deposited in *n*-butanol at 4 °C. After removing the organic solvent, the brown gummy residue was redissolved in *n*-butanol and used for further characterization by TLC, HPLC, and GC/MS. We used a preparative Novapak HR C18 (300 × 19 mm, 6 μm) column (Waters, Eschborn, Germany), using isocratic elution with acetonitrile: 25 mM sodium acetate (9: 1) (pH 5.7) at a flow rate of 1 ml/min. The chromatogram showed 6 peaks, which were collected as 6 fractions.

Alternatively, 65 g of bulbs from Carlton narcissi (including all parts except the outer dry scale and the outermost living scale) were ground in 50 ml *n*-butanol:water (1:1) for one minute in a cooled Bühler HO4 homogenizer (Bühler, Bodelshausen, Germany). The sample was centrifuged at 3000 rpm (1620 g) for 10 min at room temperature. The supernatant was dried using a rotation film evaporator (Büchi Rotavapor R-114, Flawil Switzerland, with a Heto vacuum evaporation system, Allerød, Denmark), steadily increasing the water bath temperature from 35 to 65 °C leaving a pale yellow syrup. The syrup was dissolved in 50 ml 5% HCl in water, and extracted three times with 40 ml chloroform. The pH of the aqueous phase was brought to 10 using 25% ammonia, and the aqueous phase was three times extracted with 40 ml chloroform. Conc. HCl was added to the aqueous phase until the pH was 7. The aqueous phase was extracted another three times with 40 ml *n*-butanol. The *n*-butanol phases were collected, dried, and redissolved in 3 ml *n*-butanol. Some remaining salts were removed by adding 10 ml of water. The *n*-butanol phase was dried and redissolved in 3 ml *n*-butanol. A brown syrup containing the active compound deposited in *n*-butanol at 4 °C. After removing the organic solvent, the brown gummy residue was used for subsequent TLC, reverse phase HPLC, GC/MS, and LC/MS analysis. For NMR, the material was dissolved in deuterated DMSO.

4.6. Analytical methods

All solvents used were analytical grade quality, HPLC solvents were HPLC grade quality, water was purified using a Milli-Q system (Millipore, Etten-Leur, The Netherlands). TLC was performed on silicagel-coated glass plates (10 × 10 cm, thickness silica layer 0.2 mm, average pore diameter 60 Å, Merck), which had been dried for 60 min at 60 °C, using methanol:chloroform (3:1) as mobile phase. Spots were stained with iodine (3 min at room temperature).

Reverse phase HPLC on daffodil mucilage was performed with a Waters MSDS 600E HPLC system equipped with a Waters 990 photodiode array detector (Waters, Eschborn, Germany) using a LiChrospher 100 RP 18 (250 × 4 mm, 5 μm) silica column (Merck) under isocratic conditions with 0.1% formic acid:methanol (7:3) at a flow rate of 0.7 ml/min. Absorbance was measured at 230.5 nm.

For quantitative determination of narciclasine in various mucilage samples, a reverse phase Nova-pak C18 (150 × 3.9 mm, 4 μm) column (Waters, Eschborn, Germany) was applied using isocratic elution with acetonitrile:25 mM sodium acetate (9:1) (pH 5.7) at a flow rate of 1 ml/min. A calibration curve was made with pure narciclasine.

LC/MS experiments were performed using a Waters LC Module I Plus (Waters, Eschborn, Germany) HPLC system, equipped with variable wavelength detector, coupled to an MD 800 quadrupole particle beam mass spectrometer (Fisons, Altrincham, UK). EI-MS was performed at 70 eV.

GC/MS was performed on a Carlo-Erba Mega system coupled to a QMD-1000 Mass Spec-trometer (Carlo Erba, Helsinki, Finland) using a DB-1 (20 m × 0.185 mm, 0.4 μm) column (Agilent Technologies, Amsterdam, Holland), and helium as a carrier gas. Samples were either directly injected or silylated prior to injection. When directly injected, the probe temperature was increased by 50 °C/min from 60 °C to 300 °C, where it was held for 5 min. The silylated sample (1 μl) was injected using a 1:30 split ratio and injection temperature of 300 °C. The column temperature was increased by 10 °C/min from 150 °C to 300 °C, and the final

temperature was held for 10 min. EI-MS was performed at 70 eV.

NMR spectra were recorded at 400 and 500 MHz using a JEOL JNM GX-400 and a JEOL Eclipse + 500 NMR spectrometer (JEOL, Eching, Germany). Tetramethylsilan served as internal standard.

4.7. Statistics

Results were compared by analysis of variance using the GENSTAT V program (Rothamsted, U.K.) and F test at $P < 0.05$. All treatments with chemicals included ten replicate flowers. The experiments were repeated at least once.

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