Title: Relative expression of putative genes involved in galanthamine and other Amaryllidaceae alkaloids biosynthesis in *Narcissus* field and *in vitro* tissues

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ABSTRACT

The *Narcissus pseudonarcissus* cv. Carlton contains Amaryllidaceae alkaloids namely galanthamine, lycorine, homolycorine, narciclasine, which are noted for their pharmaceutical properties such as for the treatment of early to mid-stage Alzheimer's diseases, cancer, tumor etc. Alkaloid biosynthesis using plant *in vitro* systems has been considered as a tool for drug discovery and the pathways are starting to be understood but still far from complete. Therefore, the study was emphasized to observe the relative expressions of putative genes involved in the biosynthetic pathway leading to the Amaryllidaceae alkaloids in field grown bulbs and developing cell culture systems in *Narcissus*.

MS media fortified with growth regulators were used for the development of tissue culture from Carlton twin-scale explants. MS medium with high auxin, 20 mg/l NAA was the best medium for callus growth and maintenance while media with low auxin, 4 mg/l NAA and MS basal media gave the maximum bulblets. Field tissues showed a higher amount of galanthamine content; i.e. basal plate (1050-1310 µg Gal/g FW) and bulb (980-1150 µg Gal/g FW) than the culture derived samples; callus (1.0-7.0 µg Gal/g FW) and bulblets (12-215 µg Gal/g FW) on a fresh weight (FW) basis. GC-MS chromatograms of samples under study also showed the presence of other important alkaloids i.e. lycorine, homolycorine, lycorenine, haemanthamine, crinamine, lycoramine and tazettine. RNA extracted from *in vitro* callus, bulblets and field grown bulb, basal plate were used for PCR to detect the relative expression of putative genes; P450, *PAL*, *TYDC* and *NpO₄OMT* normalized to actin. The selected transcripts for P450s and *TYDC* were expressed in both field and *in vitro* tissues. Higher expressions of PAL were observed in calli than field samples. The expression of *Np*N4OMT was notably higher in field samples than *in vitro* tissues. Therefore, *in vitro* tissues could be a good source for the reproducible and easy extraction of alkaloids from plants.

Keywords: *Narcissus*, galanthamine, alkaloids, *in vitro*, P450 (Cytochrome P450), PAL (Phenyl ammonia lyase), TYDC (Tyrosine decarboxylase) and NpO4OMT (norbelladine 4′-*O*-methyltransferase).

1. Introduction

Plants are characterized to biosynthesize a huge diversity of secondary metabolites leading to more than 200,000 plant natural products [1] and secondary metabolism in plants plays a major role in plant survival [2]. The genus *Narcissus* is among the estimated 75 genera and about 1100 species within the monocotyledon family Amaryllidaceae that includes daffodils [3, 4]. The daffodil (*Narcissus*) cv. 'Carlton' contains galanthamine as the major alkaloid in bulbs [5]. The alkaloids found in Amaryllidaceae plants are known as Amaryllidaceae alkaloids and are of twelve distinct types as determined by skeletal characterization [3] including lycorine, lycorenine, homolycorine, haemanthamine, tazettine, narciclasine, mesembrine, cherylline, oxomaritidine, epimaritidine, montanine and galanthamine. Galanthamine has marketing authorization in the UK for the symptomatic treatment of mild to moderately severe dementia of the Alzheimer's type [6]. Lycorine is a powerful inhibitor of cell growth and division with demonstrated antitumor activity and narciclasine has highly promising anti-cancer properties [7, 8].

Plants are the most cost-effective source for many medical drugs. If the biosynthetic pathway of plant derivatives is understood it is possible to determine starting substances and use the insights from enzymatic pathways for their chemical synthesis [9]. All Amaryllidaceae alkaloids are derived from the aromatic amino acids phenylalanine and tyrosine, which produce the common precursor 4′-O-methylnorbelladine [8]. Molecular knowledge of Amaryllidaceae alkaloid biosynthesis would enable rational approaches to the optimization of commercial alkaloid production [7]. Putative genes including *PAL, NMT, P450, TYDC* and *OMT* [8] and other potentially important candidate genes involved in Amaryllidaceae

alkaloids biosynthesis have been identified in *Lycoris* [10]. Phenylalanine ammonia lyase (*PAL*) is the key enzyme that catalyzes the first step in phenyl propanol pathway, which supplies the precausors for a vast of secondary metabolites including flavonoids [11] and is also considered important regulation point between primary and secondary metabolism [12]. L-tyrosine decarboxylase (*TYDC*) catalyzes the formation of tyramine and dopamine and represents the first steps in the biosynthesis of the large and diverse group of alkaloids [13] including the Amaryllidaceae alkaloids [14]. One candidate gene, norbelladine 4'-O-methyltransferase (*Np*N4OMT) has been identified for the methylation of norbelladine to 4'-O-methylnorbelladine and genes that co-express with it could be identified and used as candidates for the other steps in the proposed Amaryllidaceae alkaloids biosynthetic pathway [15]. Plant P450s catalyze a wide variety of reactions to produce primary and secondary metabolites like alkaloids, terpenoids, lipids, including plant hormones [1]. *PAL* and *TYDC* act at the initial stages, while P450s are involved later in intramolecular C-C phenol coupling reactions (*ortho-para'*; *para-para'* and *para-ortho'*) of the alkaloid biosynthesis pathway (Figure 1) [7, 13].

The amount of alkaloid in daffodil plants might depend on tissue specific distribution [5]. *Narcissus* bulbs are the main source for alkaloid extraction and some cultivars and species contained more than 0.1% (DW basis) of galanthamine in their bulbs [16]. *In vitro* tissue and tissue-based systems have been proposed for commercial applications such as production of metabolites and clonal propagation [17, 18]. Undifferentiated callus or suspension cultures have been mainly used for the production of alkaloids. However commercial success has been very narrow because most secondary metabolites are produced only in very small amounts in undifferentiated or unorganized cells [19]. MS [20] basal media supplemented with growth regulators positively influence the somatic embryogenesis in *Narcissus* [21] and auxin such as NAA (naphthalene acetic acid) had a significant effect on callus like

proliferation [22] as well as shoot multiplication in combination with cytokinin such as BA (benzyl acetic acid) [23]. For their simple structure, calli might be a good experimental system to study the biosynthesis of alkaloids, though they were not the best system for alkaloid production [24]. The present study would lead to employ the plant physiology and genetics of *Narcissus* in order to obtain *in vitro* tissues in *Narcissus*; to determine the amount of alkaloid and to assess the expression level of putative genes in field and tissue culture derived samples involved in the biosynthetic pathway of alkaloid production.

2. Materials and methods

2.1. Derivation of field and in vitro tissues

Narcissus pseudonarcissus bulbs from variety 'Carlton' supplied by New Generation Daffodil Ltd. United Kingdom were used for field and tissue culture. Bulbs were kept under cold treatment (4^{o}C) for at least one month before hot water treatment (52^{0}C for 1h) and field planting. For field sample (bulb and basal plate) collection the bulbs were harvested, cleaned and stored at -80^{0}C. For tissue culture, the hot water treated bulbs were cut into quarters and surface sterilization was performed using Domestos bleach solution (20%) for 30 min then rinsed (6×) with sterile distilled water. The disinfected bulbs were cut as twin-scales (cut from the basal part of bulbs with 2 or 3 scales of 0.8-1.0 cm in size) in a laminar hood. Among the 24 twin-scale explants obtained from each bulb, 12 twin-scales were cultured on MS basal media, 6 on high auxin (MSM1) and 6 on low auxin (MSM2) media, incubated at 24 ± 2 ^{o}C, 12 h photoperiod. (Table 1). MS media, ascorbic acid and all the growth regulators were purchased from Duchefa Biochemie (Netherlands), polyvinylpyrrolidone from Sigma-Aldrich (UK) and yeast extract from Thermo Scientific (Germany).

