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Influence of plant growth regulators on *in vitro* biomass production and biosynthesis of cytotoxic Amaryllidaceae alkaloids in *Caliphuria tenera* Baker

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ABSTRACT

Plants of the Amaryllidoideae subfamily are characterized by the presence of isoquinoline alkaloids called Amaryllidaceae alkaloids. These compounds have exhibited a broad spectrum of pharmacological effects, including anti-Alzheimer and anti-cancer. In this study, *Caliphruria tenera* Baker, Amaryllidaceae, an endangered and endemic species, was selected to evaluate the effect of growth regulators on plant micropropagation, biomass increase, and production of cytotoxic alkaloids. Different growth regulators were used individually and mixed, and the Amaryllidaceae alkaloids were identified and quantified by GC-MS. The maximum number of *in vitro* shoots (4.8) was recorded with 6-benzylaminopurine (BAP) (1 mg/L) + Kinetin (KIN) (1 mg/L) + Naphthaleneacetic acid (NAA) (0.2 mg/L). In addition, nineteen alkaloids were identified as crinine, galantamine, lycorine, narciclasine, and tazzetine-type alkaloids in cultivated and micropropagated plants. In general, the biomass was maximized with the growth hormone combination BAP + KIN + NAA, but the biosynthesis of alkaloids was minimized. However, the combination BAP + NAA showed the greatest productivity of trisphaeridine (632.01 μg/g). The growth regulators showed also a positive effect on the production of lycorine-type alkaloids with a content of 396.33 μg/g for KIN, 142.38 μg/g for BAP, and 41.67 μg/g for NAA. All alkaloid fractions were evaluated for their antiproliferative effects against human lung carcinoma (A549) and human keratinocytes (HaCat) cells. The alkaloids produced in the BAP + NAA treatment present a potent and selective cytotoxic against the A549 cell line at 30 μg/mL. This study shows that micropropagation is a promising approach for the production of cytotoxic Amaryllidaceae alkaloids in *C. tenera* without affecting natural populations.

1. Introduction

Plants of the Amaryllidaceae family belong to the Asparagales order and they consist of bulbous flowering plants (Torras-Claveria et al., 2017). The current classification includes about 1650 species in 73 genera from three subfamilies: Agapanthoideae, Allioideae and Amaryllidoideae (Chase et al., 2009). The Amaryllidoideae is mainly distributed in tropical and holarctic areas with considerable

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diversity in Africa and South America, followed by Asia and Australia (Meerow and Snijman, 1998; Torras-Claveria et al., 2017). Several molecules with bioactive potential are found in the Amaryllidaceae family (Nair et al., 2016). Specifically, the Amaryllidaceae alkaloids are a distinctive chemotaxonomic feature of plants of the Amaryllidoideae subfamily, with 636 identified alkaloids to date (Berkov et al., 2020).

The Amaryllidaceae alkaloids have exhibited a broad spectrum of pharmacological effects. However, the cholinesterase (AChE) inhibitory activity and anticancer are the most relevant. Thus, some Amaryllidaceae alkaloids showed inhibitory *in vitro* AChE and butyrylcholinesterase (BuChE) activity (Cortes et al., 2018), which raises a pharmacological potential for the treatment of Alzheimer's disease (AD) (Hulcová et al., 2019). Galanthamine (Razadyne®), a selective reversible inhibitor of AChE, is an approved drug for the symptomatic treatment of AD (Marucci et al., 2021; Rountree et al., 2013). On the other hand, lycorene and lycorine presented *in vitro* antitumor activity against glioblastoma, melanoma, and non-small-lung cancers (Lamoral-Theys et al., 2009); 5,6-dihydrobicolorine significantly inhibited proliferation of human liver hepatocellular cells HepG-2 (Bozkurt et al., 2017), and 8-*O*-demethylmaritidine presented cytotoxicity effect against human cervical cancer (SiHa) and human epidermoid carcinoma (KB) cells (Katoch et al., 2020). This has strongly motivated the selection of Amaryllidaceae alkaloids with different structural types for the determination of their cytotoxic effects.

In Colombia, 18 genus and 48 species for Amaryllidoideae subfamily have been reported, of which 27 species are natives and 9 endemics. The Western and Eastern Mountain range and the biogeographic Chocó present the greatest diversity of these species (Alzate et al., 2019). The *Caliphruria* genus presents 4 native species to northern Peru and low montane forests in Colombia: *Caliphruria hartwegiana* Herb, *Caliphruria karsakoffii* (Traub) Meerow, *Caliphruria subedentata* Baker and *Caliphruria tenera* Baker, the last two are endemic to Colombia (Calderón, 2003). *C. tenera* is possibly reported as extinct species (Romero et al., 2008). However, some specimens have been collected and preserved through university initiatives. So, it could be interesting to develop alternatives that allow propagation for the conservation of germplasm, without intervening in the wild populations. In this way, some Amaryllidaceae species have been the subject of micropropagation studies: *Eucharis x grandiflora* Planch. & Linden (Guerrero-Valencia et al., 2021), *Hippeastrum vittatum* (L'Hér.) Herb (Zayed et al., 2011), *Narcissus pseudonarcissus* L. (Ferdausi et al., 2021), *Narcissus tazetta* L. (Khonakdari et al., 2020), and *Rhodophiala pratensis* (Poepp.) Traub (Trujillo-Chacón et al., 2020). However, the species of the *Caliphruria* genus are devoid of related research.

