SUPPLEMENTARY MATERIAL

Aporphine alkaloid contents increase with moderate nitrogen supply in *Annona diversifolia* Saff. (Annonaceae) seedlings during diurnal periods

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Abstract

Aporphine alkaloids are secondary metabolites that are obtained in low levels contents from species of the Annonaceae family. Nitrogen addition may increase the alkaloid content in plants. However, previous studies published did not consider that nitrogen could change the alkaloid content throughout the day. We conducted this short-term study to determine the effects of nitrogen applied throughout the diurnal period on the aporphine alkaloids via measurements conducted on the roots, stems and leaves of *A. diversifolia* seedlings. The 60-day-old seedlings were cultured with the addition of three levels of nitrogen (0, 30 and 60 mM), and alkaloid extracts were analysed using high-performance liquid chromatography. The highest total alkaloid content was measured in the treatment with moderate nitrogen supply. Further, the levels of aporphine alkaloids changed significantly in the first few hours of the diurnal period. We conclude that aporphine alkaloid content increased with moderate nitrogen supply and exhibited diurnal variation.

Keywords: Liriodenine; Atherospermidine; Lysicamine; Diurnal variation; Hydroponic

3. Experimental

3.1. Plant material and growth conditions

A. diversifolia Saff. Seedlings were obtained from seeds (500) germinated in a growth chamber (Convion® CMP4030), using a paper sheet technique (Ministerio de Agricultura 1976).

The seedlings with cotyledonary leaves (10 days, n=320) were grown in hydroponic culture using distilled water (pH 6.91±0.07) until they achieved three true leaves (60 days). These seedlings were grown in a growth chamber (Convion® CMP4030) under controlled conditions (relative humidity 70-80%; and temperature 30±2°C) and a 12-h photoperiod for which the light (24.42±10.51 $\mu\text{mol m}^{-2} \text{S}^{-1}$) was generated using Sylvania® Octron T8 lamps and measured using a LI-COR® LI-250 light metre. These seedlings were used in the nitrogen supply test.

3.2. Nitrogen treatment test

The nitrogen supply tests were performed under controlled conditions using hydroponic culture in a growth chamber (see plant material and growth conditions). Three treatments with zero, moderate and high nitrogen supply (0, 30 and 60 mM N, respectively) were tested using Murashige and Skoog nutrient solutions (Table S1). The 60 mM N treatment was used for reference (Murashige & Skoog 1962) and the 0 and 30 mM N treatments were modified Murashige and Skoog media. The decrease in potassium from the use of KNO₃ in the modified 0 and 30 mM solutions was proportionately offset using KOH. The pH of each nutrient solution was adjusted to 6.0. The *A. diversifolia* Saff. seedlings (320) were fertilised with 15 L of nutrient solution. The seedlings were maintained under these conditions for 0, 2, 4, 6, 8, 12 or 24 h (0 h corresponding to a 7:00 am), and five samples were harvested at the end of each time period. The nutrient solution was monitored at each sampling period using a conductivity metre (YSI Incorporated® 85) and potentiometer (Thermo Scientific® Orion 3-Star). The harvested plant material was dissected from roots, stem and leaves, and then dried at room temperature for alkaloid extraction. The experiment was performed using three replicates.

3.3. Alkaloids extraction and determination

The alkaloid extracts from different organs were obtained from seedlings sample (n=5). The dried and powdered samples (n=5) were moistened with a saturated solution of Na₂CO₃ for 48 h and then allowed to dry at shade at room temperature (26±2.5°C, 55.5±12 μM.m⁻².s⁻¹, 64.55±9.8% RH) for 48 h. The total alkaloid extraction was performed following De la Cruz and González-Esquinca (2012).

Due to the low yield of alkaloids in seedlings, the plant roots of adult *A. diversifolia* (1495 g) were used to obtain and determine the structure of two of the alkaloids, and the third alkaloid, lysicamine, was donated by Dr Diego Cortes. The roots were collected from Ursulo Galvan Chiapas, México in August 2010. The plant material was allowed to dry at shade at room temperature (26±2.5°C, 55.5±12 μM.m⁻².s⁻¹, 64.55±9.8% RH) for 48 h. The roots dried was powdered and then defatted for 24 h using hexane. Total alkaloid extraction was performed following De la Cruz and González-Esquinca (2012). Total alkaloids (2.2698 g) were obtained from dried and powdered roots. Liriodenine was obtained in solid yellow precipitate form. Specifically, the liriodenine was purified using filtration and then successive crystallization with CHCl₃ to obtain yellow needles (1.1698 g); m.p. 280–282°C; UV λ max 248, 272, 310, 414; ¹H NMR (CDCl₃, 300 MHz): δ 8.90 (d, J=5.4 Hz, H5), δ 8.66 (dt, J=7.95 Hz, H11), δ 8.59 (dd, J=7.95, 0.9 Hz, H8), δ 7.79 (d, J=5.19 Hz, H4), δ 7.58 (dt, J=7.8, 0.9 Hz, H10), δ 7.52 (dt, 7.94, 1.22 Hz, H9), δ 7.20 (s, H3), δ 6.39 (s, 2H).