2.2. Calculations and data interpretation

The results of *in vitro* tissue responses (callus and bulblets) are represented in percentage. As for example, the percentage of callus and bulblets were calculated based on the number of

explants developed to callus or bulblets to the total number of explants incubated on the respective media (MS, MSM1 and MSM2).

Representative equations are as follows:

$$\text{Percent (\%) of callus} = \frac{\text{total number of callus obtained from explants grown on MSM1 or MSM2}}{\text{total number of explants incubated in MSM1 and MSM2}} \times 100$$

$$\text{Percent (\%) of bulblets} = \frac{\text{total number of bulblets obtained from explants grown on MS or MSM1 or MSM2}}{\text{total number of explants incubated in all three media (MS, MSM1, MSM2)}} \times 100$$

2.3. GC-MS analysis

After plant sample (bulb, basal plate, callus and bulblets) and standard preparation (galanthamine was used as external and codeine as internal standard) following the method described by Ferdausi et al., [25]. **The calibration curves were prepared by plotting the galanthamine peak areas (TIC) versus the standard of galanthamine molarities (0.0002671 M, 0.0001336 M and 0.00002671 M). Standards were analyzed in triplicate with *Narcissus* tissue samples and showed high linearity over the range of galanthamine molarities with correlation coefficient (R^2) of 0.902 to 0.954.**

All samples were analyzed in the Centre of Proteome Research, University of Liverpool using GC-MS. Automated injection of samples to the Micromass GCT instrument and data collection was performed in Centre of Proteome Research. Database software, 'the NIST mass spectral library' that was accessed via the Waters Mass Lynx software was used for the identification and quantification of galanthamine and detection of other alkaloids or related compounds. The database contains the mass spectra; compound structures, and retention index information that allow identifying compound in a single step.

2.4. Semi quantitative RT-PCR

2.4.1. RNA extraction and cDNA preparation

About 100 mg of field and *in vitro* tissues ground in liquid nitrogen were used for RNA extraction. The innuPREP Plant RNA Kit (Analytic Jena, Germany) was used to isolate total RNA from the samples according to the manufacturer's instructions. The quality and quantity of isolated RNA was determined using a NanoDrop Spectrophotometer (Thermo, USA) and samples with a ratio (260/280) from 2.0 to 2.1 were selected for cDNA preparation using the

Reverse Transcription Kit (QuantiTect, QIAGEN, UK) following the manufacturer's protocol.

2.4.2. Primers and PCR reaction

Gene specific primers (Table 2) were designed by Pulman [26]. Primers were purchased from Eurofins Genomics (Germany) and used in PCR reactions. Reaction mixtures (10 µl) were prepared with 1µl cDNA template, 1 µl forward (10 µM) and reverse (10 µM) primers, 5 µl BioMix Red (Bioline, UK) and 2 µl PCR water. The following conditions were used for the PCR reactions: pre-denaturation at 94^{o}C (1 min) followed by 35 cycles consisting of 20 s at 94^{o}C (denaturing), 20 s at 57^{o}C (annealing), 30 s at 72^{o}C (extension) and a final extension for 5 min at 72^{o}C. The PCR products were separated on 1% (w/v) agarose gel, stained with Midori Green (Nippon Genetics Europe GmbH, Germany) and recorded using a gel documentation system (U:Genius, Syngene, UK).

The callus and bulblets were initiated from three separate bulbs and thus provided independent biological replicates. PCR reactions were carried out in triplicate, as technical replications. Carlton bulb and basal tissues were used as controls for each PCR reaction.

2.4.3. Statistical analysis

PCR products were analyzed using Metamorph (Molecular Devices, USA) for the quantification of relative band intensities. All band intensity values were normalized against Actin band intensities for each gel image. One way ANOVA was performed using Microsoft Excel 2010 and the confidence level for mean was 95% for the analyses of all sample band intensities.

3. Results and Discussions

3.1. Tissue culture derived materials

After about four to eight weeks of culture initiation, some twin scale (Figure 2a) explants had developed into undifferentiated callus (Figure 2b). In addition, explants started to give rise to

177 small bulblets (Figure 2c) directly from the base of twin-scale. The results showed that twin-
178 scales could be used as effective explants for *in vitro* callus and bulblet production in *N.*
179 *pseudonarcissus* cv. Carlton, as reported for other species of the same genus [23, 27, and 28].
180 Formation of small bulblets was observed after eight weeks of culture on MS media
181 supplemented with auxin and cytokinin, from the base of scale explants of *N. papyraceus* cv.
182 Shirazi [27]. After nine weeks of culture, callus and tiny bulb-like structures appeared at the
183 base of the twin-scale explants in *N. asturiensis* [29]. The similar duration (4 to 8 weeks) was
184 also required for callus and small bulblets formation from twin-scale explants of *N.*
185 *pseudonarcissus* cv. Carlton in this study.

186 *3.2. Effect of different media and growth regulators on callus induction*

187 MS basal and modified MS media with both high (MSM1) and low (MSM2) auxin were
188 tested for callus induction. No callus was found from MS basal medium. The maximum
189 callus induction was recorded from high auxin (MSM1) medium (67% of total explants
190 cultured on MSM1 and MSM2) than the low auxin (MSM2) (13% of total explants cultured
191 on MSM1 and MSM2) (Figure 3). Some previous studies showed the similar findings; MS
192 medium containing high auxin was the most suitable medium for callus induction and
193 proliferation derived from bulb explants of *N. tazetta* [22, 28]. Calli derived from bulb
194 explants of *Narcissus tazetta* showed the highest growth rate and proliferation when cultured
195 on MS medium supplemented with high NAA (3 mg/l) and low BAP (1.5 mg/l) [28].
196 Interestingly, Carlton twin-scale explants completely failed to induce callus on MS medium
197 (without growth regulators) which was previously reported in *N. confusus in vitro* cultures
198 from media lacking auxins [30], which indicates the vital role of growth regulators in tissue
199 differentiation.

200 *3.3. Effect of growth regulators on callus growth and maintenance*

Calli (1.0 cm×0.8cm) obtained from MSM1 and MSM2 media were sub-cultured on the same media for six to eight weeks and the size became approximately 1.5 cm×1.0 cm. Calli (1.5 cm×1.0 cm) were again cut into several small pieces (1.0 cm×0.3 cm) before second sub-culture. After another 4 weeks about 60 pieces of callus obtained from the high auxin (MSM1) medium were tested in two different media combinations for further proliferation and maintenance. Among 60 calli (0.5 cm×0.7 cm), 30 were incubated on high auxin (MSM1) medium (control); 15 were cultured on MS medium supplemented with 10 mg/l 2, 4-D and 15 on MS medium with 8.0 mg/l 2, 4-D [31]. Figure 4 represents the three media combinations, where calli on the control medium showed higher proliferation (73% of total calli inoculated) than on the media with 10 mg/l 2, 4-D (53% of total calli inoculated) and 8.0 mg/l 2, 4-D (33% of total calli inoculated).

The best survival, callusing and bulb formation in *N. pseudonarcissus* have been reported when *in vitro* grown shoots were sub-cultured in MS media supplemented with high auxin (2, 4-D), low cytokinin (BAP) and 6% sucrose [32]. Another study also showed that the callus and bulblets formation was high in *Narcissus* with high auxins (2, 4-D, NAA and picloram) [22]. In our study the medium with high auxin showed better callus induction, maintenance and proliferation. Therefore, high auxin (20 mg/l NAA) media was used for further sub-culture and calli maintenance.

