

Effective in vitro bulbification and alkaloid dereplication by GC-MS of Chilean *Zephyranthes splendens*, an endemic species of the Andes Mountains

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Abstract

The indigenous Chilean *Zephyranthes splendens* is a representative of the subfam. Amaryllidoideae (Amaryllidaceae) and is endemic to the South-Central foothills of the Andes Mountains. The plants of this subfamily are well known for producing bioactive alkaloids from the isoquinoline-type skeleton. For the first time it is established an *in vitro* micropropagation and bulbification protocol for *Z. splendens* and the alkaloid pattern of its *in vitro* cultures are characterized. To optimize the plant *in vitro* growth on the MS medium, sucrose concentrations and combinations of growth regulators (BAP and NAA) were evaluated. It was observed that the combination 2 mg/L BAP - 0.2 mg/L NAA promoted highly efficient direct organogenesis, while 90 g/L sucrose favoured bulb proliferation. Kinetin concentration and culture time were also evaluated for bulb development. Higher kinetin concentrations resulted in a significant increase in bulb volume over time, while sucrose promoted shoot development when modulated by kinetin. Dereplication of alkaloids by GC-MS has shown different amounts of alkaloids between wild bulbs and bulbils, the latter showing reduced metabolic activity with lower variability of alkaloids possibly due to their juvenile phase. The juvenile vegetative period along with a diminished photosynthetic process may have played a role on the metabolic processes in *in vitro* bulblets. Altogether, the results highlight the potential of *Z. splendens* bulblets to produce alkaloids under controlled *in vitro* conditions, providing a methodological basis for their biotechnological exploitation.

Key message

High efficiency in organogenesis and bulbs proliferation of *Z. splendens* was achieved whereas the alkaloid profile was characterized in *in vitro* bulbils, which produce and accumulate alkaloids according with its juvenile stage.

I. Introduction

The subfamily Amaryllidoideae (Amaryllidaceae family, Monocotyledon group) comprises *ca.* 59 genera and more than 800 species distributed from warm to temperate region worldwide (2017; Berkov et al. 2020; Stevens 2021). The Amaryllidoideae is a well-known source of ornamental plants being marketed worldwide due to the beauty of its flowers, generally called "Amaryllis". The American Continent is considered a secondary center of diversification in Amaryllidaceae species (Ito et al. 1999; Chase et al. 2016), and Chile has approximately 119 species distributed into 12 genera, mainly concentrated in the Mediterranean area between the Coquimbo and Metropolitan Regions (Rodriguez et al. 2018). In traditional medicine, the Amaryllidaceae species have demonstrated a remarkable potential on managing many health ailments, such as urinary and parasitic infections, renal and hepatic conditions, mental illness, and tumour/cancer (Tallini et al. 2018b). Chemically, these activities have been assigned to a consistent and exclusive group of isoquinoline-like alkaloids known as Amaryllidaceae alkaloids (AA). Galanthamine, isolated from the Caucasian species *Galanthus woronowii* in 1940s, is a successful example of AA that reached clinical use being currently prescribed to the management of mild to moderate Alzheimer's disease (Kaur et al. 2022).

The biosynthesis of alkaloids in the subfamily Amaryllidoideae comes from the shikimate pathway through metabolic routes of the aromatic amino acids L-phenylalanine (L-Phe) and L-tyrosine (L-Tyr). The biosynthesis of AA is ruled out by a seven-step chloroplastic pathway, which eventually yields norbelladine derivatives after the condensation of the protocatechuic aldehyde and tyramine from L-Phe and L-Tyr metabolic routes, respectively (Desgagné-Penix 2020). A specific methylation of the hydroxy group at C4' in norbelladine provides the key intermediate precursor 4'-O-methylnorbelladine (Desgagné-Penix 2020). The response of individual genes of the shikimate pathway is closely related to variations of light and nutrient availability (Tako and Rook 2013).

To generate changes in the response of genes related to the alkaloid production, biotechnological tools such as tissue cultures play significant role for further exploitation of plants through the adoption of *in vitro* culture techniques. Plant biotechnology applying tissue cultures and totipotent plant cells offers an opportunity to develop mass production systems for commercial species and/or primary/secondary metabolites for supplying industrial demand. The *in vitro* culture techniques offer an alternative for exploration of resources and collection from natural environment by multiplication and biomass production under controlled conditions, thereby avoiding the threat of extinction to which many species are exposed (Arencibia et al. 2022).

Traditional tissue culture approaches for AA production have been studied and improved, not reaching a commercial exploitation though (Piątczak et al. 2014; Arencibia et al. 2018). In general, for the production of pharmaceutical compounds, the use of heterologous expression systems is recommended, but this may not be reasonable for AA considering their complex metabolic pathways (Naeem and Khan, 2013).

The Amaryllidaceae *Zephyranthes splendens* is a species endemic to Chile, distributed in the central part of the country, from the Coquimbo Region to the Maule Region. This bulbous plant grows on rocky slopes and well-drained soils at a Mediterranean biome featured by rainy winters and dry summers. Although it is not listed as an endangered species, its habitat faces significant threats due to urban sprawl, agriculture and overharvesting of bulbs for ornamental purposes. In Chile, its conservation status varies locally, with some populations protected in natural areas, while others are in decline due to habitat fragmentation (Ministerio del Medio Ambiente, Chile, 2024). The importance of conserving this species has been highlighted not only for its ecological value as a source of nectar for pollinators, but also for its potential source for bioactive alkaloids.

Thus, considering that *in vitro* culture technique represents a useful alternative for gathering plant material to explore natural resources, the aim of this study was to establish an efficient micropropagation system for the recovery of the endemic Chilean species *Zephyranthes splendens* and evaluate the alkaloid constituents of wild bulbs and *in vitro* bulblets by gas chromatography-mass spectrometry (GC-MS). These micropropagation systems offer opportunities for the production of biomass for ornamental purposes, germplasm banks for the support of law 20.283 and the programmes created by CONAF for the reintroduction of natural populations in altered ecosystems (CONAF 2023a;

Biblioteca del Congreso Nacional 2023b) or plant material to delineate biosynthetic pathways that provide bioactive alkaloids of medicinal interest.

II. Materials and Methods

2.1 Plant Material

Bulbs and seeds of *Zephyrantes splendens* (formerly *Rhodophiala splendens*), were collected from different populations located around Parque Ingles (-35.47349309758782, -70.98736284627748), Molina, Region del Maule, Chile (Fig. 1).

2.2 Micropropagation experiments

Z. splendens plants were collected and identified at flowering period followed by seed collection in January 2021 and February 2022, respectively. A total of 250 seeds were placed in the culture after removal of the seed coat.

The seed disinfection was carried out using firstly Tween 20 (5 min) followed by 0.5% NaClO (3 min) and 1% NaClO (2 min), with distilled water rinses (3 min) applied between each sanitizer solution as described previously with some modifications (Alexopoulos et al. 2022). The explants were then dried on sterile paper and placed in culture tubes containing 30 g/L of sucrose MS medium at pH 5.7–5.8, solidified with 8,8 g/L (approximately 0,8%) agar without growth regulators. Finally, the explants were maintained for 8 weeks at $23 \pm 1^\circ\text{C}$ and a photoperiod of 16/8h (light/dark) under a combination of natural light and cool white fluorescent tubes at a light intensity of $60 \mu\text{M m}^{-2}\text{s}^{-1}$.

For bulblet formation, MS nutrient medium supplemented with different concentrations of sucrose (30 as a control, 60, 90, and 120 g/L) without growth regulators, was used. The growth conditions were maintained as described above. Fifteen explants were evaluated in triplicate for each sucrose concentration.

After two months, the bulbified seedlings were cut lengthwise into 4–6 parts for multiplication of the *in vitro* material. Explants were cut while keeping the basal meristem, and a generous portion of tissue and grown on MS medium supplemented with 30 g/L sucrose, 8,8 g/L agar was treated with and different combinations of plant growth regulators (PGRs) to assay their effects on the plant propagation as follows: a control treatment (T0) without PGRs; treatment T1 used with 1 mg/L of 6-benzylaminopurine (BAP) and without the addition of naphthaleneacetic acid (NAA). In treatment T2, 1 mg/L of BAP and 0.5 mg/L of NAA were added to the nutrient medium, while in T3, the concentration of BAP was maintained at 1 mg/L and NAA was increased to 1 mg/L. For treatments T4, T5, and T6, the BAP concentration was increased to 2 mg/L, combined with NAA concentrations of 0 mg/L, 0.2 mg/L, and 1 mg/L, respectively. Fifteen explants (in triplicate) were used for each combination of PGRs, and the best combination for plant regeneration was chosen for direct organogenesis.