Different applications such as the conservation and propagation of valuable, rare, and endangered plants, have been also used for biomass production and bioactive constituents (Kumar et al., 2020). Thus, *in vitro* cultures of Amaryllidaceae plants have been considered a biotech alternative for the production of Amaryllidaceae alkaloids, where a positive effect on the stimulation of the alkaloid biosynthesis pathway is reported, at the same time that it is a friendly technique with the conservation of the species (Stanilova et al., 2010). Therefore, the present research examines the effect of growth regulators on plant micropropagation, biomass production, and biosynthesis of cytotoxic alkaloids in *C. tenera*. The latter, to the best of our knowledge, was performed for the first time in Amaryllidaceae alkaloids-producing species. The evaluation of the antiproliferative effects against human lung carcinoma (A549) of the alkaloid fractions obtained after growth regulator treatments is of particular interest.

2. Materials and methods

2.1. Chemicals

The reagents 6-benzylaminopurine (BAP), kinetin (KIN), naphthaleneacetic acid (NAA), Murashige and Skoog (MS), sucrose, Tween® 20, agar, ammonium hydroxide, sulphuric acid, dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol- 2 yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco modified eagle medium-high glucose (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine, trypsin-EDTA solution 10x, the reference standards lycorine, codeine, doxorubicin, and the mixture of alkanes C7–C40 were purchased from Sigma–Aldrich (St. Louis, USA) and PhytoTech Labs® (Kansas, USA). Sodium hypochlorite was purchased commercial grade from local distributors.

2.2. Plant material

The plant grown under greenhouse conditions were obtained from the "Álvaro José Negret" Botanical Garden, located in Popayán (Colombia). The plant was in flowering stage and with the formation of some bulbs, which were collected. This species was taxonomically identified in the herbarium of the University of Antioquia, and a specimen with the identification 5400 Alzate is preserved. This research was carried out with the authorization of the Ministry of Environment of the Republic of Colombia through the Access Contract for Genetic Resources and Derived Products No. 328 of 2022.

2.3. Nursery preconditioning stage

The bulb obtained from the mother plant was taken to the greenhouse and planted in a pot with a substrate of 50% black soil mixed with 20% peat, 10% coconut fiber and 10% rice husks. It was planted in humid chamber conditions at a temperature of 28 °C ± 3°, relative humidity of 80% ± 5%, and natural photoperiod (12/12 h). After 12 months, the vegetative cycle was completed, and the bulbs were formed; these were used for *in vitro* multiplication and bulblets production processes and others were used directly for alkaloid extraction assays.

2.4. Preparation of explants and establishment of in vitro culture

The aerial part and roots of the plants were removed, until the bulbs were ready to be washed with detergent and running water, eliminating soil excess. The disinfection process was then carried out with 2% iodinated soap for 10 min, the disinfectant was re-

moved with deionized water, and the bulbs were dehydrated for 5 days. Under completely aseptic conditions, the bulbs were disinfected with 70% ethanol for 2 min, then they were submerged in 1% sodium hypochlorite with Tween 20 for 20 min. Subsequently, a cross-section was made and the portion containing the basal plate of the bulb was selected and treated with 1% sodium hypochlorite with Tween® 20 for 3 min, and the disinfectant excess was removed with sterile deionized water. The "Twin-scales" technique with modification was used to obtain the explants (Rice et al., 2011). In all cases, a longitudinal section of the explants was made. The explants were planted in culture medium MS "Murashige and Skoog (1962), supplemented with sucrose (30 g/L), growth regulators, and agar (2.4 g/L). The culture medium was adjusted to pH 5.7 ± 0.2 and autoclaved at 121 °C for 20 min. Incubation was carried out for 8 weeks under total darkness conditions at a temperature of 24 ± 3 °C.

2.5. Plant multiplication

The *in vitro* multiplication was carried out according to previous reports with some modifications (Ferdausi et al., 2020). Plants obtained from the establishment culture were subcultured on MS medium supplemented with vitamins, sucrose (30 g/L), agar (2.4 g/L), and the growth regulators NAA, BAP, and KIN, in the conditions shown in Table 1. Incubation was carried out under photoperiod conditions of 16/8 h light cycle natural/dark at an average temperature of 26 ± 2 °C with a humidity of 80–90%. The subcultures were performed every 30 days. The percentage of shoot induction per explant and length (cm) were recorded after 8 weeks of incubation and alkaloid extraction was performed. All treatments were performed in triplicate, including control.

2.6. Extraction of alkaloids

The field and micropropagated plants were washed with drinking water. After that, the bulbs were cut into 3 cm pieces, and the bulblets obtained *in vitro* were cut into 0.5 cm portions. The plant material was dried at 40 °C for 24 h. The dried and ground plant material was extracted with methanol, evaporating the solvent at reduced pressure. The extract was dissolved in 10 mL 2% H_2SO_4, neutral compounds were removed with hexane (3 × 10 mL), and the pH of the aqueous fraction was adjusted in the range of 9–10 with 25% ammonium hydroxide. The alkaloids were extracted with chloroform (3 × 50 mL). The organic solvent was evaporated using a rotary evaporator. Finally, 5 mg of extract was dissolved in 500 μL methanol for analysis in GC-MS.

2.7. Analysis of alkaloids

The analysis of Amaryllidaceae alkaloids from *C. tenera* by GC-MS was performed according to the previously described method (Cortes et al., 2018, 2019). The alkaloid fraction (AF) (1 μL of the sample) was injected into an Agilent 7890 Gas Chromatograph equipped with a 5975C selective mass detector and electronic impact (EI) operating to 70 eV in splitless mode, programmed to acquire signals in scan mode between 40 and 400 Da. The separation of the alkaloids was performed with an HP-1 MS capillary column (30 m x 0,25 mm x 0,25 μm) with a carrier gas flow (Helium) of 1 mL/min. The temperature ramp was as follows: 100–180 °C at 15 °C/min, 180–300 °C at 5 °C/min, and 10 min hot at 300 °C. The temperature of the injector was kept at 250 °C.