3.4. Effect of different media and growth regulators on bulblet initiation from explants

Figure 5 shows that the initiation of small bulblets directly grown from the base of twin-scale explants, was influenced by low concentration of auxin (MSM2) (51% of total explants inoculated in all three media) and MS basal medium (48% of total explants inoculated in all three media). Whereas, a lower percentage (11% of total explants inoculated in all three media) were obtained from high auxin (MSM1) medium (Figure 5). Similar results were observed in several previous studies of *Narcissus* such as low amounts of auxin (1 to 5 mg/l

NAA) stimulating bulb formation in *Narcissus spp.* [29, 33]. The best result for shoot proliferation was found in MS medium supplemented with low auxin, especially a NAA to a high cytokinin from *in vitro* bulb scale culture of *N. asturiensis* [29]. Another study revealed that the combination of NAA and BA showed shoot and bulblet proliferation in several Amaryllidaceae species [17]. That indicates a lower concentration of auxin facilitates the function of cytokinin and the appropriate combination of both is mandatory for inducing differentiation such as shoot or bulblet formation [34].

3.5. Quantification of galanthamine from field and tissue culture derived samples

The GC-MS showed that the Carlton basal plate contained the highest (1050-1310 µg Gal/g FW) amount of galanthamine followed by bulb (980-1150 µg Gal/g FW); a trace amount of galanthamine (1.0-7.0 µg Gal/g FW) was quantified in callus and direct bulblets grown from the base of twin-scales contained low amount (**12**-215 µg Gal/g FW) of galanthamine (Table 3). Previous findings on the amount of galanthamine from field grown bulb (2000-2800 µg/g Gal DW) of *Narcissus* 'Carlton' showed higher amounts than our findings [35]. This increased amount could be due to the dry weight (DW) basis quantification. Similar findings were observed from *in vitro* cultures of *N. confusus*; where callus had the lowest galanthamine content (0.03 µg/g DW) followed by shoot-clumps or bulblets (0.14 µg/g DW) and plantlets (1.43 µg/g DW) [36]. Another study also has reported the similar pattern of lower Gal content in callus (30-60 µg/g DW) than *in vitro* shoots or bulblets (110-130 µg/g DW) of *N. confusus* [37]; that indicates the increased production of galanthamine is closely related with cellular differentiation.

3.6. Identification of other alkaloids and related compounds from GC-MS chromatograms

Along with galanthamine and the internal standard **codeine, lycoramine** (galanthamine type), crinamine (haemanthamine type), lycorine and lycorenine (homolycorine type) were found as the top matched compounds from the GC-MS chromatogram of Carlton bulb extracts (**Figure**

6a). The Carlton basal plate chromatogram **(Figure 6b)** also showed top matched mass spectra of related compounds such as lycoramine (Galanthamine type), haemanthamine, pancracine (narciclasine and montamine type) and lycorenine (homolycorine type). The chromatogram for callus extracts **(Figure 6c)** confirms that callus is the lowest galanthamine producing tissue but it could contain other alkaloids such as tazettine/ pretazettine. The chromatograms of small direct bulblet extracts showed the presence of galanthamine. However, the top matched compound for bulblets obtained from chromatogram mass spectra was crinamine (haemanthamine type) as shown in **Figure 6d**.

Same or similar alkaloids have been previously identified from *Narcissus* species [38, 39]. Lycoramine and homolycorine have been previously identified through GC-MS in *N. pseudonarcissus* cv. Carlton bulb extracts [5, 40]. Lycorine and homolycorine type (homolycorine, lycorenine) alkaloids were obtained from the norbelladine precursor by *ortho-para'*; which were mainly detected in Carlton field samples; haemanthamine type (crinamine), narciclasine type (pancracine) and tazettine type (tazettine, pretazettine) by *para-para'*, which were detected in both Carlton field and *in vitro* tissues; and galanthamine type (galanthamine, lycoramine) by *para-ortho'* phenol oxidative coupling, which were detected in both Carlton field and *in vitro* tissues, have also been previously reported in various *Narcissus* species [38]. Both field and *in vitro* grown samples derived from *Narcissus* could be a good source of other Amaryllidaceae alkaloids such as lycorine, homolycorine and sanguinine (galanthamine type) [41] and their quantification through GC-MS is also possible using authentic standards of the related compounds [42]. In present study the quantification of the detected alkaloids could not be quantified due to lack of reference standards but their presence was observed from the GC-MS chromatograms which could provide a future prospect for the quantification and isolation of these alkaloids in *Narcissus*.

3.7. Relative expression of gene specific transcripts implicated in alkaloid biosynthesis

All seven gene specific transcripts (Table 2) were tested for field (bulb and basal plate tissue) and *in vitro* cultured calli and bulblets to assess the relative expression of putative genes involved in alkaloid biosynthesis in *Narcissus*. The calli and bulblets were obtained from three different bulbs (bulbs 1, 2 and 3) where field tissues were used as control. Figure 7 and 8 show the relative expressions, normalized to actin (Figure 9), for the seven transcripts.

The transcripts for P450s (HDA57 and Daff 88927) were expressed almost in all tissue types; both field and *in vitro* grown samples (**Figure 7a, b, c and d**). The only exception was bulb samples (**Figure 7d**) showed no expression of Daff 88927. Daff 88927 primer was designed from *Narcissus* cv. Andrew's Choice bulb sequence [26], it could be the probable reason of low or no expression of Daff 88927 in Carlton field samples (**Figure 7c and d**). As P450s regulate the final steps of alkaloid biosynthesis (Figure 1) and all our samples under study showed different levels of galanthamine content (Table 3) along with the presence of other alkaloids (Figure 6), therefore, these low to high expression of P450s transcripts are in accordance with the GC-MS findings.

Higher expression of both *PALs* was observed in calli (**Figure 7f and h**) than field-grown bulb and basal plate. Low or no expression of PAL1 was identified in field tissues (**Figure 7e and f**) and neither PAL1 nor PAL2 were expressed in bulblets obtained from bulb 2 (**Figure 7e and g**). **Figure 8a, b, c and d** showed that the *TYDC* transcripts were present almost in all tissue types, at different levels of expression. Both TYDC1 and TYDC2 showed higher expression in calli (**Figure 8b and d**) than bulb and basal plate tissues but lower level in bulblets (**Figure 8a and c**). *PAL* and *TYDC* regulate the initial steps of alkaloid biosynthesis (Figure 1) and their expression might be related to cellular differentiation i.e. more expressed in undifferentiated or unorganized (callus) tissues than organized (filed/bulblets) tissues.

The expression level of *Np*N4OMT was notably higher in field-grown bulb and basal tissues than *in vitro* grown bulblets and calli (**Figure 7e and f**). *Np*N4OMT regulates the later step

(Figure 1) leading to alkaloid biosynthesis and its expression might also be related to cellular differentiation i.e. high expression in organized or differentiated tissues (field and bulblets) which leads to the higher accumulation of galanthamine in field samples and *in vitro* bulblets than callus (Table 3).

In this study, differential expression of putative genes involved in the biosynthesis of alkaloids in *Narcissus* was obtained from two conditions i.e. field and *in vitro* tissues (callus and bulblets). **It was observed that the expression of genes involved in the biosynthesis of alkaloids were present in both conditions** i.e. cytochrome *P450s, OMTs, PAL and TYDC* [38, 39] which were reported in previous studies in different plant materials including *Narcissus pseudonarcissus* [13]. However, transcripts (i.e. cytochrome P450s and *OMTs*) involved in the Amaryllidaceae alkaloid biosynthesis was reported earlier by Kilgore et al. [15, 43] were mainly up-regulated in field samples. Hence, a deep transcriptome analysis of *Narcissus* basal plate (highest galanthamine producing tissue) and callus (lowest galanthamine producing tissue) [44] would lead to further knowledge about the differential genes or transcripts expression related to alkaloid biosynthesis in *Narcissus*.