The bulb formation of the explants was evaluated after two months by measuring the bulb height and width with a Vernier calliper, and the best combination of PGRs was selected considering the multiplication of the explants. The growth rate data for the different combinations of growth regulators were normalized to the ranges of 0 and 1 and statistically processed.

Bulbs of *in vitro* seedlings resulting from the above experiments were cut longitudinally into at least 4–6 parts and placed in culture medium to multiply the material *in vitro*. Then, the plantlets were regenerated again and then bulbified and rooted with 1 g/L activated charcoal to assess their adaptability under greenhouse conditions, in accordance with previous studies (Berkov et al. 2021a).

2.3 Optimizing bulblet development on *in vitro* culture

The two sucrose concentrations that allowed for the greatest bulblet development were selected for joint evaluation with kinetin (0, 0.5, 1.0 and 2.0 mg/L). Fifteen individuals were selected in triplicate for each kinetin and sucrose treatment (90 and 120 g/L). The variables of length, width and number of shoots of the *in vitro* cultures were measured using a Vernier calliper. In addition, the ovoid volume was determined using the equation $V = \frac{4}{3}\pi * \left(\frac{\omega}{2}\right)^2 * \frac{h}{2}$, taking the width as the radii of the horizontal sections (ω) and the height (h) as the vertical axis. Measurements were performed on days 5, 13, and 34 of culture with sucrose and kinetin.

2.4 *Ex vitro* adaptation

The resulting plants from previous experiments with well-developed bulbs were rooted using 1 g/L of activated charcoal to assess their adaptability under greenhouse conditions according to Berkov et al. (Berkov et al. 2021a). The plants were individually transferred to trays in a mixture of peat and perlite (3:1) and monitored for three months.

2.5 Extraction and quantification of alkaloids

The plant material was washed under running water, cut, frozen at -0°C, and freeze-dried. After freeze-drying, the bulbs were crushed and stored at -20°C. Approximately 5 g of shredded bulb (wild plants and bulblets) were weighed. Subsequently, 10 mL of methanol (PA grade) were added, sonicated for 30 min (10 min rest), filtered, and evaporated under reduced pressure. The filtered cake was subjected to a new maceration process with 10 ml of methanol (PA), 30 min of sonication, and 24 h of rest. Subsequently, the material was filtered, combined with the first dried crude extract, and evaporated under reduced pressure. The last maceration process, using 30 min of sonication along with 24 h rest was repeated one more time, and the filtered material was combined with the first two crude extracts and evaporated again under reduced pressure.

For preparation of alkaloid fraction, the dried crude methanolic extracts from wild bulbs and bulblets were solubilized separately in 20 mL of H₂SO₄ solution (2% v/v) and subjected to the cleaning-up process using 15 ml of diethyl ether (three times) and 15 ml of ethyl acetate (once), which allowed the

partitioning of apolar and neutral metabolites from the plant extract. The organic phase was discarded, and the acidic aqueous solution was then alkalinized with NH_4OH (25%, up to pH–9–10) and partitioned with 15 ml of ethyl acetate (three times), which were pooled, dried with anhydrous sodium sulphate, and evaporated using a rotary evaporator under reduced pressure. At the end, one enriched-alkaloid extract from wild bulbs and bulblets were obtained.

To quantify the individual constituents of the wild bulb extract, a calibration curve of galanthamine (10, 20, 40, 60, 80, and 100 mg/mL) and codeine (50 mg/mL) as an internal standard was used. The peak areas were manually obtained considering selected ions for each compound (usually the base peak of their MS, i.e., m/z 286 for galanthamine and 299 for codeine). The ratio between the values obtained for galanthamine and codeine was plotted against the corresponding concentration of galanthamine to obtain the calibration curve and its linear equation ($y = 0.0632x - 0.4562$; $R^2 = 0.9977$). All data were standardized to the area of the internal standard (codeine), and the equation obtained for the calibration curve of galanthamine was used to calculate the amount of each alkaloid. The results are expressed as mg of galanthamine equivalent (mg GAL), which is related to the alkaloid extract weight.

For the alkaloid analysis of the enriched alkaloid extract from the *in vitro* bulblets, only the total ion current (TIC) of each identified alkaloid was considered. The proportion of each component is expressed as a percentage of the total alkaloid TIC. The area of the GC-MS peak depends not only on the concentration of the corresponding compound but also on the intensity of its mass spectral fragmentation, and the quantification in both wild bulbs and *in vitro* bulblets (with and without an internal standard, respectively) were not absolute. However, this methodology is considered suitable for comparing the specific alkaloid amount in wild bulbs and for the qualitative comparison of identified alkaloids between wild bulbs and *in vitro* bulblets.

2.6 Alkaloids dereplication

Alkaloid dereplication from the enriched-alkaloid extract from wild bulbs and *in vitro* cultured bulblets of *Z. splendens* were carried out by GC-MS analysis. The samples were prepared using 5 mg of the extract solubilized in methanol (HPLC grade) and filtered using 0.22 mm FTPE syringe filter. EI-MS spectra were obtained on an Agilent 6890N GC 5975 inert MSD operating in EI mode at 70 eV (Agilent Technologies, Santa Clara, California, USA) using a DB-5 MS column (30 m x 0.25 mm x 0.25 μm , Agilent Technologies) with an injector temperature of 280°C. The temperature program was as follows: 100–180 °C at 15°C min^{-1} , hold for 1 min at 180 °C and 180–300 °C at 5°C min^{-1} , and held for 10 min at 300°C. The flow rate of the carrier gas (helium) was 0.8 ml min^{-1} , and a split ratio of 1:20 was used. The alkaloids were identified by comparing their GC-MS spectra and Kovats retention indices (RI) with our in-house library database. This library has been continually updated and reviewed with alkaloids isolated by our group and identified using other spectroscopic techniques, such as NMR, UV, CD, and MS. The mass spectra were deconvoluted using AMDIS 2.64 software (NIST). The Kovats retention indices (RI) of the compounds were recorded using a standard calibration mixture of *n*-hydrocarbons (C9-C36).

2.7 Statistical analysis

Bulb height, width, number of aerial shoots and number of roots were measured as quantitative variables. After checking the normality of the data with the Shapiro-Wilk test and the homogeneity of variance with Levene's statistic, which was significant in both cases ($p < 0.05$), the Kruskal-Wallis test was applied to detect differences between treatments. Multiple comparisons were performed using Dunn's test. For the interaction model between factors, a Kruskal-Wallis test was established for three factors (sucrose, kinetine, and time) that were significant ($p < 0.05$). All graphs and statistical analysis were performed using RStudio software (v2024.09.0 + 375).

The Benjamini–Hochberg (BH) method was used to control the false discovery rate in the analysis of multiple comparisons. This approach began by obtaining the p -values of all statistical tests performed, which were ordered from lowest to highest. A rank was then assigned to each p -value, starting from 1 for the smallest value. The significance level (α) was set at 0.05. The p -value corresponding to each test was compared with the criterion $\frac{i}{m} * \alpha$, where i is the range of the p -value and m is the total number of comparisons. The p -values that met this criterion were considered significant. This procedure effectively identified the relevant results in the context of the analysis, thus minimizing the risk of false positives in the findings. For the analysis of bulblet growth and development, additive and interaction models were evaluated through R.

III. Results

3.1 Establishment of *in vitro* culture

In the propagation protocol proposed herein, the removal of the seed coat provided 90% of the seedlings without fungal or bacterial contamination and improved the overall morphogenic response rate of the seeds. Short exposure times of seed to NaClO were sufficient to achieve adequate disinfection and avoid the inhibition of germination and sprouting.

Despite the delay in morphogenic responses, the plants regenerated *in vitro* from seeds germinated at a range of two weeks to two months after introduction onto the MS medium. Variations in sucrose concentration allowed bulb differentiation, and the optimal concentration of 90 g/L was selected for *in vitro* bulblet formation. Once bulblets were formed, tissue multiplication and subsequent *in vitro* regeneration showed an adequate morphologic differentiation using treatment T5 (2 mg/L BAP and 0.2 mg/L NAA), even though no statistical difference were observed among all the treatments. In addition, the multiple cultures were regenerated after 2–4 weeks for all treatments. All the results are summarized and shown at Fig. 2.