2.8. Determination of the alkaloid profile

In the process of alkaloids identification, the fragments of the mass spectra of each molecule were compared with the "Amaryllidaceae Alkaloid Spectroteca, Agro Bio Institute (Bulgaria)" database and with reported data in scientific journals (Berkov et al., 2020). The Kovats retention rates (RI) of the compounds were recorded with a standard calibration mixture of n-hydrocarbons (C7–C40). The percentage of total ion current (TIC) was determined for each alkaloid. The abundance of each compound was calculated using codeine (50 μg/mL) as an internal standard. The proportion of each compound in the tested extracts was called the percentage of the total alkaloid content. The area of GC-MS peaks depends not only on the concentration of the related compounds but also on the intensity of their mass spectral fragmentation.

2.9. Assay for cell viability

2.9.1. Cell lines

The epithelial cell line of human lung carcinoma A549 (ATCC, CCL-185 TM) and human keratinocytes HaCat (ATCC® PCS-200-011™) were cultured in Dulbecco Modified Eagle Medium-high glucose (DMEM) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, 2 mM L-glutamine in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Table 1
Treatments with growth regulators.

Treatments	Regulator
T1	BAP 1 mg/L
T2	KIN 1 mg/L
T3	NAA 0.2 mg/L
T4	BAP 1 mg/L + NAA 0.2 mg/L
T5	KIN 1 mg/L + NAA 0.2 mg/L
T6	BAP 1 mg/L + KIN 1 mg/L + NAA 0.2 mg/L
C[a]	Control

BAP: 6- benzylaminopurine, KIN: Kinetin, NAA: Naphthaleneacetic acid.
[a] Shoots obtained without growth regulators.

2.9.2. Cytotoxicity assay

Cells were seeded in a 96-well plate at the density of 2 × 106 cells/mL. The 3-(4,5-dimethylthiazol- 2 yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to indicate the cell viability. In this experiment, cells were incubated with AF (30 μg/mL) of each treatment for 24 h. The AF was dissolved in dimethyl sulfoxide (DMSO) with a final concentration in the culture medium of 0.1% (v/v). After incubation, 10 μL of 5 mg/mL MTT dye was added to each well, and cells were incubated for 2 h at 37 °C. Finally, the supernatant was removed, 100 μL of DMSO was added to dissolve the reduced formazan and the absorbance of each sample was recorded on a microplate reader at 540 nm. The control group consisting of untreated cells was considered as 100% of viable cells. Results are expressed as the percentage of viable cells when compared with control groups.

2.10. Statistical analysis

The experimental design was completely randomized with six treatments and one control without growth regulators. All the experiments were carried out in triplicate with five experiments for each replicate. In the alkaloid extraction process, an equal number of *in vitro* shoots (n = 15) was considered for all treatments. In addition, the fraction of alkaloids was obtained from total shoots. Data normality was performed using the Shapiro-Wilk test ($P > 0.05$), and homoscedasticity using the Levene test ($P > 0.05$). Data were analyzed using an analysis of variance (ANOVA) and means were compared by the Tukey test at the significance level of $p < 0.05$. The statistical analysis was performed using the R Wizard 4.3 y R Studio 3.6.2 statistical package. Cell viability results were evaluated by one-way analysis of variance (ANOVA) followed by the Newman-keuls multiple-comparison test, using the GraphPad Prism 5.0 data analysis system.

3. Results and discussion

3.1. Establishment of in vitro culture

Ten bulbs obtained from the mother plant were used as a source of planting material. The technique of using twin scales allowed obtaining up to 12 explants from each basal disc of the bulb, all of them competent for multiplication processes. Twin scales are frequently used in the micropropagation of Amaryllidaceae species since meristems already exist where the leaves attach to the basal plate. These give rise to spontaneously produced shoots, i.e., without the addition of hormones (Fennell et al., 2001).

3.2. Effect of growth regulators on the micropropagation of C. tenera

For the micropropagation of *C. tenera*, different growth regulators were evaluated in the culture medium (Table 1). According to the results presented in Fig. 1, statistically significant differences between treatments were observed. The highest number of *in vitro* shoots (4.8) was recorded with the growth regulator combination BAP (1 mg/L) + KIN (1 mg/L) + NAA (0.2 mg/L) followed by 3.6 with BAP (1 mg/L) + NAA (0.2 mg/L). Treatments 1 and 5 exhibited 2.1 and 2.2 *in vitro* shoots respectively. The samples with KIN (1 mg/L), NAA (0.2 mg/L), and control (without growth regulator) did not present statistically significant differences with 1.4 *in vitro* shoots. In general, the best treatment included BAP cytokinin. This has a fundamental effect on cell cycle regulation and different processes of development. BAP is a stable cytokinin, it is tolerant to photooxidation and promotes the synthesis of other hormones (Vylíčilová et al., 2020). This is in agreement with the observed results in the proliferation of *Narcissus tazetta* (Amaryllidaceae),