4. Conclusions

Comparing the amount and presence of different alkaloids in *Narcissus* tissues originated from two growth environments (field versus *in vitro*) and different tissue types (organized and unorganized) would provide a well-adapted knowledge for insight into the plant biology related to alkaloid production in *Narcissus*. The putative genes for the alkaloid biosynthesis were highly expressed in field samples which also showed the accumulation of high galanthamine than the *in vitro* tissues. However, *in vitro* bulblets and callus also showed the different levels of expressions of the putative genes along with trace or low amount of galanthamine. The findings of the expression levels of putative genes involved in alkaloid biosynthesis would provide a rational for future transcriptome analysis to determine the

differential expression and identification of genes involved in alkaloid biosynthesis in *Narcissus*.

CRediT author statement

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Table 1 Media compositions for *N. pseudonarcissus* cv. Carlton tissue culture (Per Litre).

Media	Composition
MS (basal)	MS 4.30 g + sucrose 50 g + agar 8 g
MSM1 (modified MS) high auxin medium	MS (basal) + Yeast Extract (YE) 100 mg + Vitamin C (VC) 50 mg + Polyvinylpyrrolidone (PVP) 30 mg + Kinetin (KN) 0.5 mg + Benzyl Amino Purine (BAP) 1.5 mg + Naphthalene Acetic Acid (NAA) 20 mg
MSM2 (modified MS) low auxin medium	MS (basal) + Yeast Extract (YE) 100 mg + Vitamin C (VC) 50 mg +Polyvinylpyrrolidone (PVP) 30 mg + Kinetin (KN) 0.5 mg + Benzyl Amino Purine (BAP) 1.5 mg + Naphthalene Acetic Acid (NAA) 4 mg

Table 2 Primers used for RT-PCR.

Name	Forward Sequences (5'-3')	Reverse Sequences (5'-3')	Length
ACTIN (Housekeeping)	GATAGAACCTCCAATCCAAACACTA	GTGTGATGTGGATATTAGGAAGGAC	25
HDA57 (P450)	ATTTCTCAGCGAGAGCCAAG	CTCCAATTTCTTGGCATGGT	20
Daff 88927 (P450)	CAGTTGGTTTAATTCATCTCTGCTT	ATGACAGAATTCTAGCAGCTTTGTT	25
PAL1	ATGGGAATAAGGAAAAGATGAAAAC	CACAAACCGATACAAAGGATAACAC	25
PAL2	GGGAATAAGGAAAAGATGAAAACAC	GATACAAAGGATAAGACCTGCACTC	25
TYDC1	TGGTTTTAATATTGTGGGTTTCAAT	TTCACTAGCTGTGCCTTGAATTACT	25
TYDC2	GTAATTCAAGGCACAGCTAGTGAAG	ATAAACCACAAGCTTTTCAAGTGAT	25
*Np*N4OMT	AAGACCTGTACGACCATGCA	ATCCACCTCATCTTCCGGAC	20

Primer sequences and designs [26]

Table 3 Amount of galanthamine in *N. pseudonarcissus* cv. Carlton field and *in vitro* sample

Narcissus samples	Bulbs	µg Gal/g FW ± SD
Carlton bulb (field)	Bulb 1	**1110 ± 91.92**
	Bulb 2	**980 ± 86.59**
	Bulb 3	**1150 ± 70.30**
Carlton basal plate (field)	Bulb 1	**1250 ± 48.04**
	Bulb 2	**1050 ± 44.33**
	Bulb 3	**1310 ± 75.56**
Callus (*in vitro*)	Bulb 1	**1.0 ± 1.17**
	Bulb 2	**7.0 ± 6.94**
	Bulb 3	**7.0 ± 3.36**
Direct bulblets from twin-scale (*in vitro*)	Bulb 1	**90.0 ± 28.13**
	Bulb 2	**12.0 ± 7.23**
	Bulb 3	**215 ± 302.9**

Values expressed as µg Gal/g FW (fresh weight) ± SD, n = 3.

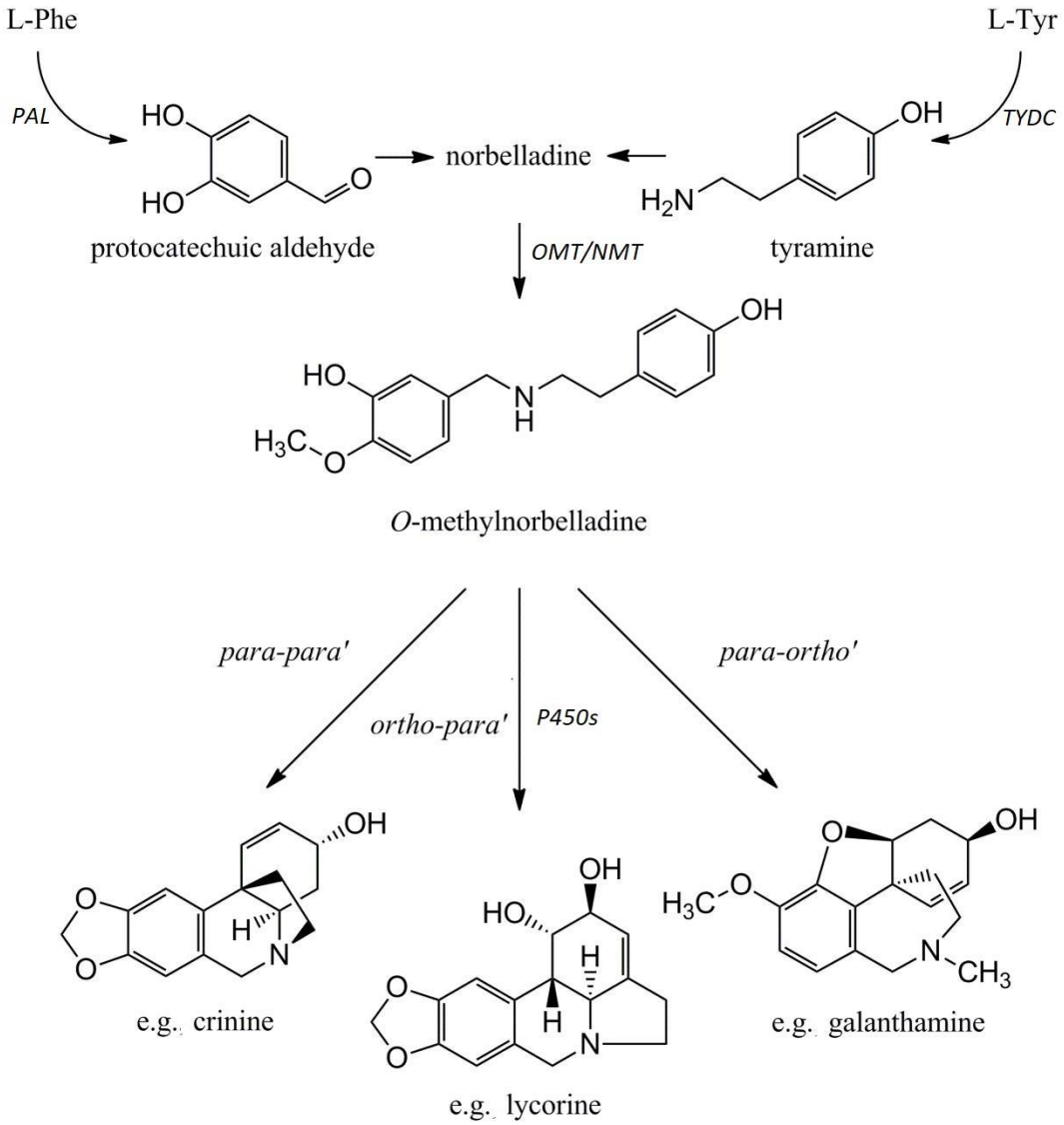


Fig. 1. Schematic overview of Amaryllidaceae alkaloid biosynthesis [postulated from 7].

