

ALKALOIDS FROM SEVERAL SUBCULTURES OF *Erythrina americana* Miller CALLUSES

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ABSTRACT

Isoquinoline alkaloids were detected in calli and seeds from the species *Erythrina americana* Miller, which were identified using liquid chromatography coupled with mass spectrometry. We studied the effect of subcultures on production of alkaloids from *E. americana* making seven subcultures of calli. Comparison of the methanolic extract obtained from seeds and cotyledons using the seven callus subcultures from *E. americana* Miller showed the presence of mainly α and β erythroidines.

ADDITIONAL KEY WORDS: Leguminosae, cotyledons, seeds, alkaloids, calli erythroidines, liquid chromatography-mass spectrometry.

ALCALOIDES OBTENIDOS DE VARIOS SUBCULTIVOS DE CALLOS DE *Erythrina americana* Miller

RESUMEN

Se detectaron alcaloides del tipo isoquinolinico en callos y semillas de la especie *Erythrina Americana* Miller, los cuales fueron identificados por la técnica de cromatografía de líquidos acoplada a espectrometría de masas. Se estudió el efecto de los subcultivos en la producción de alcaloides de *E. americana* Miller, realizando siete subcultivos de callos. La comparación del extracto metanólico obtenido a partir de semillas y de cotiledones con los de los siete subcultivos de callos de *E. americana* Miller mostró la presencia principalmente de las eritroidinas α y β .

PALABRAS CLAVE ADICIONALES: Leguminosae, cotiledones, semillas, eritroidinas, cromatografía de líquidos-espectrometría de masas.

INTRODUCTION

Alkaloids have been known to man for several centuries and have been used for human welfare. Among various groups of secondary metabolites, alkaloids are the most extensively investigated compounds. Of all the known natural products, about 20 % are classified as alkaloids. The biological activity of some of them has been investigated but only a few are produced commercially. These pharmacologically active compounds are extracted from plants, e.g. the alkaloids from *Erythrina americana* Miller, which are a series of spirocyclic-isoquinoline alkaloids found in all parts of the plant, but mainly in the seeds (García-Mateos *et al.*, 1998).

The tetracyclic *Erythrina* alkaloids have been known due their pharmacologic effects on the peripheral nervous system as a muscle relaxant (Unna and Greeslin, 1945; Lehman, 1937) and their action on the central nervous system (Pick and Unna, 1945). It was demonstrated that the effects produced by these alkaloids were similar to curare alkaloids (Lehman, 1937; Pick and Unna, 1945) used as adjuvants in surgical anesthesia (Payne and Foley, 1992).

The systematic revision of 51 *Erythrina* species showed that all alkaloids from this group have paralyzing activity to varying degrees (Folkers and Unna, 1938). Of all the alkaloids tested, α -erythroidine and β -erythroidine

showed the highest activity. β -erythroidine and its more potent derivative, dihydro- β -erythroidine, have been used as muscle relaxants in a number of clinical applications (Payne, 1991; Lozoya and Lozoya, 1982).

The *Erythrina* alkaloids have been studied extensively by GC/MS, but the use of Liquid Chromatography-mass spectrometric (LC/MS), it has not been attempted for identifying these alkaloids at the present. The method will allow to identify other structures of alkaloids in *E. americana* Miller, as well as help in their quantification and the establishment of the optimal conditions of analysis.

Considering the interest and applications of these alkaloids, the purpose of the present study was to identify by LC/MS the presence of alkaloids through the first seven subcultures of calluses. It was also explored whether calluses can still produce alkaloids in more advanced subcultures and the structural variation in these alkaloids.

MATERIALS AND METHODS

Plant material

Seeds of *E. americana* Miller were gathered at random on ten trees in Tepetlixpa, Estado de Morelos, Mexico. A "Voucher" specimen of the plant was deposited in the CHAPA Herbarium (Especialidad de Botánica, Colegio de Postgraduados) under the number 112. The authenticity of the collected material was certified by the Curator of the Herbarium of the Colegio de Postgraduados (Montecillo, Mexico).