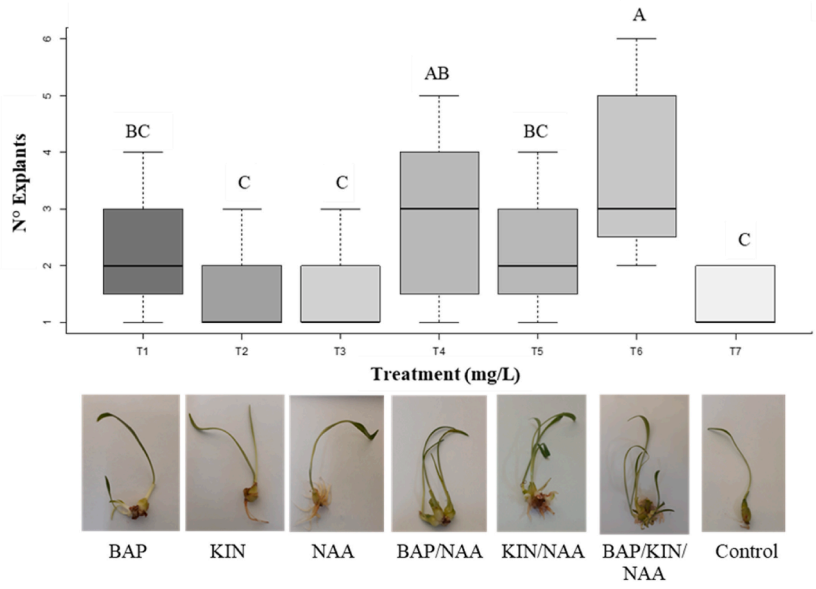

Fig. 1. Effect of growth regulators on the formation of *in vitro* shoots in *C. tenera* explants. Different letters indicate significant differences at the level of ($P < 0.05$) according to Tukey's test. For a detailed description of treatment codes see Table 1.

where interaction between growth regulators, such as BAP with photoperiod conditions, improved biomass production (Khonakdari et al., 2020). The combination between BAP cytokinin and NAA auxin had also a positive effect on the formation of shoots in this species, according to what was observed in treatments 4 and 6. These results show the potential of micropropagation techniques where it was shown that between 4.8 and 3.6 shoots (microbulblets) can be produced every 30 days, which implies that in six months there will be more than 2000 bulblets per explant (Fig. 2).

3.3. Effect of growth regulators on the directed production of Amaryllidaceae alkaloids in C. tenera

The alkaloids in the Amaryllidoideae subfamily were classified into 42 skeleton types. However, these alkaloids found in most of the genera of the subfamily are divided into 9 main skeleton types (Berkov et al., 2020). With a good distance, galantamine-type alkaloids have been the most investigated for their biotechnological production (Stanilova et al., 2010). Thus, a relationship between 6-benzylaminopurine (BAP) concentration (3 mg/L) and biosynthesized galantamine in *Narcissus confusus* Pugsley has been reported (Codina, 2002). In *Leucojum aestivum* L., naphthaleneacetic acid (NAA) regulators combined with BAP produced galantamine (Diop et al., 2006). In *Narcissus tazetta*, the production of galantamine and lycorine has been observed in presence of NAA and BAP (Khonakdari et al., 2020). Considering that growth regulators have been used as elicitors to stimulate the production of secondary metabolites in Amaryllidaceae plants, *in vitro* shoots from *C. tenera* were cultured on different regulators supplemented media, and the effects on Amaryllidaceae alkaloids were studied based on the main skeleton types (Table 2). The analysis of the alkaloid profile was carried out by GC-MS in bulbs obtained by cultivation under greenhouse conditions and by micropropagation. These alkaloids' presence is reported in relative abundance and alkaloid concentration in μg/g dry weight (μg/g DW).

The different growth regulator treatments increased trisphaeridine biosynthesis, compared to the conventionally propagated plants under "greenhouse conditions". Trisphaeridine was present in all treatments in different amounts (Table 2), and the combination BAP + NAA showed the greatest productivity of trisphaeridine (632.01 μg/g). This result correlates with the increased production of Amaryllidaceae alkaloids in *Rhodophiala bifida* cultivated in the presence of BAP + NAA (Reis et al., 2019). Thus, the relevance of the biosynthetic pathway of narciclasine-type alkaloids was observed, the skeleton type to which trisphaeridine belongs (Fig. 3). On the other hand, lycorine-type alkaloids, formed through an *ortho-para* oxidative phenol coupling in the general precursor 4′-*O*-norbelladine (Fig. 3), were the most abundant within the Amaryllidaceae alkaloids. In this study, the growth regulators showed a positive effect in the production of lycorine-type alkaloids with a content of 396.33 μg/g for KIN, 142.38 μg/g for BAP, and 41.67 μg/g for NAA, compared to the observed content in the control (without growth regulator). However, treatment with KIN showed a qualitative and quantitative increase in lycorine-type alkaloids, compared to these reported for the other growth regulators (Table 2). The alkaloid with the highest relative abundance was 11,12-dehydroanhydrolycorine with values between 8.56 and 31.42% in *in vitro* culture treatments and 9.62% in plants growing under greenhouse conditions. Although this treatment presented low *in vitro* shoots (Fig. 1). Some cytokines stimulated the production of specific Amaryllidaceae alkaloids. Previous reports showed the combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and BAP promoted the production of lycorine (Subramaniam et al., 2014).