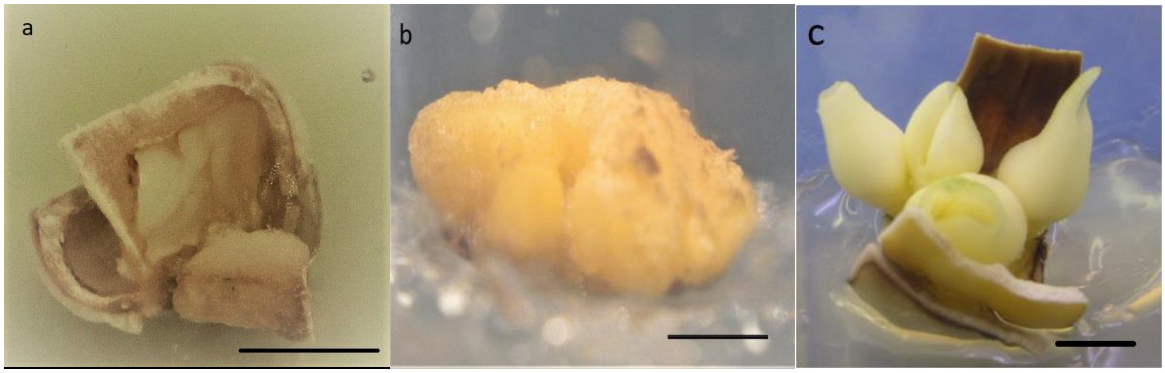


Fig. 2. Cultured samples from *N. pseudonarcissus* cv. Carlton: (a) Twin-scale explant (b) callus from MSM1 medium after 8 weeks, (c) bulblets directly grown from base of twin-scale from MSM2 medium after 8 weeks of incubation. Scale bars: 0.5 cm.

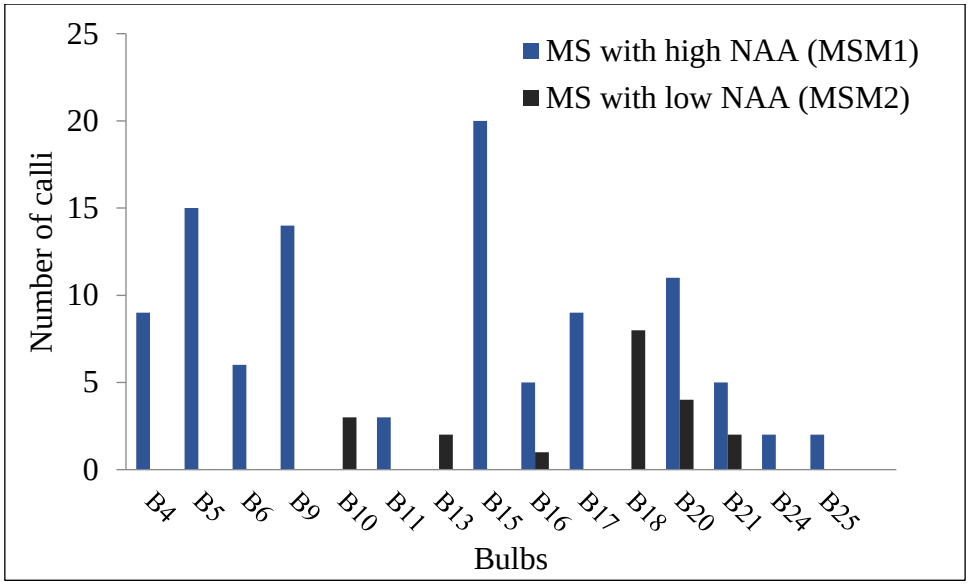
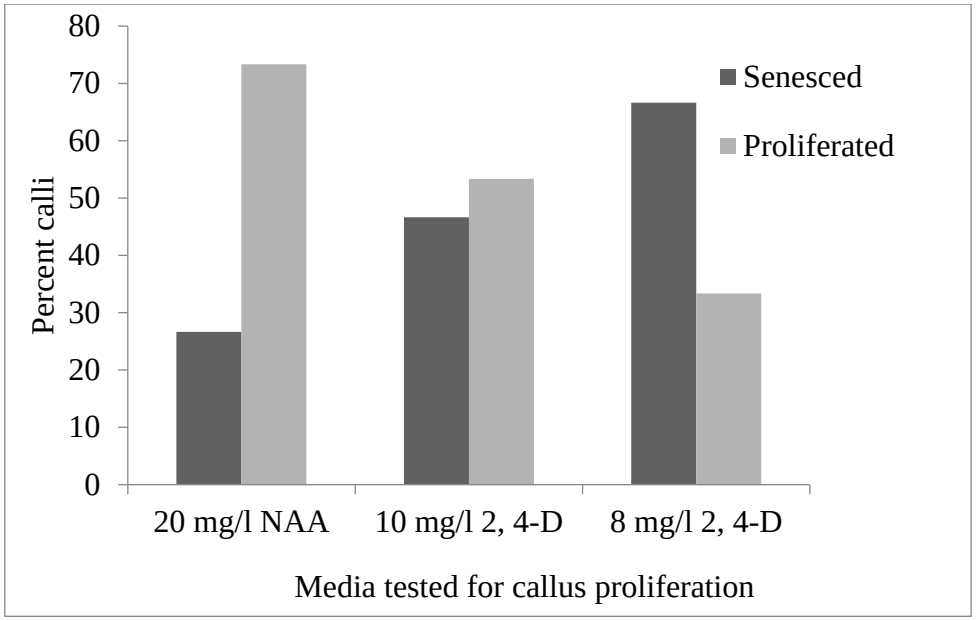


Fig. 3. Effect of different media combinations and bulbs on callus induction after twelve weeks of incubation. Multiple bars indicating the response of bulbs in both media with the absence of bars indicating no callus was obtained, due to either contamination or senescence.



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571 **Fig. 4.** Effect of growth regulators (20 mg/l NAA, 10 mg/l 2, 4-D and 8.0 mg/l 2, 4-D)
572 supplemented MS media on callus proliferation/maintenance after six weeks of sub-culture.