Other aporphine alkaloids were obtained from the remaining extract (1.1 g) using a chromatographic column packed with silica gel 60 HF254 (Merck, 2×40 cm of 0.063-0.2 mm). The column was eluted stepwise using an eluent system comprised of n-hexane:ethylacetate:methanol of increasing polarity. During the elution procedure, 471 fractions of 100-mL each were obtained. The presence of alkaloids in the fractions during the column elution was monitored using thin-layer chromatography and Dragendorff reagent. An alkaloid mixture (120 mg) was obtained from fractions 148-321 (n-hexane:ethyl acetate 5:5, 3:7). The alkaloid mixture was separated using thin-layer chromatography plates (Sigma-Aldrich, aluminium foil backing, 20×20 cm; 25 mm) impregnated with a mixture of 8% ethanol-oxalic acid and eluted with CHCl₃: MeOH 9:1 to obtain red needles (4 mg) with m.p. 216-220°C; UV λ max (CHCl₃): 246, 280, 312, 440; ¹H NMR (CDCl₃, 300 MHz): δ 8.876 (d, J=5.1 Hz, 1H), δ 8.55 (dd, J=7.5, 1.2 Hz, 1H), δ

8.53 (d, J=7.8 Hz, 1H), δ 8.131 (d, J=5.1 Hz, 1H), δ 7.71 (dt, J=7.5, 1.2 Hz, 1H), δ 7.49 (dt, J=8.2, 1.0 Hz, 1H), δ 6.28 (s, 2H) and δ 4.258 (s, 3H).

The reference alkaloid for lysicamine was provided by Dr Diego Cortes (Universidad de Valencia, España) and used for quantification of lysicamine in seedlings.

3.4. Quantification of aporphine alkaloids using high-performance liquid chromatography analysis

Liriodenine, lysicamine and atherospermidine were quantified by liquid chromatography using a high-performance liquid chromatography system (Perkin Elmer® HPLC-UV/Vis, Series NCI 900) and a Spheri-5 amino reverse phase column (Perkin Elmer®, 100×4.6 mm; 5- μ m particle diameter). The mobile phase consisted of chloroform/methanol (96:4, isocratic), and the flow rate was 0.3 mL.min⁻¹; the column temperature was maintained at 30°C. UV detection was conducted at 300 nm. The absorption spectra were obtained using a spectrophotometer (Thermo Electron Corporation® model Genesys 10 UV). The aporphine alkaloid reference samples in this system had retention times of 3.5±0.1; 4.0±0.2 and 4.7±1.0 mL.min⁻¹ for atherospermidine, lysicamine and liriodenine, respectively. Alkaloid calibration curves were constructed by analysing stock solutions containing 1000, 875, 750, 625, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 and 3.9 μ g.mL⁻¹ using the Turbochrom Work station (Perkin Elmer). Atherospermidine, lysicamine and liriodenine curves were linear in this area, with r² values of 0.94, 0.97 and 0.99, respectively. The alkaloid organ extracts (roots, stems and leaves) were dissolved at a concentration of 1 mg.mL⁻¹, and then 20 μ L of each solution was analysed using high-performance liquid chromatography. The alkaloid contents were quantified by converting high-performance liquid chromatography data to numbers of molecules using the following equations:

$$(\text{g Alkaloid}/\text{FW Alkaloid})(\text{Avogadro constant}/\text{Developmental time})$$

3.5. Statistical analysis

The effect of nitrogen on total alkaloids was analysed as the sum of average content of the three aporphine alkaloids in roots, stems and leaves at each time period. Then, an ANOVA (Tukey-HSD test, P <0.05) was conducted. The average content of the aporphine

alkaloids (n=3) were subjected to an ANOVA (Tukey-HSD test, $P < 0.05$) to determine the effect of nitrogen in various plant organs and their diurnal variation. In addition, a correlation analysis was performed to determine the relationship between aporphine alkaloid content in plants organs, nitrogen treatments and alkaloids variation during the diurnal period. Statgraphics Centurion XV was used for the analyses.

References

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Table S1. Salt composition of the nutrient solutions used for nitrogen treatment test.

Figure S1. Total alkaloid content during the day in *Annona diversifolia* Saff. with different nitrogen supply.

Figure S2. Nitrogen treatments effect on liriodenine in *Annona diversifolia* organs during the day.

Figure S3. Nitrogen treatments effect on lysicamine content in *Annona diversifolia* organs during the day.

Figure S4. Nitrogen treatments effect on atherospermidine content in *Annona diversifolia* organs during the day.

Supplementary Tables

Table S1. Salt composition of the nutrient solutions and modified solutions used for nitrogen treatment test.

Salts	Treatment		
	60 Mm N ^a	30 mM N ^b	0 mM N ^c
NH ₄ NO ₃	1650	816	-
KNO ₃	1900	971	-
KOH	-	515	1054
CaCl ₂ ·2H ₂ O	440	440	440
MgSO ₄ ·7H ₂ O	370	370	370
KH ₂ PO ₄	170	170	170
FeSO ₄ ·7H ₂ O	27.8	27.8	27.8
Na.EDTA	37.3	37.3	37.3
MnSO ₄ ·H ₂ O	16.9	16.9	16.9
ZnSO ₄ ·7H ₂ O	8.6	8.6	8.6
KI	0.83	0.83	0.83
CuSO ₄ ·5H ₂ O	0.025	0.025	0.025
CoCl ₂ ·6H ₂ O	0.025	0.025	0.025
H ₃ BO ₃	6.2	6.2	6.2
NaMoO ₄ ·H ₂ O	0.25	0.25	0.25

Note: Values represent the salt concentration (ppm). ^aHigh nitrogen level using Murashige and Skoog solution for reference. ^bModerate nitrogen level using Murashige and Skoog media modified. ^cZero nitrogen level using Murashige and Skoog media modified.

Supplementary Figures

Figure S1. Total alkaloid content during the day in *Annona diversifolia* Saff. with different nitrogen supply.

Figure S2. Nitrogen treatments effect on liriodenine in *Annona diversifolia* organs during the day.

Figure S3. Nitrogen treatments effect on lysicamine content in *Annona diversifolia* organs during the day.

Figure S4. Nitrogen treatments effect on atherospermidine content in *Annona diversifolia* organs during the day.