The crinine-type alkaloids represent the enantiomeric series α (haemantamine alkaloids) and β (crinine alkaloids), generated from the *para-para'* coupling in the general precursor 4′-*O*-norbelladine (Fig. 3). The accumulation of these compounds decreased in the different reproduced treatments in *in vitro* conditions (8.03–56.24 μg/g DW), compared to plants growing under greenhouse conditions (1906.62 μg/g DW). The greatest level of haemantamine (1114.10 μg/g DW) was obtained in plants growing under greenhouse conditions, compared to those with treatments BAP + NAA and BAP + Kin + NAA (28.91 and 56.24 μg/g DW, respectively) (Table 2). However, it was observed that buphanisine, an alkaloid absent in plants growing in the field, was detected in the BAP + NAA and KIN + NAA treatments. On the other hand, the galantamine-type alkaloids generated from a *para-ortho'* coupling (Fig. 3) were presented in percentages below 2%, suggesting that these are trace compounds in *Caliphruria* species. Nevertheless, individual cytokinins

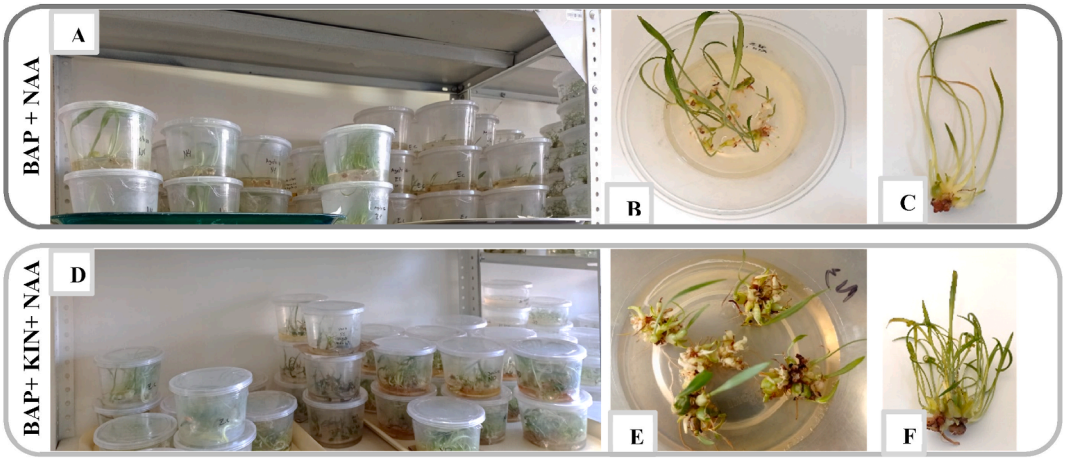

Fig. 2. Biomass production of *C. tenera*. **A.** and **D.** Multiplication of microbulblets after 30 days of incubation. **B.** and **C.** Effect of BAP + NAA on the multiplication process. **E.** and **F.** Effect of BAP + KIN + NAA on the multiplication process. For a detailed description of treatment codes, see Table 1.

Table 2
Identification of alkaloids occurring in *C. tenera* by GC–MS.

N°	*Caliphruria tenera*				Greenhouse plants[b]		*In vitro* cultures[c]													
	Alkaloid	RI[a]	M^+	m/z (relative intensity %)			BAP		KIN		NAA		BAP + NAA		KIN + NAA		BAP + KIN + NAA		T0	
					µg/g DW[d]	% relative[e]	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative
	Lycorine type				**880.64**	**27.16**	**142.38**	**51.96**	**396.33**	**50.33**	**41.67**	**36.15**	**49.08**	**6.76**	**24.87**	**21.17**	**69.18**	**26.52**	**1.52**	**5.19**
1	Lycorene	2194	255 (52)	254(100), 239 (8), 227(17), 226 (20), 211(15), 183(14)	nd	nd	nd	nd	nd	nd	1.89	1.64	49.08	6.76	1.22	1.04	nd	nd	1.52	5.19
2	5,6-Dihydrobicolorine	2263	239 (44)	238(100), 180 (15), 152(7), 166 (5), 139(10), 118 (6).	372.95	11.50	7.83	2.86	80.97	10.28	3.40	2.95	nd	nd	13.59	11.57	nd	nd	nd	nd
3	11,12-Dehydrolycorene	2301	252 (100)	253(42), 252 (100), 224(19), 166(14), 152(9), 139(9)	19.78	0.61	2.56	0.93	11.45	1.45	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
4	Anhydrolycorine	2436	251 (41)	250(100), 220 (2), 192(14), 191 (12), 165(4), 124 (8)	nd	nd	18.20	6.64	11.71	1.49	1.50	1.30	nd	nd	nd	nd	4.09	1.57	nd	nd
5	2-O-Acetyllycorine	2468	329 (100)	328(17), 270(7), 269(11), 268 (41), 252(12), 250 (7)	nd	nd	17.36	6.34	42.87	5.44	4.90	4.25	nd	nd	nd	nd	nd	nd	nd	nd
6	11,12-Dehydroanhydrolycorine	2535	249 (59)	248(100), 190 (26), 163(8), 123 (6), 95(14)	311.94	9.62	84.18	30.72	247.39	31.42	29.98	26.01	nd	nd	10.06	8.56	65.09	24.95	nd	nd
7	Lycorine	2686	287 (20)	268(20), 250 (10), 227(61), 226(100), 211 (5), 147(11)	166.72	5.14	12.25	4.47	1.94	0.25	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
8	Pseudolycorine	2736	289 (26)	289(26), 270 (18), 229(67), 228(100), 147 (9), 111(10).	9.25	0.29	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Crinane type				**1906.62**	**58.82**	**16.46**	**6.00**	**52.76**	**6.70**	**8.85**	**7.68**	**31.75**	**4.37**	**17.75**	**15.11**	**56.24**	**21.56**	**8.03**	**27.35**
9	Buphanisine	2349	285 (59)	273(54), 272 (43), 254(40), 215(100), 157 (39), 129(35), 128(55), 115 (64)	nd	nd	nd	nd	nd	nd	nd	nd	2.84	0.39	3.36	2.86	nd	nd	nd	nd