Preparation of the material

The seeds were washed and were surface sterilized with 70 % EtOH solution for 20 min, and with 50 % NaClO for 15 min. They were rinsed with 4 X 200 ml autoclaved distilled water. The seeds were scarified with concentrated H_2SO_4 for 30 min at 20 °C and then rinsed with 5 X 50 ml distilled water. Thereafter, fifty seeds were imbibed and incubated for 48 h under aseptic conditions on moist cotton in glass jars at 27 °C under the light, seeds coats were eliminated. The seeds were incubated at 27 °C with 16 h photoperiod and light intensity of 59.83 mmol·m⁻²·s⁻¹. After 72 h germination was observed and 100 cotyledons were separated for further subculture.

Calluses culture

The cotyledons were aseptically removed and placed in 50 ml glass jars, and obtained forty jars with four explants each one, containing 20 ml of modified Murashige-Skoog medium (Murashige and Skoog, 1962), supplemented with 3 % sucrose, 1 mg·liter⁻¹ 2,4-D, and 0.5 mg·liter⁻¹ benzyladenine (García-Mateos *et al.*, 1998). The pH was adjusted to 5.7. The medium was autoclaved for 20 min at

120 °C and solidified with 6 g·liter⁻¹ of agar. Callus appeared within 10 days when the explants were maintained at 24 °C with a 16 h light and 8 h dark period. Young and healthy calluses were then subcultured at four week intervals, to obtain approximately an average of 200 g of tissue for the extraction of alkaloids. A total of seven subcultures were done. From subcultures 1, 3, 5, and 7 healthy callus were selected for analysis. If differentiation was evident, differentiated and undifferentiated materials were separated by hand, dried at 30 °C, and milled separately.

Extraction of the alkaloids

Alkaloids were extracted from tissue (seed, cotyledons y calluses) as described by (Millington *et al.*, 1974; Games *et al.*, 1974). First, the seeds were milled and extracted with hexane by Soxhlet for 48 hours. Alkaloids in the hexane fraction were washed with 2 % sulfuric acid (3 X 50 ml), and the aqueous acidic phase adjusted to pH 8 using solid $NaHCO_3$. Finally, extraction with CH_2Cl_2 (3 X 100 ml) gave the hexane fraction of "free" alkaloids. The defatted flour of each fraction was next extracted in a Soxhlet for 48 hours with MeOH, the extract evaporated under vacuum, and the residue taken up in 2 % sulfuric acid. The acidic solution was extracted with CH_2Cl_2 to remove traces of fat. The aqueous phase was basified with $NaHCO_3$ to pH 8, and was extracted with CH_2Cl_2 (3 X 100 ml) to give a methanolic fraction of the "free" alkaloids. The solvent was evaporated and the residue was dried in a desiccator and weighed. Tissue of cotyledons and subcultures of calluses were treated in the same way as the seeds, except that free alkaloids in hexane were not obtained.

Identification of alkaloids

The alkaloids were identified previously by TLC using silicagel 60 GF₂₅₄ plates (Merck, USA.) with mobile phase 8:2 (v:v) dichloromethane:methanol. They were identified previously on basis of colour reaction after treatment with chromogenic reagents (e.g. Dragendorff's Reagent) and co-chromatography with authentic samples of *Erythrina* alkaloids obtained in others studies (García-Mateos *et al.*, 1998; 1999).

Liquid chromatography-mass spectrometry

LC-MS was used to analyze the alkaloid fractions. The LC-MS/MS system consisted of a high performance liquid chromatograph (Waters 600 E pumps and controller) interfaced to a quadrupole ion trap mass spectrometer (Thermo Finnigan LCQ classic) via an atmospheric chemical ionization (APCI) source. The alkaloids were separated on a 250 mm x 4.6 mm (i.d.) Supelco Discovery C-18 (5 μ m) column, using a 1 ml·min⁻¹ linear mobile phase gradient programmed from three solvent reservoirs, A (0.1 % ammonium acetate, pH 7.4), B (methanol) and C (acetonitrile), as follows: t = 0 min, 75 % A, 20 % B, 5 % C; t = 10 min, 50 % A, 45 % B, 5 % C; t = 15 min, 50 % A, 45 % B, 5 % C. The vaporizer of

the APCI source was set to 450 °C with sheath and auxiliary nitrogen gas pressures of 80 and 20 psi, respectively, and the needle current was 5 mA; the heated capillary temperature was 150 °C. The MS was programmed to survey ions with a mass to charge ratio (m/z) of 150-500 and subject to the most intense ion in the survey scan to MS/MS analysis and MS/MS/MS analysis (using the most intense product ion the MS/MS scan). The collision energy was 40 % using an ion isolation width of 3 amu.