3.2 Effect of sucrose concentration on the micropropagation of *Z. splendens*

For micropropagation of *Z. splendens*, different concentrations of BAP and NAA were evaluated in the culture medium. Once the seedlings responded to the culture medium, they were transferred to new

culture media with different concentrations of sucrose as the carbon source (Fig. 3, Table 1). The best results were achieved after one month of cultivation on MS medium supplemented with 90 and 120 g/L sucrose (Fig. 3a-c). This step was the key to raise *in vitro* biomass (bulbification) and multiply the number of explants. Once the bulblets reached a size of around to 0.8 to 1.0 cm of thickness (Fig. 3d,e), they were cut and multiplied at the rate of 1:4–1:6.

The effects of different concentrations of BAP and NAA on the direct organogenesis of Amaryllidaceae explants allowed us to define a suitable protocol for the regeneration of explants from tissue cultures.

Table 1
Morphological response to sucrose concentrations during *in vitro* bulbification of *Z. splendens* after one month of cultivation.

MS medium + Sucrose (g/L)	Bulb width (cm)	Height (bulb height) (cm)	N° of shoots on root	N° of aerial sprouts	Induction of bulblet formation
30 (control)	0.30 ^a ± 0.10	0.93 ^a ± 0.21	4.33 ^a ± 1.15	1.67 ^a ± 0.58	-
60	0.33 ^a ± 0.06	0.80 ^a ± 0.10	3.33 ^a ± 0.58	1.33 ^a ± 0.58	-
90	0.43 ^a ± 0.06	0.90 ^a ± 0.01	3.67 ^a ± 0.58	1.67 ^a ± 0.58	+
120	0.77 ^b ± 0.15	1.53 ^a ± 0.12	4.33 ^a ± 0.58	1.33 ^a ± 0.58	+

* The table shows the mean ± standard deviation. Different letters indicate significant differences according to Dunn’s test (p < 0.05).

The induction of bulbs from *in vitro* culture did not stimulate spontaneous roots formation during the multiplication phase; however, it produced a completely new explant response after two weeks, as shown in Fig. 3-C. The complete regeneration response was due to previous explant bulbification because the growth medium lacked growth regulators.

3.3 Effect of the interaction of sucrose concentrations and kinetin on plant bulblet development

The experimental design employed additive and interactive models to systematically evaluate how sucrose concentrations and kinetin levels independently and jointly influence *Z. splendens* bulblet development *in vitro* (Fig. 4). This approach allowed to distinguish between individual factor effects and potential synergistic interactions. The Kruskal-Wallis non-parametric analysis revealed highly significant differences in ovoid volume development across treatment groups ($\chi^2 = 42.053$, $p = 0.00895$), demonstrating that the experimental treatments produced distinct morphological outcomes. Subsequent post hoc analysis using the Benjamini-Hochberg method for multiple comparisons identified several statistically significant pairwise differences. Most notably, the combination of 2 mg/L kinetin

with 120 g/L sucrose produced ovoid volumes that were significantly larger ($p < 0.05$) than those observed in the 0 mg/L kinetin + 90 g/L sucrose treatment group, suggesting a potentially optimal concentration range for bulblet development.

Further examination of individual factors showed that kinetin concentration alone had a marked influence on ovoid volume (Fig. 5). The 2 mg/L kinetin treatment consistently resulted in a significant larger volume compared to both the 0 mg/L and 1 mg/L treatments, indicating a dose-dependent response. The interaction model provided additional insights, revealing significant two-way interactions between kinetin and time, as well as between sucrose and time. These interactions showed a progressive greater ovoid volume at the 34-day measurement point compared to the 13-day evaluation, suggesting a temporal component to the growth response. Paradoxically, the three-way interaction between sucrose, kinetin, and time failed to reach statistical significance, suggesting that while these factors independently influence the development, they do not appear to act synergistically in determining ovoid volume.

Using the summative model, the sucrose concentration exerted a significant positive effect on shoot number ($p < 0.05$) and kinetin demonstrated a significant negative relationship with shoot proliferation ($p < 0.05$). Time of culture showed a negative trend in shoot production ($p = 0.0734$). For the summative model of shoot number, a $R^2 = 0.109$ was calculated.

Throughout the interaction model ($R^2 = 0.1579$), sucrose alone showed a positive effect on shoot number ($p < 0.01$), confirming its role as a growth promoter. Two noticeable negative interactions were observed i.e. (i) between sucrose and kinetin ($p < 0.05$), where kinetin presence attenuated sucrose's beneficial effect, and (ii) between sucrose and time ($p < 0.05$), indicating sucrose's positive influence has progressively diminished during the culture (Fig. 6).

For ovoid volume development, linear models revealed distinct patterns. Kinetin concentration showed a significant positive relationship ($p = 0.0028$), while time demonstrated an even stronger effect ($p = 9.55 \times 10^{-7}$), emerging as the dominant growing factor. Noteworthy, sucrose alone showed no significant effect on this parameter. The model explained 24.2% of volume variability ($R^2 = 0.242$), highlighting the crucial role of time in ovoid expansion, although other factors may also contribute to growth regulation.

The interaction model ($R^2 = 0.2787$) explained almost 28% of the observed variability in ovoid volume development, representing a 15% improvement over the main effects models alone. Time was the only statistically significant factor ($p = 0.040$) in this multivariate analysis, with non-significant interaction terms ($p > 0.05$) for sucrose and kinetin combinations. This statistical pattern strongly suggests that temporal factors operate independently of nutritional-hormonal interactions in mediating bulb expansion.

During the critical acclimatization phase, 93% of transplanted seedlings successfully developed three key survival indicators: (1) protective tunicate bulb coverings, (2) robust brown root pigmentation, and

(3) active meristem growth points. The transition protocol proved particularly effective for root system establishment, with secondary root emergence occurring within 10–14 days post-transfer (Fig. 7).

In vitro multiplication revealed distinct developmental phases. While primary bulb induction occurred within 4 weeks, root initiation showed a consistent 14-day lag period across all treatments. The most effective biomass production combined MS basal medium with 120 g/L sucrose over 4–6 week culture periods, yielding 3.2-fold increases in fresh weight compared to standard protocols.

3.4 Alkaloid biosynthesis in wild-type and *in vitro* bulbs of *Z. splendens*.

Concerning the alkaloid content of both wild-type and *in vitro* bulbs of *Z. splendens*, eighteen alkaloids were identified by means of GC-MS analysis throughout the comparison with the authentic standards from our in-house library (Section 2.6). Furthermore, other three compounds were tentatively identified or had a skeleton assigned after the analysis of their EI mass spectral data with those reported as typical fragmentations of Amaryllidaceae alkaloids under GC-MS conditions (Table 2, Fig. 8). Two compounds showed EI-fragmentation of Amaryllidaceae alkaloids but cannot be identified. The 21 alkaloids assigned were sorted into 6 skeleton-types along with one exception classified as miscellaneous (compound **8**). Quantitative analysis using I.S. in the wild bulb extract revealed seven alkaloids synthesized from the *para-para'* phenolic coupling route, 85.75 mg GAL/g in total, which were categorized as follows: three alkaloids assigned as haemanthamine- (**6**, **11**, and **15/15a**), two as tazettine- (**12/12a** and **18**), one as a narciclasine- (**1**) and one as a montanine-type representative (**10**). From the *ortho-para'* phenolic coupling, the alkaloids **7**, **9**, **13**, **14**, **16**, **17**, and **19** were identified and the compound TI2897 was assigned as a lycorine-type derivative, totalizing 57.74 mg GAL/g. The galanthamine-type skeleton, the only series coming from the *para-ortho'* phenolic coupling, totalized 24.52 mg GAL/g split into five components, identified as the alkaloids **2**, **3**, and **5**, along with the alkaloid **4** tentatively assigned as 3-epilycoramine and the component TI2590, which displayed EI-mass fragmentation of a galanthamine-type derivative. The alkaloid content including the two components not identified (N.I. A and B) reached 191.47 mg GAL/g in the wild bulb extract.