(*continued on next page*)

Table 2 (*continued*)

N°	*Caliphruria tenera*				Greenhouse plants[b]		*In vitro* cultures[c]											
	Alkaloid	RI[a]	M^+	m/z (relative intensity %)			BAP		KIN		NAA		BAP + NAA		KIN + NAA		BAP + KIN + NAA		T0	
					µg/g DW[d]	% relative[e]	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative
10	Crinine	2404	271 (100)	270(19), 228 (30), 200(38), 199(97), 187 (84), 129(36), 128(50), 115(56)	133.16	4.11	1.47	0.53	4.72	0.60	1.87	1.62	nd	nd	5.08	4.32	nd	nd	nd	nd
11	8-O-Demethylmaritidine	2467	273 (100)	230(26), 202 (26), 201(86), 189(61), 175 (26), 174(19), 129(22), 128(24)	282.05	8.70	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
12	Haemantamine	2585	301 (11)	272(100), 257 (9), 240(21), 225 (8), 211(18), 181 (31), 153(15)	1114.10	34.37	14.99	5.47	48.04	6.10	6.98	6.06	28.91	3.98	9.31	7.93	56.24	21.56	8.03	27.35
13	Hamayne	2668	287 (5)	258(100), 242 (16), 212(12), 211(17), 186 (18), 181(21), 153(11), 128 (18)	377.31	11.64	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Galanthamine type				**55.07**	**1.70**	**2.18**	**0.80**	**4.77**	**0.61**			**12.96**	**1.79**						
14	Galanthamine	2340	287 (77)	286(100), 270 (13), 244(25), 230(14), 216 (35), 174(35)	55.07	1.70	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
15	Sanguinine	2355	273 (100)	272(83), 256 (22), 212(23), 202(54), 166 (19), 160 (77).	nd	nd	2.18	0.80	4.77	0.61	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
16	N-Formylnorgalanthamine	2521	301 (100)	270(27), 243 (84), 239(15), 211(17), 128 (39), 115(42)	nd	nd	nd	nd	nd	nd	nd	nd	12.96	1.79	nd	nd	nd	nd	nd	nd
	Tazettine type				**10.34**	**0.32**														
17	Tazettine	2593	331 (20)	316(12), 298 (20), 247(100), 227(14), 201 (21), 181(19), 152(15), 115(27)	10.34	0.32	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Narciclasine type				**371.07**	**11.45**	**109.84**	**40.09**	**333.52**	**42.36**	**64.75**	**56.18**	**632.01**	**87.08**	**74.83**	**63.72**	**135.45**	**51.92**	**19.81**	**67.47**

(*continued on next page*)

Table 2 (*continued*)

N°	*Caliphruria tenera*				Greenhouse plants[b]		*In vitro* cultures[c]													
	Alkaloid	RI[a]	M^+	m/z (relative intensity %)			BAP		KIN		NAA		BAP + NAA		KIN + NAA		BAP + KIN + NAA		T0	
					µg/g DW[d]	% relative[e]	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative	µg/g DW	% relative
18	Trisphaeridine	2215	223 (100)	222(41), 167 (12), 165(11), 164(22), 138 (31), 111(12)	371.07	11.45	109.84	40.09	333.52	42.36	64.75	56.18	632.01	87.08	74.83	63.72	135.45	51.92	19.81	67.47
	Miscellaneous				**18.21**	**0.56**														
19	Demethylismine	2192	224	243(22), 225 (21), 224(100), 167(10), 166 (15), 154(11), 77 (12)	18.21	0.56	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[a] RI: Kovats retention index.
[b] Bulbs from plants under greenhouse conditions.
[c] ANA:1-naphthaleneacetic acid (0.2 mg/L); BAP:6-benzylaminopurine (1.0 mg/L); KIN: kinetin (1.0 mg/L); T0: control.
[d] Quantitative values obtained by response factor of codeine internal standard.
[e] Percentages of relative peak area of compounds in the analyzed samples. nd: not detected.

