Alkaloid Production in *Crinum moorei* Cultures

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The alkaloids cherylline (**1**), crinamidine (**2**), crinine (**3**), epibuphanisine (**4**), lycorine (**5**), powelline (**6**), undulatine (**7**), 1-epideacetylbowdensine (**8**), and 3-*O*-acetylhamayne (**9**) were identified in the in vitro propagated bulblets of *Crinum moorei*. In addition, crinine, powelline, and undulatine were detected in the solidified Murashige and Skoog (MS) medium. The identity of the alkaloids was confirmed by comparing retention times and mass spectra with known samples. Light, as well as benzyladenine (BA) and charcoal supplementation of the tissue culture medium, influenced the levels of specific alkaloids in both the bulblets and media.

Crinum moorei Hook. f. (Amaryllidaceae) is one of five indigenous species used in both medicinal and veterinary practices in South Africa.[1] To date, 21 alkaloids of the Amaryllidaceae type have been isolated from *C. moorei*,[2,3] several of which have known microbiological and pharmacological effects.[4] One such alkaloid—galanthamine—has attracted the most attention because of its potential use in treating Alzheimer's disease.

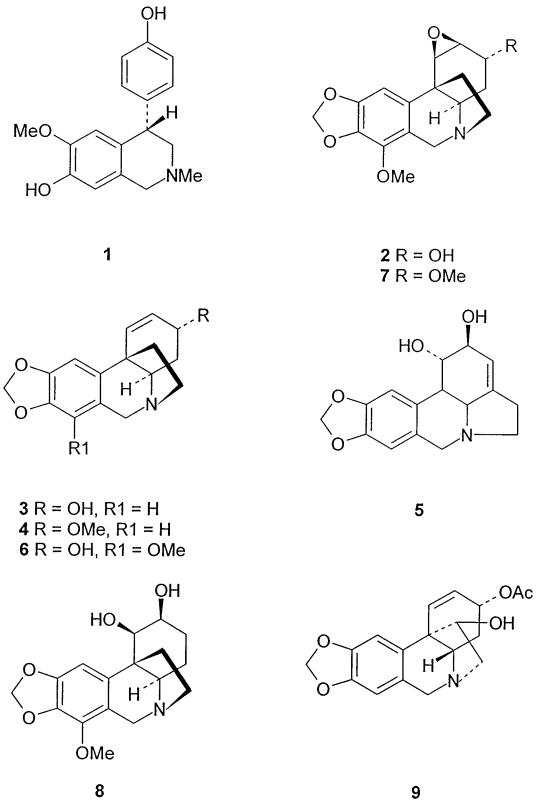
Exploitation by both the horticultural and medicinal plant trade has placed enormous pressure on *C. moorei* plants growing in the wild. They are legally protected because they are rare.[5] Conservation efforts are further exacerbated by the fact that *Crinum* species are difficult to propagate using conventional means.[6]

Cell, tissue, or organ cultures provide alternatives to the whole plant for molecule hunters and may guarantee constant and stable supplies of alkaloids.[7,8] Five Amaryllidaceae alkaloids are reportedly produced in vitro: galanthamine, *N*-formylnorgalanthamine, haemanthine, tazettine,[9] and pancratistatin.[10]

The aims of this study were 2-fold: first, to determine whether bulblets of *C. moorei*, produced in vitro, were capable of synthesizing alkaloids, and second, to establish whether their production could be increased by manipulating the culture environment. The presence of the alkaloids in the culture medium was also investigated, since this has important implications for product recovery.

Bulblets grown in vitro were initiated from twin scales of mature *C. moorei* bulbs. Although one to four shoots formed in the axes of the bulb scales, only one or two developed into bulblets within 27 weeks. Nine alkaloids were identified in extracts of 1-year-old *C. moorei* bulblets including cherylline (**1**), crinamidine (**2**), crinine (**3**), epibuphanisine (**4**), lycorine (**5**), powelline (**6**), undulatine (**7**), 1-epideacetylbowdensine (**8**), and 3-*O*-acetylhamayne (**9**). Three of the alkaloids present in the bulblets (**3**, **6**, and **7**) were also released into the medium (Table 1).

Only compounds **2**, **6**, and **7** were detected in *C. moorei* bulblets grown in the dark, and at much lower concentrations than in the light (Figure 1, Supporting Information). Higher concentrations of **6**, **8**, and **9** were recorded in bulblets grown on benzyladenine (BA)-supplemented media compared to the control. Greater amounts of **1**, **3**, **4**, **5**, and **7** were present in bulblets on the medium containing activated charcoal, and only **1** occurred in significantly greater amounts compared to the control (Figure 1, Supporting Information).