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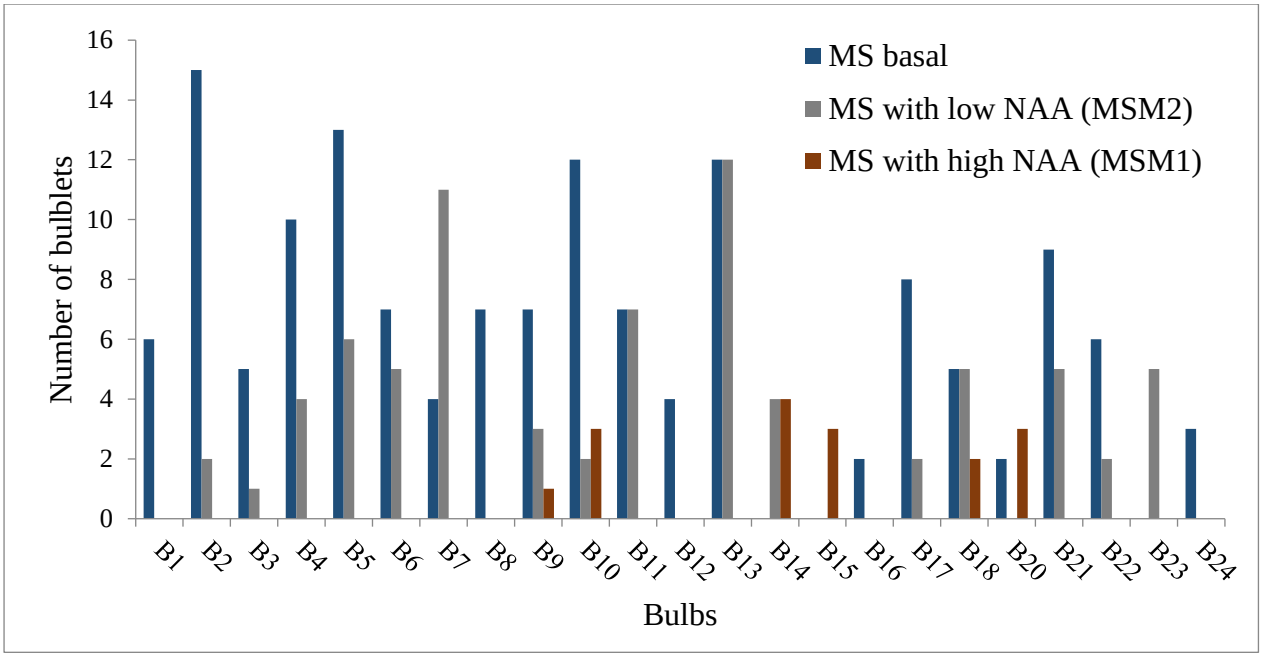
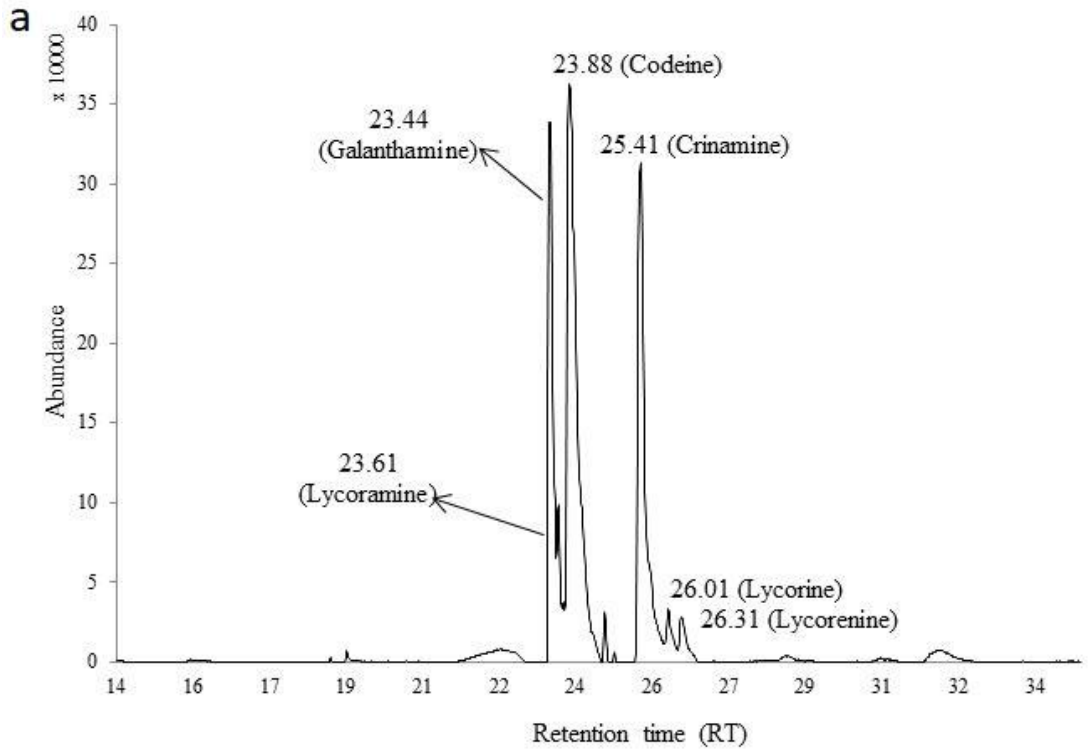


Fig. 5. Effect of different media combinations and bulbs on bulblets initiation after twelve weeks of incubation. Multiple bars indicating the response of bulbs in multiple media with the absence of bars indicating no bulblets were obtained, due to either contamination or senescence.


a
x 10000
Abundance
23.88 (Codeine)
23.44
(Galanthamine)
25.41 (Crinamine)
23.61
(Lycoramine)
26.01 (Lycorine)
26.31 (Lycorenine)
Retention time (RT)

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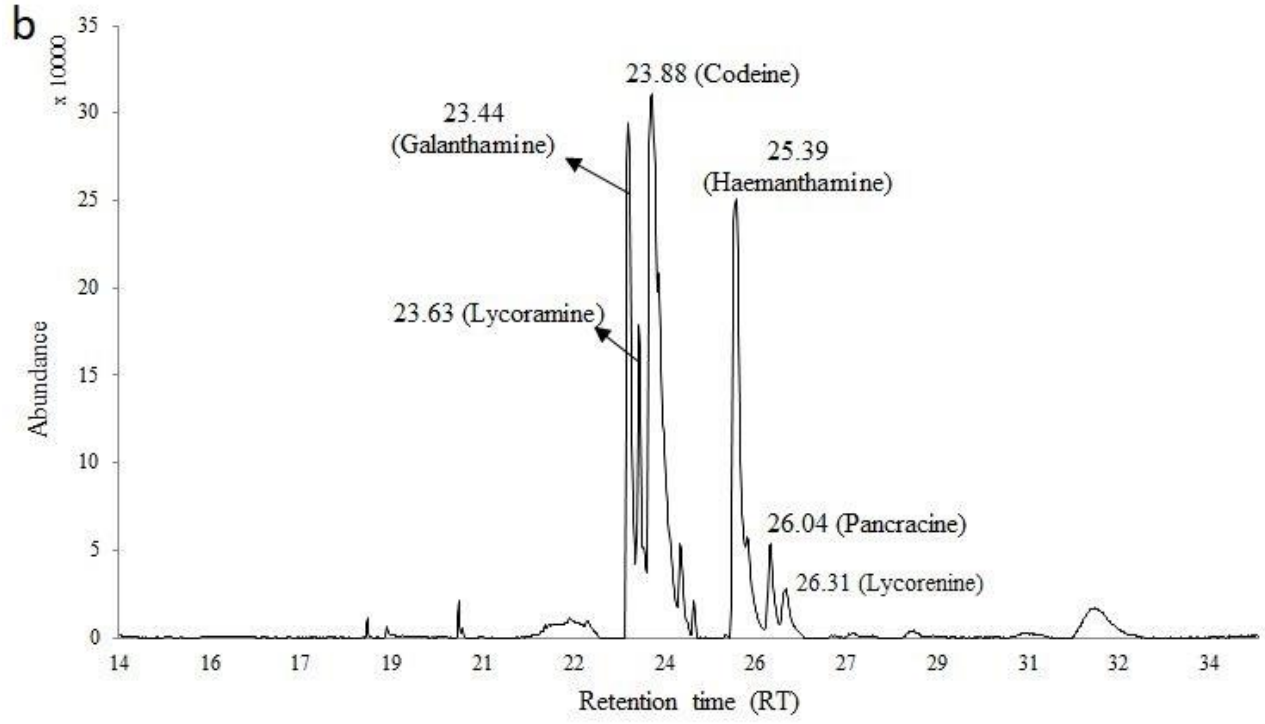
b
x 10000
Abundance
23.88 (Codeine)
23.44
(Galanthamine)
25.39
(Haemanthamine)
23.63 (Lycoramine)
26.04 (Pancracine)
26.31 (Lycorenine)
Retention time (RT)