The alkaloids were identified by comparing the various CID spectra and retention times with isolated standards. The identification of alkaloids was done by comparison with authentic samples, interpretation of mass spectral characteristics and use of reference data.

RESULTS AND DISCUSSION

The development of the calluses were followed carefully, the yields of the extract and fractions of alkaloids from the tissues analyzed, were expressed as crude alkaloids mg·100 g⁻¹ of dry weight of plant material. The methanolic extracts of the calluses *E. americana* Miller were higher

than those obtained from seeds. The total alkaloid content was higher in cotyledons than in seeds, whereas in the subcultures the content was low (Table 1). This could be due to the presence of 2,4-D in the medium, as it has been described that this auxin can inhibit the production of alkaloids (Merillon and Ramawat, 1999). We used 2,4-D as an auxin in the culture medium based on the work of Meyer and Staden (1991) who first described culture *in vitro* of *Erythrina*. It was observed that the concentration tested (1 mg·liter⁻¹) had no inhibitory effect on the growth of the calluses nor in the accumulation of alkaloids in spite of the indications that showed that 2,4-D inhibited the accumulation of alkaloids *in vitro* (Merillon and Ramawat, 1999).

It also observed that the “free” alkaloids fraction was higher than the liberated alkaloids fraction in all the samples analyzed as it has been observed in previous works, perhaps, due to the technical of analysis by LC/MS (García-Mateos *et al.*, 1999; 1998; 1996). The proportions of alkaloids in the calluses analyzed, also in cotyledons and seeds are shown on Table 2. In these respect, the cotyledons were the tissues with higher amount of alkaloids compared to the calluses.

TABLE 1. Characteristics and content of alkaloids in the fractions from seeds, cotyledons and calluses of *Erythrina americana* Miller.

	Seeds	Cotyledons	Subculture 1	Subculture 3	Subculture 5	Subculture 7
Water content (%)	nd	70.76	94.24	95.90	94.89	94.20
Fresh weight (g)	nd	65.00	193.28	381.10	217.50	129.48
Dry weight (g)	92.98	19.00	11.10	15.60	11.10	7.50
Methanolic extract ^y	23.50	38.94	63.06	48.71	49.54	40.05
Fraction free alkaloids ^x	0.56	0.84	0.13	0.12	0.89	0.10
Fraction liberated alkaloids ^x	0.06	0.13	0.04	0.10	0.02	0.04
Total alkaloids ^x	0.61 ^y	0.97	0.17	0.22	0.91	0.14

^yExtract (g) obtained from 100 g dry tissue.

^xAlkaloids (mg) present in 100 g dry tissue.

nd: It was not determinated.

TABLE 2. Percentage content of alkaloids in seeds, cotyledons and calluses from *Erythrina americana* Miller.

Alkaloid	Alkaloid content (%) ^z					
	Seeds	Cotyledons	Subculture 1	Subculture 3	Subculture 5	Subculture 7
Erysodine	traces					6.74
Erysovine	1.38					10.00
Erythraline	traces	14.85	0.94			
Eritrinine		1.81	6.61	3.50	0.75	
8-Oxo-erythraline		Traces				
Erythratidine			3.09	3.09	1.23	
Erythartine-N-oxide		1.75	0.39	0.39		
α-Erythroidine	40.77	45.51	43.26	43.26	45.58	38.55
β-Erythroidine	58.73	36.08	49.75	49.75	52.44	44.71

^zThe relative proportions of the alkaloids were calculated from the area of the LC peaks. Traces were considered $P \leq 0.01$. The structures of the alkaloids are show in Figure 2.