Compounds **3** and **6** were released into the medium containing BA (Table 1), although this was not statistically different from the control. Smaller amounts of **3** and **6** were present compared to the quantities of these alkaloids extracted from the bulblets. Compound **7** was detected in the control medium (Table 1) but at levels not significantly different from the other treatments.

Of the nine alkaloids identified in the bulblets of *C. moorei*, 3-*O*-acetylhamayne (**9**) is reported to occur here for the first time. All the others are known to occur in *C. moorei* plants collected from the wild.[2] Compound **2**, the most abundant alkaloid in the bulblets, also gave the highest yields for mature plants.[11] It is, however, difficult to make a comparison of alkaloid yields in in vitro cultures

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Table 1. Types and Quantities of Alkaloids Present in *Crinum moorei* Bulblets and in Different Culture Media Supporting the Growth of the Bulblets[c]

	bulblets of *C. moorei* (mg 100 g^{-1} dry weight)				medium (mg 100 g^{-1} dry weight)			
Crinum alkaloid	control	dark	BA	charcoal	control	dark	BA	charcoal
cherylline (**1**)	1.03[b]	0[b]	0[b]	6.9[a]				
crinamidine (**2**)	203.49[a]	7.41[b]	137.54[a]	192.95[a]				
crinine (**3**)	34.71[a,b]	0[b]	25.46[a,b]	49.55[a]	0[a]	0[a]	13.34[a]	0[a]
epibuphanisine (**4**)	0.59[a,b]	0[b]	0[b]	2.46[a]				
lycorine (**5**)	5.64[a,b]	0[b]	6.13[a,b]	9.07[a]				
powelline (**6**)	34.94[a]	3.9[b]	46.84[a]	28.71[a]	0[a]	0	2.35[a]	0[a]
undulatine (**7**)	31.82[a,b]	1.74[b]	3.4[b]	88.68[a]	0.39[a]	0[a]	0[a]	0[a]
1-epideacetylbowdensine (**8**)	35.78[a]	0[b]	62.56[a]	32.82[a,b]				
3-*O*-acetylhamayne (**9**)	29.82[a,b]	0[b]	55.47[a]	25.97[a,b]				
total	377.82[a]	13.05[b]	337.4[a]	437.11[a]				

[a,b]Different letters show significant differences between treatments at the 5% level (ANOVA). [c] Control, MS medium + light; dark, MS medium + dark; BA, MS medium + 2 mg/L benzyladenine + light; charcoal, MS medium + 5 g/L activated charcoal + light.

with those of field-grown and mature, flowering-size plants since plant age, flowering, and seasonal effects are known to influence the type and quantity of alkaloids present.[11,12]

Fewer alkaloids were present in the different media and at concentrations lower than those detected in the whole bulblets of *C. moorei*. For *Narcissus confusus*, though, higher concentrations of galanthamine-type alkaloids were found in the medium.[13]

Compared to bulblets grown in the light, those kept in the dark contained almost no alkaloids. Light is an important trigger for secondary metabolite synthesis in several plant species where synthesis does not take place in the dark.[14−16] Galanthamine production was similar in both the light and dark in *Narcissus confusus*, although that of *N*-formylnorgalanthamine occurred preferentially in the dark.[17] In addition to the inductive effects of light on secondary metabolite production, light also regulates the mechanisms for secretion[14] either by suppressing the release of alkaloids into the medium (in cases where it occurs preferentially in the dark) or by promoting it.[17]

Including BA in the medium in this study resulted in an increase in the production of three out of the nine alkaloids. Cytokinins are known to stimulate or enhance the production of a wide range of secondary metabolites[18] including alkaloids.[19] They are important in the repression or derepression of enzyme production and in influencing the rate of product turnover.[20] The addition of cytokinins to media to promote growth, thus, has the added benefit of either inducing alkaloid biosynthesis, as in the case of berberine production—where BA resulted in the rapid conversion of the precursor L-tyrosine into berberine—or maintaining alkaloid productivity, especially in tissue cultures capable of producing isoquinoline-like alkaloids.[21] The effects of cytokinins, like BA, on alkaloid release into the medium are, however, poorly recorded in the literature. Where it does occur is mostly in suspension cultures or liquid media.

Levels of some *Crinum* alkaloids (**1**, **3**, **4**, **5**, and **7**) increased in charcoal-supplemented media. No other workers have demonstrated the effects of activated charcoal on secondary metabolite synthesis despite the fact that it is frequently added to tissue culture media for various reasons,[22] including the induction of root and bulblet formation and superior growth.