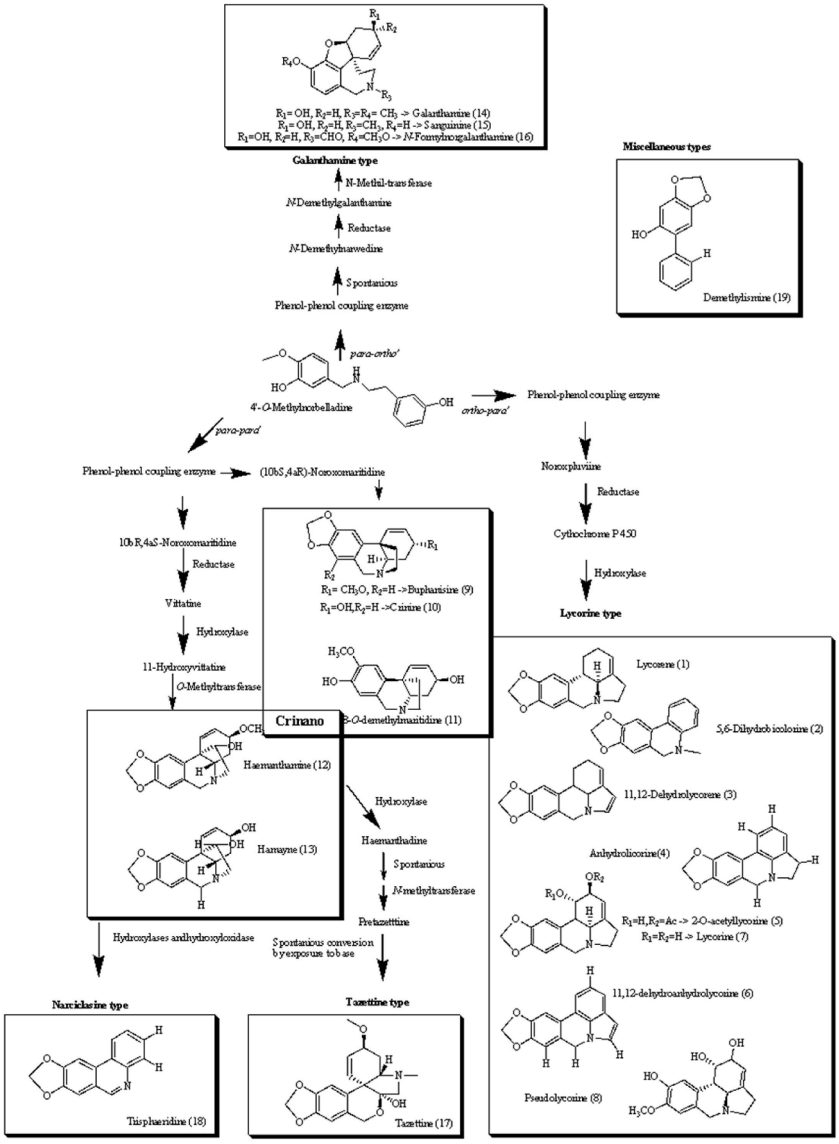

Fig. 3. Biosynthetic pathway of Amaryllidaceae alkaloids.

stimulated the production of sanguinine (2.18 μg/g DW for BAP and 4.77 μg/g DW for KIN), and growth regulator combination stimulated the production of *N*-formylnorgalanthamine (12.96 μg/g DW for BAP + NAA). Ultimately, this was considered the best treatment for producing a qualitative increase in crinine-type and galanthamine-type alkaloids.

The relationship between the content of alkaloids and the treatments is also shown in Fig. 4. In the heat map, it can be observed that the intense colors represent the greater presence of alkaloids. The trisphaeridine alkaloid was the most representative of this species in the applied treatments, while haemantamine was negatively affected by growth regulators. When the alkaloid production was analyzed, the cytokinins BAP and KIN produced alkaloids in common and similar abundances. This suggests that the enzymes responsible for the Amaryllidaceae alkaloids biosynthesis were expressed in some cases, and diminished in others, signifying the differentiation in the alkaloid production and the non-linear response between the different types of Amaryllidaceae alkaloids. This effect has been observed in the production of alkaloids with different combinations of growth hormones. For example, vincristine and vinblastine in *Catharanthus roseus* (Oudin et al., 2007; Mekky et al., 2018), the related sceletium-type compounds in *Narcissus pallidulus* (Berkov et al., 2021), and tropane alkaloid in *Duboisia leichhardtii* (Yamada and Endo, 1984), just to mention a few examples. These results confirmed that growth regulators play an important role in the production of alkaloids; and that the use of auxins and cytokinins, individually or in combination, alter both the growth of the plant and the production of secondary metabolites (Jamwal et al., 2018). This is the first time it is reported a chemical study related to secondary metabolites in *C. tenera* and the micropropagation of the species, contributing to the chemical characterization of plants of the Amaryllidaceae family and an alternative for the mass production of *C. tenera*.

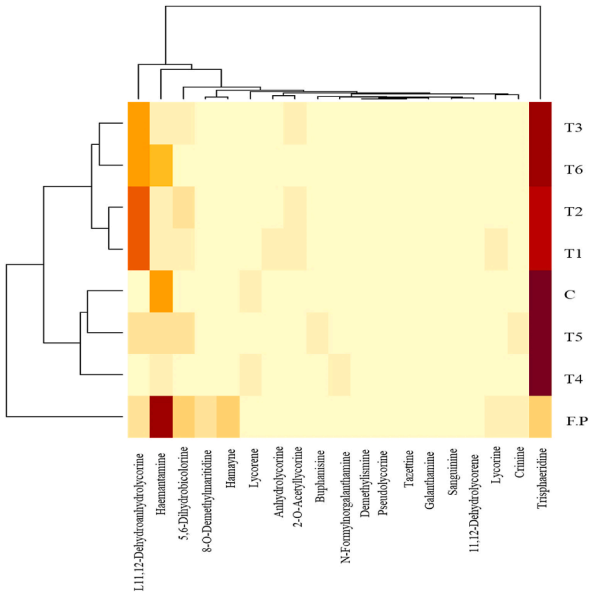

Fig. 4. Relationship between growth regulators and Amaryllidaceae alkaloids production. For a detailed description of treatment codes, see Table 1.