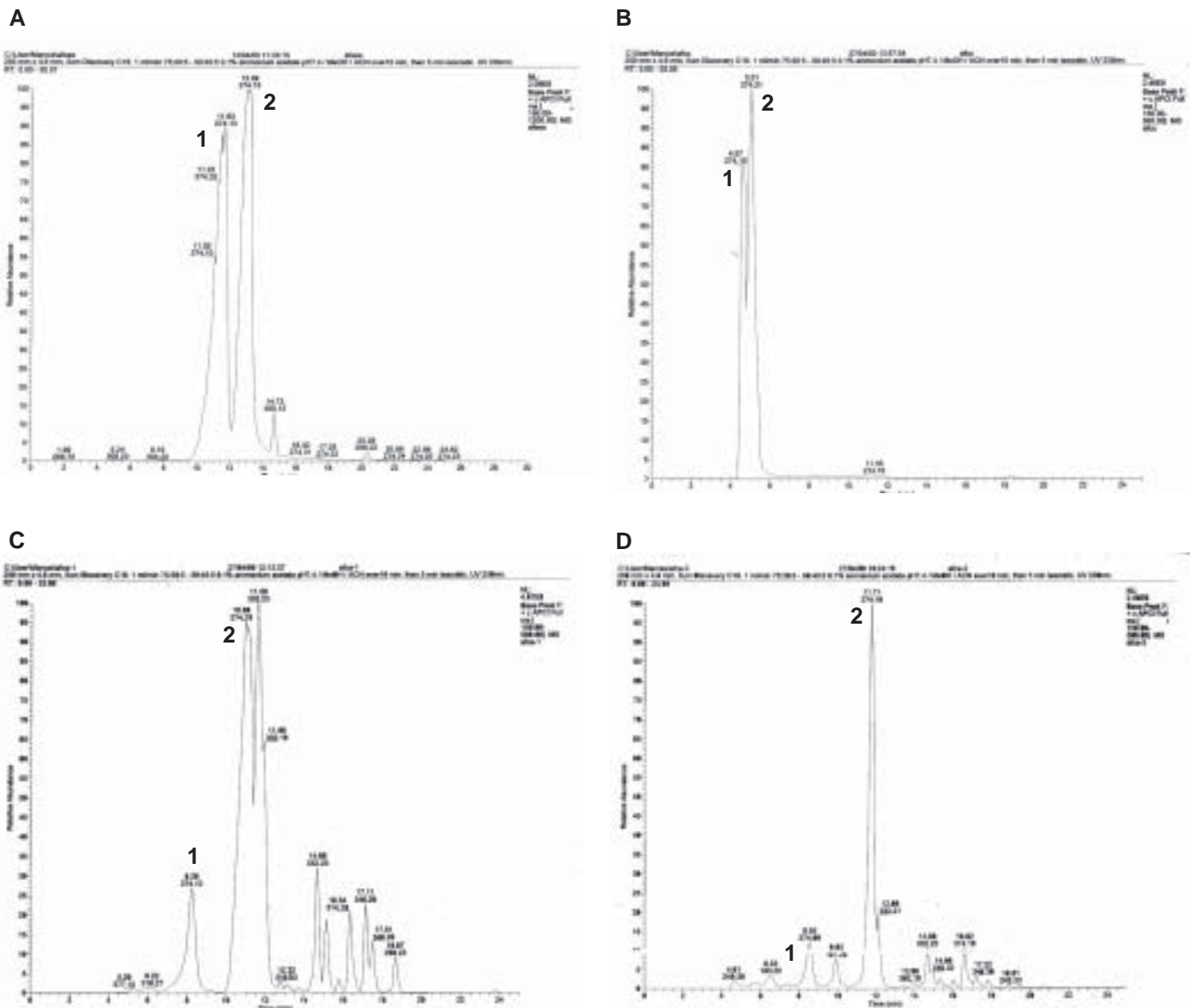
The methanolic fraction of the “free” alkaloids in seeds, cotyledons and calluses (from each subculture) were analyzed by a liquid chromatography system equipped with a mass spectrometer as detector. With the use of LC/MS, it was possible to detect some of the alkaloids no described in previous work (García-Mateos *et al.*, 1999). The relative retention times of the analysis by LC of the identified alkaloids are shown on Table 3. Each sample of calluses showed 4 to 6 peaks depending on the subculture sequence (Figure 1).

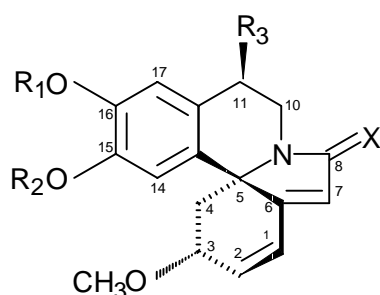
Several interesting aspects were observed in relation to the alkaloids analyzed. The lactonic alkaloids were detected in all the tissues analyzed (seeds, cotyledons and calluses) in comparison with previous work (García-Mateos

TABLE 3. Relative retention times of the analysis by LC of the alkaloids from *Erythrina americana* Miller.