Including BA and charcoal in the MS medium affected both the total amount of alkaloids produced and the composition of the alkaloids. Whereas alkaloid production increased in the charcoal treatment, there was a general decrease in BA-supplemented media. The composition of the culture medium also affected the production of certain alkaloid ring types. Cherylline (**1**) yields were significantly higher in the charcoal treatment compared to the control. BA, however, reduced the yields of **2** and **7** and improved those of **6**, **8**, and **9**. Statistical analysis showed that much of the variation between treatments was not significant. Since the bulblets were initiated from twin scales derived from more than one bulb, genetic differences may account for some of the variation.

Thus, alkaloid production in *C. moorei* cultures is triggered by manipulating the physical and nutritional environment under artificial conditions for growth, just as in vivo studies have shown that Amaryllidaceae alkaloid content is ecogeographically determined.[23] The advantage of using an in vitro system is that the process is more precisely defined with the added potential of producing alkaloids on a larger scale. Of the nine alkaloids extracted from the bulblets of *C. moorei*, crinine and lycorine have known pharmacological and microbiological activity.[4]

Experimental Section

General Experimental Procedures. The Varian 3300 gas chromatograph was equipped with FID and NPD detectors and a DB-5 capillary column (30 m × 0.32 mm i.d. × 0.25 mm film thickness; J&W Scientific, CA) and was linked to a Hewlett-Packard 3395 integrator. Nitrogen was the carrier gas with a head pressure of 40 kPa. Oven temperatures were set initially at 220 °C, for 1.5 min, and then increased to 270 °C at a rate of 3 °C per min. The injector and detector temperatures were 270 and 300 °C, respectively.

To identify alkaloids **1**−**9**, the retention time and mass spectra were compared with that of known samples. These were isolated from *C. bulbispermum* and *C. moorei*[2,24] and the mass spectra determined using a Hewlett-Packard gas chromatographic mass spectrometer (HP 5988A). The amount of each alkaloid present in the sample was calculated by converting the area under each peak to a concentration value using regression plots defined for each alkaloid.[11]

Plant Material: Explant Selection and Decontamination. Flowering-size bulbs of *C. moorei* were obtained from nursery stock at the National Botanical Gardens Pietermaritzburg and their identity confirmed by Mr. Brian Tarr. A voucher specimen (Elgorashi2 NU) was deposited in the University of Natal Herbarium, Pietermaritzburg. The dry outer scales, roots, and tunica were first removed and the bulbs then washed under running tap water. The bulbs were divided, radially, into eight segments prior to decontamination. Sporekill (1%) was used as a presterilization treatment in which the segments were soaked for 30 min. The bulbs were then dipped in 70% EtOH for 1 min and transferred to 3.5% NaOCl for 30−35 min. Twin scales were excised and consisted of two adjacent scales, measuring 15 mm × 3 mm, conjoined by the basal plate.

Cultures: Bulblet Production. Twin scales were placed on a basal Murashige and Skoog (MS) medium[25] in the light (70.7 μmol·m^{-2}·s^{-1}; 16 h light and 8 h dark) at 25 °C for bulblet

production. Other twin scales were placed in the dark, on benzyladenine-supplemented (BA) medium (2 mgL) or on charcoal-supplemented medium (5 gL). For comparative purposes, the control bulblets were kept in the light on MS medium with no hormone or charcoal supplements.

Drying and Extraction. A method for the extraction and separation of Amaryllidaceae alkaloids by gas chromatography, developed by Bastos et al.[26] and modified by Elgorashi et al.,[27] was used to analyze the alkaloids in *C. moorei* cultures. *C. moorei* bulblets and media were dried at 55 °C and the bulblets then ground to a fine powder and weighed. Both the bulblets, weighing between 0.04 and 0.15 g, and media were extracted in dilute acid by adding 5 mL of 0.05 N HCl and shaking (150 rpm) at 40 °C for 2.5 h. The solution was centrifuged (3600 rpm) for 5 min before adding 1 mL of 0.3 N NaOH and 4 mL of chloroform to 3 mL of the extract. After centrifuging (4500 rpm) for 5 min, the chloroform layer containing the extract was filtered through anhydrous sodium sulfate. This was air-dried. Methanol (100 μL) was added to the dried residue. A 1 μL sample was injected into the gas chromatograph for analysis. For each of the three samples per treatment, two injections were made and the mean concentration was calculated.

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Supporting Information Available: The effects of dark, benzyladenine (BA), and activated charcoal on alkaloid levels in *C. moorei* bulblets are presented in Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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