The Total Ion Current (TIC) were used for the comparison between the wild type and the *in vitro* bulbs of *Z. splendens*. Only eight alkaloids were identified in bulblet extract and all of them were also shared with the wild type. Great differences were observed into the relative percentage of the identified alkaloids from both plant material. The alkaloid **10** was the main alkaloid in bulblet (31.7%) and all the other alkaloids displayed less than 5% of TIC. From the wild type, the alkaloids **11/12** and **10** were the main compounds, displaying 26.5 and 16.9% of TIC, respectively. The striking differences were noticed respect to the other alkaloids in the wild bulb extract, where five components reached percentages varying from 5 to 10% of TIC, suggesting a greater metabolic activity in the wild bulbs. All the remaining alkaloids reached percentages less than 5% or even found as traces. Noteworthy, the chromatogram from the wild bulbs showed the presence of the basal metabolite γ -sitosterol in a low percentage, in opposite to of that observed in bulblet extract where a mixture of basal metabolites grasped more than 50% of the TIC.

Table 2

Alkaloids present in wild bulbs and bulblets of *Z. splendens* by GC–MS. The alkaloid content is represented as mg galantamine equivalents/g dry bulbs in the wild bulbs and as a percentage of TIC in the wild bulbs and bulblets of *Z. splendens*. The numbers in parentheses indicate the structures shown in Fig. 8.

Components	RI	Wild bulb		Bulblet
		mg GAL/g	% of TIC	% of TIC
Trisphaeridine (1)	2290	5.46	tr	-
Galanthamine (2)	2388	5.38	tr	2.98
Lycoramine (3)	2409	6.44	1.43	2.02
3-Epilycoramine* (4)	2430	5.74	tr	3.49
N-Demethylgalanthamine (5)	2439	6.59	1.62	-
Vittatine/Crine (6)	2469	7.25	2.49	-
Anhydrolycorine (7)	2798	5.55	tr	-
Galanthindole (8)	2493	tr	tr	-
TI2590	2590	5.75	tr	-
11,12-Dehydroanhydrolycorine (9)	2601	6.86	1.97	tr
Montanine (10)	2618	12.65	9.57	2.08
Haemanthamine (11)	2627	18.24	16.91	31.71
Tazettine / Pretazettine (12/12a)	2641	25.58	26.54	1.29
Hippamine (13)	2662	5.84	tr	-
Galanthine (14)	2688	10.17	6.32	-
11-Hydroxyvittatine (15) / Hamayne (15a)	2701	11.01	7.42	-
N.I. A	2735	7.17	2.38	-
Lycorine (16)	2742	11.71	8.34	3.22
9-O-Methylpseudolycorine (17)	2769	5.90	tr	-
3-Epimacronine (18)	2800	5.56	tr	-
Pseudolycorine (19)	2815	5.67	tr	-
N.I. B	2876	10.91	7.30	-
TI2897	2897	6.04	tr	-
Basal Metabolite (γ -Sitosterol)	-	-	3.53	-

Components	RI	Wild bulb		Bulblet
		mg GAL/g	% of TIC	% of TIC
Other basal metabolites	-	-	-	52.71
Total		191.47		

* Proposed structure-type according to the fragmentation pattern; RI: Kovats Retention Index; N.I.: not identified; tr: traces (≤ 5.00 mg/g). TI2590 (galanthamine type) and TI2897 (lycorine type) are alkaloids and can be included in the % and discussion.

IV. Discussion

4.1. Tissue culture and bulb development

4.1.1 *In vitro* bulb development

In contrast to conventional strategies for *in vitro* multiplication of bulblets, the vertical bulblet cutting strategy applied herein showed a rapid response in regenerating new explants, with signs of shoot regeneration one week after transfer. Several studies have shown that sucrose is essential for bulblet morphogenesis, while starch is essential as a source of emergence and new bulblet development (Yang et al. 2017; Peng et al. 2021; Guo et al. 2022). This suggests that lower concentrations of growth regulators are appropriate for micropropagation of *Z. splendens* explant regeneration. The asynchrony in primary bulb induction at 4 weeks and the delay in root initiation of 14 days after multiplication observed in this work, these developmental patterns observed in other monocot species were also reported (Li et al. 2021). The root initiation delay may reflect an evolutionary adaptation prioritizing storage organ formation before root investment, a strategy documented by (Berkov et al. 2021a), in a water-stressed environment. The sucrose concentration threshold (120 g/L) aligned with (Dang et al. 2022; Li et al. 2023) findings on osmotic potential requirements for bulb maturation, although our auxin-free conditions demonstrated that process can occur through carbohydrate signalling alone.

Other studies have established methodologies for crop production using callus formation (indirect organogenesis) or increasing the number of shoots directly from the establishment of explants *in vitro* instead of using bulblets as explants for multiplication (Sellés et al. 1999; Berkov et al. 2021a; Trujillo Chacón et al. 2023). In addition, it has already been reported that some auxin analogues, such as NAA, indole propionic acid (IPA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 4-iodophenoxyacetic acid, can play a role in regulation of bulblet initiation or development (Shu et al. 2024). Other studies have compared the effects of growth regulators (IBA and NAA) on root tissue development. For example, the use of IBA resulted in the highest rooting of the explants when compared with NAA in the eastern hybrid bulbous species *Lilium cv.* Ravenna (Rafiq et al. 2021). It should be noted that exogenous auxins sometimes promote an increase in scale rot rather than scale propagation or bulblet development, as observed in *Hippeastrum vittatum* (Amaryllidaceae) (Zhang et al. 2013). In previous research, many authors have shown that bulblet development is a finely controlled process

under the influence of growth hormones, cytokinins and gibberellins (Li et al. 2021; Wu et al. 2021; Prasad 2022).

The observed responses in ovoid volume development highlight the complex interplay between nutritional and hormonal regulation during *Z. splendens* bulblet morphogenesis. The superior performance of the 2 mg/L kinetin treatment aligns with established principles of cytokinin action in storage organ development, where moderate concentrations usually optimise cell expansion and biomass accumulation. The absence of a significant three-factor interaction between sucrose, kinetin and time suggests that they operate through independent physiological mechanisms for bulblet formation. Sucrose mainly influences bulblet development due to its role as a carbon and energy source, while kinetin may act by modulating cell division and expansion pathways. The temporal progression of volume increases implies an accumulation effect, where sustained exposure to favourable conditions produces progressively better results.

The opposite effects of sucrose and kinetin on shoot proliferation were also significant. While the positive influence of sucrose follows the expected patterns of carbohydrate-enhanced growth, the inhibitory effect of kinetin contrasts with cytokinins as shoot-promoting hormones. This paradoxical result may reflect species-specific responses or threshold effects, where cytokinin levels suppress meristematic activity. Similar phenomena have been documented in *Narcissus* (Rahimi Khonakdari et al. 2020), where high concentrations of cytokinins induced a developmental shift from shoot formation to the development of storage organs. The significance of the time factor ($p = 0.0734$) in shoot reduction could indicate a gradual depletion of resources or the initiation of phase change processes *in vitro*.

4.1.2 Linear models applied to explaining factors

From a practical perspective, these results suggest different optimisation pathways depending on propagation requirements. For bulblet growth, a kinetin concentration of 2 mg/L seems optimal, especially when combined with extended growing periods. For shoot multiplication, lower kinetin levels combined with higher sucrose concentrations are preferable. The modest R^2 value underlines the need to identify additional physiological control factors, along with light potency, variations in temperature regimes or endogenous hormone levels. Future studies should explore these variables while investigating the molecular mechanisms underlying sucrose-kinetin antagonism. These results on the interaction of different factors for bulb development *in vitro* represent a substantial advance in our understanding of *Z. splendens* micropropagation and provide a solid basis for further refinement of the protocol. The sucrose-kinetin antagonism in shoot proliferation suggests metabolic trade-offs, in which kinetin may redirect resources towards storage organ development rather than shoot formation. The decay that occurs over time in sucrose efficiency probably reflects both carbohydrate depletion and progressive metabolic changes during prolonged cultivation, similar to that described by (Sellés et al. 1997).

When assessing ovoid volume development, two key processes emerge: (1) the role of kinetin in regulating tissue expansion and (2) the cumulative effect of time on tissue growth. The absence of

significant effects of sucrose suggests that its influence may be indirect or mediated by unmeasured factors and variables. The moderate explanatory power of the models ($R^2 = 0.1579\text{--}0.242$) indicates several avenues of investigation for future studies.