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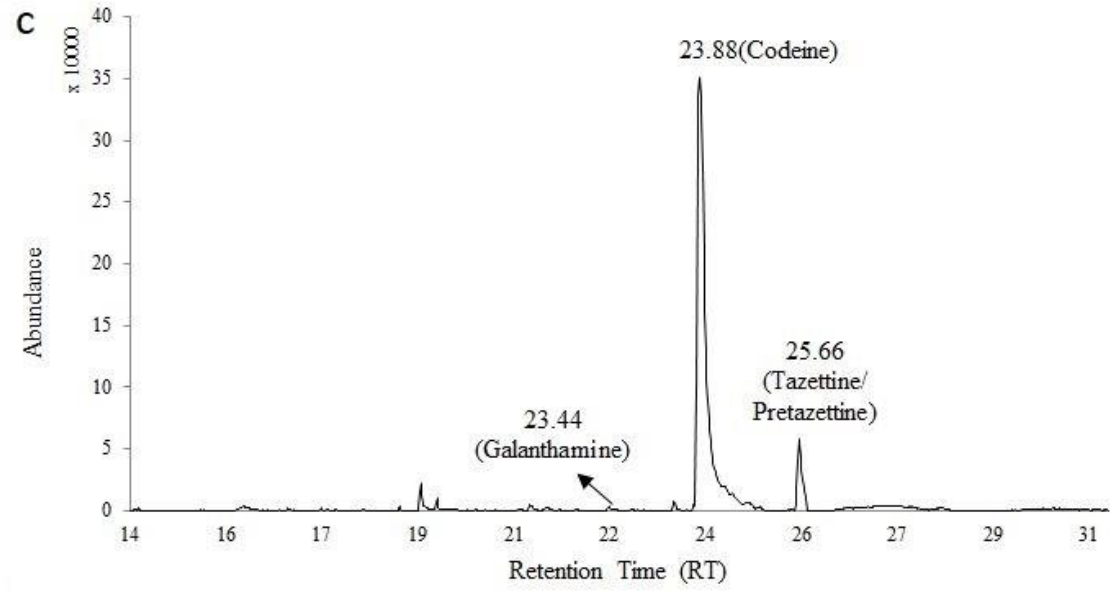

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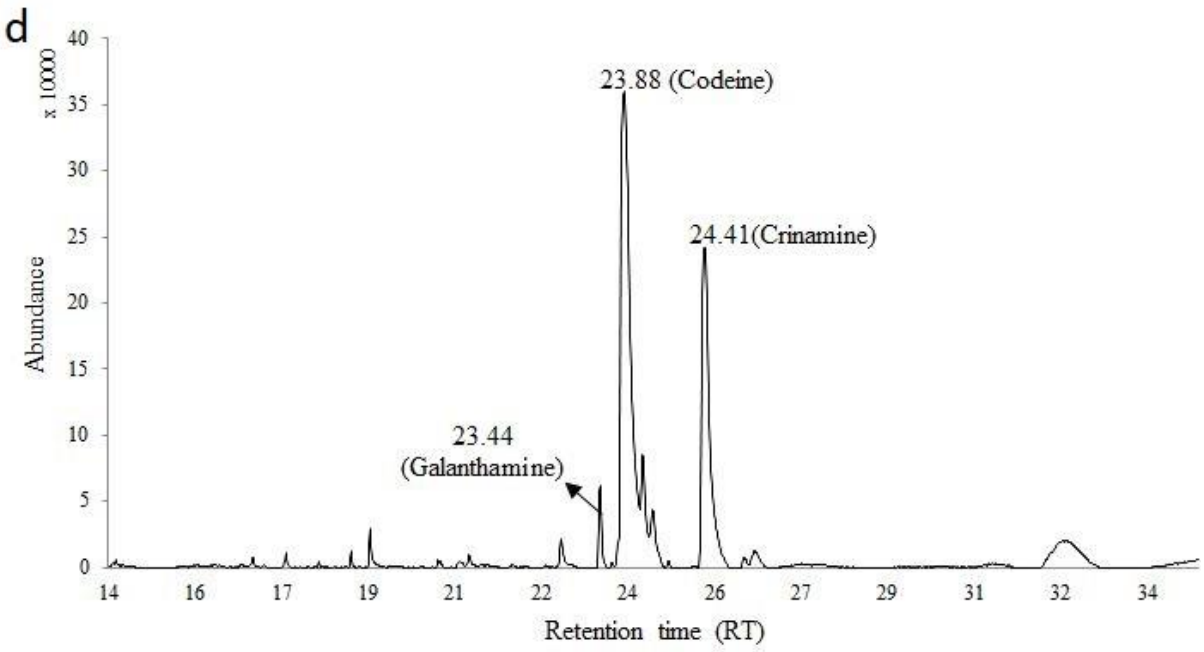

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608 **Fig. 6.** GC-MS chromatograms of alkaloid extract of field grown Carlton bulb (a); Carlton
609 basal plate (b); tissue culture derived callus (c) and bulblets (d) showing signals (Retention
610 time) of galanthamine, codeine (internal standard) and other alkaloids and/or their
611 derivatives.