Alkaloid	Retention times ^a
Erysodine	1.23
Erysovine	1.30
Erythraline	1.89
Erythrynine	1.69
8-Oxo-erythraline	1.67
Erythratidine	1.29
Erythartine-N-oxide	1.76
α -Erythroidine	1.00
β -Erythroidine	1.16

^aExpressed with respect to the retention time of α -Erythroidine





Dienoid alkaloid	R ₁	R ₂	R ₃	X
Erysodine	H	Me	H	H
Erysovine	Me	H	H	H
Erythraline	-CH ₂ -		H	H
8-Oxo-erythraline	-CH ₂ -		H	O
Erythrynine	-CH ₂ -		OH	H

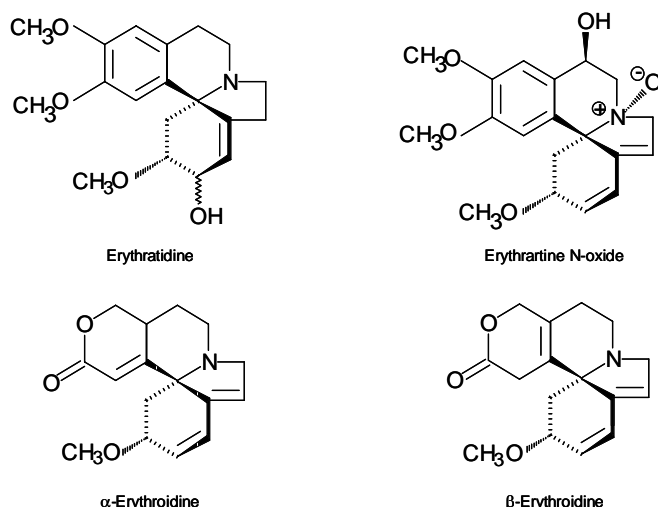


FIGURE 2. Structures of alkaloids identified in seeds, cotyledons and calluses from *Erythrina americana* Miller.

et al., 1999), they are not detected in calluses, probably by the low sensitivity of the equipment used in that work.

The first subculture showed the presence of α -erythroidine, β -erythroidine, erythratidine, erythrynine, erythartine-N-oxide and erythraline. The third subculture contained α -erythroidine, β -erythroidine, erythratidine, erythrynine and erythartine-N-oxide. The fifth subculture was similar, but the n-oxide alkaloid was absent. The last subculture contained erythroidines and erysodine and erysovine (Figure 2). In this case the presence of a N-oxide from cal-

lus suggest a considerable difference in alkaloid content when an intact plant is compared with tissue cultures derived from it.

The identification of these alkaloids was based on the well known patterns in other species of the genus (Aguilar *et al.*, 1981; García-Mateos *et al.*, 1996) where the lactonic alkaloids are the most abundant apart of minor amounts of erysodine, erysovine, erythrynine and erythraline. The online spectra library also helped to support these observations. It is well known that these lactonic alkaloids are the main alkaloids in the seeds of *E. americana* Miller and was observed a similar tendency in tissue culture in which β -erythroidine was more abundant than α -erythroidine, (Figure 1). In calluses more alkaloidal structures with high level of oxidation were detected compared to seeds. The LC-MS as a reliable method compared with TMS derivatives by GC-MS certainly supports these observations.

The application of this method allowed the identification of major content of alkaloids and structures not identified in calluses and seeds of the specie of *Erythrina americana* Miller (erythraline, 8-oxo-erythraline, eritrynine and erythratidine), not reported in the literature.

Also, in this study it was observed that the accumulation of alkaloids and growth of the calluses mainly those related to the erythroidines were present still in the seventh subculture step in MS medium with 2,4-D as auxin. With this in mind, we are further investigating in detail the culture conditions to increase the accumulation of these alkaloids and support their demand in biotechnology or clinical applications.

CONCLUSIONS

In this study lactonic alkaloid α - and β -erythroidine were found. The last one is responsible for the pharmacological activity found in previous studies. The number of alkaloids detected were reduced when the number of subcultures *in vitro* increased. The higher content of alkaloids was found in the first subculture.

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