The summative model indicated that sucrose concentration exerted a significant positive effect on shoot number ($p < 0.05$), with increased carbohydrate availability promoting greater shoot formation. In contrast, kinetin showed a significant negative relationship with shoot proliferation ($p < 0.05$), with increasing concentrations of cytokinin paradoxically reducing shoot number. Growing time showed a non-significant negative trend ($p = 0.0734$), potentially indicating a gradual decline in shoot production capacity over prolonged growing periods. Although the relatively low explanatory power of the model ($R^2 = 0.109$) suggests that other unmeasured factors contribute to the variability of shoot proliferation, it provides clear evidence of the opposite effects observed for sucrose and kinetin on this developmental parameter. The interaction model evaluating the combined effects of sucrose and kinetin over time revealed several significant patterns in shoot development. Statistical analysis showed that sucrose alone had a strong positive effect on shoot number ($p < 0.01$), confirming its role as a growth promoter. However, we observed two significant negative interactions: (1) between sucrose and kinetin ($p < 0.05$), where kinetin attenuated the beneficial effects of sucrose, and (2) between sucrose and time ($p < 0.05$), indicating that the positive influence of sucrose progressively decreased during cultivation. The explanatory power of the model ($R^2 = 0.1579$) and like the summative model suggests that other unmeasured factors are likely to influence shoot proliferation dynamics. The model results challenge conventional assumptions about sucrose-kinetin synergy in bulb development. Instead, our data support the temporal dominance hypothesis (Wu et al. 2021), according to which developmental phase transitions overcome the combinatorial effects of PGRs. The high acclimation success rate (93%) - particularly without dormancy interventions - suggests that our protocol effectively mimics natural seasonal transitions, as (Prasad 2022) observed in related geophytes.

4.2. Alkaloid biosynthesis in both wild and in vitro bulblet of *Z. splendens*

4.2.1. Assignment of the alkaloids (dereplication)

For the identification of the alkaloids, GC-MS technique was used and the match between EI-mass fragmentation and the RI compared to the data from our in-house library allow the identification of the analyte (Section 2.6). Then, 18 alkaloids were identified although some distinctiveness from a dereplication procedure using GC-MS must be pointed out. First and foremost, the alkaloid **12** is an artifact formed from **12a** after acid-base extraction and/or when it is analysed under GC-MS condition. The strong alkaline environment or the high temperature of a GC-MS columns provide conditions for the epimerization from **12a** to **12** (Wildman and Bailey 1969; de Andrade et al. 2012). Further, vittatine and crinine (**6**) are both enantiomers and show quite similar mass fragmentation by EI ionization and RI, which prompt the use of other instrumental technique for the unambiguous identification of these both alkaloids. The 3-hydroxy epimers **15** and **15a** also show a quite similar RI and EI-mass fragmentation and the presence of one of them or both cannot be resolved by GC-MS without a previous process like

derivatization, for example. Noteworthy the presence of alkaloid **8**, which was assigned as “miscellaneous” since its structure may be related with catabolic product from isoquinolines (Bastida et al. 2006).

Moreover, some alkaloids detected using GC-MS could be rationalized according to their EI-mass fragmentation pattern. The alkaloid **4** was tentatively identified a 3-epilycoramine since its EI-fragmentation pattern was very similar of those observed by **3**, although they showed different retention index. The difference of their RI of 21 units is similar to that observed between galanthamine and epigalanthamine (Berkov et al. 2013) indicating that **4** most probably is 3-epilycoramine. Likewise, the alkaloid named TI2590 also showed a typical fragmentation pattern of galanthamine-type alkaloids without a double bound at C4-C4a like in compounds **3** and **4** (Berkov et al. 2012). As the substitution at the *N* greatly influence the fragmentation pattern, we suggest that TI2590 have a substitution at C3. The difference between the M^+ of compound **3** (m/z at 289) and TI2590 (m/z 345) indicates a substituent with 56 amu. The fragmentation pattern of TI3897 indicates a lycorine type compound with substituents at C1, C2 and C3 (Berkov et al. 2021b). Further isolation and NMR spectroscopy of these alkaloids would lead to their complete structural identification.

4.2.2. Alkaloid content in the wild *Z. splendens*

Some species from the *Rhodophiala* genus have been now catalogued as *Zephyranthes* according to the last taxonomical evidence supporting the transfer (García et al. 2019). Then, *Z. splendens* is the former *Rhodophiala splendens* and a population collected in 2016 in Las Trancas (Bio- Bio Region, Chile) has been evaluated in its alkaloid content by GC-MS using the same quantification methodology applied here for *Z. splendens* wild bulbs. Comparing both populations, the species from Bio-Bio Region produced 161.9 mg GAL/g while *Z. splendens* from Maule synthesized 191.47 mg GAL/g (Tallini et al. 2018a). Although the content in *Z. splendens* from Maule was little higher, the content was quite similar of those observed in other Amaryllidoideae species from the Central Region of Chile (Tallini et al. 2018a). Twenty alkaloids were identified in the species from Bio-Bio but only eight alkaloids were common with *Z. splendens* from the Maule Region. The undefined components in both populations were also unique. In terms of skeleton-type biosynthesis, the content was quite similar for *para-para'* and *ortho-para'* phenolic coupling varying at the range of 70.3–85.75 mg GAL/g and around 50.0 mg GAL/g, respectively. However, the production of galanthamine-type derivatives that feature the *para-ortho'* phenolic coupling was only observed in the species from the Maule Region. It sets a precedent for evaluation of other *Z. splendens* populations into the Central Region of Chile to delineate this striking biosynthetic distinction. This chemotypic variation have been observed in other Amaryllidoideae genus, as the case of *Galanthus* populations growing in Bulgaria showing a great variability in galanthamine production (Berkov et al. 2011).

4.2.3. *Z. splendens*: wild bulbs x bulblets

For the comparison between the wild and *in vitro* bulbs of *Z. splendens* studied here, only the relative percentage of TIC was used. The bulblets of *Z. splendens* produced a lower content of alkaloids than the

wild bulbs due to its juvenile vegetative period. The high percentage of the TIC observed for the basal metabolites is suggestive of a low abundance of alkaloids, which is also evidenced by the low number of identified components (only 8 alkaloids). Furthermore, a deep analysis of the identified alkaloids in both wild and *in vitro* bulbs of *Z. splendens* also confirm the lower metabolic activity of the latter. Precisely, from *para-para'* phenolic coupling, since **11** was the main alkaloid and **12/12a** and **10** were detected in a very small percentage in *in vitro* bulblets of *Z. splendens*, the compound **12/12a** was the main metabolite followed by **11** and **10** in wild bulbs of this species. The switch between the main components in both extracts can be explained by the biosynthetic route of alkaloids in Amaryllidaceae. The hemanthamine-type skeleton (compound **11**) is primarily formed from *para-para'* phenolic coupling followed by montanine and tazettine (as compounds **10** and **12/12a**, respectively), as in wild bulbs, since the alkaloid **12/12a** is the main component, but with a remarkable percentage for **9** and **10** (Table 2). Otherwise, the *in vitro* bulblet synthesizes **11** as the main compound and **10** and **12/12a** in a clearly lower percentage, which is suggestive of a lower metabolic activity, since the later steps of the route have not been activated during the juvenile vegetative period to synthesize the montanine- and tazettine-type skeletons. Similarly, the alkaloids **13** and **14** are closer to the end steps of the route that arises from the *ortho-para'* coupling since they need more methylations steps to be synthesized. Again, they were not detected in *in vitro* bulblets but were present in the wild bulb extract. These results suggest lower metabolic activity in *in vitro* bulblets than in wild bulbs, which may be explained by the juvenile vegetative period of the bulblets compared to the flowering period of the wild type, in addition to the reduced photosynthetic process in plants grown under *in vitro* conditions.

V. Conclusions

For the first time an *in vitro* micropropagation and bulbification protocol is established for the endemic Andean species *Z. splendens*. The combination of 2 mg/L of BAP and 0.2 mg/L of ANA was shown to promote highly efficient direct organogenesis, while 90 g/L of sucrose favoured bulb proliferation. Furthermore, higher concentrations of kinetin resulted in a significant increase in bulb volume in response to culture time, while sucrose promoted shoot development when modulated by kinetin. Additionally, alkaloid production by *Z. splendens* bulbils is confirmed to a lesser extent and variability probably due to their juvenile vegetative period. Notably, the bulbils synthesized the first pre-alkaloid of more complex metabolites from each phenolic oxidative coupling to 4'-*O*-methylnorbelladine, indicating that bulbil metabolism responds to their vegetative period, in contrast to the flowering period of a wild-type species. Optimization of the *in vitro* photosynthetic process of plantlets with bulbils will be a next challenge that needs to be further evaluated. Overall, the technique applied for the production and multiplication of bulbils, together with the maintenance of the alkaloid production capacity in the generated bulbils, highlights the potential of Amaryllidaceae species for biotechnological applications in the *in vitro* production of bioactive alkaloids.