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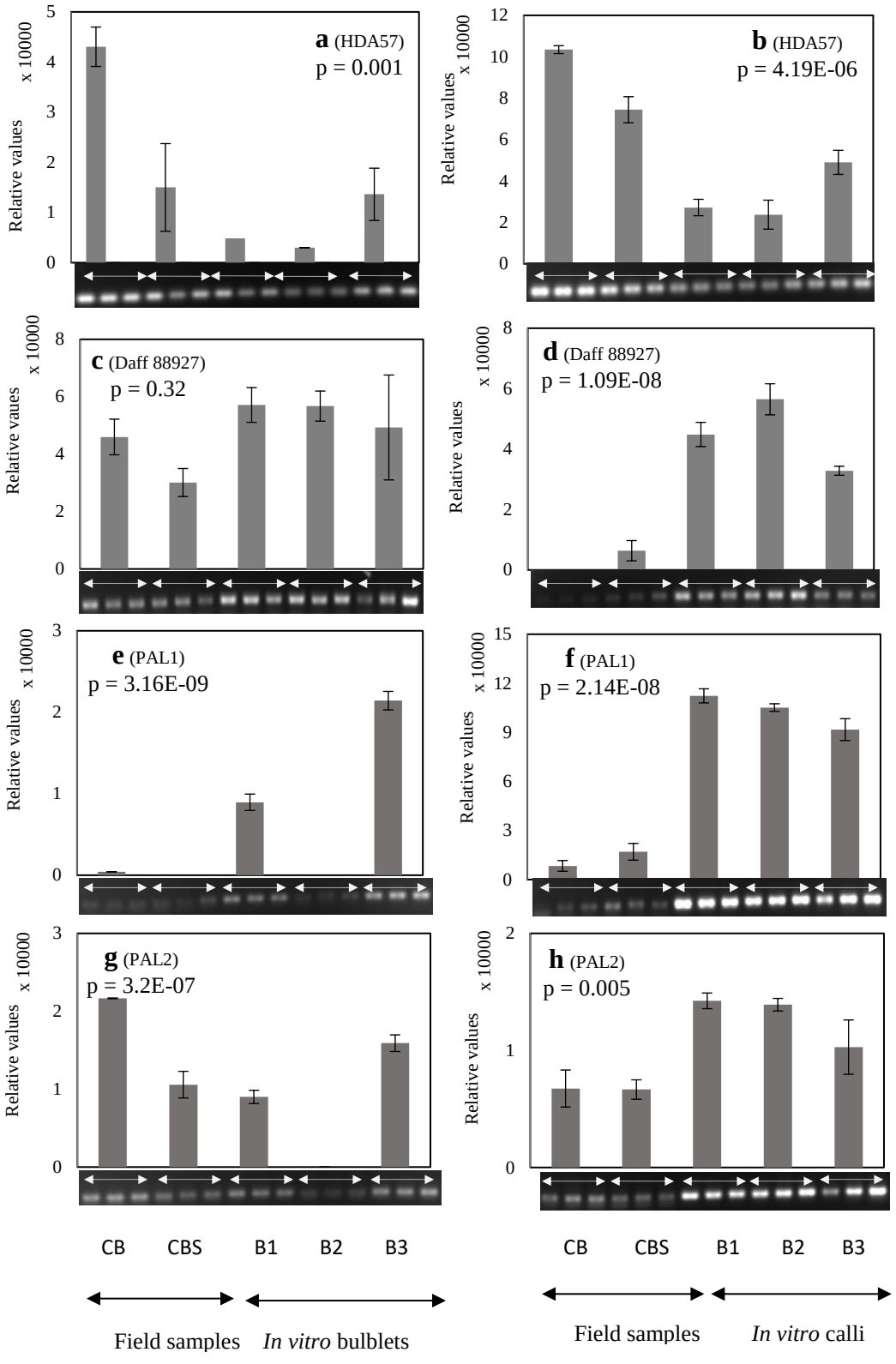
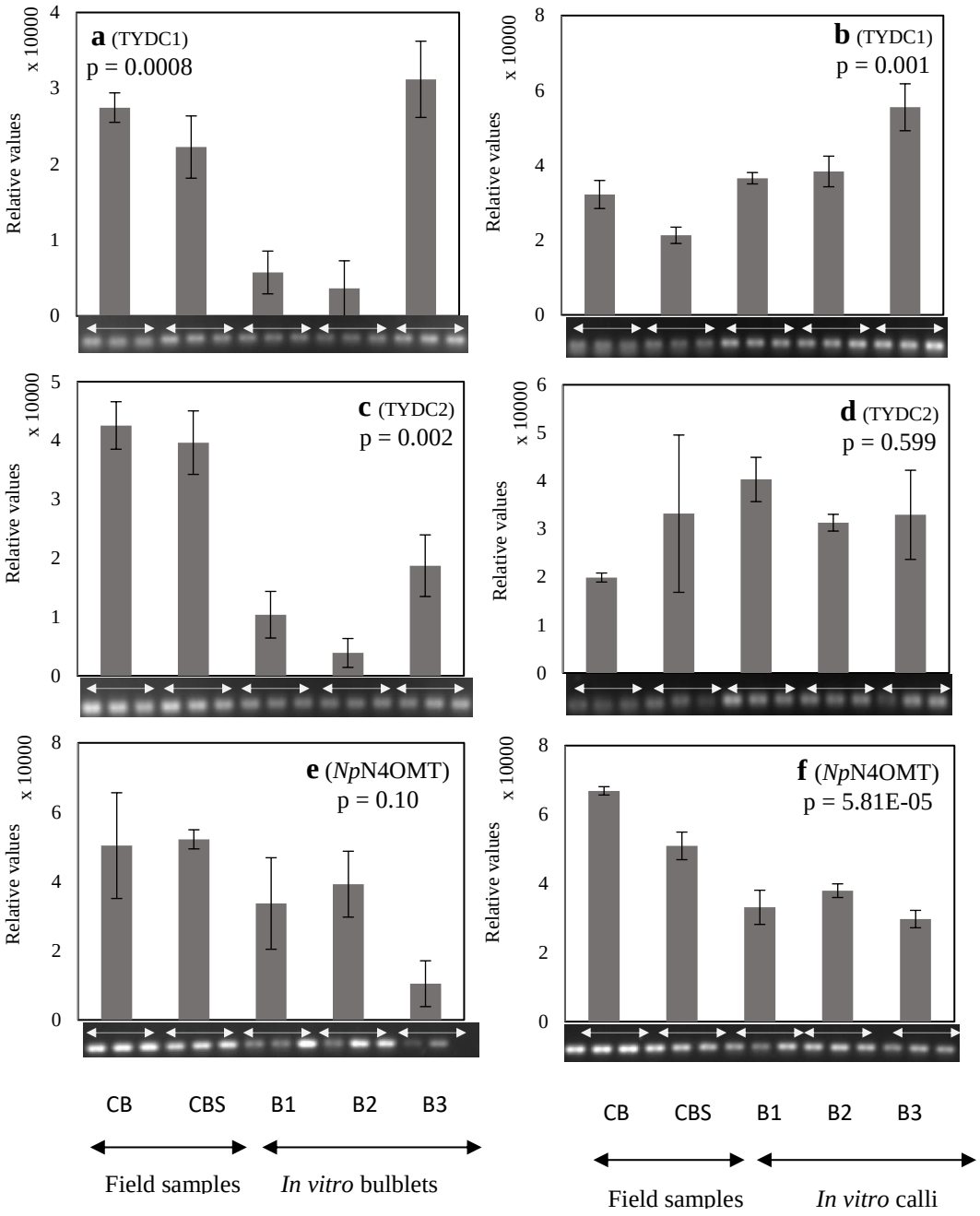


Fig. 7. Relative expression levels P450s and *PAL* transcripts to ACTIN: HDA57 **(a and b)**; Daff 88927 (**c and d)**; PAL1 **(e and f)** and PAL2 **(g and h)** with their corresponding gel images (3 replicates); CB=Carlton bulb, CBS=Carlton basal plate, different bulbs (B1, B2 and B3). Error bars = SE. The p-values of the ANOVA between the different samples are shown in the figure. **Confidence level for mean was 95%.**



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632 **Fig. 8.** Relative expression levels of *TYDC* and *Np*N4OMT transcripts to ACTIN: TYDC1 **(a**

633 **and b);** TYDC2 **(c and d);** *Np*N4OMT **(e and f)** with their corresponding gel images (3

634 replicates); CB=Carlton bulb, CBS=Carlton basal plate, different bulbs (B1, B2 and B3).

635 Error bars = SE. The p-values of the ANOVA between the different samples are shown in the

636 figure. **Confidence level for mean was 95%.**

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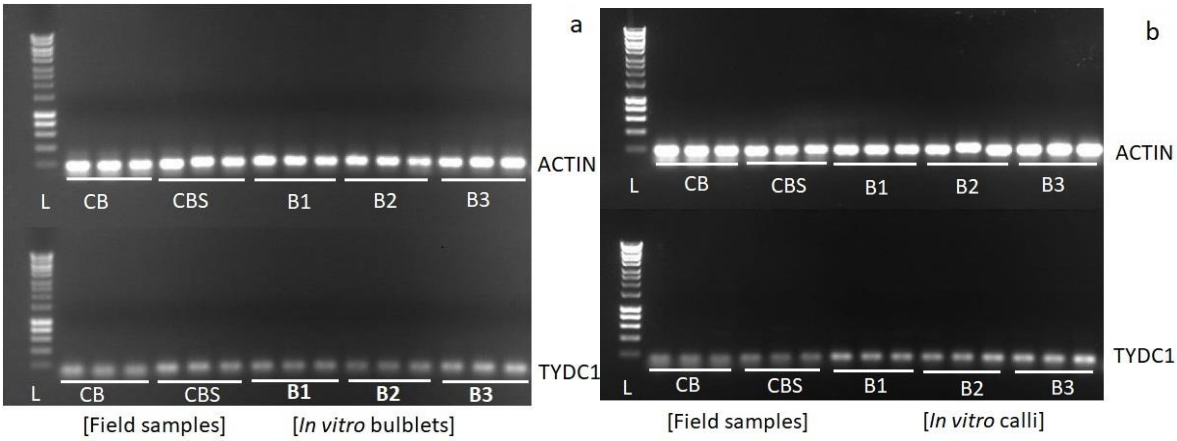

Fig. 9. Representative gel images of relative expression levels of TYDC1 transcript to ACTIN; (a) field samples and *in vitro* bulblets and (b) field samples and *in vitro* calli; CB=Carlton bulb, CBS=Carlton basal plate, three different bulbs (B1, B2 and B3), L=Ladder.