3.4. Cytotoxic activity in the tumor cell line of alkaloid fractions obtained with different growth regulators

There is a need for the continuous production of anticancer alkaloids, which may be achieved using a range of biotechnological approaches (Changxing et al., 2020). In the present study, cytotoxicity assays of the alkaloid fractions (AF) obtained with different regulators were performed to potential anticancer effect against the cell line of human lung carcinoma A549, using human keratinocytes HaCat as control (Fig. 5). The cell viability differed according to the treatments with statistically significant differences ($p < 0.01$, $p < 0.001$). The AF obtained with NAA (49.71%) and BAP + NAA (63.38%) exhibited the greatest activity with cell viability close to 50% at 30 μg/mL. The cell viability of AF from plants growing under greenhouse conditions was 63.11% at 30 μg/mL. Therefore, although the last plants produce cytotoxic alkaloids, their production is stimulated with NAA treatment which promotes the production of lycorine-type alkaloids with *ortho-para'* orientation (Fig. 3). The cell viability of HaCat was 64.37%, showing low selectivity of this alkaloid fractions. In A549 cells, lycorine showed low cellular viability (66.66%). However, lycorine also shows low selectivity in A549 cells due to increased cytotoxicity in HaCat cells with 26.93% viability. Therefore, the other lycorine-type alkaloids present in the NAA treatment, such as 11,12-dehydroanhydrolycorine, anhydrolycorine, and 2-*O*-acetyllycorine could also be responsible for the toxicity of this AF and molecules with potential cytotoxic activity in lung cancer. Recently, lycorine, as well as structural derivatives, have shown decreased proliferation, invasion, neovascularization, and metastasis *in vitro* and *in vivo* models of lung cancer (Zhao et al., 2020).

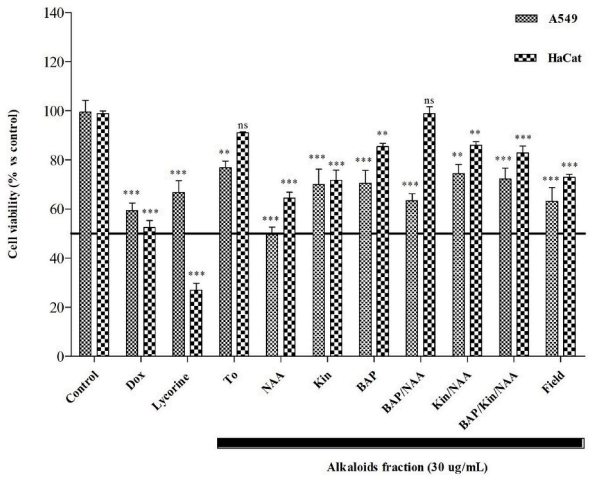

Fig. 5. Cell viability of the alkaloids fraction against human lung carcinoma (A549) and human keratinocytes (HaCat) cells. The experiments were performed in triplicate. Data are presented as the mean ± SD from three separate experiments. In A549 and HaCat cells, the treatments showed a significant difference with a $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control cells. ns: no significant difference, control (DMSO 0.1%), doxorubicin (Dox, 5 μg/mL), lycorine (15 μg/mL).

The alkaloidal fractions of BAP + NAA and plants growing under greenhouse conditions showed cell viability in A549 cells of 63.38% and 63.11% respectively, without significant difference. However, the selectivity of the alkaloids produced in BAP + NAA against A549 cells stands out, due to high cell viability (98.77%) in HaCat control cells. The BAP + NAA treatment promoted the production of buphanisine, and in less quantity of haemantamine, two crinane-type alkaloids of the *para-para'* coupling. Although little is known about the cytotoxic effects of buphanisine, some crinane-type alkaloids, such as acetoxycrinamine, haemanthamine, and haemanthidine, have shown potent anti-proliferative activity in A549 lung cancer cells with chemoresistance to standard cytotoxic pharmacological treatments (Feng et al., 2011; Koutová et al., 2021). Therefore, the alkaloids produced in the BAP + NAA treatment would be an alternative to the production of Amaryllidaceae alkaloids with selective cytotoxic potential in lung cancer cells.

To our knowledge, *C. tenera* does not present pharmacological studies, but cytotoxicity has been reported against A549 cells of other Amaryllidaceae species. In this way, some alkaloids have been found in *Crinum latifolium*, *Haemanthus humilis*, and *Lycoris radiata* with potential anti-proliferative via screening against A549 cells (Chen et al., 2018; Feng et al., 2011; Masi et al., 2019). In general, the findings have allowed positioned the crinane, homolycorine, lycorine, and narciclasine-type alkaloids, as the most desirable cytotoxic compounds in studies of the Amaryllidaceae, and consolidated the plant family Amaryllidaceae as a rich and diverse source of antiproliferative isoquinoline alkaloids (Kornienko and Evidente, 2008; Nair and Van Staden, 2021; Nair et al., 2016).

In conclusion, this study determined that the combination of the growth regulators BAP + NAA and BAP + Kin + NAA favored plant proliferation with the formation of 3.6–4.8 bulblets per month, which implies that in six months there will be more than 2000 bulblets per explant, demonstrating the potential of this technique for bulblet production. In the case of greenhouse-grown materials, twelve months were required to produce ten bulblets. The BAP + NAA combination showed the highest trisphaeridin productivity (632.01 μg/g), which corresponds to twice that produced by greenhouse plants. Similarly, a positive effect on the production of lycorine-type alkaloids was obtained with a content of 396.33 μg/g for KIN, 142.38 μg/g for BAP, and 41.67 μg/g for NAA, which was absent in greenhouse bulbs. In this way, the *in vitro* propagation protocol of *C. tenera* could be applied to other species of the Amaryllidaceadea family to multiply and optimize the extraction of alkaloids. Finally, cell viability results showed that the alkaloids produced in the BAP + NAA treatment present cytotoxic potential and selectivity against A549 cell lines. Therefore, the alkaloidal fraction of BAP/NAA could be fractionated and purified in further studies to determine the compound(s) responsible for the biological effects. The latter is currently underway in our laboratory.

Declarations of competing interest

None.

Data availability

Data will be made available on request.

Acknowledgments

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