Abbreviations

BAP 6-Benzylaminopurine

NAA Naphthaleneacetic acid

PGRs Plant Growth Regulators

RI Kovats Retention Index.

EI-MS Electron Ionization Mass Spectrometry

AA Amaryllidaceae Alkaloids

FTPE Polytetrafluoroethylene

Declarations

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Data availability: Data will be made available on request.

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Figures

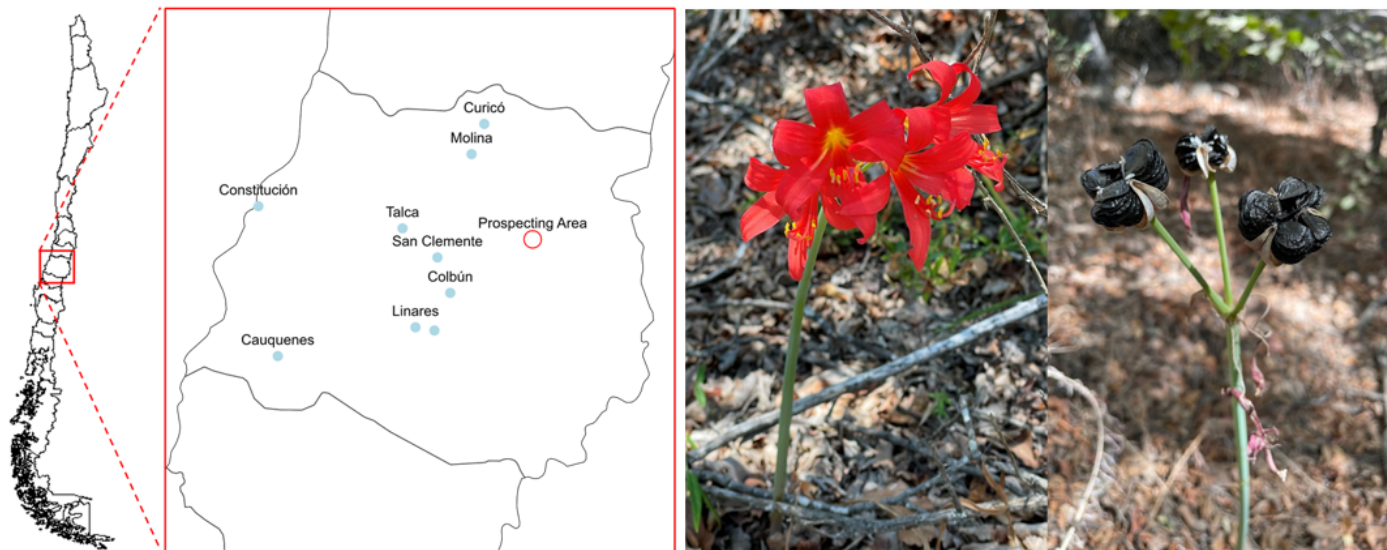


Figure 1

Geographical location of *Z. splendens* populations in the Maule Region of Chile

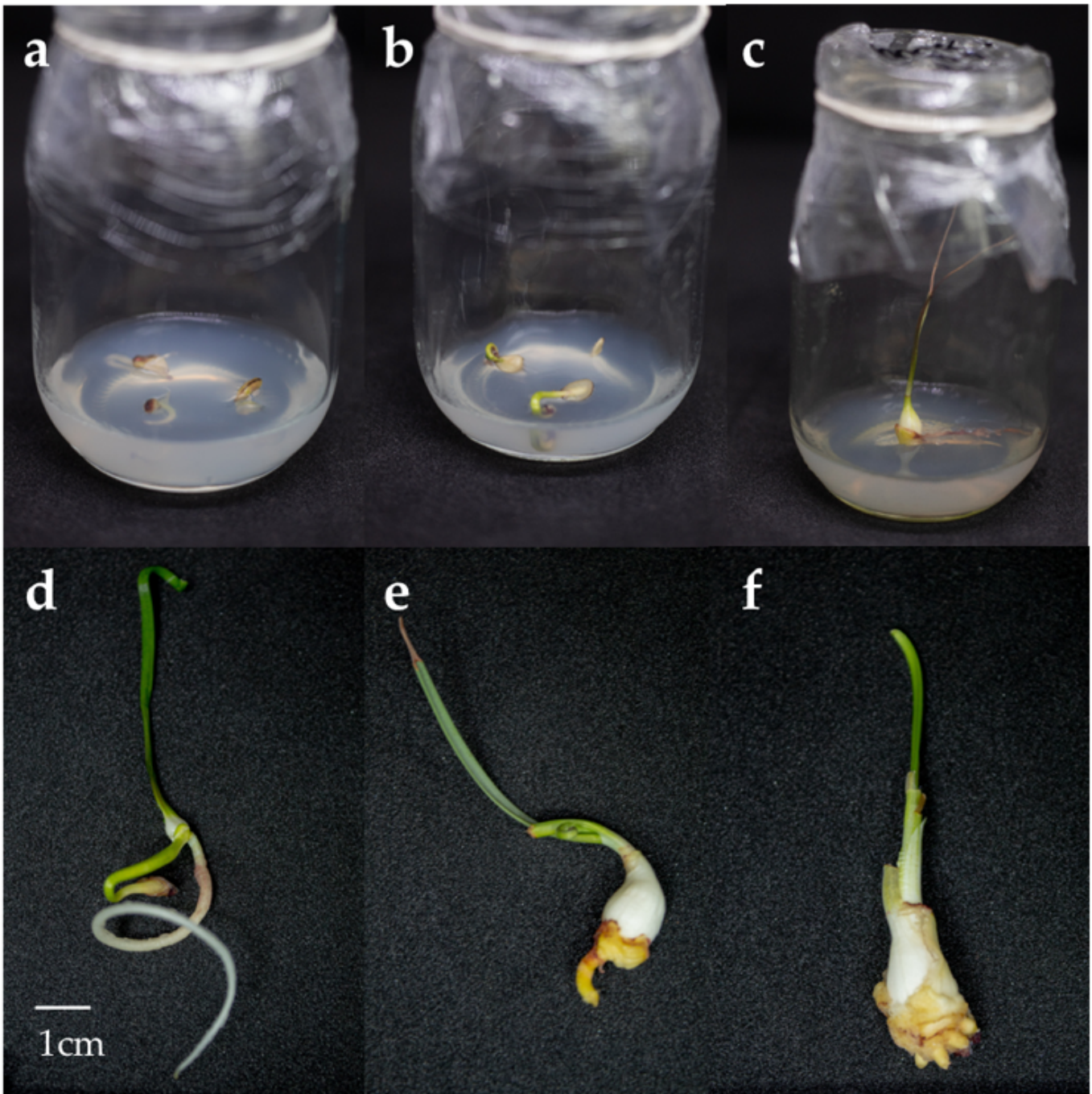


Figure 2

In vitro introduction scheme from *Z. splendens* seeds **a**. Seed coat removal and endosperm culture under *in vitro* conditions **b**. First morphogenic responses of seedlings without PGRs **c**. Initiation of bulb growth on MS medium with different concentrations of sucrose **d**. Seedling obtained after one month under *in vitro* conditions (control) **e**. *In vitro* bulbing with 90 g/L sucrose **f**. *In vitro* bulbing with 120 g/L sucrose

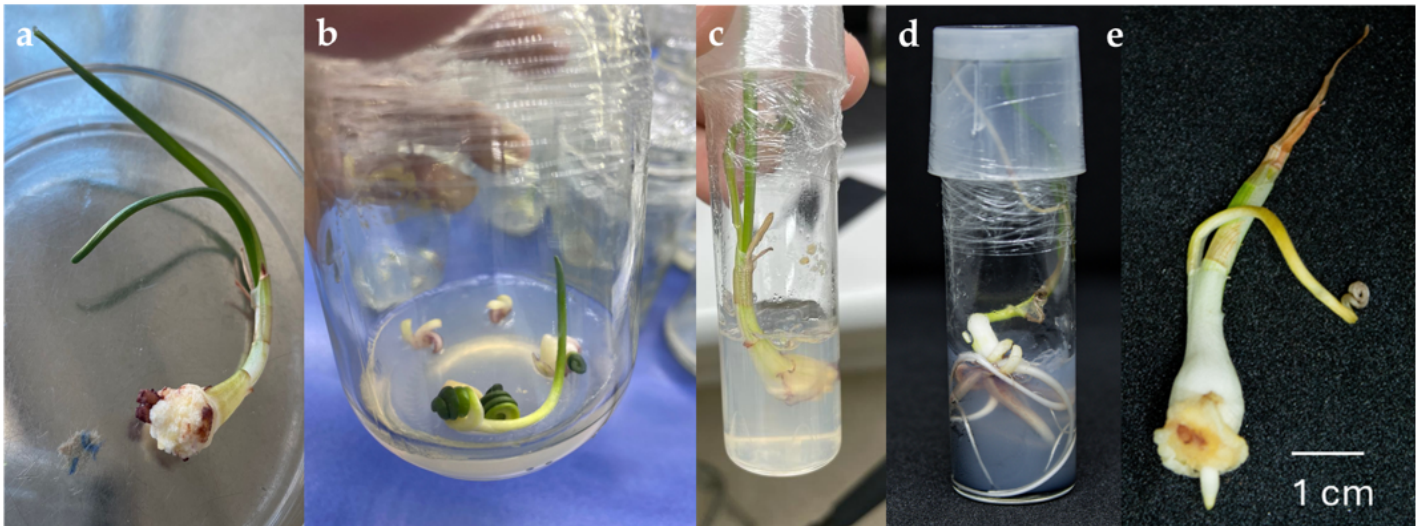


Figure 3

Multiplication of *Z. splendens* bulblets. **a.** *In vitro* bulbification with 120 g/L sucrose, **b.** Multiplication of explants (1:6) from bulblets, **c.** Regeneration of multiplied explants on MS medium supplemented with 90 g/L sucrose without PGRs, **d.** Individualization and rooting of regenerated seedlings with 1g/L activated charcoal, **e.** Regenerated plants from multiplication and bulbed with 90 g/L sucrose

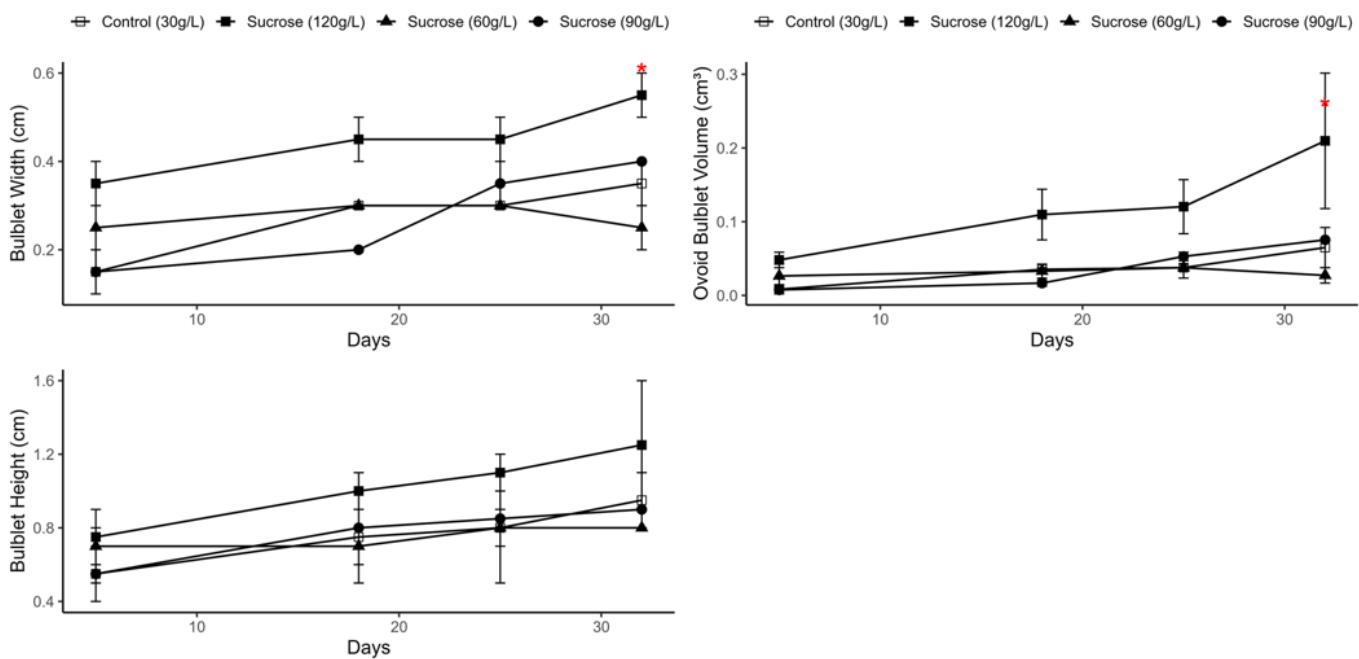


Figure 4

Changes in bulb width and height after one month of cultivation. The values represent the mean of three independent experiments. Bars represent the standard error (n = 15 for each treatment)

*Significant difference at $p < 0.05$.

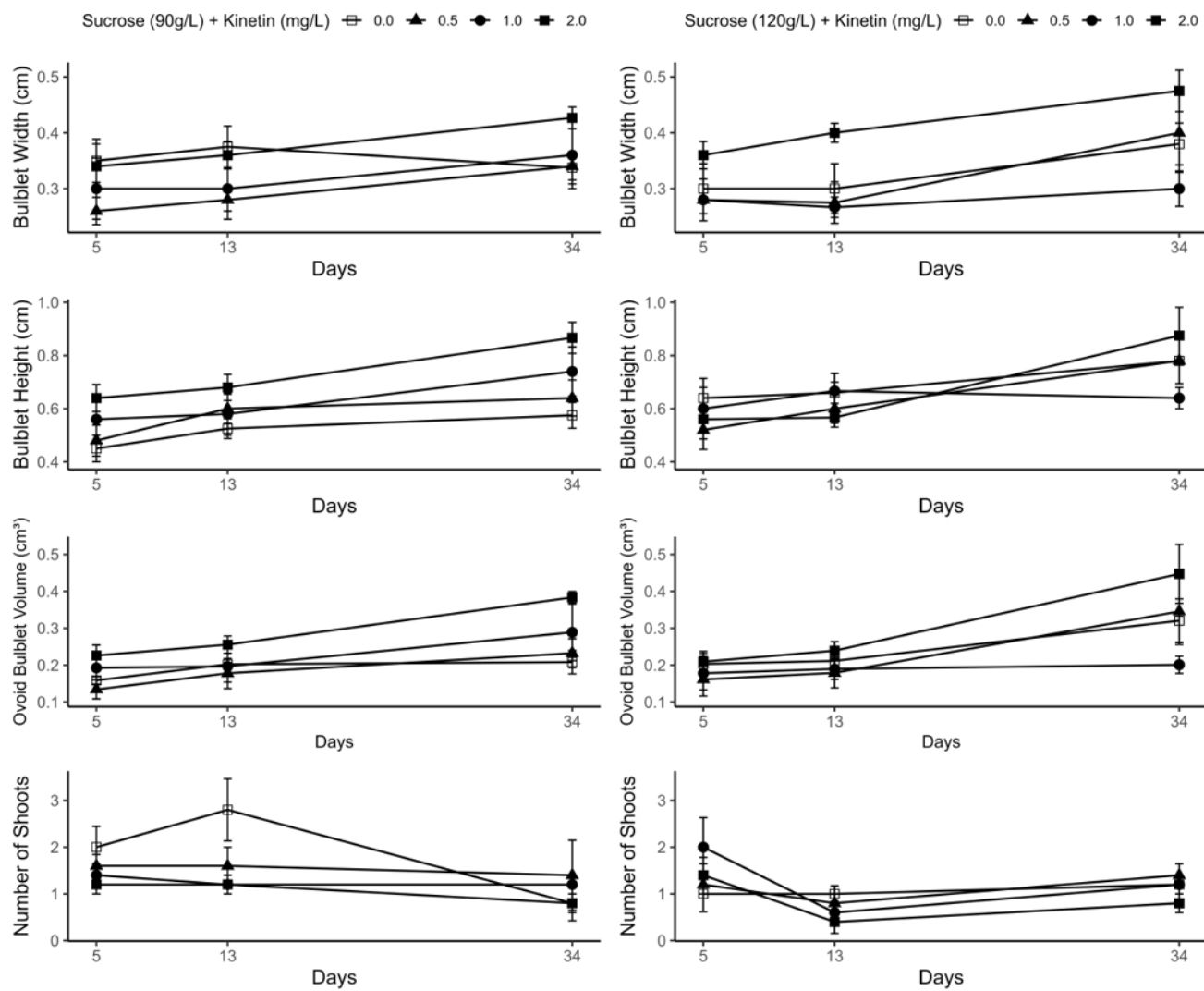


Figure 5

Effect of kinetin and sucrose interaction on bulblet optimization development in *Z. splendens* explants

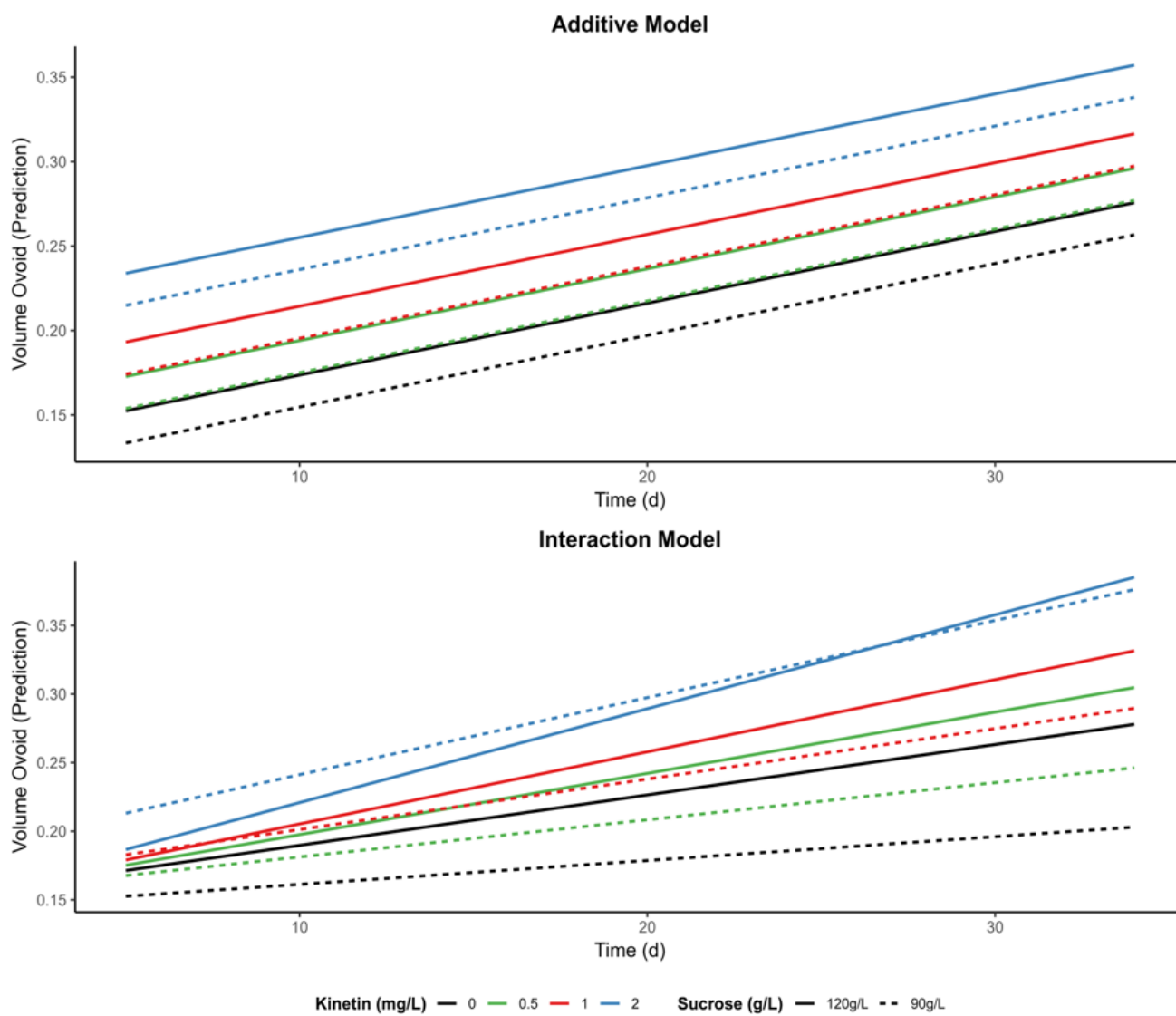


Figure 6

Interaction between Kinetin and Sucrose in ovoid volume optimization in *Z. splendens* explants. The intersection of the straight lines shows that the treatments behaved similarly over time



Figure 7

Physiological response to greenhouse conditions **a.** Plant generated on *in vitro* culture **b.** Roots response after 4 weeks of culture in *ex vitro* conditions **c.** Activated roots after 8 weeks of growth **d.** Plants established under greenhouse conditions after 8 weeks

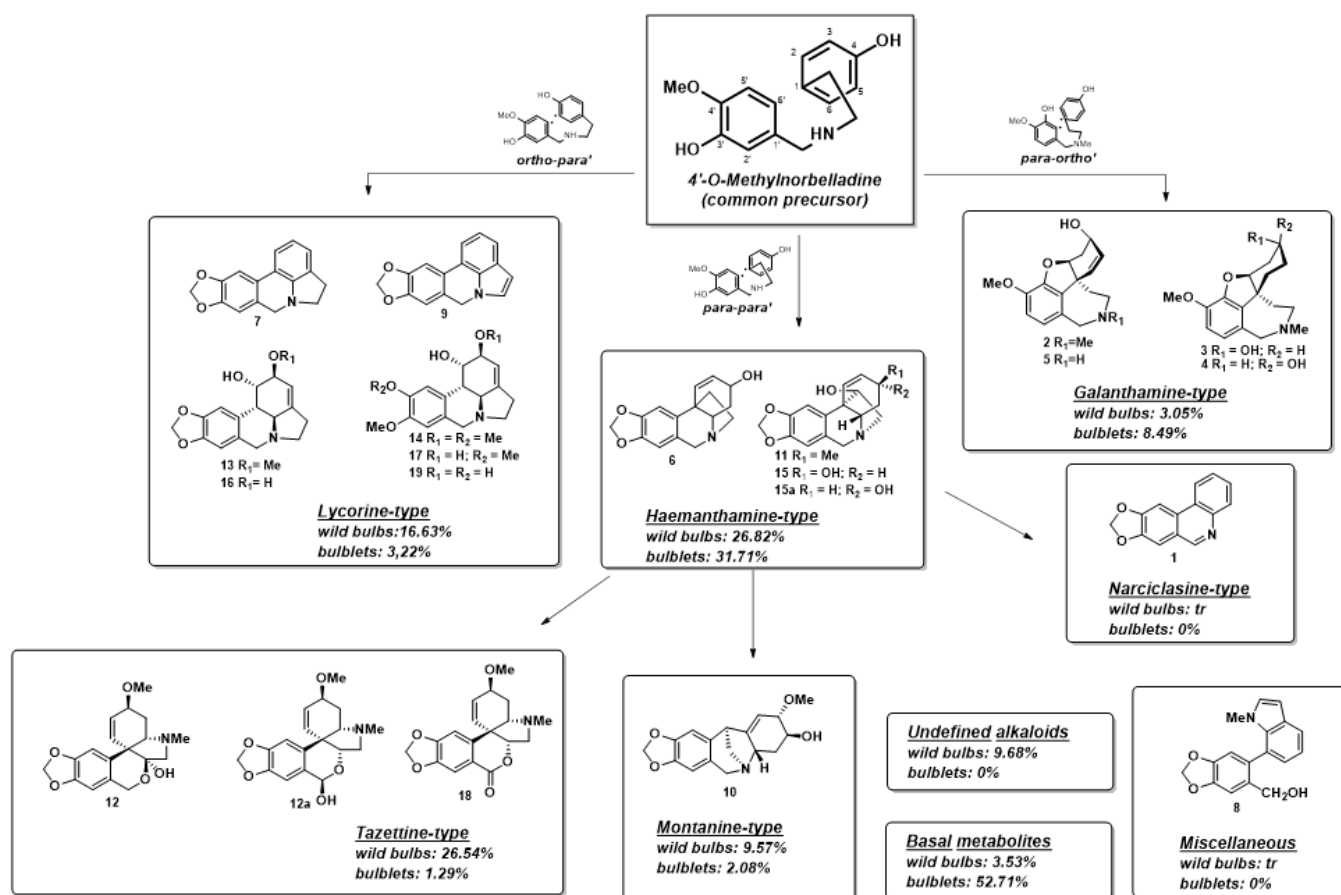


Figure 8

Structures of alkaloids identified in *Z. splendens*.